An SV40 Transformation Revertant Due to a Host Mutation: Isolation and Complementation Analysis

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We have isolated an SV40 transformation revertant cell line, CL1L, by selection for normal cells whose growth is inhibited under low serum conditions. This line expresses a single, wild-type copy of large T antigen, yet is not transformed. It is not retransformable by transfection of SV40 DNA or infection with a recombinant retrovirus encoding large T antigen. Resistance to transformation therefore appears to be due to a cellular mutation. Fusion of CL1L cells to normal 3T3 cells or SV40-transformed cells results in somatic cell hybrids that are transformed, indicating that resistance is recessive. In addition, fusion of CL1L cells to another SV40 transformation-resistant line, A27, results in transformed hybrids, indicating the existence of discrete complementation groups with respect to SV40 transformation. © 1992 Academic Press, Inc.

INTRODUCTION

The disruption of normal cell growth control by SV40 large T antigen requires the interaction of T antigen with proteins which normally regulate cell growth and division. Although some of these proteins, such as p53, pRb, and p107/p120 (referred to herein as p120), have been identified and their genes cloned and characterized (Linzer and Levine, 1979; Lane and Crawford, 1979; DeCaprio et al., 1988; Ewen et al., 1989; Dyson et al., 1989), they alone cannot account for large T antigen's transforming ability. For example, some mutant T antigens which still bind these proteins are defective for transformation (Rutila et al., 1986; Manfredi and Prives, 1990; Yaciuk et al., 1991), and mutants which do not bind these proteins can sometimes still transform (Srinivasan et al., 1989; Tack et al., 1989; Ludlow et al., 1990). Thus, at least one more host function must be involved in transformation. Additionally, one would like to know what steps beyond binding of T antigen to host proteins lead to the transformed state.

All of the proteins mentioned above were recognized by their presence in immunoprecipitates of T antigen. While such biochemical analysis of T antigen complexes is a powerful approach toward identifying such

factors, it can be limited in that it only allows detection of proteins which are stably associated with T antigen. Proteins which interact with T antigen on a transient basis and proteins which act downstream of those actually bound to T antigen probably escape detection in these assays.

In attempts to identify these types of factors, a number of laboratories, including ours, have taken genetic approaches toward the study of transformation by T antigen. Specifically, we have been interested in isolating murine cell lines which are resistant to transformation by large T antigen. Such cell lines presumably contain a cellular mutation which renders them resistant. One such line, A27, was isolated by introducing T antigen into 3T3 fibroblasts under nonselective conditions and screening for cells that express T antigen but are not transformed (Ryan et al., 1985). Another genetic approach involves the isolation of transformation revertants, derivatives of transformed cell lines which appear to be normal due to host mutations (Vogel et al., 1973; Maruyama et al., 1982; Bauer et al., 1987). In this report, we describe the isolation and characterization of one such revertant cell line, CL1L. CL1L cells express a functional T antigen (T+), are transformation negative (tr−), and are specifically resistant to retransformation by SV40. Fusion of CL1L to normal and transformed cells indicates that the defect in CL1L is recessive. Of particular interest, fusion of CL1L to A27 yields transformed cells, indicating the existence of at least two complementation groups with respect to SV40 transformation susceptibility.

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MATERIALS AND METHODS

Cell lines

All cell lines except retrovirus producer lines were maintained in minimal Eagle's medium (MA Bioproducts) supplemented with 0.2 mg/ml NaHCO₃, 0.29 mg/ml glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Transformed and revertant cells were maintained in 10% fetal calf serum (MEM-10); and BALB/c 3T3 cells in 20% fetal calf serum (MEM-20). Retrovirus producer cell lines (Brown et al., 1986) were maintained in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% calf serum (DMEM-10)..The transfer of dominant selectable markers into cells for use in fusion experiments was carried out by transfection of pSV2neo (Southern and Berg, 1982) or pY3 (Blochlinger and Diggelmann, 1984), followed by selection in 400 to 600 μg/ml G418 (GIBCO) or 300 μg/ml hygromycin B (CalBiochem), respectively. Well-isolated, drug-resistant colonies were picked and subcloned by end point dilution.

Isolation of revertants

To isolate serum revertants, A209R1B1 cells were plated on Day 0 at a density of 1 × 10⁴ cells/cm² in MEM-10. On Day 1 the medium was changed to MEM-1, and on Day 2 one of two drugs was added: 200 μg/ml fluorodeoxyuridine (FUdR) in the presence of 2 mg/ml uridine, or 1 μg/ml colchicine. Excess uridine was added during FUdR treatment to prevent the incorporation of FUdR into RNA. After a 6-day exposure to the drugs, cells were trypsinized and plated in MEM-10. Surviving colonies were subcloned by limiting dilution. To isolate density revertants, A209R1B1 cells were plated on Day 0 at a density of 6 × 10⁴ cells/cm² and exposed to drugs for 3 days.

Transfections

Calcium phosphate–DNA precipitates were made and added to cells as previously described (Imperiale et al., 1983). Approximately 18 to 24 hr later the medium containing the precipitate was removed and the cells were fed with MEM-10. After an additional 24 hr, cells were split into assays as described under Results.

Transformation and growth assays

Monolayer overgrowth assays, measurements of cell growth in 10% FCS and 1% FCS, and focus formation assays were performed as described (Brockman, 1978; Christensen and Brockman, 1982). Assay dishes were stained with 0.1% Wright's stain in methanol. Soft agar assays were carried out as described

previously (Brockman, 1978; Christensen and Brockman, 1982), with the following modifications. Assays on the original 56 candidate revertant cell lines were performed in low-melting-point agarose (LMPA), which was used at a concentration of 0.625% in the base layer and 0.412% in the cell overlay layer. The assays were fed weekly with overlay mixture, and the dishes were scored after 2 weeks. All subsequent assays used low EEO agarose (Boehringer-Mannheim) at 0.5% in the base layer and 0.33% in the cell overlay layer. These assays were fed weekly with overlay mixture and scored after 5 weeks. Colonies were scored positive if they were ≥0.01 inches in diameter.

Southern blots

Southern blots were performed using 0.8% agarose gels as previously described (Bender and Brockman, 1981).

Plasmid DNA preparation

Plasmid DNA was prepared by standard methods (Maniatis et al., 1982).

Retroviral infections

Twelve hours prior to infection the producer cells, at 80% confluence, were fed fresh DMEM-10. The day of the infection, the medium from the producer lines was collected, polybrene was added at 8 μg/ml, and the mixture was passed through a 0.45-μm low-protein-binding filter. Cells at approximately 50% confluence were infected with 2 ml of the harvested retroviral medium for 2 hr at 37° with gentle rocking every 20 min. The dishes were then fed with 8 ml of MEM-10, moved to 33°, incubated for 2 days, and split into assays. Infected cells (1/1000 or 1/500) were plated into 60-mm dishes in 500 μg/ml G418 to determine the efficiency of the retroviral infection. Cells to be assayed for transformation were split either 1/100 or 1/50 from the infected dish into 100-mm dishes and grown in MEM-10 plus 500 μg/ml G418. After 2 weeks these cells were placed into soft agar assays, picked to establish clonal cell lines, or used for immunoprecipitations. The remainder of the original dish of infected cells was placed directly into focus or 1% serum assays.

Cell fusions

The day before the fusion, equal numbers of cells from both parental lines were plated into each of three 10-cm² wells in MEM-10 at 37°. Fusion was carried out the next day, when the cell monolayers had reached confluence. Medium was removed and the cells were exposed to 1 ml of 50% 2× MEM, 5% FCS,

0.3 mg/ml glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 50% (v/v) polyethylene glycol 600 (Sigma) for 40 sec followed by rapid dilution with 4 ml MEM-5. The cells were then washed four times with 2 ml MEM-5, fed with MEM-10, and moved to 33°. Twenty-four hours later the cells were fed with fresh MEM-10. The next day cells from the three wells were trypsinized, pooled, and plated into two 150-cm² flasks in MEM-10. The following day selection was started with 450 μg/ml G418 and 300 μg/ml hygromycin B. Equal numbers of surviving colonies were pooled and placed into transformation assays.

Immunoprecipitation analysis

Confluent 100-mm dishes of cells were washed once with DMEM without methionine (DMEM met−) and fed with 5 ml of labeling medium (10% FCS, 10% DMEM, and 80% DMEM met−) containing ³⁵S-labeled methionine and cysteine (Translabel, ICN Radiochemicals) at 60 μCi/ml. Cells were labeled at 37° for 23 hr. After labeling, the cells were washed twice with ice-cold PBS and lysed at 4° for 30 min in 1 ml lysis buffer (250 mM NaCl, 0.1% NP-40, 50 mM HEPES, pH 7.0) (Whyte et al., 1988) containing 5 μg/ml each of PMSF, leupeptin, and aprotinin. The lysates were cleared by centrifugation at 16,000 g for 2 min and stored at −70°. Immunoprecipitations were carried out overnight at 4° in 0.5-ml reactions containing 250 μl lysate, 250 μl lysis buffer, and excess antibody. Complexes were recovered by incubating with 150 μl of 6% CL-4B protein A–Sepharose (Pharmacia) in lysis buffer for 30 min, with rocking. Samples were washed five times with 1 ml lysis buffer, and immunoprecipitated proteins were released by boiling and separated by electrophoresis through 8% SDS–polyacrylamide discontinuous gels (Laemmli, 1970). Gels were fixed for 30 min in 30% methanol, 10% acetic acid and then treated with Amplify (Amersham) for 30 min. After drying under vacuum at 80°, the gels were exposed to Kodak XAR film at −70°.

RESULTS

Generation and characterization of SV40 revertants

In order to increase the chances of obtaining revertants due to mutations in cellular genes, we chose a parental transformed cell line containing multiple copies of the SV40 early region. Use of such a parental line would be expected to lower the frequency of reversion resulting from loss of T antigen coding regions. This line, A209R1B1, is an SV40-transformed BALB/c 3T3 line that expresses at least two distinct transformation-competent large T antigens (Brockman, 1978; Bender

TABLE 1

GROWTH ASSAYS OF REVERTANT CELL LINES

Cell line	Monolayer overgrowth[a]	Growth in soft agar[a]
Expt 1		
3T3	—	0.01
A209R1B1	0.190	0.27
CL1F	0.002	0.04
CL1L	<0.004	0.08
Expt 2		
3T3	—	0.01
A209R1B1	0.200	0.48
CL1E	<0.002	0.03
FH1B	<0.002	0.13
Expt 3		
3T3	—	0.01
A209R1B1	0.1	0.7
FH2B	0.02	0.8
FH2K	0.025	0.1

[a] Efficiency of plating is the number of colonies/number of cells seeded into the assay.

et al., 1983). To isolate revertants, A209R1B1 cells were subjected to the negative selection protocols discussed under Materials and Methods. These conditions allow for growth of transformed cells, which are then selectively killed by the cytotoxic drugs colchicine or FUdR. Colchicine disrupts microtubule polymerization and kills cells by inhibiting cytokinesis (Borisy and Taylor, 1967) and is more toxic to SV40-transformed cells than normal cells (Vogel et al., 1973). FUdR prevents thymidine synthesis by inhibiting thymidylate synthetase (Rueckert and Mueller, 1960). Two different selection protocols were employed with each drug in an effort to generate revertants caused by distinct cellular alterations: cells plated at high density in normal concentrations of serum, or low density in low serum, were selected with each drug.

Fifty-six potential revertant cell lines were isolated using the four variations of the selection scheme. This represents a reversion frequency of about 1 × 10⁻⁵, which is within the range reported by others (Culp and Black, 1972; Vogel and Pollack, 1973). These cell lines were screened for the presence of large T antigen by indirect immunofluorescence, the ability to overgrow preformed monolayers of BALB/c 3T3 cells, and growth in LMPA. Six cell lines had the desired phenotype, T⁺/tr⁻. These fell into two classes: colchicine serum revertants, designated CL (Colchicine, Low density), and FUdR density revertants, designated FH (FUdR, High density) (Table 1). All six produce large T antigen which localizes to the nucleus. Five exhibit at least partial reversion in both growth parameters.

FH2B, however, fails to form colonies on preformed monolayers, yet grows as well as A209R1B1 in LMPA.

To examine the integrity of the early region sequences in the revertant lines, cellular DNA from these lines and A209R1B1 was digested with HpaII and BamHI, enzymes which separate the early region of the SV40 genome from the late region, and analyzed by Southern blotting using a probe that consists of the entire SV40 genome. The results from four of the cell lines are shown in Fig. 1A. CL1E, CL1F, CL1L, and FH1B all contain an intact SV40 early region, as indicated by a diagnostic band of 3056 nucleotides. All of these cell lines therefore contain early region sequences that are capable of encoding a wild-type size T antigen. The large T antigen produced by the six cell lines was examined by immunoprecipitation of [^{35}S]methionine-labeled proteins with hamster anti-SV40 tumor serum (Fig. 1B). All six lines produce a large T antigen of 94 kDa, the same size as wild-type T antigen. The cellular protein p53 coimmunoprecipitates with T antigen in all the revertant lines. This analysis suggests that there is no gross alteration of the T antigens produced by these six cell lines.

Retransformation with wild-type T antigen

Even though the large T antigens expressed by these lines appear to be wild type, viral mutations could inactivate the transforming ability of T antigen without altering its size, association with p53, or subcellular localization. To test whether these cell lines are true cellular revertants, we asked whether they could be retransformed by introducing a wild-type copy of large T antigen. The revertants were cotransfected with a plasmid encoding T antigen, pSVB3, and a plasmid encoding a selectable marker, pSV2neo, at a 10:1 ratio and assayed for the ability to overgrow preformed 3T3 monolayers. Cells that survived selection in G418 were plated into transformation assays. A plasmid encoding an activated c-Ha-ras gene, pEJ6.6 (Shih and Weinberg, 1982), was transfected independently to ascertain the specificity of transformation resistance of the revertants and to ask whether they are capable of exhibiting the transformed phenotype. The FH2K cell line was difficult to transfect and was dropped from the study. CLIF, FH1B, and FH2B were retransformed by T antigen, but CL1L and CL1E were not (Table 2). The efficiency of transfection of all the cell lines was the same, so differences in DNA uptake cannot explain the results. In addition, all of the cell lines were transformed by ras. CL1L and CL1E were next tested for the ability to be retransformed in a focus formation assay. Cells were cotransfected with pSV2neo and either pSVB3 or pEJ6.6 and assayed for focus formation. Both lines were transformable by ras and resistant to SV40 (data not shown). Since CL1L grew to a lower cell
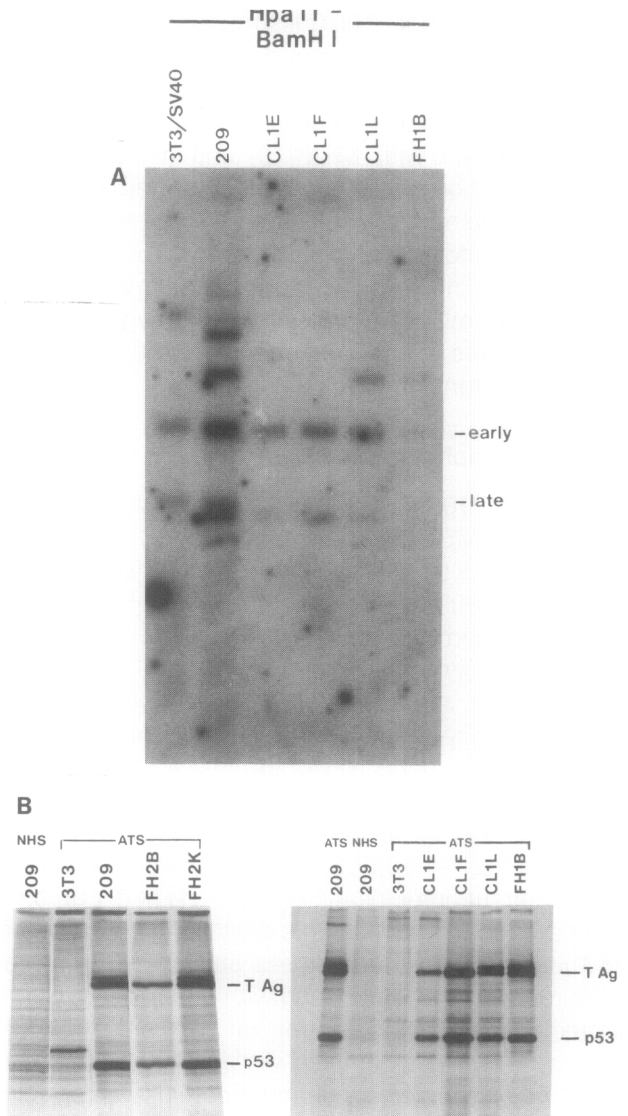

Fig. 1. Analysis of SV40 sequences and T antigen expression in revertant cell lines. (A) Southern blot analysis. Genomic DNA from the four cell lines was digested with HpaII and BamHI, electrophoresed, and probed with the entire SV40 genome. The lane marked 3T3/SV40 is a reconstruction in which SV40 genomic DNA was mixed with 3T3 genomic DNA and analyzed. 209 represents the A209R1B1 line. The positions of bands corresponding to the early and late regions are shown. (B) Immunoprecipitation analysis. ^{35}S-labeled proteins were immunoprecipitated with anti-T antigen hamster ascites (ATS) or normal hamster serum (NHS) and analyzed on 8% SDS gels.

density in monolayer culture than CL1F, indicating the tightest growth control, further examination was restricted to CL1L.

Growth parameters of the transformation revertant CL1L

Additional growth parameters of the CL1L cell line were examined to determine whether other properties

TABLE 2

RETRANSFORMATION OF REVERTANT CELL LINES

Cell line	SV40[a]	ras[a]
3T3	2.5	1.3
CL1L	0.1	20.0
CL1E	<0.1	1.4
CL1F	0.6	1.7
FH1B	5.5	6.0
FH2B	0.5	9.0

[a] EOP on preformed 3T3 monolayers is expressed as the number of colonies/number of cells plated × 100.

of CL1L had also reverted. The morphologies of confluent monolayers of A209R1B1, CL1L, and 3T3 cells are shown in Fig. 2A. A209R1B1 cells are spindle-shaped, rounded, refractile, and grow in a disorganized, dense configuration that is typical of transformed cells. 3T3 cells are flat, nonrefractile, and form well-ordered monolayers. The morphology of CL1L cells is indistinguishable from that of 3T3 cells. By this criterion, CL1L is therefore classified as a revertant. As shown in Table 3, the efficiency of plating (EOP) of CL1L cells in soft agar is much more like that of 3T3 cells than that of A209R1B1 cells. Photographs of representative fields of soft agar assays in Fig. 2B show colonies of CL1L cells to be slightly greater in size than colonies of 3T3 cells (which remained as single cells), but much smaller than the colonies of A209R1B1 cells. Even though the CL1L cell line appears to have some capacity to form anchorage-independent colonies, this capacity is much less than that of A209R1B1 cells. Therefore, CL1L has reverted as judged by this parameter. Saturation density in 10% FCS and the ability to grow in 1% FCS were also examined (Table 3). Once again, CL1L cells behave like normal cells. Since the CL1L cell line was originally isolated as a serum revertant, it is especially significant that CL1L cells do not grow in 1% serum.

Characterization of the CL1L T antigen

One possible explanation for the inability of the transfected SV40 early region to retransform CL1L is that the T antigen expressed by CL1L might be defective and dominant over the exogenously introduced T antigen. Southern blot analysis showed that CL1L contains a single copy of the early region (data not shown), so we cloned a genomic fragment containing this copy into a plasmid vector to create pSVCL1L. This plasmid, or one expressing wild-type T antigen (pSVB3 or pJYM), was transfected into 3T3 cells to assess the relative transforming ability of the CL1L T antigen. pSVCL1L transforms 3T3 cells with at least wild-type efficiency as judged by focus formation, ability to grow in soft agar, and growth in low serum (Table 4). Indeed, cells receiving the CL1L T antigen had a higher EOP in soft agar than cells that received wild-type T antigen. By these three criteria, then, the CL1L-encoded large T antigen appears to be a fully functional transforming protein.

Transformation resistance of CL1L

To more closely examine the transformation resistance of CL1L, we infected cells with a retrovirus expressing a large T antigen cDNA to efficiently introduce T antigen into the cells (Brown et al., 1986). CL1L and 3T3 cells were infected with this virus (TEXS) or a control virus lacking the T antigen cDNA (SVX), and the infected cells were assayed for three growth parameters (Table 5). The TEXS virus induced anchorage-independent growth of 3T3 cells to a significantly higher extent than it did of CL1L cells. This is most clearly seen by comparing the relative EOP of TEXS-infected cells to that of SVX-infected cells for both cell lines: the TEXS EOP:SVX EOP for 3T3 cells is approximately 20 times higher than that for CL1L. In addition, the TEXS virus induced a significantly larger overall colony size in 3T3 cells than it did in CL1L cells and induced many microcolonies in 3T3 cells which were not scored as positive since they were smaller than colonies of uninfected CL1L cells (Fig. 3). Thus, the change in the soft agar growth phenotype of CL1L cells after TEXS infection is not as great as that observed with 3T3 cells. We also examined the behavior of the infected cells in focus formation in 5 and 1% serum (Table 5). Again, the two cell lines behaved quite differently. TEXS-infected 3T3 cells formed aggressive foci in monolayers in 5% serum which were obvious by 13 days postinfection. CL1L cells infected with TEXS never formed foci in monolayer culture, although they did grow to a slightly higher density than SVX-infected CL1L cells. In 1% serum, TEXS-infected 3T3 cells formed foci but at a level lower than that in 5% serum. No growth of CL1L was observed in 1% serum after infection with either the TEXS or the SVX virus. We conclude that although soft agar growth of CL1L cells can be increased slightly by T antigen, these cells are not capable of being fully retransformed.

Specificity of CL1L transformation resistance

To further define the specificity of transformation resistance, the CL1L cell line was transfected with a small panel of oncogenes: a polyoma middle T antigen cDNA (pPyMT1); an activated c-Ha-ras gene (pEJ6.6); and a viral src gene (pMvsrc). Transfected cells were

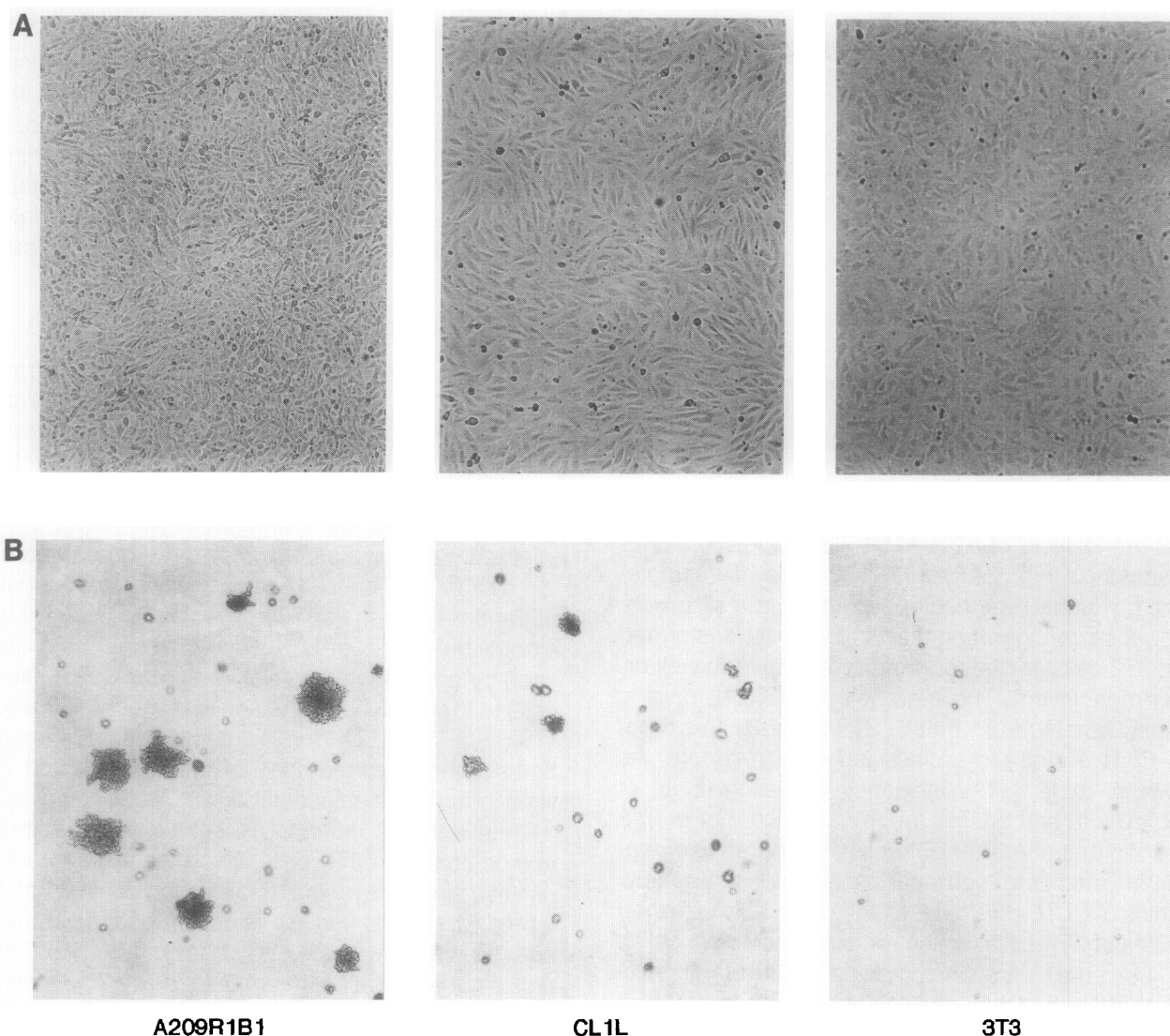A209R1B1   CL1L   3T3

FIG. 2. Morphology and growth of CL1L cells. (A) Photographs of confluent monolayers of cells. (B) Photographs of soft agar colonies.

assayed for growth in soft agar and focus forming ability in 5 and 1% FCS (Table 6). All three oncogenes induced the growth of CL1L cells in soft agar. Soft agar colonies of CL1L cells transfected with middle T and v-src were quite large, showing that CL1L can exhibit the full transformed phenotype in soft agar (Fig. 4). All of the plasmids were also able to confer focus forming ability on CL1L in 5% serum, although to various extents: the effects on CL1L of middle T antigen and ras were much greater than the effects of v-src. This difference was also seen in 1% serum assays, where middle T antigen and ras induced CL1L cells to form foci, but v-src did not.

Binding of T antigen to cellular proteins in CL1L

An obvious candidate for a cellular mutation in CL1L would be one of the T antigen-binding proteins which have been shown to play a role in SV40 transformation. We therefore examined the cellular proteins that are bound to large T antigen. CL1L cells or A209R1B1 cells were metabolically labeled and proteins were isolated, immunoprecipitated with the anti-T antigen antibody PAb419, and subjected to SDS–PAGE. In precipitates from both CL1L and its parental cell line one finds p53 and a protein with an approximate molecular weight of 120 kDa (Fig. 5). This is probably the p107/p120 protein that binds to the pRb binding domain of large T antigen (Ewen et al., 1989), since a large T antigen mutant in this domain does not complex with this protein and it does not comigrate with pRb (D.C.K. and M.J.I., in press). Although pRb may be present in these precipitates just above the T antigen band, experiments to prove that this is the Rb protein were inconclusive. Nevertheless, there is no obvious defect in

TABLE 3

Growth of CL1L in Soft Agar and Saturation Density
in 1% or 10% Serum

Cell line	EOP in soft agar[a]	Saturation density[b] in	
		1% Serum	10% Serum
A209R1B1	3.84 (192)	34.0×10^4	97×10^4
CL1L	0.04 (2)	4.4×10^4	15×10^4
3T3	<0.02 (0)	3.9×10^4	13×10^4

[a] EOP is the number of colonies/number of cells plated × 100. The number in parentheses is the average number of colonies scored of 5000 cells plated per dish.
[b] Saturation density is the number of cells/cm².

binding to proteins known to be involved in SV40 transformation.

The phenotype of CL1L is recessive and defines a complementation group

To determine if the defect in CL1L is dominant or recessive, CL1L subclones expressing selectable markers were fused to normal cells (BALB/c 3T3) or to transformed cells (A209R1B1) expressing different selectable markers. Somatic cell hybrids were selected and plated into soft agar to test for anchorage-independent growth (Table 7). Fusion of CL1L to normal 3T3 cells resulted in hybrids that have a much higher EOP than either parental cell line. Some of these hybrid colonies in soft agar were larger than colonies formed by A209R1B1, indicating that these fused cells are fully transformed. Fusion of CL1L cells to transformed A209R1B1 cells also resulted in hybrids with an EOP greater than that of CL1L cells and comparable to that

TABLE 4

Transformation of BALB/c 3T3 Cells by the CL1L Large T Antigen

Plasmid	Soft agar[a]	Foci in[b]	
		5% Serum	1% Serum
pSV2neo	<0.01	2	0
pSVB3	0.29	18	ND
pJYM	0.31	ND	25
pSVCL1L	0.53	18	24

Note. The relative transfection efficiencies, as judged by the number of G418[r] colonies, were comparable for all samples.
[a] EOP is as defined in Table 3. Value is average of two to four experiments.
[b] Average number of foci per 100-mm dish (5% serum) or 60-mm dish (1% serum) from two experiments.

TABLE 5

Growth of Retroviral Infected Cells in Soft Agar,
and Focus Formation in 5% and 1% Serum

Cell line	Virus	Soft agar	Foci in	
			5% Serum	1% Serum
3T3	TEXS	1.35	243	61
	SVX	0.006	1	0
CL1L	TEXS	0.062	0.7	0
	SVX	0.005	0	0

Note. See Table 4 for definitions. Soft agar and 5% serum values are averages of three experiments. The 1% serum assay is one experiment.

observed when 3T3 cells were fused to transformed cells. The defect in CL1L could be complemented by normal cells and does not suppress the phenotype of a transformed cell; therefore, it appears to be recessive. Fusion of the CL1L cell line to another T$^+$/tr$^-$ cell line, A27, was also performed to test for complementation. The EOP in soft agar of these hybrids indicates that the two cell lines complement each other (Table 7). Fusion of each cell line to itself did not result in transformation. The CL1L × A27 hybrids form soft agar colonies that are fully transformed, as judged by their size.

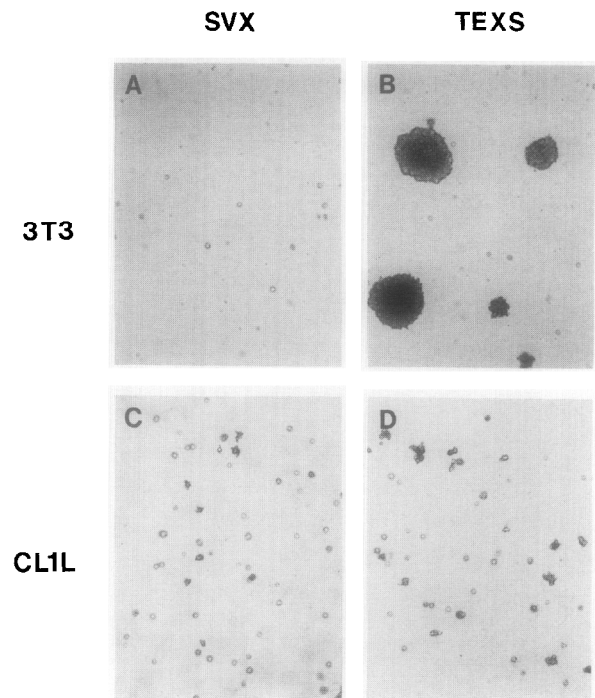SVX   TEXS

FIG. 3. Growth of retroviral-infected cells in soft agar. Soft agar colonies of (A) SVX-infected 3T3; (B) TEXS-infected 3T3; (C) SVX-infected CL1L; and (D) TEXS-infected CL1L.

TABLE 6

TRANSFORMATION OF CL1L BY OTHER ONCOGENES

Plasmid	Soft agar	Foci in	
		5% Serum	1% Serum
pSV2neo	0.05	1.2	2.8
pMvsrc	0.34	29.0	0
pEJ6.6	0.60	167.3	49.0
pPyMT1	0.86	312.0	93.5

Note. See Table 4 for definitions. Soft agar and 5% serum values are averages of three to four experiments. The 1% serum values represent one to two experiments.

DISCUSSION

Expression of SV40 large T antigen in nonpermissive cells triggers cell division and morphological changes through its interaction with a number of host factors and the subsequent activation or suppression of pathways controlled by these factors (Green, 1989). In the present study, we have isolated a cell line, CL1L, which appears to carry a recessive mutation in such a factor. The evidence for such a mutation is threefold. First, CL1L expresses a T antigen which is fully capable of transforming cells, yet CL1L exhibits the growth phenotypes of a normal cell. Second, CL1L is resistant to retransformation by exogenously added large T antigen but not other representative oncogenes. Third, hybrids formed by fusing CL1L with either normal or transformed cells are themselves transformed. In addition, the normal phenotype is stable: rerevertants arise

at a frequency of 2.5×10^{-7}, although these cells rarely grow as aggressively as the parental cell line, A209R1B1, when plated in soft agar.

We examined the function of the T antigen expressed in CL1L by cloning out the single SV40 early region from CL1L and testing its ability to transform 3T3 fibroblasts. We have extensively mapped the SV40 sequences in CL1L and find no evidence for a second copy of any part of the early region. Thus, it appears that one of the two copies of the early region originally present in A209R1B1, the parental cell line, was lost during isolation of CL1L. Nonetheless, the CL1L T antigen transforms at least as well as wild-type T antigen on a molar basis. Although the level of T antigen in CL1L is less than that expressed in A209R1B1, it is more than sufficient to transform 3T3 cells (G.H.S., Ph.D. thesis). In addition, the fact that fusion of CL1L cells to 3T3 cells yields transformed hybrids indicates that the CL1L T antigen is fully functional. Finally, CL1L T antigen is fully capable of forming complexes with p53 and p120. Detection of T antigen–pRb complexes in rodent cells has been technically difficult, even in cells expressing very high levels of T antigen, in a number of laboratories including our own (J.B.C., unpublished data). We do not think, however, that the defect in CL1L involves pRb, for the following reasons. We know that the T antigen in CL1L can bind pRb since it can transform normal cells (Table 4). Thus, one would have to argue that the pRb in CL1L is mutated such that it blocks cell growth and is unresponsive to T antigen. If this were the case, one would expect this unresponsive pRb to be dominant over wild-type pRb,

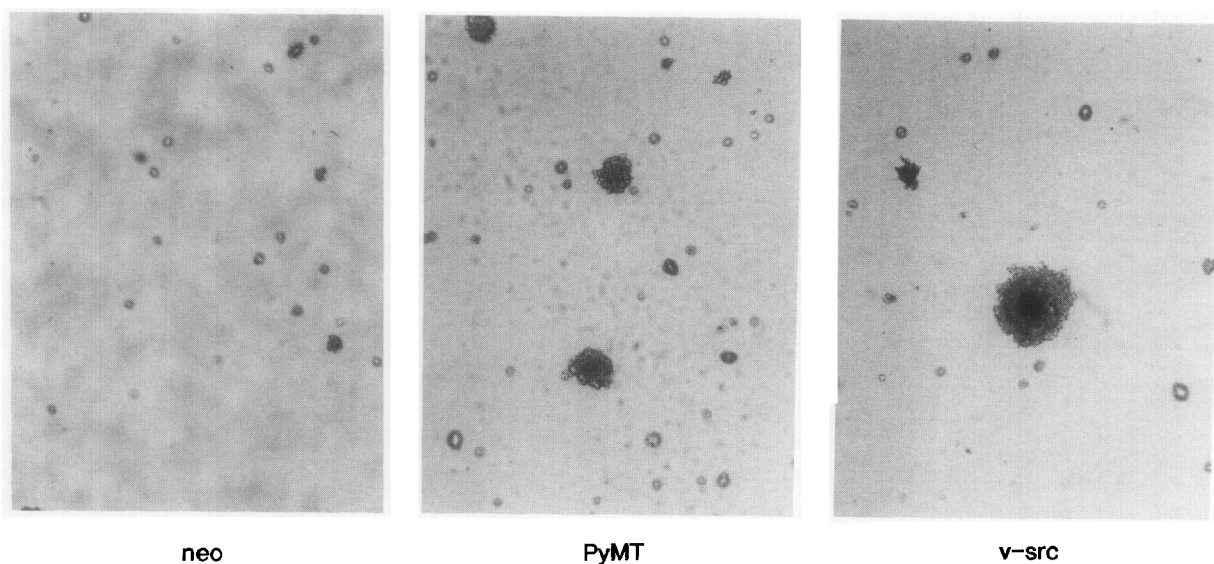neo   PyMT   v–src

FIG. 4. Soft agar growth of oncogene-transfected CL1L cells. Colonies of cells transfected with control plasmid (neo), polyomavirus middle T antigen (PyMT), or v-*src* are shown.
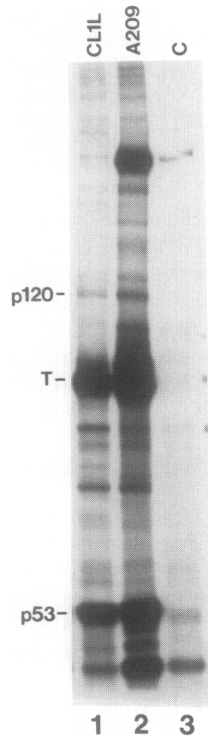
FIG. 5. T antigen-binding proteins in CL1L cells. ^{35}S-labeled proteins from: lane 1, CL1L immunoprecipitated with PAb419; lane 2, A209R1B1 with pAB419; lane 3, A209R1B1 with C2-5, an isotype-matched control monoclonal specific for herpes simplex virus type 2 glycoprotein C. The high-molecular-weight band in lane 2 is a nonspecific band sometimes seen in A209 precipitates.

which is presumably inactivated when it is bound to T antigen (Buchkovich et al., 1989; DeCaprio et al., 1989). Our data, however, show that fusion of CL1L to normal cells, which express wild-type pRb, results in transformation (Table 7). Although this does not formally rule out a mutation in the Rb gene in CL1L, the possibility of such a mutation is clearly inconsistent with current available data. Since evidence does exist for an additional host function in SV40 transformation (Rutila et al., 1986; Manfredi and Prives, 1990; Yaciuk et al., 1991; Michael-Michalovitz et al., 1991), it is possible that the mutation in CL1L may define the gene encoding that function.

When exogenous T antigen is introduced into CL1L by infection with a recombinant retrovirus, growth in soft agar is stimulated somewhat, and the cells grow to a higher density in monolayers. These retroviral-infected cells do not, however, form soft agar colonies as large as those formed by infected 3T3 cells, nor do they form foci in monolayers or grow in low serum. This indicates that the mutation in CL1L differentially affects these three parameters of the transformed state, namely anchorage independence, loss of contact inhibition, and serum independence. In addition, the intro-

duction of exogenous T antigen appears to have an effect upon anchorage-independent growth, but the infected cells do not form large colonies in soft agar, presumably due to the cellular mutation. One possible explanation for this is that T antigen might play two roles in transformation: (1) stimulating progression through the cell cycle and (2) inducing expression of the characteristic transformation phenotypes. In this model the presence of additional T antigen in CL1L might stimulate extra rounds of division but not allow the full phenotypic changes which define the transformed state, perhaps as occurs in abortive transformation. A prediction of this model is that other agents which stimulate cell division might have the same effect.

The finding that the normal growth phenotype of CL1L is recessive opens the possibility of identifying the gene involved by standard DNA transfection protocols: genomic DNA or cDNA expression libraries from transformation-competent cells should be able to complement the defect. Although this type of analysis requires single-gene defects, the frequency at which CL1L arose, approximately 10^{-5}, indicates that the involvement of more than one gene is unlikely. Moreover, although such a frequency appears high for a recessive mutation, one must remember that the parental cells, being transformed, have likely suffered extensive chromosomal loss and rearrangement and may therefore be haploid at many loci. Since the phenotype of CL1L is most striking in low serum, such conditions should provide a powerful selection in a complementation assay.

TABLE 7

GROWTH IN SOFT AGAR OF SOMATIC CELL HYBRIDS

Cells	EOP
A209Hym1A	0.98
CL1LNeo	0.03
3T3Hym4	0.01
3T3Neo	0.01
A27SVX2	0.01
A209Neo × A209Hym1A	0.76
CL1LHym × CL1LNeo	0.02
A27Hym1B × A27SVX2	0.01
CL1LNeo × 3T3Hym4	0.28
CL1LNeo × A209Hym1A	0.40
3T3Neo × A209Hym1A	0.70
CL1LHym × A27SVX2	0.53

Note. EOP is the number of soft agar colonies/number of cells plated × 100. The drug resistance of each cell line is indicated by the suffixes Hym and Neo for hygromycin and G418 resistance, respectively. A27SVX2 is also G418 resistant.

A number of groups have studied cell lines in which the expression of SV40 T antigen does not correlate with the growth phenotype, due to a cellular mutation. Pollack and co-workers previously described a number of revertant cell lines (Pollack *et al.,* 1968; Vogel and Pollack, 1973; Vogel *et al.,* 1973; Vogel and Pollack, 1974), as did Maruyama *et al.* (1982). At that time, however, tools for molecular analysis were unavailable. More recently, the Graessmann group has identified a revertant line, Rev2, which appears to be due to a recessive mutation (Bauer *et al.,* 1987). Studies on tsA N-type and A-type transformants, cells expressing a temperature-sensitive T antigen which do and do not return, respectively, to a normal growth phenotype when grown at the nonpermissive temperature, indicate that the phosphorylation pattern of T antigen correlates with the growth phenotype (Knippschild *et al.,* 1991). Specifically, it was found that return to growth control was accompanied by a shift to an underphosphorylated form of T antigen in the N-type cells at the nonpermissive temperature, whereas the T antigen in the A-type cells retained the same phosphorylation pattern at both temperatures. This may be significant in that it appears that this same underphosphorylated form of T antigen has recently been found in Rev2 cells (Deppert *et al.,* 1991). It will therefore be of interest to examine the phosphorylation of T antigen in CL1L cells.

The most provocative result of these studies is the complementation of the CL1L and A27 cell lines. This indicates that the defects in these two transformation-resistant lines fall into separate complementation groups. Since A27 cells were isolated under nonselective conditions and therefore were never transformed, whereas CL1L is a transformation revertant, it is possible that the two cell lines harbor independent mutations in genes which affect, respectively, establishment and maintenance of the transformed state. Alternatively, the two defects may relate to the two functions proposed for T antigen above. It would be interesting to test other revertants in complementation assays to obtain an idea of how many host functions might be involved in transformation by SV40. The further study of transformation-resistant cell lines such as CL1L and A27 should provide valuable information about the pathways involved in SV40-mediated transformation.

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