Immortalization of Primary Rat Cells by Reciprocal Complementations of Human Papillomavirus Type 16 E7 and Adenovirus Type 5 E1A Mutants

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ABSTRACT

Primary baby rat kidney (BRK) cells were transfected with two different non-immortalizing mutants of adenovirus type 5 (Ad5) E1A(s) and/or human papillomavirus type 16 (HPV16) E7(s) to test whether immortalization was induced by the genetic complementation. Co-transfection of an RB-nonbinding E1A mutant (*dl*922/947) and N-terminal E7 mutant (2PRO) formed transformed foci on BRK cells, but a combination of an N-terminal E1A mutant (NT*dl*598) and RB-nonbinding E7 mutant (24GLY) did not. However, NT*dl*598 plus 24GLY as well as *dl*922/947 plus 2PRO produced immortalized BRK cell lines. The cell lines immortalized by *dl*922/947 plus 2PRO showed a morphology similar to those immortalized by Ad5 E1A ; while cell lines immortalized by NT*dl*598 plus 24GLY showed a morphology similar to those immortalized by HPV16 E7. These results suggest that the RB-binding regions of Ad5 E1A and HPV16 E7 are mutually replaceable, and that the N-terminal function of E1A is essential for focus formation and maintenance of transformation morphology. A combination of RB-nonbinding mutants, E1A *dl*922/947 plus E7 24GLY, was able to immortalize BRK cells when introduced together with the Ad5 E1B 19K gene. The Ad5 E1B 55K and Bcl-2, which also possess anti-apoptotic activity, did not increase colony formation of and/or immortalize BRK cells when co-transfected with *dl*922/947 plus 24GLY. In a CAT assay E1B 19K, but not Bcl-2, enhanced E2F-dependent CAT transactivation by Ad5 E1A or *dl*922/947. Thus, it is suggested that E1B 19K might complement RB-nonbinding mutants by means of two activities, suppression of apoptosis and enhancement of E2F-dependent transactivation.

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Key words : Adenovirus type 5 (Ad5) E1A, Adenovirus type 5 (Ad5) E1B 19K, Human papillomavirus type 16 (HPV16) E7, Immortalization, Complementation

INTRODUCTION

Adenovirus (Ad) E1A and E7 genes of human papillomavirus type 16 (HPV16) and HPV18 share various biological and biochemical activities. These include induction of cellular DNA synthesis (1-4), immortalization of primary rat and/or human cells (5-7), *ras* collaborative transformation of primary rat cells (8-11), transactivation of Ad E2 promoter (9,12,13) and abrogation of c-*myc* induction in kerationocytes by TGFβ (14-16). Most, if not all, of these activities are closely associated with interactions of the viral oncoproteins with cellular protein RB, p107 and G1 cyclins (17-22).

The E1A regions required for *ras* collaborative transformation comprise the nonconserved amino terminus together with conserved region 1 (CR1) and CR2, which coincide with p300- and RB family-binding domains (10,17,18,23). The N-terminal 37 amino acids of HPV16 E7 are homologous to amino acid residues 39-49 and 116-137 of Ad5 E1A (9). Functional analysis using chimeric E7 genes which were constructed from high-risk HPV16 and low-risk HPV6 E7s revealed that *ras*-collaboration activity of HPV16 E7 was localized in the amino acid residues 17-29 within the region homologous to Ad5 E1A (24,25). Thus, it is suggested that the common mechanisms are used in immortalization by E1A or E7, which involves their association with a set of cellular genes including RB (26,27).

On the other hand, Ad E1A shows growth-supporting and -inhibitory activities which HPV E7 does not possess. The Ad5 E1A gene forms transformed cell foci characteristic of E1A on primary rat cells (5) and induces apoptosis in various types of cells depending on the N-terminal region and/or CR1 and CR2 domains (28,29). The HPV16 and HPV16 E7 gene can form foci on some of the established rodent cells (30,31), but does not form foci on primary rat cells nor induce apoptosis in cultured cells like E1A.

To elucidate functional similarities and differences between Ad5 E1A and HPV16 E7, two different mutants of the viral oncogenes were co-transfected to primary rat cells to examine whether immortalization could be induced by their genetic complementation. It was found that the N-terminal function of E1A and E7 was different in focus-forming ability, and that Ad5 E1B 19K was able to complement the RB non-binding mutants of E1A and E7. The possible function of E1B 19K in the complementation of RB non-binding mutants will be discussed.

MATERIALS AND METHODS

Plasmids

p5XhoC, p5SstE, pXbaC, and pcD2-16E7 carry Ad5 whole E1, Ad5 E1A and Ad5 E4 regions and HPV16 E7 gene, respectively (32-35). NT*dl*598 and *dl*922/947 are recombinant plasmids containing Ad5 E1A genes with deletions at the N-terminus and in the CR2 of Ad5 E1A, respectively (10). Neither of the mutants can form complexes with p300 (NT*dl*598) or RB families (*dl*922/947) (17) nor transform primary baby rat kidney (BRK) cells in collaboration with the activated *ras* gene (10). HPV16 E7 mutants 2PRO and 24GLY contain Pro2 and Gly24 instead of His2 and Cys24, respectively and cannot transform NIH3T3 cells nor primary BRK cells in collaboration with the activated *ras* gene (36,37). 24GLY does not bind RB, while 2PRO does (38).

pcD2-2PRO and pcD2-24GLY were constructed by inserting the whole E7 sequence from 2PRO and 24GLY separately into an SV40-based expression vector pcD2-Y (35,39). pcD2-12S was constructed by inserting the sequence of Ad5 E1A 12S cDNA (40) into the pcD2-Y. Ad5 E1B-containing plasmids, pCMV19K and pCMV55K, in which production of E1B 19K and 55K is driven by the cytomegalovirus (CMV) early promoter and enhancer, contain Ad5 E1B 19K and 55K cDNAs, respectively (41). Plasmid *dl*922/947-E1B was constructed from p5XhoC by substituting the CR2 fragment by the corresponding sequence of *dl*922/947. E2CAT-ATF$^-$A20 contains CAT gene under the adenovirus early promoter E2 from which the ATF site was deleted (42,43). pCH110 eukaryotic assay vector encodes a β-galactosidase fusion protein (Pharmacia). Bcl-2 expression plasmid pCMV-Bcl-2 was constructed by inserting the 1.0 kb *Eco* RI fragment prepared from pB4 (44) into the CMV-based expression vector pEV3S (45).

PCR primers

A sense primer, 5'-CGT AAC TTG CTG GAA CAG AGC-3' (nucleotide position [n.p.] 1753 - n.p. 1773, 46) and an antisense primer, 5'-TTC TTG TGT CTG ACA ACC GC-3' (n.p. 2105 - n.p. 2086) were used to detect Ad5 E1B 19K mRNA specifically by RT-PCR (Fig. 3, A). A sense primer for Ad5 E1B 55K mRNA is 5'-AGA TCT GGA AGG TGC TGA GG-3' (n.p. 3328 - n.p. 3347) and an antisense primer is 5'-AAC GAG TTG GTG CTC ATG GC-3' (n.p. 3625 - n.p. 3606) (Fig. 3, A). The oligonucleotide primers, 16-C, 5'- AAA TAG ATG GTC CAC TG -3' (n.p. 671 - n.p. 688, 47) and 16-3', 5'- GAT TAT GGT TTC TGA GAA -3' (n.p. 860 - n.p. 843), were used to detect the 204 base-pair (bp) E7 sequence of HPV16 (Fig. 4, A). A sense primer, #1, 5'- AAA ATG AGA CAT ATT ATC TG -3' (n.p. 557 - n.p. 576) and an antisense primer, #3, 5'- GAT CGA TCA CCT CCG GTA CA -3' (n.p.

923 - n.p. 904) can amplify 378 bp band from wild-type (wt) E1A but not from *NTdl*598 (Fig. 5, A). A sense primer , #4, 5'- GAT CGA TCT TAC CTG CCA CG -3' (n.p. 916 - n.p. 935) and an antisense primer, #6, 5'- CTT ATG GCC TGG GGC GTTT -3' (n.p. 1546 - n.p. 1528) can amplify 385- and 525-bp DNA fragment from 12S and 13S Ad5 E1A and NT*dl*598 but not from *dl*922/947 (Fig. 5, A).

Cells

Primary BRK cells were prepared as described before (7). Briefly, after rat kidneys were removed from 3- to 5-day-old F-344 rats and minced, cells were dispersed by treatment in Dulbecco modified Eagle's medium (DME) containing 500 μg/ml collagenase and 3.3 μg/ml dispase for a total of 90 min at 37℃. Suspended cells were plated at 2.0 to 4.0 X 10^4 cells/cm^2 in DME supplemented with 5 % fetal bovine serum (FBS), 30 μg/ml streptomycin and 40 μg/ml penicillin. All the cells and cell lines were cultured in the conditions described above.

DNA transfection and establishment of cell lines

DNA transfection was performed according to the standard calcium-phosphate coprecipitation technique of van der Eb and Graham (48) with 15 % glycerol treatment (49). For focus formation, primary BRK cells subconfluently growing in 6 cm dishes were transfected with the HPV16 E7 and/or Ad5 E1A plasmid(s), and cultured for two to three weeks. For colony formation, BRK cells subconfluently growing in 10 cm dishes were transfected with the HPV16 E7 and/or Ad5 E1A plasmid(s), split equally among five 10 cm dishes 12 to 18 hr after transfection and cultured in DME with 5 % FBS, antibiotics and 150 μg/ml G418.

For establishment of BRK cell lines, G418-resistant colonies were isolated separately using cloning cylinders and cultured in a 24-well plate. When cells had grown over 50 % of the well surface, they were detached and transferred to a 6-well plate. After they had grown over 50 % on the surface of the 6 well plate, they were transferred to a 10 cm dish. Since BRK cell clones that reached confluence in the 10 cm dish always became passageable without difficulty (7 and present work), we considered them to be immortalized.

RT-PCR

For RT-PCR, 5.0 μg of total RNA was treated with 5U of RNase free DNase I (Promega) at 37℃ for 30 min. Reverse transcription was carried out at 37℃ for 60 min in 33 μl of a reaction mixture containing 2 μg of DNase-treated RNA, 60 μg/ml oligo $d(T)_{18}$ primers, 80 μg/ml bovine serum albumin, 1.8 mM each dNTP, 45 mM Tris-HCl (pH 8.3), 68 mM KCl, 9 mM $MgCl_2$, 15 mM DTT, and 50 units of Moloney Murine Leukemia Virus reverse transcriptase (Pharmacia).

After the reverse transcriptase was inactivated at 94℃ for 5 min, PCR was performed according to the method of Saiki *et al.* (50) with a modification (51). One tenth of the product was amplified in 25 μl of a reaction mixture containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 1.0 μM each primer, 0.2 mM each dNTP, 0.01% (W/V) gelatin and 0.625 unit of Taq polymerase overlaid with mineral oil. They were subjected to 30 cycles of amplification using a thermal cycler. Each PCR cycle included one min at 94℃, 2 min at 55℃ and 2 min at 72℃. After amplification, 10 μl of the reaction products were fractionated on 3 % or 1 % agarose gel and then visualized by staining with ethidium bromide.

Transient CAT expression assay

CAT assay was performed according to the method of Gorman *et al.* (52). CV1 cells were transfected with E2CAT-ATF⁻A20 and pCH110 in the presence or absence of viral or cellular genes. Transfection was carried out as described above and transfected cells were cultured for 70 hr. Then, each cell extract was subsequently assayed for both CAT and β-galactosidase activities. The β-galactosidase activity, used as an internal control in each dish, was measured according to the mothod of Nielsen *et al.* (53). 30 μl of cell extract was added to 500 μl of buffer Z (10 mM KCl, 1 mM $MgSO_4$, 50 mM 2-mercaptoethanol, 100 mM sodium phosphate, pH 7.5) and preincubated at 37℃ for 5 min. Then 100 μl of *o*-nitrophenyl-β-D-galactopyranoside (Sigma, 4 mg/ml in 100 mM sodium phosphate, pH 7.5) was added and the mixed solution was incubated at 37℃ for 3 hr. After the reaction was terminated by Na_2CO_3 , absorbance at 420 nm was measured. To assay the CAT activity, 70 μl of each cell extract was incubated with ^{14}C-chloramphenicol and 4 mM acetyl CoA (Pharmacia) in 250 mM Tris-pH 7.8 at 37℃ for 30 min. After analysis by ascending thin layer chromatography, the conversion rate was determined using the image analyzer (Fuji BAS2000).

RESULTS

Focus formation of primary BRK cells by E1A and/or E7 mutants

Ad5 E1A gene forms transformed cell foci on primary BRK cells as shown in Fig.1, A, while HPV16 E7 does not (Table 1). In order to test if the E1A mutants can be complemented by other E1A or E7 mutants, BRK cells were co-transfected with Ad5 E1A and/or HPV16 E7 mutants (Table 1). None of the Ad5 E1A mutants, one of which is defective in the N-terminus (NT*dl*598) and another one in the CR2 domain (*dl*922/947), formed foci on BRK cells (Table 1). These two E1A mutants complemented each other to form foci on primary BRK cells (Fig. 1, B, Table 1). Co-transfection of E1A *dl*922/947 and the N-terminal

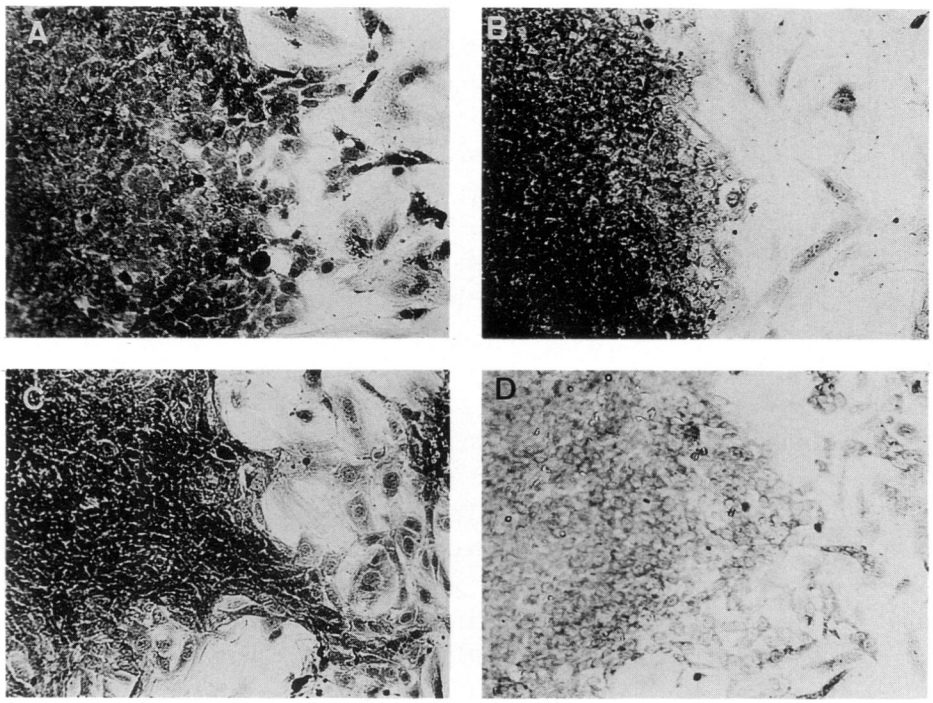


Fig. 1 Morphology of transformed cell foci on BRK cell culture. An edge of a focus induced by transfection of wt Ad 5 E1A (p5SstE) (A), E1A NT*dl*598 + E1A *dl*922/947 (B), E1A *dl*922/947 + E7 2PRO (C), E1A *dl*922/947 + E7 24GLY (D) (see also Table 1). Cells were fixed by methanol and stained by Giemsa.

Table 1 Focus formation of primary BRK cells by wild-type and mutant Ad5 E1A and HPV16 E7 genes.

Transfected DNA	Number of foci [1]		Establishment [2]
	Exp I	Exp II	
Ad5 E1A	57	51	2/5
HPV16 E7	0	0	N.A.[3]
*dl*922/947	0	0	N.A.
*dl*922/947 + HPV16 E7	19	N.T.[3]	N.T.
*dl*922/947 + 2PRO	14	32	2/5
*dl*922/947 + 24GLY	(3)[4]	(6)	0/5
NT*dl*598	0	0	N.A.
NT*dl*598 + HPV16 E7	0	0	N.A.
NT*dl*598 + 2PRO	0	0	N.A.
NT*dl*598 + 24GLY	0	0	N.A.
NT*dl*598 + *dl*922/947	30	34	2/5

1) Subconfluent primary BRK cells in 6 cm dishes were transfected with various combinations of E1A and E7 mutants. Two dishes of cells were transfected in each transfection.
2) Number of cell lines established / number of foci isolated.
3) N.A.: not applicable. N.T.: not tested.
4) Small E1A-specific foci.

mutant of HPV16 E7 (2PRO) also produced transformed cell foci characteristic of Ad E1A (Fig. 1, C, Table 1). Transformed cell lines were established from *dl*922/947 + 2PRO foci as well as from wt E1A or NT*dl*598 + *dl*922/947 foci with almost the same efficiency (Table 1). These findings suggest that the function of the RB-binding domain of E1A which is defective in *dl*922/947 can be substituted not only by the NT*dl*598 but also by the corresponding region of HPV16 E7.

Co-transfections of E1A NT*dl*598 and wt HPV16 E7, 2PRO, or RB-nonbinding mutant of E7 (24GLY) all failed to produce foci on primary BRK cells (Table 1). This suggests that the function(s) of the N-terminal region of Ad E1A is required for focus formation and is not present in HPV16 E7. Tiny foci were repeatedly observed in primary BRK cells when transfected with two RB-nonbinding mutants (*dl*922/947 + 24GLY) (Fig. 1, D, Table 1). However, they were not established into cell lines (Table 1).

Immortalization of primary BRK cells by E1A and/or E7 mutants.

Primary BRK cells were transfected with an E7 plasmid containing a neomycin resistant gene (*Neo*^{r}) and one of the E1A or E7 mutants. The transfected cells were cultured in the G418-containing media to test if potentially immortalized cell colonies could be generated by complementation of E1A and/or E7 mutants. None of the mutants produced G418-resistant or immortalizable cell

Table 2 Colony formation of primary BRK cells by transfection of wild-type and mutants Ad5 E1A and HPV16 E7 genes.

Transfected DNA [1]	Number of G418-resistant colonies		Establishment [2]
	Exp I	Exp II	
pcD2-Y	0	0	N.A.[3]
pcD2-Y + 2PRO	5	4	0/ 5
pcD2-Y + 24GLY	0	0	N.A.
pcD2-Y + NT*dl*598	0	0	N.A.
pcD2-Y + *dl*922/947	1	4	0/ 5[4]
pcD2-Y + *dl*922/947-E1B	5	9	2/10
pcD2-Y + E1B	0	0	N.A.
pcD2-16E7	9	6	5/ 5
pcD2-2PRO	2	2	0/ 5[4]
pcD2-2PRO + NT*dl*598	3	2	0/ 5[4]
pcD2-2PRO + *dl*922/947	16	12	2/ 5
pcD2-24GLY	0	0	N.A.
pcD2-24GLY + 2PRO	5	2	1/ 5
pcD2-24GLY + NT*dl*598	3	2	3/ 5[4]
pcD2-24GLY + *dl*922/947	5	4	0/ 6
pcD2-24GLY + *dl*922/947-E1B	10	12	5/10

1) Subconfluent primary BRK cells in 10 cm dishes were transfected with a pcD2 plasmid containing *Neo*^{r} (4.0 μg) with or without a plasmid containing E1A or E7 mutant (8.0 μg), split and cultured in G418-containing media.
2) Number of cell lines established / number of colonies isolated.
3) N.A.: not applicable.
4) Results of additional experiments were included.

colonies, when they were transfected with *Neo*^{r}-containing vector pcD2-Y (Table 2). Combinations of two N-terminal (pcD2-2PRO + NT*dl*598) or two RB-nonbinding (pcD2-24GLY + *dl*922/947) mutants did not produce immortalizable cell colonies (Table 2). However, when the N-terminal mutant of HPV16 E7 (pcD2-2PRO) was co-transfected with the RB-nonbinding mutant of E1A (*dl*922/947), immortalizable G418-resistant colonies were generated (Table 2). These cell lines (BRK2/922 cell lines) showed a morphology indistinguishable from those immortalized by wt E1A (Fig. 2, C, D). Saturation densities of BRK2/922 cell lines were not significantly different from those of the cell lines immortalized by Ad5 E1A (data not shown). These data suggest that the function of the RB-binding region of E1A can be replaced by the corresponding region of HPV16 E7 in not only focus formation but also immortalization of primary BRK cells.

A combination of 24GLY and NT*dl*598, which failed to form foci on BRK cells (Table 1), was able to produce immortalizable cell colonies (Table 2). All the cell lines immortalized by 24GLY + NT*dl*598 (BRK24/598 cell lines) showed a morphology similar to those immortalized by wt HPV16 E7 (Fig. 2, A, B). Saturation densities of BRK24/598 cells were lower than BRK5E1A cells (data not shown). Thus, it is suggested that the N-terminal function of E1A is essential for the maintenance of the morphology characteristic of E1A transformants and the function is not contained in HPV16 E7.

A combination of RB-nonbinding mutants (E1A *dl*922/947 + E7 24GLY), which produced abortive foci on primary BRK cells (Table 1, Fig. 1, D), did not produce immortalizable G418-resistant colonies (Table 2). However, when *dl* 922/947 was combined with Ad5 whole E1B sequence (*dl*922/947-E1B) and transfected to primary BRK cells, immortalized cell lines were obtained (Table 2). Co-transfection of 24GLY with *dl*922/947-E1B enhanced immortalization efficiency (Table 2).

E1B 19K can complement RB-binding region

Because primary BRK cells were immortalized by transfection of *dl*922/947-E1B, we examined which E1B gene, 19K- or 55K-encoding gene could contribute to immortalization (Table 3). Primary BRK cells were transfected with pcD2-24GLY, and one of the E1A or E7 mutants in the presence or absence of pCMV19K or pCMV55K, and cultured in the G418-containing media. As a result, potentially immortalized cell colonies appeared when pCMV19K was co-transfected but did not when pCMV55K was co-transfected (Table 3).

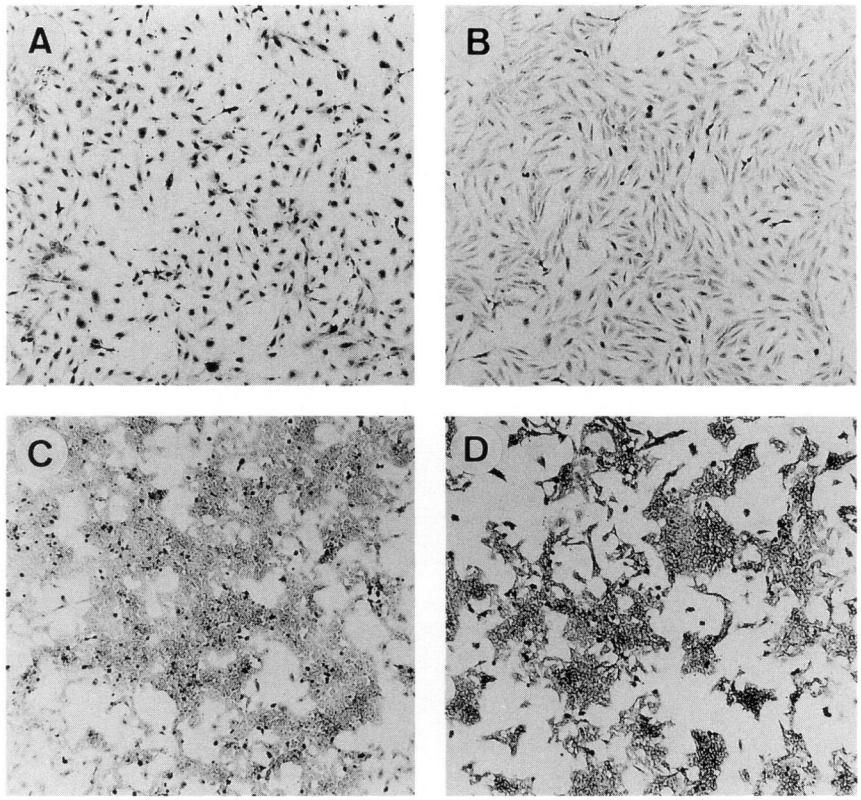


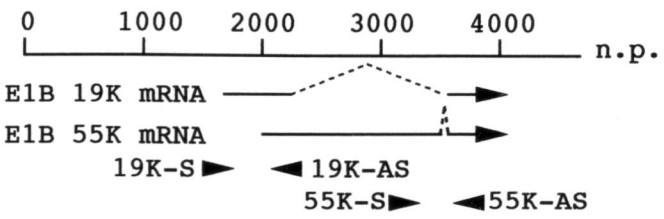
Fig. 2 Morphology of immortalized BRK cell lines. Two independent cell lines immortalized by E1A NT*dl*598 + E7 24GLY (A) (B) and E1A *dl*922/947 + E7 2PRO (C) (D) were shown (see also Table 2). Cells were fixed by methanol and stained by Giemsa.

Table 3 Colony formation of primary BRK cells by transfection of Ad5 E1A and HPV16 E7 genes.

Transfected DNA[1)]	Number of G418-resistant colonies			Establishment[2)]
	Exp I	Exp II	Exp III	
pcD2-24GLY	0	0	0	N.A.[3)]
pcD2-24GLY + 2PRO	2	2	N.T.[3)]	N.T.
pcD2-24GLY + NT*dl* 598	2	3	N.T.	3/ 5
pcD2-24GLY + *dl*922/947	2	5	N.T.	0/ 5
pcD2-24GLY + *dl*922/947-E1B	10	20	77	5/10
pcD2-24GLY + *dl*922/947 + pCMV19K	4	6	19	4/10
pcD2-24GLY + *dl*922/947 + pCMV55K	1	2	8	0/10[4)]

1) Subconfluent primary BRK cells were transfected with a pcD2 plasmid containing *Neo*^{r} (4.0 μg) and plasmid(s) containing E1A or E7 mutant (8.0 μg), with or without a Ad5 E1B plasmid (8.0 μg), split and cultured in G418-containing media.
2) Number of cell lines established / number of colonies isolated.
3) N.A.: not applicable. N.T.: not tested.
4) Results of additional experiments were included.

A.



B.

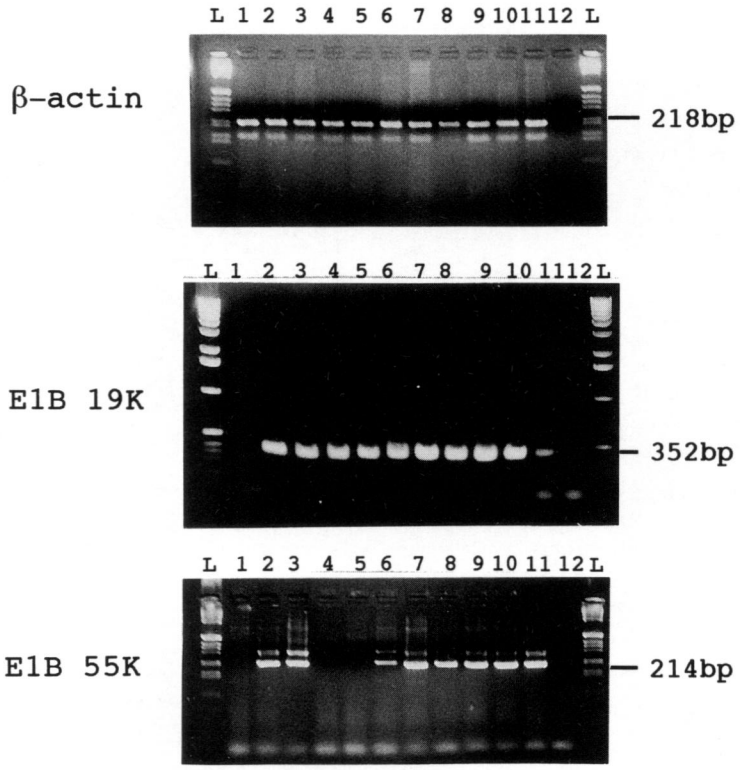


Fig. 3 RT-PCR analysis of Ad5 E1B transcripts in the immortalized BRK cell lines.

A. Schematic structure of Ad5 E1B mRNAs. The nucleotide numbering is based on the DNA sequences by van Ormondt and Galibert (46). Arrowheads represent the 3' ends of each primer.

B. (lane 1) Primary BRK cells as a negative control, (lanes 2, 3) two independent cell lines, BRK5XhoC-1 and -2 as positive controls, (lanes 4, 5) BRK24/922/19K-1 and -2 cell lines, (lanes 6-9) BRK24/922-E1B-1, -2, -3 and -4 cell lines, (lanes 10, 11) BRK922-E1B-1 and -2 cell lines, (lane 12) water, (L) GIBCO's 1kb ladder.

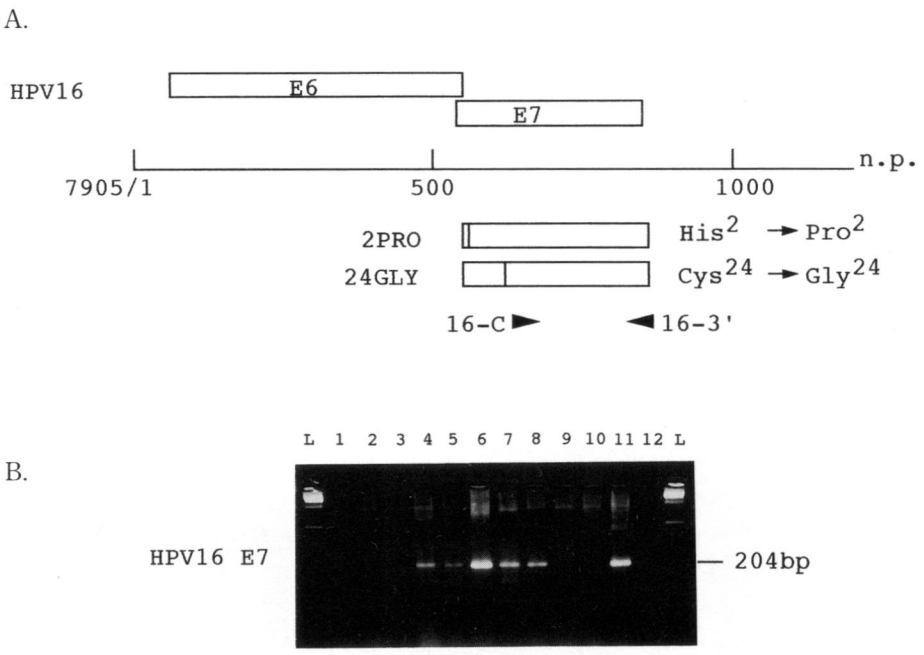


Fig. 4 RT-PCR analysis of HPV16 E7 transcripts in the immortalized BRK cell lines.
A. Schematic structure of coding regions of the HPV16 E6/E7 mRNAs and mutants. The nucleotide numbering is based on the DNA sequences by Seedorf *et al.* (47). Arrowheads represent the 3' ends of each primer.
B. (lane 1) Primary BRK cells as a negative control, (lanes 2, 3) two independent cell lines, BRK5XhoC-1 and -2 , (lanes 4, 5) BRK24/922/19K-1 and -2 cell lines, (lanes 6-8) BRK24/922-E1B-2, -3 and -4 cell lines, (lanes 9, 10) BRK922-E1B-1 and -2 cell lines, (lane 11) BRK16E7-1 as a positive control, (lane 12) water, (L) GIBCO's 1kb ladder.

Cytoplasmic RNA was prepared from BRK cell lines immortalized by 24GLY + *dl*922/947 + E1B 19K (BRK24/922/19K cell lines) and RT-PCR was carried out using HPV16 E7-, Ad5 E1A-, Ad5 E1B 19K- or E1B 55K-specific primer pairs. The E1B 19K- and E1B 55K-specific primers were expected to amplify 352 bp and 214 bp fragments which represent the 19K and 55K mRNAs, respectively. As expected, cell lines immortalized by pcD2-24GLY + *dl*922/947-E1B (BRK24/922-E1B cell lines) produced both the E1B 352 bp and 214 bp bands, HPV16 E7 204 bp and E1A 378 bp bands (Fig. 3, B, lanes 6-9 ; Fig. 4, B, lanes 6-8 ; Fig. 5, B, lanes 6-9). BRK24/922/19K cell lines contained RNAs which produce E1B 19K 352 bp, HPV16 E7 204 bp and E1A 378 bp bands (Fig. 3, B, lanes 4, 5 ; Fig. 4, B, lanes 4, 5 ; Fig. 5, B, lanes 4,5), respectively. BRK cell lines immortalized by *dl*922/947-E1B (BRK922-E1B-1 and - 2) produced PCR bands specific for E1B 19K, E1B 55K and *dl*922/947 (Fig. 3, B, lanes 10, 11 ; Fig. 5, B, lanes 10, 11).

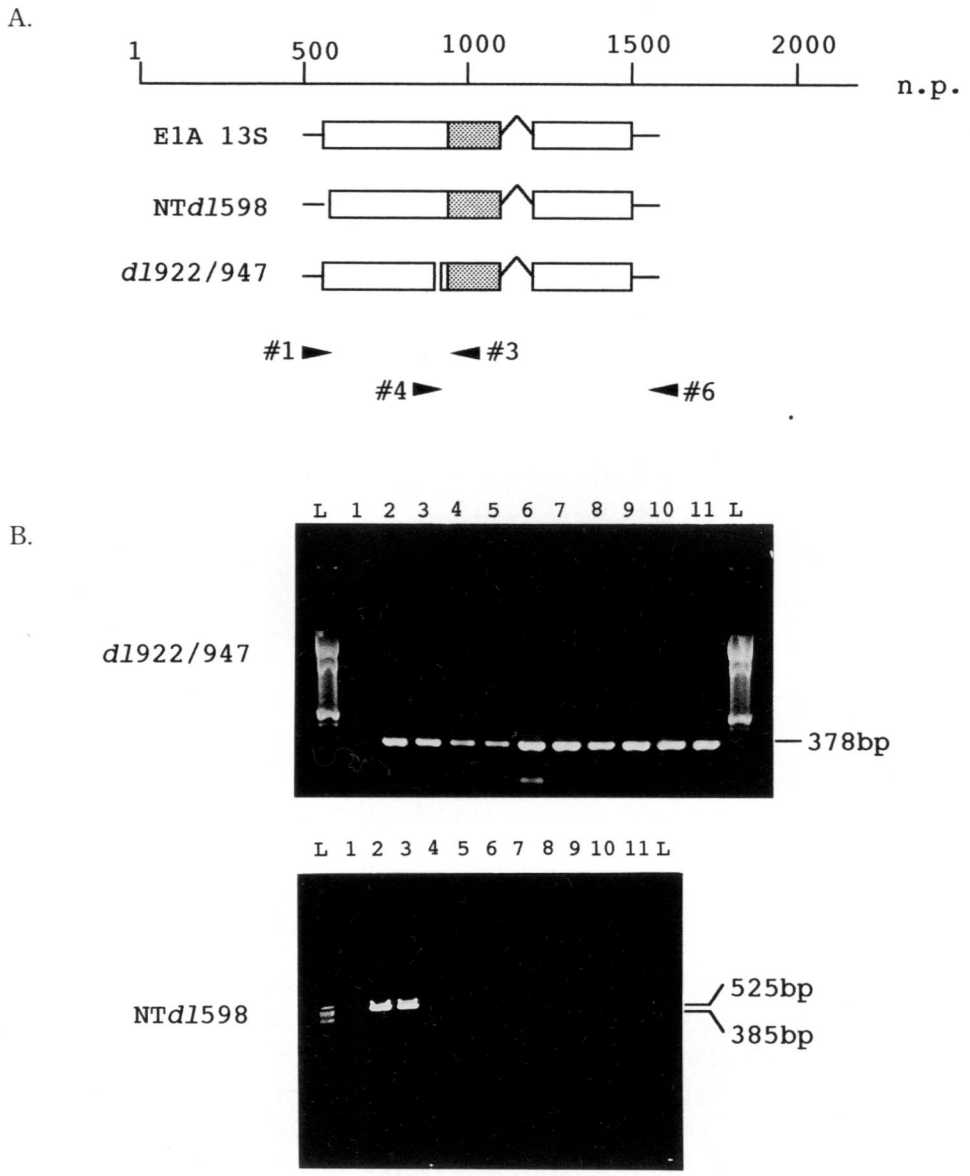


Fig. 5 RT-PCR analysis of Ad5 E1A transcripts in the immortalized BRK cell lines.

A. Schematic representations of the E1A mRNAs and mRNAs transcribed from deletion mutants of E1A are shown as symbols : ▭ , translated region of mRNA ; — , untranslated portion of mRNA ; ▩ , unique region in 13S E1A. The nucleotide numbering is based on the DNA sequences by van Ormondt and Galibert (46). Arrowheads represent the 3' ends of each primers.

B. (lane 1) Primary BRK cells as a negative control, (lanes 2, 3) two independent cell lines, BRK5XhoC-1 and -2 as positive controls, (lanes 4, 5) BRK24/922/19K-1 and -2 cell lines, (lanes 6-9) BRK24/922-E1B-1, -2, -3 and -4 cell lines, (lanes 10, 11) BRK922-E1B-1 and -2 cell lines, (lane 12) water, (L) GIBCO's 100b ladder.

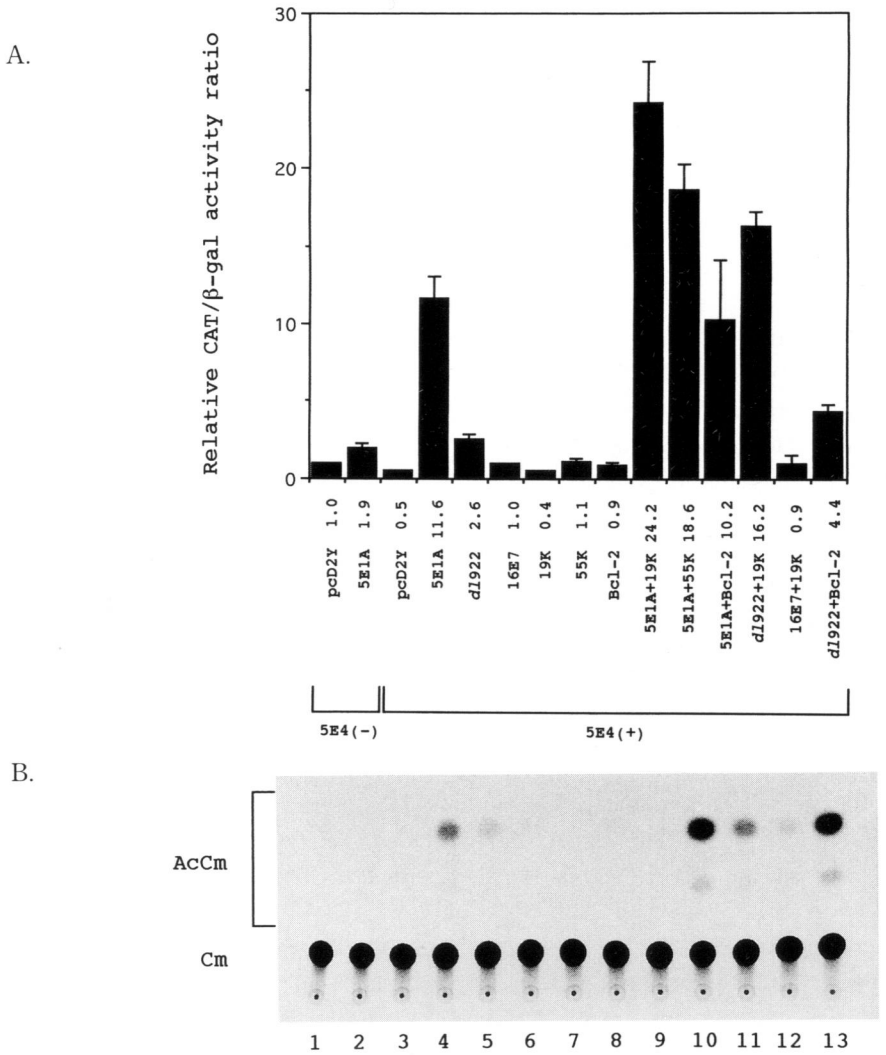


Fig. 6 Effect of E1B 19K, E1B 55K and Bcl-2 on CAT expression.

A. Diagrammatic representation of E2F-dependent CAT assay. CV-1 cells were transfected with 4.0 μg of E2CAT-ATF$^-$A20, 8.0 μg of pCH110 and 4.0 μg each of pcD2-Y or viral or cellular genes. Total amount of transfected DNA was adjusted to 24.0 μg by adding pcD2-Y. Cells were harvested 70hr after transfection. CAT assays were performed as described in the Materials and Methods. The conversion rates were determined using the image analyzer (Fuji BAS2000). (AcCm) Acetyl chloramphenicol ; (Cm) chloramphenicol. Relative CAT activities and relative β-gal activities are compared to that of E2CAT-ATF$^-$A20, pCH110 plus pcD2Y, and the results were expressed as the relative CAT activity/β-gal activity ratio (figures under the graphic chart) . Data are taken from at least two independent experiments.

B. A representative result of CAT assay. CV-1 cells were transfected with pcD2-Y as carrier DNA and/or viral gene(s) as follows in addition to E2CAT-ATF$^-$A20 and pCH110 ; (1) pcD2-Y, (2) p5SstE , (3) pXbaC (Ad5 E4) (4) pXbaC and p5SstE, (5) pXbaC and *dl*922/947, (6) pXbaC and pcD2-16E7, (7) pXbaC and pCMV19K, (8) pXbaC and pCMV55K, (9) pXbaC and pCMV-Bcl-2, (10) pXbaC, p5SstE and pCMV19K, (11) pXbaC, p5SstE and pCMV55K, (12) pXbaC, p5SstE and pCMV-Bcl-2, (13) pXbaC, *dl*922/947 and pCMV19K.

E1B 19K enhances E1A transactivation activity

Since Ad5 E1B 19K was able to complement RB-nonbinding mutants of Ad5 E1A and HPV16 E7 (Table 3), it is of interest to examine whether E1B 19K can complement the biochemical function of the RB-binding region of E1A and E7. An E2F-dependent CAT assay using E2CAT-ATF$^-$A20 plasmid (42,43) was performed for this purpose. CV1 cells were transfected with E2CAT-ATF$^-$A20, pCH110 and pXbaC (Ad5 E4) with or without E1A and E7 plasmids (Fig. 6). CAT activity of each cell extract was normalized by β-galactosidase activity (Materials and Methods).

Transfection of the E2CAT plasmid with only a carrier DNA produced a low level of CAT expression (Fig. 6, A ; B, lane 1), which was rather decreased by co-transfection of Ad5 E4 (Fig. 6, A ; B, lane 3). Ad5 E1A alone enhanced E2F-CAT activity only about two fold (Fig. 6, A ; B, lane 2). When Ad5 E4 was co-transfected with Ad5 E1A, CAT activity was increased about 12 fold compared to the case without E4 (Fig. 6, A ; B, lane 4). CAT activities obtained from co-transfection of Ad5 E4 with HPV16 E7 (Fig. 6, A ; B, lane 6), E1B 19K (Fig. 6, A ; B, lane 7), E1B 55K (Fig. 6, A ; B, lane 8), or Bcl-2 (Fig. 6, A ; B, lane 9) were not significantly different from the background level of CAT activity (without E4, Fig. 6, A ; B, lane 1).

However, when E1B 19K was co-transfected with Ad5 E1A plus Ad5 E4 (Fig. 6, A ; B, lane 10), or *dl*922/947 plus E4 (Fig. 6, A ; B, lane 13), CAT activities were further enhanced about two fold and six fold respectively, compared to the case without E1B 19K. The E1B 55K slightly enhanced (Fig. 6, A ; B, lane 11) but Bcl-2 showed no effect (Fig. 6, A ; B, lanes 12) on the level of CAT activity which was transactivated by Ad5 E1A plus E4 (Fig. 6, A ; B, lane 4). Bcl-2 slightly enhanced the CAT expression obtained from co-transfection of *dl*922/947 plus E4, but the level of the CAT activity by *dl*922/947 plus E4 plus Bcl-2 (4.4, Fig. 6, A) was lower than that by Ad5 E1A plus E4 (11.6, Fig. 6, A). Thus, E1B 19K can enhance E2F-dependent transactivation by *dl*922/947 more strongly than Bcl-2, and it is suggested that this activity of E1B 19K might be related to its complementation ability of RB-nonbinding mutants.

DISCUSSION

RB-binding domains of Ad E1A (CR2) and SV40 large T (101 to 118 residues) are replaceable in *ras*-collaborative transformation (54). In addition, the SV40 large T without the RB-binding region is able to complement an N-terminal deletion mutant of E1A (55), suggesting that the N-terminal function of E1A and SV40 large T is also replaceable. Both the Ad5 E1A and SV40 large T antigens form complexes with RB and p300 (17,18,56-58).

To investigate complementations of E1A and E7 mutants, we used NT*dl*598 as the N terminal and *dl*922/947 as the RB-nonbinding mutants of Ad5 E1A (17, 10), and 2PRO as the N-terminal and 24GLY as the RB-nonbinding mutants of HPV16 E7 (36). None of the E1A and E7 mutants immortalized primary BRK cells, which is compatible with their inert *ras* collaboration activity (10,37). Transfection of *dl*922/947 and 2PRO formed transformed cell foci on BRK cells, while NT*dl*598 and wt E7 or 24GLY did not (Table 1 and Fig. 1), suggesting that the N-terminal function of E1A which is required for complex formation with cellular p300 is essential for focus formation.

In addition to the homologous combination of E1A mutants (NT*dl*598 and *dl*922/947), heterologous combinations of E1A and E7 mutants (NT*dl*598 + 24GLY and *dl*922/947 + 2PRO) complemented each other to immortalize primary BRK cells (Table 2 and Fig. 2). Comparable with the result of focus formation, NT*dl*598 + 24GLY generated only E7-type cell lines (Fig. 2, A, B) which can easily be distinguished from E1A-type cell lines by *dl*922/947 + 2PRO (Fig. 2, C, D). These suggest again that the N-terminal function of E1A is required for focus formation and maintenance of the morphology of E1A transformants. Further analyses should be carried out to know whether the N-terminal function of E1A and E7 is different or partially overlapped and the interaction of E1A with p300 is required for focus formation.

Different from the homologous combination of E1A mutants, a homologous combination of E7 mutants (2PRO and 24GLY) hardly immortalized primary BRK cells. It is not clear why the E7 mutants can complement each other with only a low efficiency. Similar results have been reported by Davies and Vousden even in the presence of the activated *ras* gene (37).

Tiny foci were repeatedly generated by co-transfection of two RB-nonbinding mutants, E1A *dl*922/947 and E7 24GLY. Since an immortalized cell line was not established from *dl*922/947 + 24GLY transfection (Table 1 and Table 2), regions required for RB binding of E1A and E7 seem to be essential for immortalization of BRK cells. Ad E1A, SV40 large T and HPV16 E7 bind with RB to disrupt a complex between RB and transcription factor E2F and release E2F, which might drive cell cycle progress by inducing cellular genes essential for G1/S progression (26, 27, 59). However, BRK cell lines became established when cells were transfected with *dl*922/947 + 24GLY + Ad5 E1B 19K-encoding gene (Table 3). All the cell lines obtained by transfection of *dl*922/947 + 24GLY + E1B 19K were shown to contain HPV16 E7, Ad5 E1A and E1B 19K RNA, which was demonstrated by HPV16 E7-, Ad5 E1A- and Ad5 E1B 19K-specific RT-PCR (Fig. 3, 4, 5). Thus, it is suggested that E1B 19K has some function which can complement the RB-binding region of E1A and E7.

Mutational analysis of the E7 gene has demonstrated partial resolution of its transactivating and transforming activities (60), but the E1A mediated transactivation is unlikely to contribute significantly to E1A-mediated transformation (61). Therefore, it was of interest to determine the ability of the E1B 19K to modulate the transactivation potential of E1A and E7 mutants. Since E1B 19K complemented RB-nonbinding mutants in our present experiment, it is possible that the protein has some function in the process of E2F activation by the viral oncoproteins. E1B 19K but not Bcl-2 could enhance E2F-dependent transactivation through E1A or *dl*922/947 (Fig. 6). Ad5 E1B 19K had no effect on the CAT expression from SV40, c-*fos*, or rat PCNA promoters (data not shown). Thus, it is unlikely that E1B 19K can non-specifically enhance transcription from the Ad E2A upstream sequence. E1B 55K slightly enhanced E2F-dependent transactivation through Ad5 E1A (Fig. 6, A, B). The details of the molecular basis of the enhancement of the E2F-dependent transactivation by E1B 55K is nuclear.

It has been reported that the transforming activity of E1B 19K is inseparable from its protection ability from cytolysis mediated by E1A or DNA-damaging agents (62,63), suggesting that both activities are the consequences of the same function of the protein. E1B 19K is characterized as a general suppressor of apoptosis that is functionally interchangeable with Bcl-2 (61,64,65). The second E1B protein, E1B 55K, binds p53 and impairs its ability to regulate gene expression (66). As reported by Rao *et al.* (64), generation of G418-resistant colonies after transfection of E1A 12S cDNA (pcD2-12S) was increased by co-transfection of not only E1B 19K (7.8-fold) and E1B 55K (2.1-fold) but also Bcl-2 (4.5-fold)

Table 4 Effect of E1B 19K, E1B 55K and Bcl-2 genes on colony formation of primary BRK cells transfected with E1A and E7 genes.

Transfected DNA [1)]	Number of G418-resistant colonies		Total
	Exp I	Exp II	
pcD2-12S	4	11	15
pcD2-12S + pCMV19K	34	83	117
pcD2-12S + pCMV55K	10	21	31
pcD2-12S + pCMV-Bcl-2	24	44	68
pcD2-24GLY	0	0	0
pcD2-24GLY + *dl*1922/947	1	1	2
pcD2-24GLY + *dl*1922/947 + pCMV19K	9	6	15
pcD2-24GLY + *dl*1922/947 + pCMV55K	1	3	4
pcD2-24GLY + *dl*1922/947 + pCMV-Bcl-2	0	1	1

1) Subconfluent primary BRK cells were transfected with a pcD2 plasmid containing *Neo*^{r} (4.0 μg) in the presence or absence of *dl*1922/947, Ad5 E1B 19K, Ad5 E1B 55K and/or Bcl-2 plasmid (8.0 μg), split and cultured in G418-containing media.

(Table 4). However, E1B 55K did not immortalize BRK cells when co-transfected with *dl*922/947 + 24GLY (Table 3), nor Bcl-2 had no effect even on the colony-forming efficiency of BRK when co-transfected with *dl*922/947 + 24GLY (Table 4). Therefore, it is unlikely that the anti-apoptotic activity of E1B 19K alone is responsible for the RB-complementation activity. Since E1B 19K seems to have anti-apoptotic activity as well as enhancing the E2F-dependent transactivation more strongly than E1B 55K and Bcl-2 (Table 4, Fig. 6), it is suggested that the RB complementation might require both activities with specific thresholds. Further analyses, including a complementation assay of RB mutation using E1B 19K mutants and an E2F gel shift assay should be carried out to elucidate the molecular mechanism of complementation of the RB binding region of E1A and enhancement of E2F activation by E1B 19K.

ACKNOWLEDGEMENTS

We thank Dr. Harlow E for providing us with NT*dl*598 and *dl*922/947, Dr. Vousden K for 2PRO and 24GLY, Dr. Grider T for E2CAT-ATF$^-$A20, Dr. Tsujimoto Y for pB4 and Dr. Sawada Y for p5SstE and pXbaC. This work was supported in part by a grant-in-aid from The Ministry of Education, Science and Culture of Japan.

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