Immortalization of Primary Epithelial Cells Requires First- and Second-Exon Functions of Adenovirus Type 5 12S

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Immortalization of primary cells is a multistep process. The adenovirus E1A 12S gene product is a member of the class of oncoproteins that have the ability to establish primary cells as cell lines in culture. It is encoded by two exons. Extensive mutational analysis demonstrates that four regions of the E1A 12S gene, encoded by both exons, are necessary for immortalization of primary epithelial cells. Expression of two regions is necessary to activate quiescent cells into the cell cycle but is unable to extend the life span of these cells in culture and thus cannot immortalize them. These regions are encoded by the first exon. A third first-exon region, for which no function has yet been identified, is also required. These three regions are also required for 12S to cooperate with an activated *ras* gene to bring about tumorigenic transformation. The fourth region is required to maintain the cells in a proliferative mode, extend their life span in culture, and induce an autocrine growth factor. These functions are encoded by the second exon. The cells immortalized by wild-type 12S and immortalization-competent mutants retain their epithelial morphology and expression of keratin and vimentin intermediate filament proteins.

Immortalization, the ability to escape apoptosis, or programmed cell death, and grow indefinitely, has been identified as an early step in the multistep pathway of conversion of a normal primary cell to the transformed state. However, it seems that the process of immortalization itself is a multistep phenomenon (5). It corresponds to changes in the expression of a set of critical cellular genes. The existence of genetic changes that enable quiescent or senescent cells to proliferate for additional generations but not bring about permanent establishment indicates that immortalization is a complex process. The mechanism(s) by which normal somatic cells acquire unlimited proliferative potential is poorly understood. Immortalizing genes, separately or in combination, influence cellular functions to drive cells to divide autonomously or in response to self-secreted growth-promoting factors.

The study of normal epithelial cell immortalization and tumorigenic conversion has been hindered by the difficulty in maintaining primary epithelial cells in culture. However, human and rodent primary epithelial cells can be immortalized and transformed by the early E1 region of adenovirus (Ad), a DNA tumor virus (28, 32, 63, 66). Ads normally infect quiescent epithelial cells (61); thus, Ads can be used as efficient vectors to introduce recombinant molecules into epithelial cells.

The E1 region is composed of two transcriptional units, E1A and E1B, each of which encodes several mRNAs. The E1A region encodes five different mRNAs and several functions (for reviews, see references 4, 7, and 16 and references therein). The E1A 13S and 12S genes encode related polypeptides of 289 and 243 amino acids (aa), respectively, that are responsible for the establishment or immortalization of primary cells by Ad (28, 50). Cells immortalized by E1A retain many of their differentiated characteristics (14, 43, 45). Both 13S and 12S can bring about tumorigenic transformation in cooperation with other viral oncogenes,

including Ad E1B, or cellular oncogenes (16, 17, 19, 21, 30, 50).

The E1A 12S protein seems to play a major role in the stimulation of cell proliferation responses (6, 45–47). Expression of the 12S gene product is required for optimal virus production in growth-arrested permissive cells but not in actively growing cells (54). It induces cellular DNA synthesis and cell cycle progression in quiescent cells (3, 32, 43, 45, 52, 55), even in the absence of serum (44, 45). The 12S protein also induces the production of a growth factor that induces primary epithelial cells to proliferate (44, 46, 58) and F9 embryonal carcinoma cells to differentiate (43).

Much research has been done to identify the regions of E1A 12S involved in cotransformation with Ha-*ras* (24, 34, 36, 37, 51, 57, 58, 68, 70). In fact, several cellular proteins that interact with those regions of E1A required to cooperate with Ha-*ras* have been identified (12, 18, 69). Unfortunately, little attention has been given to determine the regions and functions of 12S that are required to bring about immortalization (47, 58). The 12S sequences involved in stimulating cellular DNA synthesis and proliferation have been identified (2, 37, 47, 53, 58, 71) and correspond to two of the three regions whose sequences have been conserved among Ad serotypes (33, 64). This latter function seems to be required in order for cotransformation of primary cells with an activated *ras* gene (37, 51, 53, 68).

We demonstrate here that immortalization of primary epithelial cells requires the expression of four distinct regions (I, II, III, and IV) of the 12S gene: three regions from the first exon and one region of the second exon. Two of the first-exon regions (I and II) necessary for immortalization are necessary for E1A to activate quiescent cells into the cell cycle. However, expression of these sequences can only stimulate the cells transiently and cannot extend their lifespan. The role(s) of the third first-exon region (III) is presently unknown. Expression of these same three regions is also required for complete transformation of primary cells in the presence of an activated *ras* oncogene (24, 34, 36, 37,

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51, 57, 58, 68, 70). The region (IV) of the second exon that is necessary for immortalization is required to enable the cells to escape senescence and thus maintain the cells in a proliferative mode. Region IV is required to induce the epithelial cell-specific growth factor. The region IV functions of 12S are distinct from those E1A functions required for cotransformation with an activated *ras* gene.

MATERIALS AND METHODS

Cells and viruses. Cultures of baby rat kidney (BRK) cells were prepared from 2- to 5-day-old rats (Fisher F344; Harlan, Indianapolis, Ind.) as described previously (50). Construction of the mutants and recombinant viruses containing mutated E1A genes in place of the wild-type E1A region has been described previously (10, 47, 57, 68). All of the viruses were plaque purified, and each of the virus genomes was analyzed for the presence of the respective mutation. Virus stocks were propagated, and titers were determined on 293 cells (20).

Cellular DNA synthesis and proliferation. BRK cultures in 24-well trays were incubated for 24-h intervals with 1 μCi of [³H]thymidine (33 Ci/mmol; ICN Radiochemicals, Irvine, Calif.) per ml of K1, a serum-free, hormonally defined medium (60). At the end of the labelling period, the medium was removed and the cells were washed with phosphate-buffered saline and trypsinized. The cells were harvested and lysed with a Skatron cell harvester (Skatron Inc., Sterling, Va.). Total incorporated counts were determined by liquid scintillation counting. Cell proliferation was determined by fixing cells in culture dishes with methanol and staining with 2% Giemsa stain.

Immunoprecipitations and indirect immunofluorescence. Detection of E1A polypeptides was accomplished as previously described (45) by using a pool of monoclonal antibodies (M1, M37, M53, and M73) that recognize several E1A epitopes (25), provided by E. Harlow, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. Keratin and vimentin expression were determined by fixing the cells with methanol at −20°C and processing them for indirect immunofluorescence with an antikeratin monoclonal antibody, LE61 (35), kindly provided by B. Lane, or an antivimentin monoclonal antibody (Amersham, Arlington Heights, Ill.). Photomicrographs were taken at the magnifications indicated in the legend to Fig. 5 with a Zeiss Axiophot epifluorescence microscope with Tri-X Pan film, ASA 400 (Eastman Kodak, Rochester, N.Y.).

Growth factor analysis. Conditioned media were generated and assayed for stimulatory activity as described previously (46).

RESULTS

We have previously demonstrated that expression of the E1A 12S gene can activate quiescent primary BRK cells into the cell cycle (45). It induces cellular DNA synthesis between 8 and 12 h after infection. Increased cell numbers can be detected between 48 and 72 h postinfection. The 12S polypeptide also induces the production of a growth factor that stimulates primary epithelial cells and hepatocytes to proliferate (44, 46) and F9 embryonal carcinoma cells to differentiate (43). To determine the regions and functions of the 12S gene that are necessary for it to immortalize primary epithelial cells, an extensive mutational analysis was undertaken. The maps of the mutated genes and their products are detailed in Fig. 1. The second-exon mutations were made in

two different plasmids. p12SXS is an *Xba*I-*Sac*I (nucleotides [nt] 1339 to 1767) fragment of E1A in pUC118. Mutants derived from this subclone are designated XS. p12SHBXS contains the same restriction enzyme fragment of E1A, except that the *Hpa*I site (nt1572) outside the E1A protein-coding region was changed to a *Bam*HI site in order to generate the internal deletion mutants in the second exon (HBdl). Mutants derived from this plasmid are indicated by HB. All of the mutated 12S genes were built back into an intact virus in order to perform several of the assays used in these analyses. The level of expression of each mutant polypeptide in BRK cells was at least equivalent to that of the wild-type 12S protein (Fig. 2) (68).

Two regions of the first exon (I and II) are necessary to activate quiescent epithelial cells into the cell cycle. Primary BRK epithelial cells proliferate for only 2 days after being placed in culture. After this time they quiesce and die, leaving sparse nonproliferating fibroblasts (45). To determine the role of the first exon of 12S, a series of 3′ deletions in the first exon (CTdl), in the absence of any second-exon sequences, was examined. Equal numbers of primary BRK cells were infected with each mutant virus, an intact 12S virus (12S), or Ad5 *dl*312, an E1A deletion virus (31), or were mock infected. Cellular DNA synthesis was determined by measuring the levels of [³H]thymidine incorporated over 24-h intervals at various days after infection (Fig. 3A and B). Epithelial cell proliferation was determined by cell counting and visualization, which was recorded by fixing the cultures at different days after infection and staining them with Giemsa stain (Table 1). An intact first exon alone (CTdl976) was able to induce cellular DNA synthesis within 24 h after infection and cellular proliferation equivalent to that of an intact 12S (Fig. 3B and Table 1). Mutated genes with 3′ deletions in the first exon to nt940 were also able to induce a proliferative response, although the extent was less than that induced by 12S or CTdl976. Further deletion in the 5′ direction resulted in mutants that were completely defective in these assays, resembling cells infected with *dl*312 or mock infected.

A series of mutations in the 5′ end of the first exon, in the presence of an intact second exon (Fig. 1), were also examined for their effect on the activation of quiescent BRK cells (Fig. 3A and Table 1). 5′ sequences to nt598 could be removed and not affect the ability of the 12S polypeptide to stimulate proliferation. Deletion to nt646 or further 3′ resulted in a loss of stimulation, in spite of the presence of the 3′ region of the first exon necessary for induction. Similarly, infection of BRK cells with the 30K cDNA virus in which sequences from nt637 to 854 have been spliced out (57, 62) was also unable to induce cellular DNA synthesis and proliferation.

Infection of BRK cells with viruses that encoded 12S genes with mutated second exons, but with intact first exons, also resulted in the stimulation of these quiescent cells to cycle (Fig. 3C to F and Table 2).

It has previously been demonstrated that removal of sequences between nt814 and 916 does not interfere with E1A functions (51, 68). Thus, expression of two regions of the first exon, between nt637 and 814 (region I) and nt916 and 940 (region II), was necessary and sufficient to activate quiescent epithelial cells to synthesize cellular DNA and proliferate (Table 1 and Fig. 3 and 6).

Cell cycle activation is not sufficient for immortalization. E1A 12S mutants that could activate quiescent BRK cells were analyzed for their ability to maintain these cells in a proliferative mode for extended periods of time and ulti-
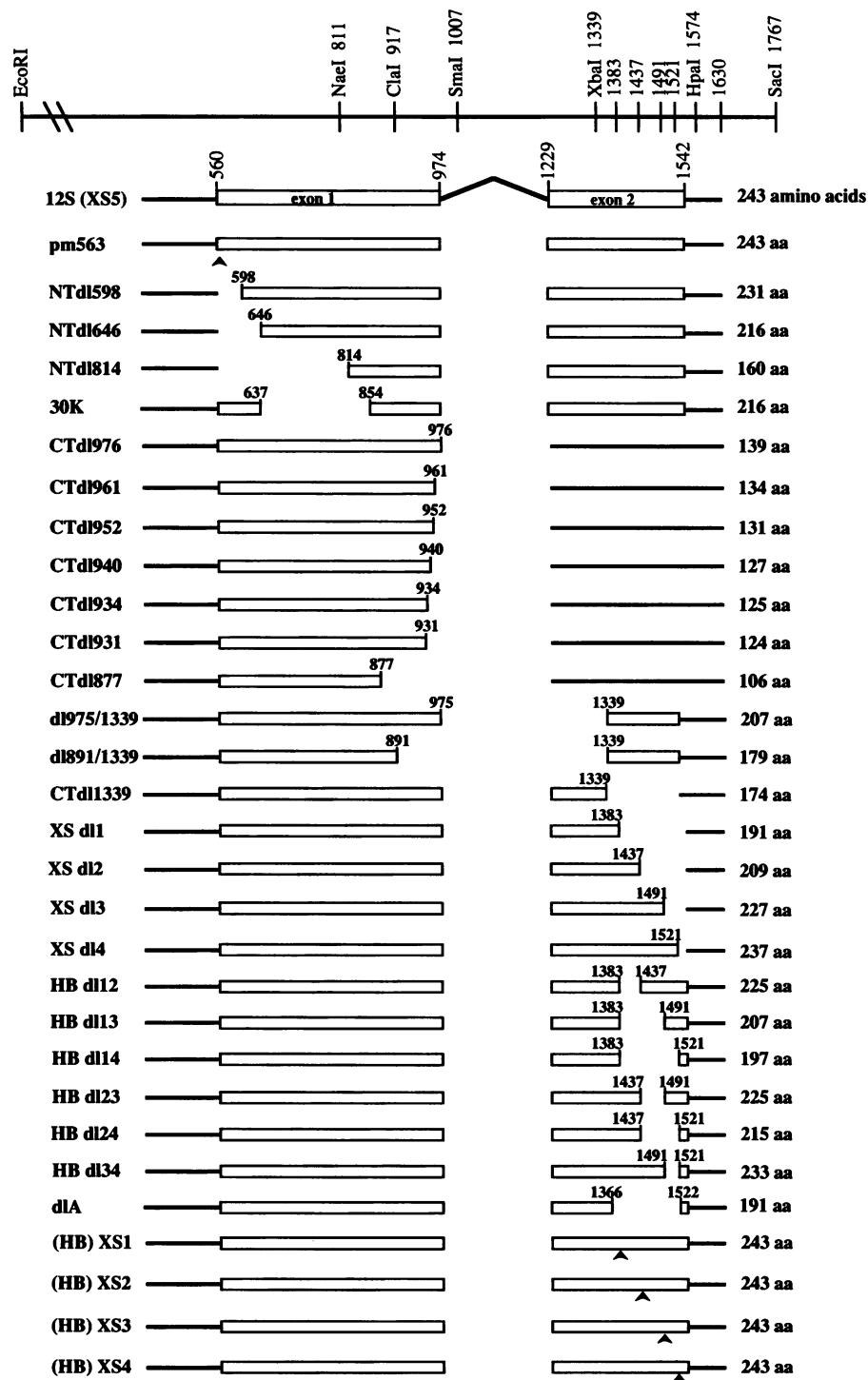
FIG. 1. Structures of the Ad5 E1A 12S mutants and their translation products. The top line is a map indicating salient restriction enzyme sites. The numbers above each line represent Ad nucleotide numbers. The lines below the top line are schematic representations of wild-type 12S (first line) and mutant transcripts. The name of each mutant is indicated at the left. XS5 is the name of the virus in which the *HpaI* site at nt1574, outside the E1A protein coding sequence, has been converted to a *Bam*HI site. HB indicates the virus in which the mutation was made in the p12SHBXS plasmid. The solid lines are the untranslated regions, the open boxes are the protein-coding regions, and the arrowheads indicate the sites of point mutations. The numbers located above the schematic representations refer to the Ad nucleotide number at the boundaries of the deletions. To the right is shown the number of amino acids encoded by each transcript.
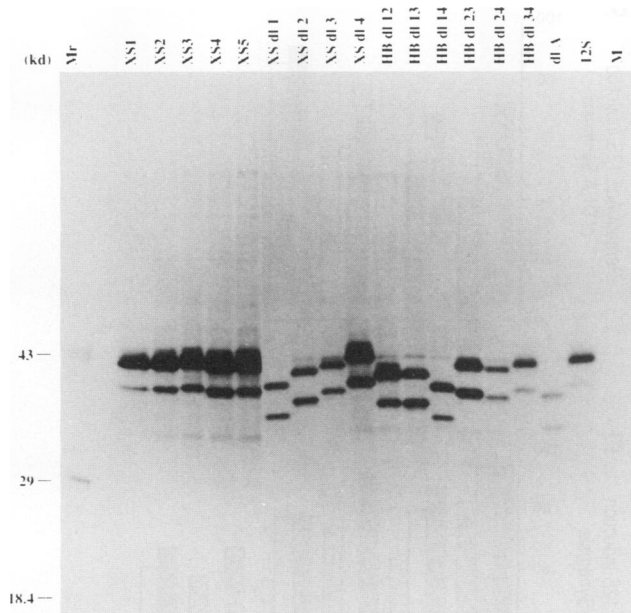
FIG. 2. Expression of polypeptides encoded by 12S second-exon mutants. Immunoprecipitates isolated from [^{35}S]methionine-labelled BRK cells infected with the indicated viruses were resolved on a sodium dodecyl sulfate–12% polyacrylamide gel, which was fluorographed. The 12S polypeptides were collected by using a pool of monoclonal antibodies recognizing several E1A epitopes (25). Molecular mass markers (Mr) are indicated at the left. The faint band in all lanes that migrates with the 43-kDa molecular mass marker is cellular actin.

mately to produce foci of cells that could be expanded into cell lines that can grow indefinitely in culture.

BRK cells infected with the first-exon CTdl mutants that were able to induce cellular DNA synthesis (CTdl976, CTdl961, CTdl952, and CTdl940) continued to synthesize DNA until about 3 days postinfection, although the levels decreased compared with those with an intact 12S virus (Table 1 and Fig. 3A). The maximum cell numbers were also obtained at 3 days, after which the epithelial cells ceased proliferating and started to die. By 6 to 7 days after infection with CTdl976, CTdl961, CTdl952, or CTdl940, there were much fewer cells than in 12S-infected dishes (Table 1). This time frame is coincident with the loss of epithelial cells from untreated, unstimulated cultures. Of course, no immortalized foci were detected at 3 to 4 weeks after infection (Table 1).

Thus, a region of the first exon can temporarily stimulate quiescent cells to proliferate but cannot prolong their life span in culture. These same mutants (CTdl976, CTdl961, CTdl952, and CTdl940) are able to cooperate with an activated ras gene to bring about tumorigenic transformation of the same cells (68), indicating that activation of the cell cycle by E1A is necessary for ras to transform primary epithelial cells.

Expression of the second exon is required for maintenance of proliferation, escape from senescence, and immortalization. Expression of the first-exon regions I and II can activate quiescent epithelial cells into the cell cycle and cooperate with Ha-ras. However, these functions were not sufficient to immortalize these cells (see above), indicating that the second exon must play a role. To determine which region(s) of the second exon is necessary for maintenance of prolifer-

ation and immortalization, BRK cells were infected with two mutants, dl975/1339 and CTdl1339, that have intact first exons and either the 3′ 203 nt or the 5′ 110 nt, respectively, of the second exon (Fig. 1). Both of these mutants were able to activate cellular DNA synthesis and proliferation (Fig. 3C and Table 2), although the level of induction by CTdl1339 was less. However, only dl975/1339 behaved like an intact 12S and gave rise to immortalized foci after 3 weeks. Thus, the C-terminal region of the 12S protein encoded by the 3′ region of the second exon has the ability to extend the response initiated by the expression of the first exon. The C terminus also contains a nuclear localization sequence that is required for efficient localization of E1A (38, 47) and therefore may affect the ability of E1A to immortalize, although it does not affect the ability of E1A to cooperate with an activated ras gene (10).

To determine whether the expression of this region alone was able to bring about immortalization, BRK cells were infected with dl891/1339 (Fig. 1), which expresses the same 3′ 203 nt as in dl975/1339 but has a deletion in the first exon that removes sequences necessary to activate the cell cycle. These cells behaved as if they were infected with dl312 or mock infected (Fig. 3C and Table 1). Thus, expression of the first-exon function(s) is a prerequisite for the expression of the second-exon function(s) to bring about immortalization.

To further identify the region(s) of the second exon that is necessary to cooperate with the first exon for immortalization, a series of point and deletion mutants were made in the 3′ end (Fig. 1). As expected, all of these mutants were able to induce a transient proliferative response on BRK cells because they have an intact first exon (Fig. 3D to F and Table 2), but only some of them were able to immortalize. All of the point mutants were capable of bringing about immortalization (Fig. 4 and Table 2). However, only two of the deletion mutants, XSdl4 and HBdl12, were able to do so, and both were less efficient than an intact 12S in producing foci of immortalized cells that could be expanded into cell lines (Fig. 4 and Table 2). XSdl4, which is missing only the very last 6 aa of the 12S polypeptide, gave rise to only one to two foci per dish, which is about 100-fold less than 12S. HBdl12 is missing an internal stretch (nt1383 to 1437) of 18 aa and gave rise to about 25 to 30% of the number of foci induced by 12S. Thus, in addition to the two regions of the first exon, a region (IV) encompassing nt1437 to 1521 of the second exon is necessary for immortalization by 12S.

These same mutants have been examined for their ability to cotransform BRK cells with Ha-ras. All of the second-exon mutants were able to do so. Furthermore, some of the mutations in the 3′ end of the second exon resulted in much more aggressive cotransformants than even an intact 12S gene (10). Subramanian and coworkers have also shown that removal of the C terminus of the 12S protein enhances the transformed phenotype (59). These hypertransforming mutants include those that are able to immortalize (XS2 and XS3) and those that are not (dl1 to dl3; HBdl13 to HBdl34). Therefore, there is no obvious correlation between the roles of the second exon in immortalization and modulation of transformation. Consistent with their ability to cooperate with Ha-ras, all of the polypeptides encoded by the second-exon mutants are able to interact with pRB105 (data not shown). pRB105 interacts with regions of the 12S polypeptide encoded by the first exon and has been shown to be necessary for E1A to cooperate with an activated ras gene (12, 69). The data presented here indicate that the mutations in the second exon do not affect binding of pRB105 and that
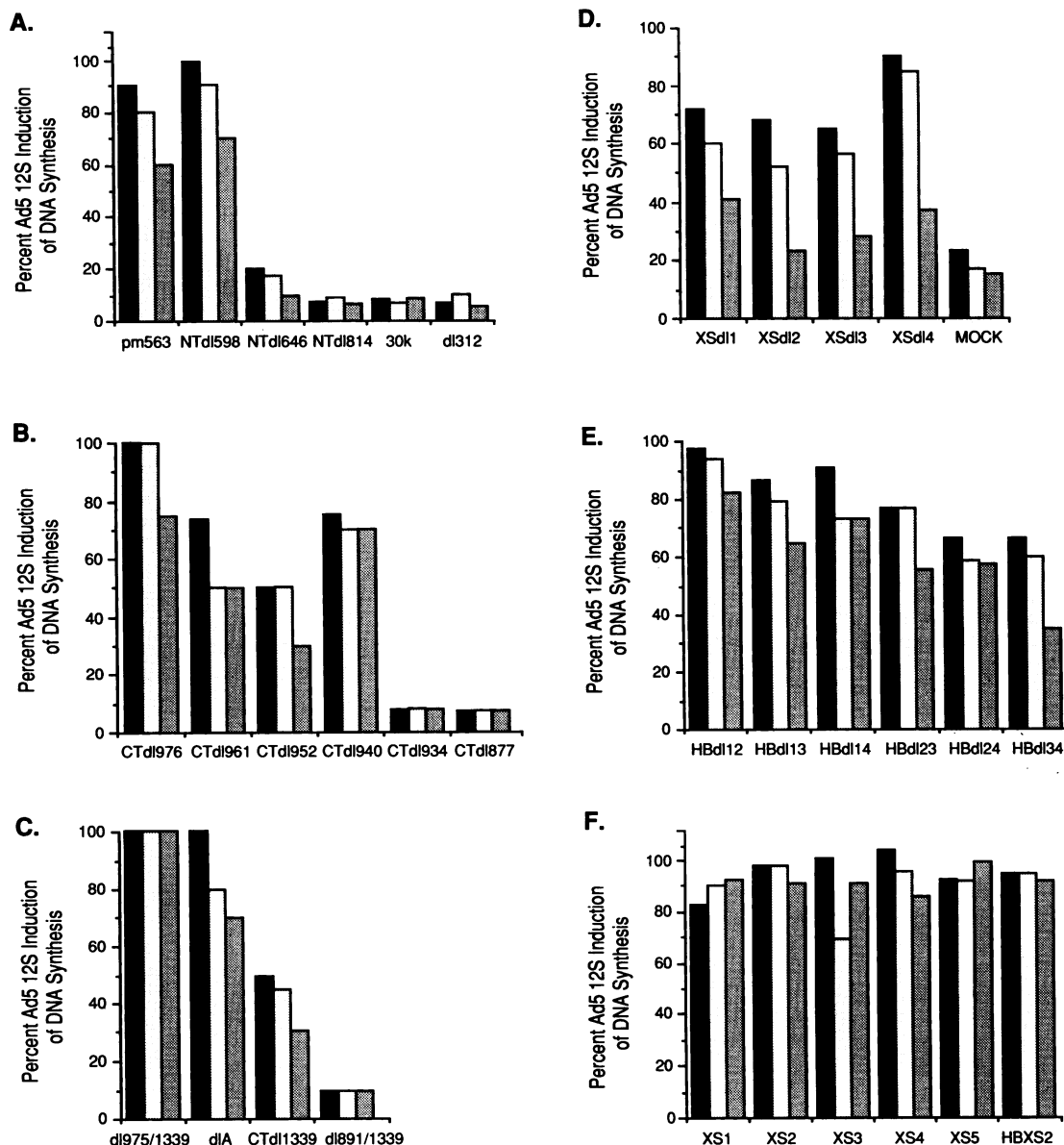
FIG. 3. Cellular DNA synthesis in mutant-infected BRK cells. At 2 days after plating, primary BRK cells were infected at an MOI of 10 with each of the mutant viruses, as indicated below each triplet of bars. Cells were labelled with [³H]thymidine for 24 h, ending at 24 (left bar in each triplet), 48 (middle bar), or 72 (right bar) h after infection. The levels of [³H]thymidine incorporated were determined and are shown as a percentage of [³H]thymidine incorporated by BRK cells infected with an intact 12S virus.

this interaction with a cellular recessive oncoprotein is not sufficient for immortalization of primary cells by E1A.

A third region (III) of the first exon is necessary for immortalization. Infection of BRK cells with *pm*563 or NTdl598 results in a transient proliferative response (Fig. 3A and Table 1) only. *pm*563 has a point mutation at nt563 that changes the second amino acid encoded by the first exon. NTdl598 has an internal deletion of 36 nt and is thus missing aa 2 to 13. Neither of these mutants is able to induce growth factor activity in primary BRK cells, but both can do so in BRK cells immortalized by 12S (47). Because *pm*563 and NTdl598 are able to activate the cell cycle and they both express an intact second exon, it was expected that they would be able to extend the life span of primary epithelial cells in culture. However, no immortalized foci have been

detected in cultures infected with either of these mutants. These data demonstrate that yet another function(s) encoded, at least in part, by the first 39 nt (nt560 to 598) of E1A is also needed for immortalization. This N-terminal region of the E1A polypeptide is needed to cooperate with an activated *ras* gene (68).

Growth factor induction requires expression of second-exon sequences. The Ad5 12S gene induces the production of a growth factor that induces primary epithelial cells and hepatocytes to proliferate in the presence or absence of serum (46). We have previously shown that expression of the second exon is necessary for its induction (47). Therefore, we examined the ability of polypeptides encoded by the genes with mutations in the second exon to induce this stimulatory factor. The same mutants that were able to bring

TABLE 1. Properties of first-exon mutants[a]

Mutant	Activation of cell cycle	Proliferation at:		Growth factor induction	Cooperation with Ha-ras[b]	Immortalization
		3 days	7 days			
pm563	+	+	↓	+[c]	−	−
NTdl598	+	+	↓	+[c]	−	−
NTdl646	−	−	−	−	−	−
NTdl814	−	−	−	−	−	−
30K	−	−	−	−	−	−
CTdl976	+	+	↓	−	+	−
CTdl961	+	+	↓	−	+	−
CTdl952	+	+	↓	−	+	−
CTdl940	+	+	↓	−	+	−
CTdl934	−	−	−	−	−	−
CTdl931	−	−	−	−	−	−
CTdl877	−	−	−	−	−	−
dl891/1339	−	−	−	(+)	−	−

[a] +, positive for the function; −, negative for the function; ↓, epithelial cells are proliferating, but to a lesser extent than at 3 days; (+), activity detected at an MOI of 50 but not at the MOI of 10 that was used in the standard assay.

[b] Data derived from the work of Whyte and coworkers (68).

[c] Data derived from the work of Quinlan and coworkers (47).

about immortalization (that is, all of the point mutants, XS1 to XS4, and only two of the deletion mutants, XSdl4 and HBdl12) were able to induce its production (Table 2). The remaining second-exon mutants were unable to induce this activity, at least not to levels we could detect, even if BRK cells were infected with greater multiplicities of infection (MOI) than were standardly used to generate the growth factor. This indicates that the region of the second exon required for immortalization (nt1437 to 1521) is coincident with that for growth factor induction and further suggests that in addition to cell cycle activation by the first exon, autocrine stimulation is important for immortalization by

TABLE 2. Properties of second-exon mutants[a]

Mutant	Activation of cell cycle	Proliferation at:		Growth factor induction	Cooperation with Ha-ras[b]	Immortalization
		3 days	7 days			
dl975/1339	+	+	+	+	+	+
CTdl1339	+	+	−	−	+	−
XSdl1	+	+	−	−	+	−
XSdl2	+	+	−	−	+	−
XSdl3	+	+	−	−	+	−
XSdl4	+	+	↓	+	+	+[c]
HBdl12	+	+	+	+	+	+[d]
HBdl13	+	+	↓	−	+	−
HBdl14	+	+	↓	−	+	−
HBdl23	+	+	↓	−	+	−
HBdl24	+	+	↓	−	+	−
HBdl34	+	+	↓	−	+	−
dlA	+	+	↓	−	+	−
XS1	+	+	+	+	+	+
XS2	+	+	+	+	+	+
XS3	+	+	+	+	+	+
XS4	+	+	+	+	+	+

[a] +, positive for the function; −, negative for the function; ↓, epithelial cells are proliferating, but to a lesser extent than at 3 days.

[b] Data derived from the work of Douglas and coworkers (10).

[c] The number of immortalized foci ranged from 1 to 5% of that produced by wild-type 12S but could be expanded and propagated as cell lines.

[d] The number of immortalized foci ranged from 25 to 35% of that produced by wild-type 12S.

12S. Whether any additional function(s) is encoded by this region is presently unknown.

To determine whether the second exon alone could induce the 12S mitogen, media from cells expressing dl891/1339 were examined for growth-stimulating activity. No activity was detected from cells that were infected at an MOI that usually produces detectable activity, i.e., MOI of 10. However, if BRK cells were infected with dl891/1339 at an MOI of 50, a low level of secreted growth factor was detectable. This further supports the finding that the C terminus of the 12S polypeptide is able to induce this growth factor. That growth factor induction alone is not sufficient for immortalization by 12S further supports the requirement for first-exon functions in immortalization.

In addition, all of the immortalized cell lines established by 12S or immortalization-competent mutants were able to grow in K1 (60), a hormonally defined, serum-free medium that contains only insulin as a mitogenic peptide (data not shown).

Epithelial cells immortalized by the wild type and immortalization-competent mutants retain differentiation markers. We have previously demonstrated (45) that BRK cells immortalized by the 12S gene retain their epithelial cell morphology and expression of keratins, the intermediate filament proteins (for a review, see reference 56) specific for epithelial cells. Kidney epithelial cells in the animal do not normally express vimentin, the intermediate filament protein specific for mesenchymal cells. However, they do coexpress vimentin and keratin when induced to proliferate in culture (26). The immortalized cell lines derived from these studies retained their epithelial cell morphology (Fig. 5 and data not shown). We examined each of the cell lines derived from BRK cells immortalized by an intact or mutated 12S gene for the expression of these two intermediate filament proteins. Expression was determined by using indirect immunofluorescence and monoclonal antibodies specific for each type. All of the established cell lines were positive for keratin and vimentin expression, as shown for cells immortalized by an intact 12S gene and the mutants XS3 and HBdl12 (Fig. 5). Thus, epithelial cells immortalized by the mutants able to do so resembled those immortalized by 12S, with respect to their expression of intermediate filament proteins.

DISCUSSION

From a genetic analysis using an extensive panel of mutants, we have demonstrated that expression of several regions of the E1A gene is required for immortalization of primary epithelial cells (Fig. 6). These include three regions of the first exon. Two of these regions, I, nt637 to 814, and II, nt916 to 940, are required to stimulate quiescent epithelial cells to proliferate. Mutations in either of these regions results in the loss of cell cycle activation and progression by the 12S gene product. Region I, nt637 to 814, contains the region identified as conserved region 1 (nt679 to 799 [33, 64]). Region II, required for the proliferative response (nt916 to 940), is contained within the region identified as conserved region 2 (nt919 to 979 [33, 64]). These two regions correspond to the pRB105 binding sites (12, 67). The proliferative response brought about by the expression of these two regions is only transient in the absence of the expression of either of the other two regions (III and IV) of 12S required for immortalization (Table 1 and Fig. 6). Thus, their function seems to be to stimulate proliferation of quiescent primary cells, but they do not extend the cell life span in culture. The
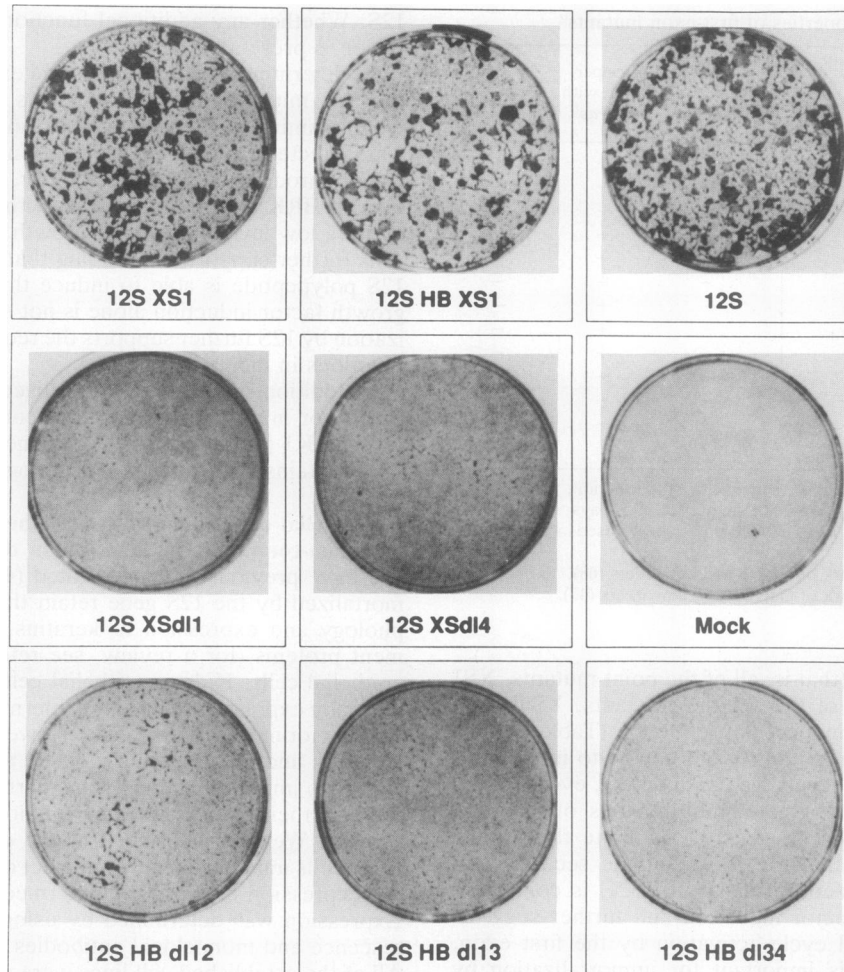
FIG. 4. Foci of immortalized cells produced by infection of BRK cells with wild-type 12S or mutant viruses. Primary BRK cells were infected at an MOI of 10 with the indicated viruses. The cultures were fixed and stained at 4 weeks after infection.

hyperproliferating cells senesce at the same time as the untreated cells.

Region III of the first exon required for immortalization is encoded by at least the first 39 nt. A function has not yet been attributed to this region (Fig. 6). However, it does bind to a 300-kDa cellular polypeptide, also of unknown function at this time (13, 69). Mutations in this region do not interfere with the proliferative response; in fact, the BRK cells proliferate even more than in the presence of an intact 12S gene. This N-terminal region is very sensitive to mutation. A single amino acid change (*pm*563) prevents immortalization (this paper) and cooperation with Ha-*ras* (68). Similarly, Subramanian and coworkers have shown that a 3-aa insertion into this region interferes with its function (58). Such sensitivity to change is interesting since the sequence of this region is not conserved among the Ad serotypes.

Expression of the three first exon regions has been shown to be necessary and sufficient for the E1A gene to cooperate with an activated *ras* gene to bring about tumorigenic transformation of primary cells (34, 36, 41, 51, 58, 68). We have demonstrated that expression of these same regions is also necessary for primary cell establishment. However, unlike cooperation with *ras*, expression of the first exon is not sufficient for immortalization by E1A (Fig. 6). This requires the expression of region IV, nt1437 to 1521 of the second exon. Although not necessary for cotransformation with *ras*, the second-exon region does play a modulating role in the transformation process (10, 59). The first exon is required to activate the quiescent epithelial cells into the cell cycle and provides an additional, unknown function. The second-exon region seems to be required to maintain the cells in a proliferative mode and to escape apoptosis (Fig. 6). The C terminus of the 12S polypeptide encoded by the 3' end of the second exon is also necessary for the 12S gene to induce growth factor production (reference 47 and this paper). The ability of the 12S gene to induce growth factor is necessary for the immortalization of primary epithelial cells (references 47 and 58 and this paper). Whether other important functions are encoded by this region is presently under investigation. Franza and coworkers (15) have previously shown that REF52 cells, an established cell line, could not be transformed by an activated *ras* gene but needed the expression of the E1A gene. In light of the many E1A regions and functions identified, it would be interesting to ascertain what E1A function(s) was required. The fact that the first exon alone can cotransform primary epithelial cells with *ras* but not immortalize them suggests that an activated *ras* must abrogate the need for the second-exon function(s)
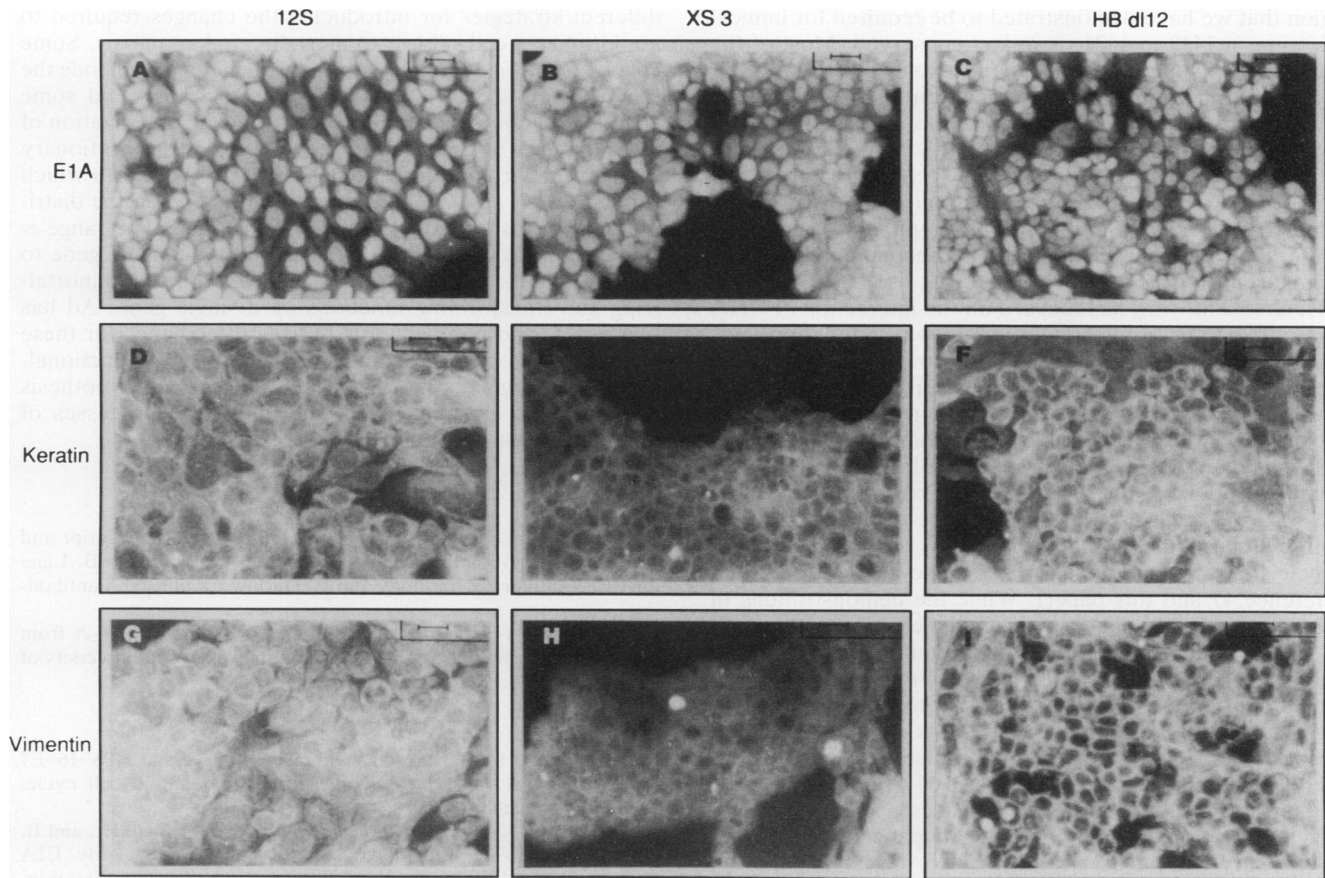
FIG. 5. Expression of E1A and keratin and vimentin intermediate filament proteins by cell lines derived from BRK cells immortalized by wild-type 12S and two immortalization-competent mutants. BRK cell lines established from primary BRK epithelial cells infected with wild-type 12S, 12SXS3, or HBdl12 viruses were processed for indirect immunofluorescence by using antibodies specific for E1A, keratin, or vimentin proteins. Original magnifications: E and H, ×400; A, B, and C, ×500; D, F, G, and I, ×640.

in the transformation pathway. Whether other cooperating oncogenes require only the first-exon functions of E1A or need second-exon sequences to cotransform is presently under investigation.

The first exon of E1A contains three highly conserved regions, two of which (I and II) are essential for immortalization and transformation by E1A. The second exon is less well conserved among the Ad serotypes. However, the
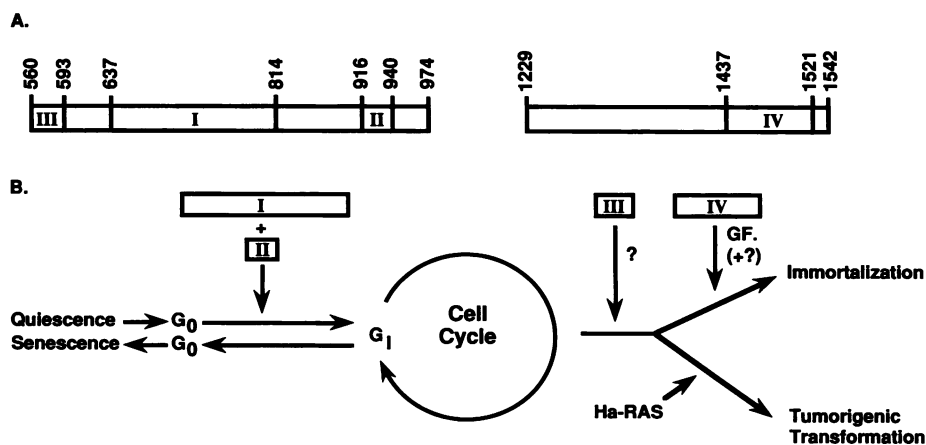

FIG. 6. Multiple regions of E1A 12S are required for immortalization. (A) Map of the E1A 12S protein indicating the four regions (I, II, III, and IV) required for immortalization of primary epithelial cells. Numbers above the schematic correspond to Ad5 nucleotide numbers of the exons encoding the regions of the polypeptide indicated. (B) Model of the roles of the four essential regions in immortalization of primary epithelial cells. GF, growth factor induction; ?, unknown function(s).

region that we have demonstrated to be required for immortalization, nt1437 to 1521, is in fact conserved. Most of the remaining sequences of the second exon of the Ad5 E1A gene are, in fact, missing altogether from the E1A genes of the other serotypes (33, 64). When the corresponding sequences are removed from the Ad5 12S gene, as in mutant *dl*975/1339, in which the 5' end of the second exon is deleted (nt1229 to 1339), there is no effect on the ability of the mutated gene to immortalize primary epithelial cells. This further indicates that the 3' end of the second exon encodes an important function(s).

Much recent work has focused on the interaction of DNA tumor virus transforming oncoproteins with cellular proteins (for a review, see reference 22) and provided many data to suggest common mechanisms of transformation among DNA virus oncogenes and among oncogenes in general. The E1A oncoprotein has been shown to form complexes with many cellular polypeptides (13, 69). Two of these have been identified: pRB105, the product of the retinoblastoma gene (12, 67), and cyclin A (18), a cell cycle-regulating protein. Both cellular proteins bind (18) to the regions of the 12S protein that are important for cell cycle activation by 12S (reference 47 and this paper). While the demonstrations of interactions of viral oncoproteins with cellular proteins thus far identified are undoubtedly important, they are not the whole story. Manfredi and Prives (39) have shown that complex formation of pRB105 and p53 to the polyomavirus and simian virus 40 large T antigens is necessary but not sufficient for immortalization or transformation, respectively. Similarly, each of the pRB105-cyclin A or p300 binding sites of the E1A proteins is necessary, but neither is sufficient for E1A to immortalize (this paper) or transform (68) primary BRK cells.

Analyses of E1A by our laboratory and several other laboratories support the model that immortalization is a multistep process involving several changes in the cell's genetic program and physiology. E1A imparts several changes to a primary cell. Some of the alterations have been more specifically defined, such as activation of the cell cycle by the first exon and induction of an autocrine growth factor by the second exon. Other alterations, such as that provided by the 5' 39 nt of the first exon and perhaps the 3' end of the second exon, are at present not identified. When we examine other viruses encoding immortalizing genes, a similar conclusion can be drawn. For example, immortalization by bovine papillomavirus and human papillomavirus requires the expression of two separate genes, E6 and E7 (8, 27, 29, 42). The E7 gene product resembles the first exon of E1A. It has three regions conserved among the human papillomaviruses, two of which resemble conserved regions 1 and 2 of the first exon of E1A (65). E7 also functionally resembles the polypeptide encoded by the first exon. E7 binds pRB105 (11); stimulates cellular DNA synthesis (1, 49), which also requires the E7 regions homologous to E1A conserved regions 1 and 2 required for cell cycle activation (49); and cotransforms with an activated *ras* gene (40).

Much less is known about E6 function, except that it is localized to the nuclear and membrane fractions of the cell and has nonspecific double-stranded-DNA-binding ability (23). Perhaps E6 is comparable to the second exon of E1A and enables an extended life span, while E7, like E1A first exon, induces hyperproliferation.

The large T antigen of polyomavirus is encoded by two exons of a single viral oncogene capable of immortalization (9, 48). Several regions and functions of this gene are also necessary for the process. Thus, different viruses have

different strategies for introducing the changes required to immortalize a cell subject to mortality and apoptosis. Some encode all of the functions on a single gene, some encode the functions on separate exons of the same gene, and some encode the functions on different genes. The organization of the functions by each virus may reflect the evolutionary stage of the virus or the particular host cell type with which it interacts. A similar situation is observed when the distribution of transforming functions is examined. The range is from all of the functions being encoded by a single gene to multiple genes. Simian virus 40 carries all of the immortalizing and transforming functions on a single gene. Ad has two genes, and polyomavirus utilizes three genes for these processes. Each of the viral oncoproteins is multifunctional. Thus, the original two cooperating oncogenes hypothesis can easily be expanded to fit the multistage processes of immortalization and tumorigenic conversion.

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