MOLECULAR AND CELLULAR BIOLOGY, Aug. 1988, p. 3191–3203
0270-7306/88/083191-13$02.00/0

Vol. 8, No. 8

Growth Factor Induction by the Adenovirus Type 5 E1A 12S Protein Is Required for Immortalization of Primary Epithelial Cells

MARGARET P. QUINLAN,†* PETER WHYTE,‡ AND TERRI GRODZICKER

Cold Spring Harbor Laboratory, P.O. Box 100, Cold Spring Harbor, New York 11724

Received 4 March 1988/Accepted 3 May 1988

The 12S protein encoded by the adenovirus E1A region induces cellular DNA synthesis in and proliferation and immortalization of primary rat epithelial cells in the presence or absence of serum. It also induces the production of a growth factor(s) that stimulates epithelial cell proliferation. We have undertaken a mutational analysis of the 12S gene to determine the sequences required for these functions. We found that a region near the C-terminus of the 12S protein was required for growth factor induction. No activities have been defined previously for this region. Furthermore, we show that growth factor production was necessary for epithelial cells to survive past their normal life span in culture and to become immortalized. The ability to induce growth factor production required prior expression of E1A activities encoded by the N-terminus of the 12S protein, including activation of quiescent cells into the cell cycle, and an unknown activity that required expression of the first 13 amino acids of the gene. In addition, examination of the subcellular localization of mutant 12S polypeptides suggested new regions that affect the nuclear localization of E1A proteins.

The growth of nontransformed mammalian cells in vitro requires culture conditions that include appropriate polypeptide growth factors that exert their effects through specific plasma membrane receptors (for review, see reference 12). Although fibroblasts and established epithelial cell lines are able to grow in standard tissue culture media, which include serum, many primary epithelial cells are, in fact, inhibited by serum components (41). This has hindered the study of epithelial cell transformation in vitro and explains why most in vitro transformation experiments have been done with fibroblasts, although malignant tumors of nonepithelial cell origin account for only 10 to 20% of human neoplasms. However, human and rodent primary epithelial cells can be transformed with adenovirus, a DNA tumor virus (13, 43, 46). In fact, adenovirus normally infects quiescent epithelial cells (42), and the expression of the adenovirus type 5 (Ad5) E1A 12S gene enables primary rodent epithelial cells to proliferate in the presence or absence of serum (26). Thus, the 12S gene provides a means of studying the changes involved in the immortalization and transformation of primary epithelial cells.

The 12S gene is a member of the adenovirus E1A transcription unit. At early times after infection and in transformed cells, two E1A transcripts, designated the 13S and 12S mRNAs, are produced. These mRNAs are translated into proteins of 289 and 243 amino acids (aa), respectively, that differ only by the presence of an additional internal 46 aa in the 289-aa protein. The products of the E1A region can immortalize primary cells (13, 22) and can cooperate with other viral genes and cellular oncogenes to transform primary rat cells (5, 8, 30).

The E1A 12S protein seems to play a major role in the stimulation of cell proliferation responses. The 12S gene product is required for optimal virus production in growth-arrested permissive cells but not in actively growing cells (21, 33). It induces cellular DNA synthesis and cell cycle progression in quiescent cells (1, 26, 33, 35). The 12S protein also immortalizes primary epithelial cells so that they retain many of their differentiated characteristics. It is able to induce DNA synthesis and proliferation of primary baby rat kidney (BRK) epithelial cells in the absence of serum (26). The 12S gene product induces the production of a growth factor(s) that induces quiescent primary BRK epithelial cells and primary rat hepatocytes to synthesize DNA and to proliferate (27; E. Lamas and M. P. Quinlan, unpublished results).

E1A proteins have activities that affect the transcription of viral and cellular genes. Expression of E1A is needed to transactivate the transcription of early viral genes; however, this activity is carried by the 46 aa unique to the 13S protein and is not required for immortalization or transformation (9, 18, 21, 22). It is known that regions within the N-terminal half of both the 12S and 13S proteins are necessary for the E1A-mediated transcriptional repression of some viral and cellular enhancers and that mutants that have lost this activity are defective for transformation and immortalization (3, 19). In fact, E1A mutants that produce N-terminal polypeptide fragments of at least 127 aa are able to cooperate with a T24 *ras* oncogene to transform primary BRK cells (47, 49).

We demonstrate here that the ability of the 12S protein to induce growth factor production and to immortalize primary BRK epithelial cells requires expression of a region near the carboxy terminus of the protein, a region heretofore not identified as having any E1A functions. We further show that the ability of the E1A 12S gene product to induce growth factor production is required to immortalize primary epithelial cells. We also show that the ability to induce growth factor production requires prior expression of activities carried by the N-terminal half of the protein. These activities include activation of quiescent cells into the cell cycle and an unknown activity associated with the first 13 aa of the 12S protein.

* Corresponding author.
† Present address: Department of Microbiology and Immunology, University of Tennessee Health Science Center, 858 Madison Ave., Memphis, TN 38163.
‡ Present address: M385, Fred Hutchinson Cancer Research Center, 1124 Columbia St., Seattle, WA 98104.

MATERIALS AND METHODS

Cells and viruses. Cultures of BRK cells were prepared from 2-day-old rats (Fisher F344; Taconic Farms) and maintained as described previously (26, 30). They were plated in Dulbecco modified Eagle medium (DMEM; Gibco Laboratories) plus 100 μg each of penicillin and streptomycin per ml and 5% fetal calf serum (FCS; Flow Laboratories).

The mutations in the NT and CT series of mutants have been described (47). All mutated plasmids were checked by restriction enzyme or sequence analysis. The mutations in the NT series were placed in a 12S background by replacing the genomic *Cla*I-*Xba*I fragment (nucleotides 917 to 1339) with the *Cla*I-*Xba*I fragment isolated from a 12S cDNA in pUC118, p12S (nucleotides 1 to 1767). The 12S CT*dl*1339 mutant was constructed by digesting p12S with *Xba*I. The ends were filled in with Klenow fragment and ligated to an *Xba*I termination linker. To build these mutated E1A regions back into viruses, the *Eco*RI-*Xba*I fragments were purified and ligated to the large *Xba*I fragment of Ad5 *dl*309 (16) DNA. The ligated DNAs were then transfected onto 293 cells to produce virus stocks as described previously (38). *dl*A was made by digesting an *Xba*I-*Sac*I (nucleotides 1339 to 1767) subclone of the 12S cDNA (p12SXS) with *Alu*I to completion and deletion of the internal *Alu*I restriction fragment (nucleotides 1366 to 1522) upon religation. The mutated fragment was used to replace the *Xba*I-*Sac*I fragment of plasmid pLE12S1A (22). To build this mutant back into virus, in vivo recombination was performed by transfecting the *dl*309 large *Xba*I fragment and the mutated plasmid onto 293 cells. Mutant viruses were identified by restriction enzyme and Southern blot (32) analyses. All virus stocks were propagated on 293 cells. Viral infections and titrations were performed as described previously (22). Micrographs were taken at 10× magnification on a Nikon microscope with TriX-Pan film, 400 ASA (Eastman Kodak Co.). Cells in culture dishes were fixed with methanol and stained with 2% Giemsa.

Cellular DNA synthesis. BRK cultures in 24-well trays were incubated with 1 μCi of [^{3}H]thymidine (20 Ci/mmol; Amersham Corp.) per ml of DMEM plus FCS for 24-h intervals. 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. Total incorporated counts were determined by liquid scintillation counting.

Indirect immunofluorescence. The indirect immunofluorescence assay was performed as described previously (26) with a pool of monoclonal antibodies (M1, M37, M53, and M73) that recognize several epitopes of the E1A polypeptides (10).

Growth factor analysis. Conditioned medium was generated and treated as previously described (27). Cells were infected with the 12S or mutant viruses. Conditioned medium was harvested at 4 days postinfection (dpi), filtered (0.2 μm pore size; Nalgene), and stored at 4°C.

RESULTS

Primary BRK cultures isolated from 2-day-old rats and plated in DMEM with 5% FCS consist of a mixture of fibroblasts and epithelial cells. Regardless of whether the cultures are maintained in this medium or in a serum-free, hormonally defined medium, K1, which was developed to optimize kidney epithelial cell growth and is inhibitory to fibroblast growth (41), the epithelial cell population only survives for 5 to 7 days after plating. However, BRK cells infected 2 to 3 days after plating with an adenovirus variant, Ad512S, in which the E1A region has been replaced by an E1A 12S cDNA, proliferate, produce a growth factor(s) that induces primary epithelial cell growth, and ultimately are immortalized (27). We analyzed several E1A mutants in order to determine the regions of the 12S gene required to express these properties. Deletions were made in various regions of the E1A gene (Fig. 1) (47). The mutated genes were reconstructed back into adenovirus and used to infect primary BRK cells isolated from 2-day-old rats. The mutants produce E1A polypeptides that showed the expected decrease in molecular weight and were expressed at levels similar to the wild-type 12S protein, as determined by immunoprecipitation and immunoblot analyses with anti-E1A monoclonal antibodies (47) (data not shown). These mutants were also tested in *ras* cotransformation assays; no differences were found in the amounts of E1A polypeptides produced by viruses that were able to transform and those that were defective for transformation (47) (data not shown). Either because the mutations were made in a 12S cDNA background or because the 13S unique region had been deleted during the construction of the mutant, none of the mutant E1A genes expressed the transactivation function of E1A. Thus, the mutant viruses were not cytotoxic, because early viral gene expression and viral DNA synthesis were not induced in infected cells.

Mutants were tested for their effects on the induction of cellular DNA synthesis, epithelial cell proliferation and immortalization, and growth factor production. The mutants fell into four different phenotypic classes. Type I mutants behaved like the wild-type 12S virus. Type IV mutants behaved like Ad5 *dl*312, which carries a large deletion of the E1A gene (16). Thus, BRK cells infected with type IV mutants resembled mock-infected cells. Type II and III mutants had some but not all of the properties of the parental 12S virus. Both types transiently induced cellular DNA synthesis and proliferation but failed to immortalize cells or produce growth factors.

Expression of the second exon of the E1A 12S gene is required to immortalize primary epithelial cells. To examine the role of the second exon of the E1A 12S gene in the immortalization of primary epithelial cell, we analyzed several adenovirus mutants that had deletions in the C-terminal region of the molecule. The deletion mutant *dl*975/1339 produces a protein that is missing the first 36 aa encoded by the second exon of E1A, although it contains the 68 C-terminal aa (Fig. 1). This mutant had the same phenotype as the parental 12S virus (type I mutant). It rapidly induced cellular DNA synthesis, as measured by the incorporation of [^{3}H]thymidine in infected BRK cultures (Fig. 2). It also induced rapid proliferation of the epithelial cell population (Fig. 3, type I). Figure 3 shows culture dishes that were fixed and stained at 3 and 6 dpi and at 4 weeks postinfection (wpi) with the wild-type 12S virus and *dl*975/1339. Representative fields of cells from infected culture dishes at 3 dpi and 4 w pi are shown in Fig. 4. These experiments demonstrated that both 12S- and *dl*975/1339-infected cells had an epithelial cell morphology and that the cells proliferated to form well-delineated islands. These cells expressed cytokeratins, as determined by indirect immunofluorescence with a monoclonal antibody which recognizes cytokeratins from most soft or simple nonsquamous epithelial cells (data not shown). In addition, *dl*975/1339-infected BRK cells produced the epithelial cell growth factor, a property unique to this mutant virus, as discussed below. The mutant *dl*975/1339, like the 12S virus, was able to immortalize, primary BRK epithelial cells.

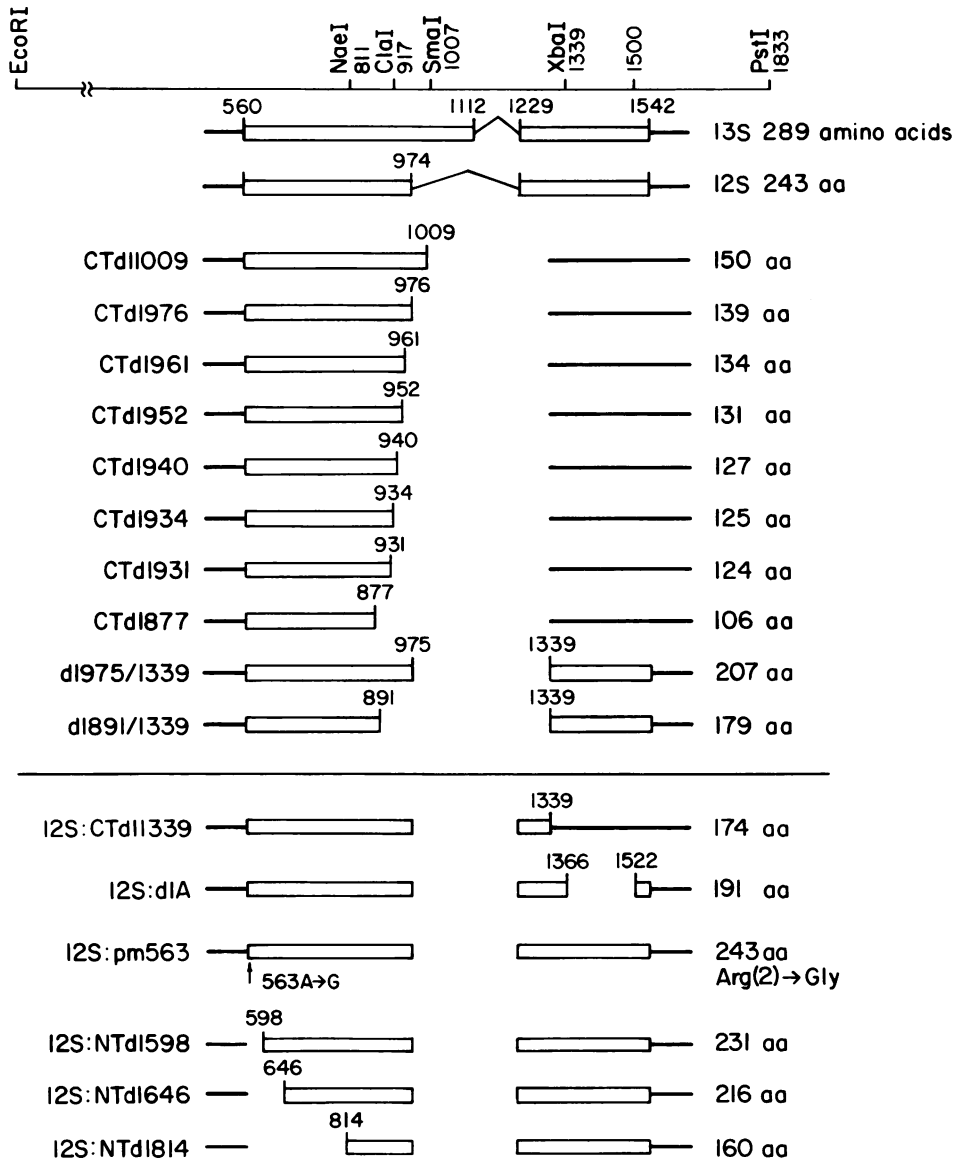

FIG. 1. Structures of the Ad5 E1A deletion mutants and their translation products. The structures of the 13S and 12S gene products are shown below the map indicating salient restriction enzyme sites. Below these are representations of the E1A gene product produced by each mutant. Each line represents a transcript: the solid lines are untranslated regions, and carets are introns. The protein coding sequences are boxed. The number in the name of each mutant represents either the last (CT) or first (NT) adenovirus nucleotide remaining after the deletion, except for pm563, where it represents the nucleotide altered by a point mutation (A to G). *dl*A was created by an in-frame deletion of an *Alu*I restriction fragment, the sites of which are indicated. To the right is shown the number of E1A aa encoded by each transcript; they do not include the linker-encoded aa Leu for the CT*dl* series and Ser for 12S CT*dl*1339.

The foci of epithelial cells that were observed at 3 to 4 wpi could be expanded in mass culture and were grown continuously for 12 months. They retained epithelial cell characteristics, including morphology (Fig. 4), poor plating efficiency, and the expression of simple nonsquamous epithelial cytokeratins. These results show that a 12S protein that is missing the first 36 aa encoded by the E1A second exon can immortalize primary epithelial cells.

The mutant CT*dl*1339 produces a 12S protein that is missing the 68 C-terminal aa (Fig. 1). This mutant also induced DNA synthesis (Fig. 2A) and cell proliferation (Fig. 3 and 4, type III), but the response was transient. After 3 dpi, no additional epithelial cell proliferation was observed, and by 6 dpi many of the cells had been lost from the culture dish (Fig. 3). No foci of epithelial cells were found at 3 to 4 wpi (Fig. 3 and 4). These data suggest that at least part of the C-terminal 68-aa region of the 12S protein is required to maintain the epithelial cells in a proliferative mode and to immortalize them.

To test this hypothesis further, we infected primary BRK cells with 12S *dl*A, a mutant in which sequences encoding 52 internal aa from the critical C-terminal 68-aa region have been removed. This deletion mutant retains 16 of the C-terminal 68 aa (including the 7 C-terminal aa (Fig. 1). The polypeptide encoded by *dl*A induced cellular DNA synthesis (Fig. 2A) and proliferation (Fig. 3 and 4, type II), but failed to extend the life span of epithelial cells in culture (Fig. 3 and 4). Thus, a region within the C-terminal portion of the 12S

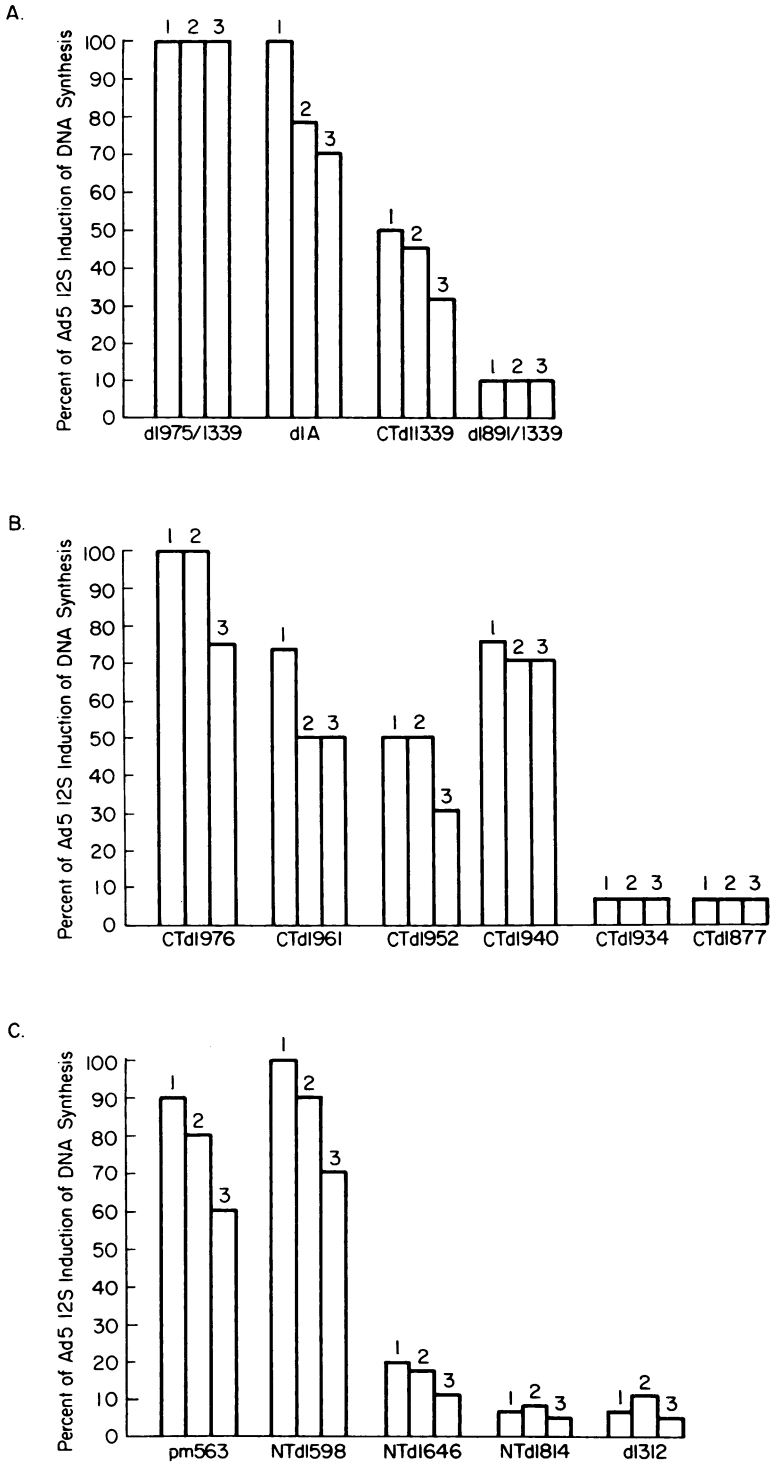

FIG. 2. DNA synthesis in mutant-infected BRK cells. At 2 days after plating, primary BRK cells were infected at a multiplicity of infection of 10 with the 12S and mutant viruses as indicated below each set of bars. Cells were labeled with [^{3}H]thymidine for 24 h, ending at the dpi indicated above each bar. The levels of [^{3}H]thymidine incorporation were determined and are shown as a percentage of the [^{3}H]thymidine incorporated by Ad5 12S-infected cells.

protein is necessary to maintain primary epithelial cell proliferation.

To determine whether the functions encoded by this C-terminal region were independent of other E1A activities, we infected BRK cells with the mutant *dl*891/1339, which is similar to *dl*975/1339 except that it is missing an additional 28 aa encoded by the 3′ end of the first exon (Fig. 1). BRK cells infected with *dl*891/1339 did not synthesize DNA or proliferate (type IV mutant, Fig. 2 to 4) and resembled *dl*312- or mock-infected cells. This suggests that the first exon en-

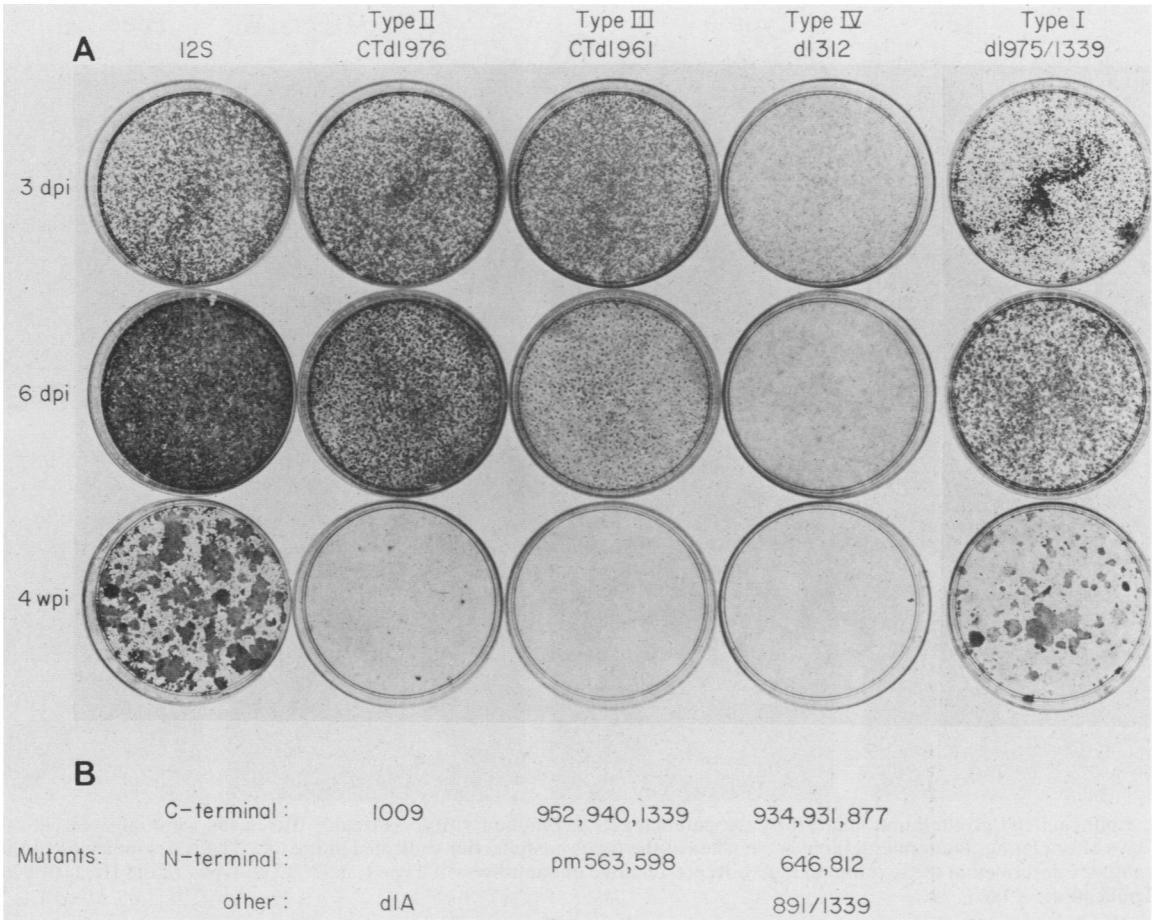

FIG. 3. (A) Proliferation of primary BRK cells infected with the parental 12S and mutant viruses. Primary BRK cells were infected at a multiplicity of 10 at 2 days after plating with the virus indicated above each vertical row. The cultures were fixed and stained with Giemsa at the times postinfection indicated at the left. (B) Below each vertical row are indicated the mutant viruses that yielded similar results.

codes additional functions that are required for the immortalization of primary epithelial cells.

Expression of the first exon of the 12S gene is required to activate quiescent primary epithelial cells. Because *dl*891/1339 failed to induce even a transient response in BRK cells and the region of the first exon that is missing in *dl*891/1339 has also been shown to be important for transformation (18, 24, 31, 47), we examined the role of the first exon in the activation of quiescent epithelial cells. BRK cells were infected with a series of CT mutants containing progressive 3′ deletions in the first exon that do not express any sequences from the second exon (Fig. 1).

CT*dl*976 produced a 139-aa E1A protein that included the region encoded by the first exon of the 12S gene (Fig. 1). CT*dl*1009 expressed an additional 11 aa encoded by the 13S unique region (Fig. 1) but did not transactivate viral gene expression (data not shown). Infection of BRK cells with these mutants resulted in the rapid induction of DNA synthesis (Fig. 2B) and epithelial cell proliferation at wild-type levels until 3 dpi (Fig. 3 and 4, type II mutants). The infected cultures resembled 12S-infected BRK cultures up to this time (Fig. 4). However, by 6 dpi, cells infected with the type II mutants ceased proliferating. The epithelial cells were subsequently lost from the dish, and no foci were found at 3 to 4 wpi (Fig. 3 and 4). These results again stress the importance of second-exon functions in maintaining epithelial cell growth. However, both the CT*dl*1009 and CT*dl*976 E1A genes carried on plasmid vectors were able to cooperate efficiently with the T24 Ha-*ras* gene to transform BRK cells in a cotransfection assay (47). Cells from several transformed lines still expressed cytokeratins, indicating that they were of epithelial cell origin (data not shown). These results suggest that an activated *ras* gene can abrogate the need for E1A second-exon activities in the transformation of BRK cells.

Mutants with larger deletions in the first exon than that in CT*dl*976 (Fig. 1, CT*dl*961, -952, and -940, type III mutants) also induced substantial DNA synthesis (Fig. 2B) and epithelial cell proliferation (Fig. 3) until 3 dpi (Fig. 3 and 4). However, after this time the epithelial cells were lost from the culture, so that by 6 dpi only a minority of the epithelial cell population was present. No foci were found at 3 to 4 wpi. Thus, type III mutants can also rescue the epithelial cells from quiescence but are unable to extend their life span in culture. The somewhat lesser ability of these mutants than of CT*dl*976 to stimulate epithelial cell growth is probably related to the fact that they have lost first-exon sequences that are important, but not essential for transformation, as measured in a *ras* cooperation assay (18, 24, 31, 47).

The mutants that produced the shortest N-terminal fragments (CT*dl*934, -931, and -877) were completely defective in the induction of cellular DNA synthesis and proliferation (type IV, Fig. 2 to 4) and failed to cooperate with a T24 Ha-*ras* gene to transform BRK cells (47). Thus, these

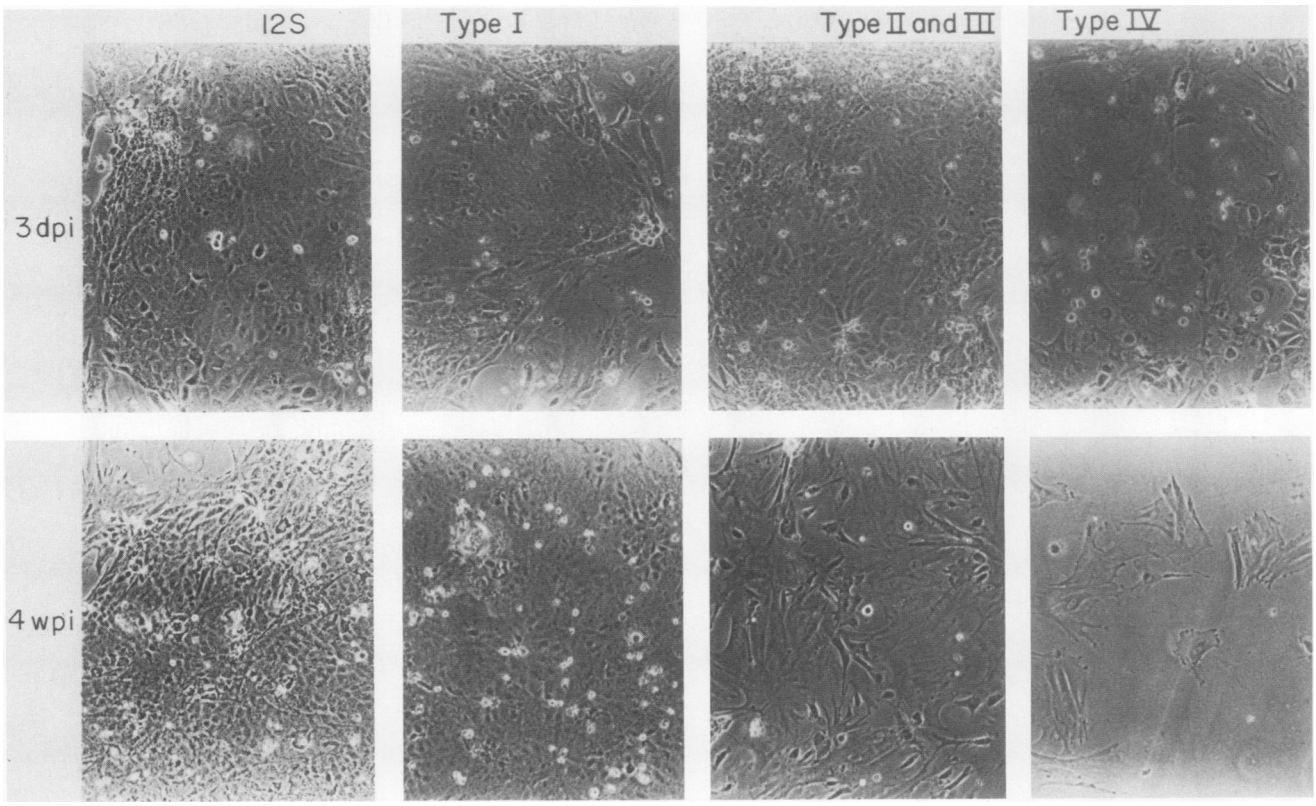

FIG. 4. Epithelial cell proliferation induced by the parental 12S and mutant viruses. Primary BRK cells were infected with the mutant viruses 2 days after plating. Photomicrographs were taken at the times postinfection indicated at the left. The types indicated at the top refer to the phenotypes described in the text and in Fig. 3. Representative mutants shown: Type I, *dl*975/1339; types II and III, CT*dl*976; type IV, *dl*312. Magnification, ×9.6.

mutants had the same phenotype as *dl*891/1339. Similar results (Fig. 2 to 4) were obtained with the mutants NT*dl*646 and NT*dl*814, in which 27 and 85 aa, respectively, were removed from the region encoded by the 5′ end of the first exon of the 12S gene (Fig. 1). This suggests that regions within the N-terminal half of the protein must be expressed for activation of quiescent BRK cells and their subsequent growth and immortalization. Consistent with these data is the observation that an adenovirus variant in which the E1A region contained a cDNA of the E1A 10S gene product (i.e., was missing the region between nucleotides 653 and 854 [36]) was also unable to induce DNA synthesis and proliferation (data not shown).

A new E1A activity requires expression of the first 39 base pairs of the 12S gene. The mutants pm563, in which aa 2 has been changed from Arg to Gly, and NT*dl*598, which is missing the N-Terminal 12 aa (Fig. 1), induced DNA synthesis to high levels (Fig. 2C). At early times after infection, they also stimulated epithelial cell proliferation as well as the 12S and *dl*975/1339 viruses did (Fig. 3). In spite of the strength of this initial response, epithelial cells ceased proliferating after 3 dpi and were lost from the culture by 6 dpi (Fig. 3 and 4, type III). Thus, the mutants were unable to immortalize epithelial cells even though they produced 12S proteins that contained the C-terminal portion of the molecule (see below). Furthermore, neither of these mutants was able to cooperate with the T24 Ha-*ras* gene in a transformation assay (47). These data suggest that an activity, in addition to induction of DNA synthesis and proliferation, is necessary for both the immortalization and transformation pathways. This activity must be carried, at least in part, by the first 13 aa of the protein.

C-terminal region of the 12S protein is required for induction of growth factors in BRK cells. We have previously described the ability of the 12S protein to induce the production of a growth factor that stimulates primary epithelial cell proliferation (27). To determine the region(s) of the 12S gene necessary for this property, BRK cells were infected with all the mutant viruses described above. Conditioned medium was harvested and tested for the ability to induce cellular DNA synthesis and subsequent cell division in primary BRK cultures, as previously described (27).

The type I mutant *dl*975/1339, which was able to immortalize BRK cells, yielded conditioned medium with stimulatory activity. The properties of the growth factors induced by *dl*975/1339 and by the parental 12S virus were the same (Table 1). Both 12S and *dl*975/1339 conditioned media were produced and were effective in stimulating epithelial cell proliferation in the presence or absence of serum (27) (data not shown). Furthermore, conditioned medium generated from 12S- or *dl*975/1339-infected BRK cells could be diluted at least 20-fold and retain stimulatory activity (Fig. 5). The conditioned medium induced by *dl*975/1339, in fact, seemed slightly more active and was more heat stable than 12S-conditioned medium (Table 1 and Fig. 5). This probably reflects the higher concentration of factor(s) in the medium.

The type II and III mutants, which were able to induce transient DNA synthesis and proliferation in infected BRK cells but failed to immortalize them, all failed to induce growth factor production in primary BRK cells (data not

TABLE 1. Properties of conditioned medium[a]

Treatment of conditioned medium	Stimulation of epithelial cell proliferation	
	Ad5 12S	Ad5 *dl*975/1339
None	+	+
Heat		
43°C, 30 min	+	+
46°C, 30 min	±	+
50°C, 30 min	−	−
Centrifugation (100,000 × *g*)		
Supernatant	−	−
Pellet	+	+
Recentrifugation of pellet + 4.0 M NaCl		
Supernatant	+	+
Pellet	−	−

[a] Conditioned medium generated in DMEM with 5% FCS was treated as indicated and then combined with an equal volume of fresh DMEM with FCS and added to primary BRK cultures 2 days after plating to assay for growth-stimulating factors. Symbols: +, ±, −, high, low, and undetectable stimulation, respectively.

shown). These mutants included the mutants that produced 12S polypeptides that were missing parts of the C-terminal portion of the protein (e.g., CT*dl*976, -*dl*1339, and *dl*A), as well as the mutants pm563 and NT*dl*598, which produced 12S proteins that were altered only at the N-terminal end of the molecule.

We examined the ability of these mutants to induce growth factor production further. We have shown previously that established BRK epithelial cell lines immortalized by the Ad5 12S virus do not constitutively produce detectable

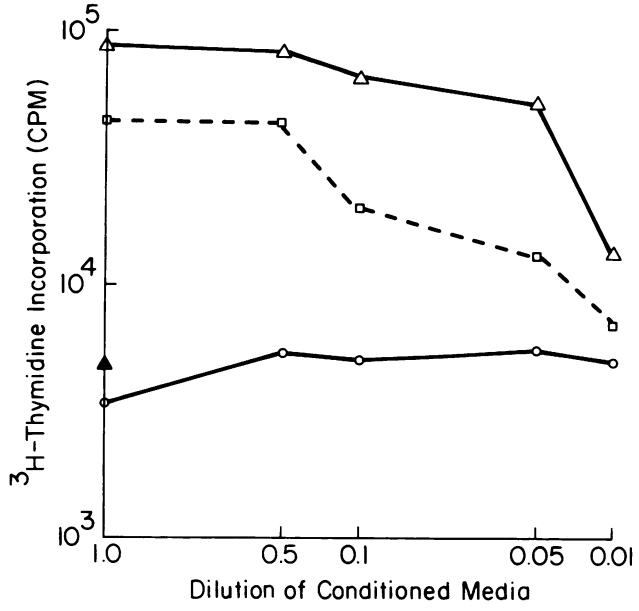

FIG. 5. DNA synthesis induced by the epithelial cell growth factor produced by BRK cells infected with 12S or *dl* virus. Primary BRK cells were infected with Ad5 12S, *dl*975/1339, or *dl*312. Conditioned medium was harvested 4 days later, filtered, diluted with fresh medium (*x*-axis), and added to new cultures of primary BRK cells. After 3 days, the cultures were incubated with [^{3}H]thymidine for 24 h, and the levels of incorporation were determined (*y*-axis). Symbols: □, Ad5 12S-conditioned medium; △, *dl*975/1339-conditioned medium; ▲, *dl*312-conditioned medium; ○, DMEM plus FCS.

levels of growth factor (27). This may be due to the fact that established epithelial cells undergo changes in tissue culture that differentiate them from the original primary cells (26, 41). However, it is possible that Ad5 12S-established BRK cells produce the stimulatory factor(s) at a level too low to be detected in the bioassay used (27). However, the growth factor(s) was produced upon superinfection of these cell lines with the 12S virus (27). The properties of the growth factor produced by cell lines after superinfection were the same as those of growth factor produced by primary cells after infection with the 12S virus (27) (data not shown). We therefore tested the ability of the type II and III mutants to induce growth factor production in one of these cell lines, BRK12-1. Conditioned medium from BRK12-1 cells infected with either pm563 or NT*dl*598 had stimulatory activity (data not shown). Thus, an established BRK cell line can complement, at least in part, the defects of the N-terminal mutants. As expected, *dl*975/1339 also induced growth factor production in BRK12-1 cells, while CT*dl*976, *dl*1339, and *dl*A were not able to do so. These data suggest that the E1A activity responsible for growth factor production requires expression of the C-terminal region of the protein, since mutants such as CT*dl*976 failed to induce growth factors even in an already immortalized cell line.

The type IV mutants NT*dl*646 and NT*dl*814, which failed to induce DNA synthesis and proliferation in BRK cells, were unable to induce growth factor production in either BRK cells or BRK12-1. It seems likely that the production of the growth factor as well as immortalization of primary epithelial cells depends on the activation of quiescent primary BRK cells. This idea is consistent with the fact that growth factor production does not occur immediately after infection. Induction of DNA synthesis by the 12S protein occurs between 8 and 12 h postinfection (hpi), while the growth factor is not produced until 24 hpi (26, 27).

Primary BRK epithelial cells are unable to survive trypsinization and replating. However, within 24 hpi with the 12S virus, the epithelial cells could be replated (Fig. 6), albeit at low efficiency, onto tissue culture dishes on which 12S-, but not *dl*312-, infected BRK cells had been growing. After trypsinization and replating, 200 to 300 very small epithelial colonies were found (data not shown). The appearance of a representative colony 2 days after replating is shown in Fig. 6. The ability of the mutant-infected BRK cells to survive passaging was examined, and only *dl*975/1339-infected cells were able to do so (Fig. 6). Whether the ability of epithelial cells to survive passage is due to the secretion of a growth factor(s) or to some other change in the cells is not known. However, for proliferating BRK epithelial cells to produce growth factors, survive passage, and become immortalized, expression of the C-terminal region of the 12S protein is required.

Subcellular localization of the mutant E1A polypeptides. E1A proteins contain a 5-aa sequence, located at the C-terminus of the polypeptide, that is involved in nuclear localization (20). It was important to determine the subcellular distribution of mutant E1A polypeptides, some of which were lacking C-terminal sequences, in order to show that the altered phenotypes did not result from lack of E1A proteins in the nucleus. Therefore, BRK cells infected with the various mutants were examined by indirect immunofluorescence with E1A monoclonal antibodies (10). Similar results were obtained with infected HeLa cells (data not shown).

All of the 12S protein in BRK cells infected with the parental 12S virus was located in the nucleus, as expected

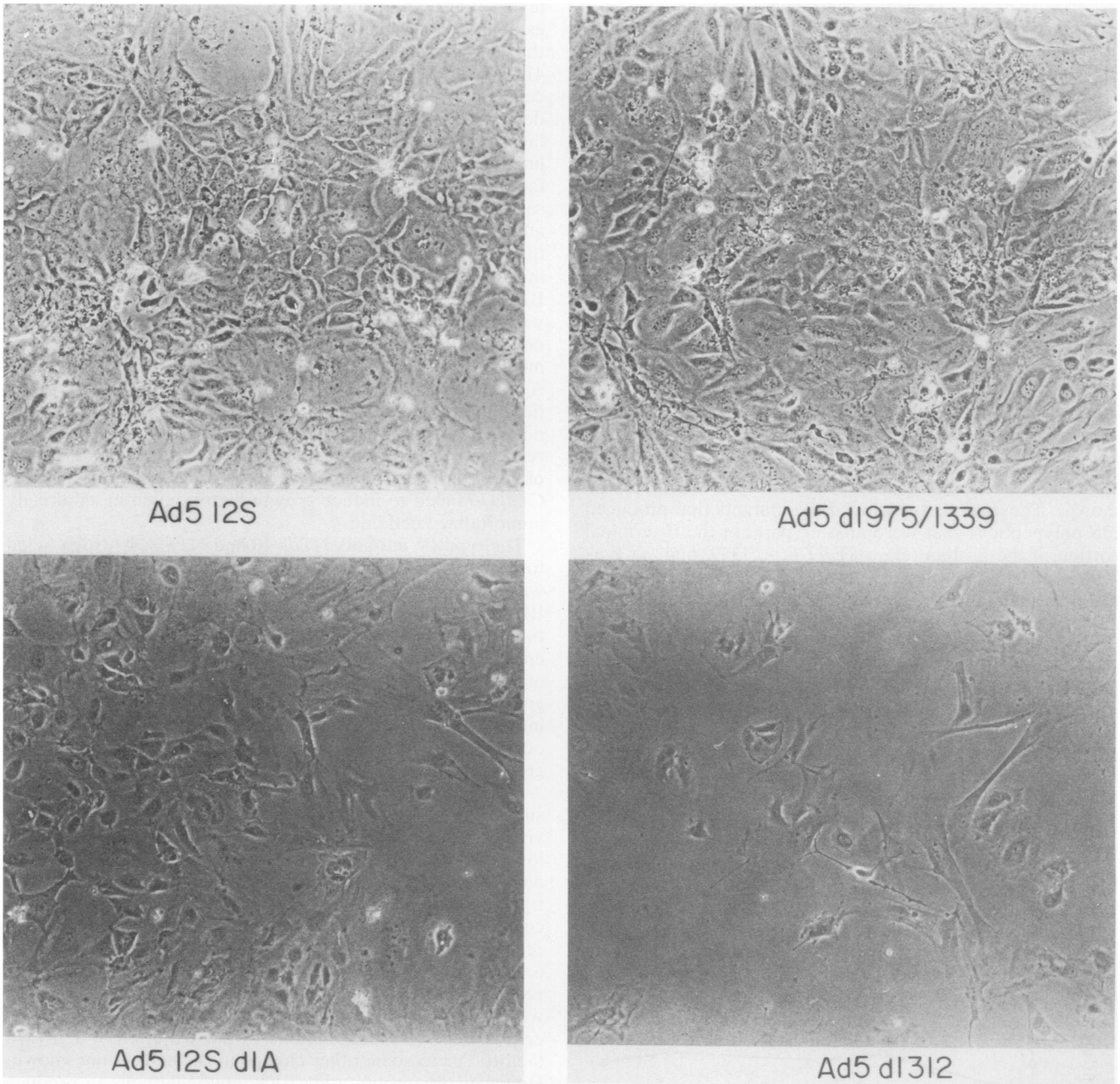

FIG. 6. Ability of primary BRK cells to be replated. Primary BRK cells were infected with the indicated viruses 2 days plating, and 24 h later, the cells were trypsinized and replated onto the same tissue culture dish. Photomicrographs were taken 3 days later. Similar results were obtained when mutant-infected BRK cells were plated onto tissue culture dishes on which 12S-infected BRK cells had been growing. Magnification, ×10.

(Fig. 7) (20). BRK cells infected with Ad5 *dl*312, which carried a large E1A deletion, showed no specific staining (Fig. 7d). Cells infected with any of the mutants in the CT*dl* series, which produced 12S N-terminal fragments, exhibited 12S polypeptides in both the nucleus and the cytoplasm (Fig. 7b; CT*dl*976). Thus, the absence of the previously described nuclear location signal did not result in exclusion of E1A protein from the nucleus. In fact, the mutant *dl*1339, which produced a 12S polypeptide lacking only the C-terminal 68 aa, exhibited more nuclear than cytoplasmic staining (Fig. 7g). The E1A polypeptides expressed by Ad5 *dl*311 (16)-infected BRK cells behaved similarly (data not shown). Ad5 *dl*311 contained an out-of-frame deletion of the region between base pairs 1281 and 1339 (3). It thus produced both 13S and 12S gene products that were lacking the same C-terminal aa as the *dl*1339 12S protein. These results show that large amounts of E1A proteins are present in the nuclei of mutant infected cells. However, it should be noted that the *dl*975/1339 12S protein, which contained the normal E1A C-terminal region, was located exclusively in the nucleus (Fig. 7c).

The 12S polypeptide expressed in d1A-infected cells was predominantly nuclear, although not completely localized to the nucleus (Fig. 7h). The d1A 12S protein contained the

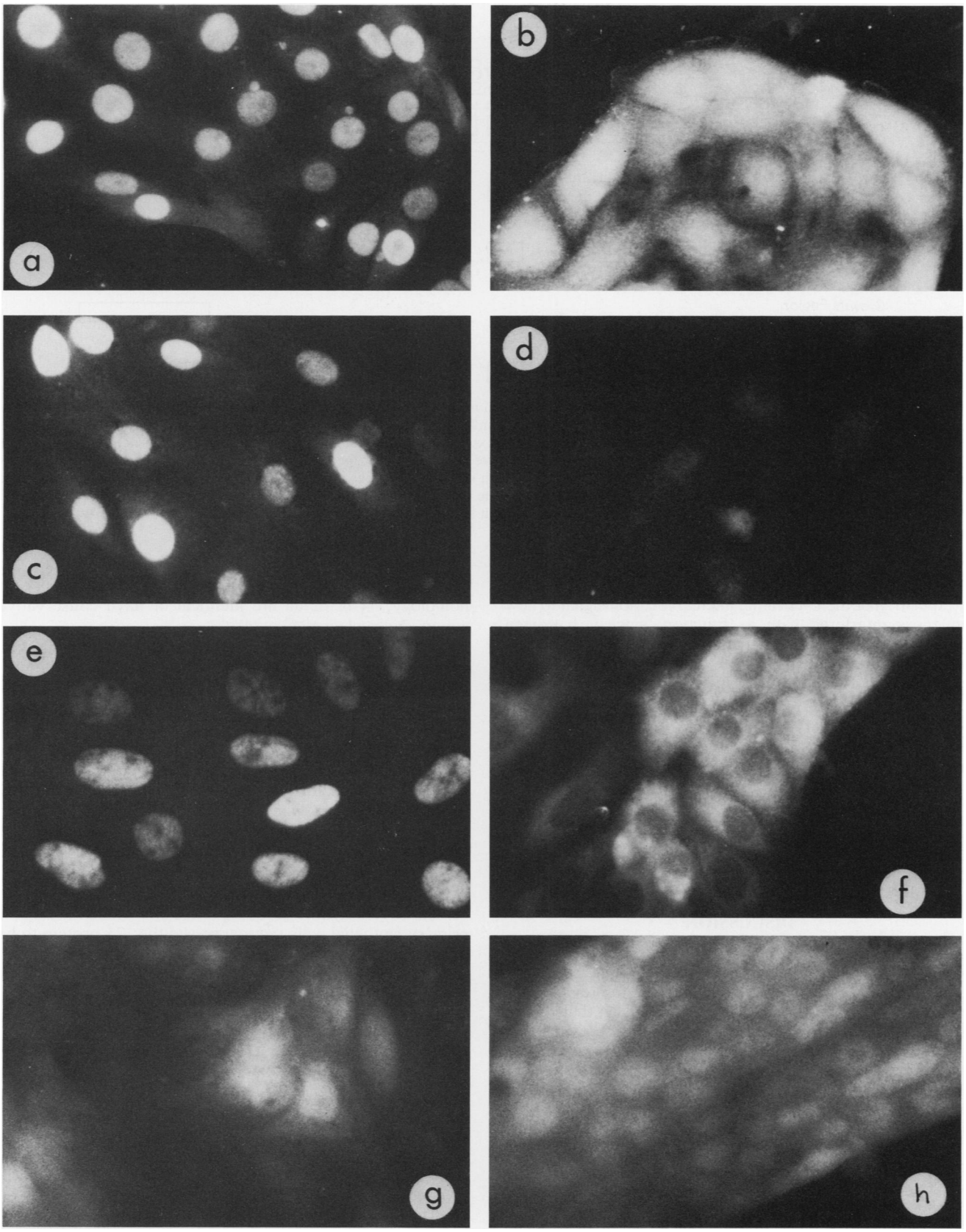

FIG. 7. Intracellular localization of E1A mutant polypeptides. Primary BRK cells were infected with the indicated viruses at 2 days postplating. At 16 hpi, the cells were fixed and processed for indirect immunofluorescence with a pool of monoclonal antibodies that recognize different epitopes of the E1A proteins. Viruses: a, Ad5 12S; b, CT*dl*976; c, *dl*975/1339; d, *dl*312, e, NT*dl*646; f, NT*dl*814; g, CT*dl*1339; h, *dl*A. Magnification, ×63.

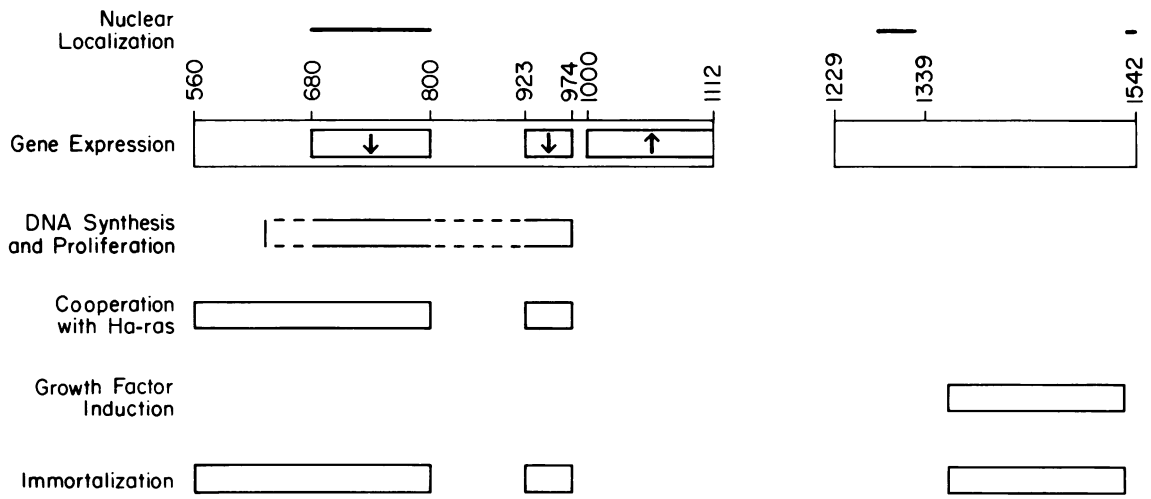

FIG. 8. Functional regions of the E1A gene. The protein-coding region of E1A is denoted by the two large open boxes. The numbers above the boxes refer to adenovirus nucleotide numbers. The smaller boxes inside indicate regions that are conserved between different adenovirus serotypes and have been shown to be important for modulating gene expression. Symbols: ↓, transrepression; ↑, transactivation. The bars above the protein-coding region indicate regions of the protein involved in the nuclear localization of E1A protein. The small open boxes delineate regions required for the functions indicated at the left of each row. Dashed lines show regions where the absolute boundaries have not yet been defined. Data for nuclear localization (20; this study); gene expression (18, 19, 24, 31), DNA synthesis and proliferation (19, 39, 49; this study), cooperation with Ha-*ras* (18, 19, 24, 31, 39, 47), growth factor induction (this study), and immortalization (31, 39; this study) were taken from the indicated references.

C-terminal nuclear location sequence. It is possible that the juxtaposition of the C-terminal nuclear location signal to new sequences interfered with its function, since it has been shown that a localization signal can be severely affected by the surrounding sequences (29).

The 12S polypeptides produced by the NT mutants pm563, NT*dl*598, and NT*dl*646 were efficiently localized to the nucleus, as shown for NT*dl*646 (Fig. 7e). However, the 12S polypeptide encoded by NT*dl*814 was located in the cytoplasm, and no staining was seen in the nucleus (Fig. 7f). A mutant, NT*dl*919, that carried an even larger 5′ deletion and initiated translation at nucleotide 919 produced a polypeptide with the same subcellular localization (data not shown). These results are particularly interesting, since the NT*dl*814 and NT*dl*919 mutant 12S polypeptides were only altered at the N-terminus.

DISCUSSION

Much attention has been given to three regions in the first exon of the E1A gene that exhibit substantial homology among different adenovirus serotypes (44; for review, see reference 23). Mutational analyses have shown that two of these regions contain sequences that are necessary for E1A-mediated transcriptional repression and cotransformation of primary cells with a Ha-*ras* gene, while the third region is required for transcriptional activation but not for transformation or immortalization (Fig. 8 and references in the legend). Our experiments show that expression of the first exon of the 12S gene induces quiescent BRK epithelial cells to synthesize DNA and proliferate, although only transiently. Our results also show that the immortalization of primary epithelial cells, the induction of an epithelial cell growth factor(s), and the alteration of the epithelial cells so that they can be passaged require E1A sequences that lie outside the first 12S exon. These sequences are located within a 156-base-pair region near the 3′ end of the second exon (Fig. 8). It should be noted that the carboxy-terminal regions of Ad5, Ad7, and Ad12 E1A proteins have significant homology; 34 of 62 aa are identical or have conservative amino acid replacements (44).

Our results suggest that induction of the epithelial cell growth factor(s) in primary BRK cells is required for immortalization by the E1A 12S protein. We have previously shown that it is the epithelial cell population that both produces and responds to this growth factor (27). This suggests that this growth factor acts in an autocrine fashion. The one mutant, *dl*975/1339, that retained the ability to induce growth factors was also able to immortalize BRK cells. None of the 12S mutants that failed to induce growth factors were able to immortalize cells, although some of them retained some of the properties of the parental 12S virus, such as the ability to cooperate with an activated Ha-*ras* gene to transform cells or the ability to induce cellular DNA synthesis and proliferation. The ability of primary epithelial cells that expressed the 12S protein to be replated could also be due to production of the epithelial cell growth factor(s). Some growth factors bind to the extracellular matrix (6), which modulates the growth responses of epithelial cells (48). We know that this growth factor is found as part of a high-molecular-weight complex (27), which may reflect an interaction with the extracellular matrix. Epithelial cells rest on a basement membrane and attach to factors provided by the serum or to factors which they produce themselves (25). The growth factor could also serve as an attachment factor, enabling the epithelial cells to attach to the culture dish. Whether induction of the growth factor(s) is the only function of the C-terminal region of the 12S protein and whether this function is required for the immortalization of all primary cell types is not known. It has been found that some regions of the *myc* gene are required for the transformation of chicken macrophages but not embryo fibroblast cells (11) and for the transformation of rat embryo fibroblasts but not Rat1A cells (37).

Although the 12S first exon is unable to immortalize epithelial cells, it is able to cooperate with an activated *ras* gene to transform primary cells. That an activated *ras* gene can abrogate the need for functions encoded by the second exon is intriguing. Since *ras* proteins seem to be involved in mediating signal transduction (40, 45), it is possible that the normal *ras* protein interacts with the receptor for this growth factor.

Our results suggest that the 12S protein must effect several changes in quiescent primary BRK cells in order for the C-terminal region to induce the growth factor(s). For example, mutants that were altered within or missing the first 13 aa of the 12S protein stimulated cellular DNA synthesis and proliferation but were unable to induce growth factors or to immortalize cells. However, they could induce a growth factor(s) in a 12S-immortalized epithelial cell line derived from BRK cells. It is clear that these mutants are not missing sequences required for growth factor induction. The fact that the mutant defect could be complemented in *trans* by an established cell line suggests that the N-terminal region of the protein carries an activity that must be expressed in order for growth factor induction to occur. This activity is also required in order for E1A to cooperate with the Ha-*ras* gene to transform primary BRK cells (47). Subramanian and co-workers recently described a mutation that alters the region between aa 18 and 20 (39). This mutant is defective for growth factor induction, immortalization, and transformation, although it can still induce cellular DNA synthesis (39).

The behavior of the mutant *dl*891/1339 (Fig. 1) suggests that quiescent cells must be activated into the cell cycle for the growth factor(s) to be induced. This mutant, which is missing internal sequences but retains the 52-aa C-terminal region of the 12S protein that is required for growth factor induction, failed to stimulate cellular DNA synthesis, proliferation, or growth factor production. We do not have direct evidence that *dl*891/1339 is not missing sequences that are part of the E1A region that is directly involved in growth factor induction. However, mutants such as CT*dl*1339 and 12S *dl*A (Fig. 1), which are only missing the C-terminal region of the 12S protein, had very different properties. They were able to stimulate DNA synthesis and transient cell proliferation as well as the parental 12S virus did even though they failed to induce the growth factor(s). Thus, it seems likely that the ability of the 12S protein to stimulate primary BRK cell proliferation is a function that is independent of its ability to induce growth factors. It should be noted that transcriptional repression activity of the 12S protein could be important for activation of quiescent primary cells, although the transactivation function is not required (Fig. 8). Whatever number of biochemical steps are involved, the N-terminal half of the 12S protein seems to be able to initiate a new program of gene expression that allows quiescent primary cells to enter the cell cycle and proliferate. Perhaps the gene(s) encoding the growth factor(s) is resistant to activation until initiation occurs.

A nuclear localization signal has been identified within the last 5 aa of E1A proteins (20). However, we found that the absence of this signal does not exclude E1A protein from the nucleus. The CT*dl* series of mutants, which do not express this sequence, produced 12S proteins that were distributed in both the cytoplasm and the nucleus. Nor does the presence of the 5 C-terminal aa ensure total nuclear localization, because the 12S *dl*A polypeptide, which contains these aa, was not located totally in the nucleus. However, it is not necessary for all E1A proteins to be localized to the nucleus in order to obtain high levels of activity. CT*dl*976, which produced a polypeptide that corresponded to the N-terminal half of the 12S protein, induced DNA synthesis and proliferation and cooperated with an activated Ha-*ras* gene as well as wild-type 12S and *dl*975/1339 did. Furthermore, there was far more E1A protein present in the nucleus of these mutant-infected cells than in the nucleus of transformed cells (unpublished observations). Thus, we believe that the inability to induce growth factor production and immortalization is not due to insufficient amounts of nuclear E1A protein, but rather to the absence of sequences encoding particular functions. It also seems unlikely that the absence of the NT*dl*814 and NT*dl*919 12S polypeptides from the nucleus contributes to the functional defects described here, since the 12S from NT*dl*646, which had the same phenotype, was efficiently localized to the nucleus. However, it is possible that in some circumstances, defective localization could affect E1A phenotypes. For example, when the NT*dl*814 and NT*dl*919 mutations were present in an E1A genomic background, the viruses were partially defective in growth on HeLa cells, while the NT*dl*646 virus was not (unpublished observations).

The presence in the nucleus of mutant E1A polypeptides that were missing the C-terminal 5 aa suggests the presence of additional nuclear localization or retention signals. Multiple signals are necessary for the efficient localization of other nuclear proteins, such as polyomavirus large T antigen (28), c-*myc* (37), and nucleoplasmin (2, 4). The possible presence of another nuclear localization signal within the 32 aa encoded by the proximal region of the second exon is suggested by the distribution of E1A proteins in cells infected with CT*dl*1339 and *dl*311. There was a greater proportion of E1A protein in the nucleus than was found in CT*dl*976-infected cells. Seven aa (202 to 208; Arg-Pro-Thr-Arg-Arg-Pro-Lys) exhibited the same basic character as has been described for other nuclear localization signals, including E1A (Lys-Arg-Pro-Arg-Pro [20]), simian virus 40 large T antigen (17), polyomavirus large T antigen (28), and nucleoplasmin (2). It is possible that these aa represent a potential localization signal.

The exclusive cytoplasmic localization of the NT*dl*814 and NT*dl*919 12S polypeptides, which were only missing aa from the N-terminus, and the presence in the nucleus of proteins encoded by the CT*dl* series suggest that a localization or retention signal may be encoded in the first exon of the E1A gene. Since the 12S polypeptide produced by NT*dl*646 was completely localized to the nucleus, this signal is presumably encoded within the region between nucleotides 645 and 814. However, at this time we cannot rule out the possibility that the mutations caused conformational changes that affected the localization of E1A polypeptides. Experiments are in progress to determine the contribution(s) of these additional regions of E1A to nuclear localization and retention.

ACKNOWLEDGMENTS

We thank Carmella Stephens and Ed Harlow for the Ad5 and Ad5 10S viruses. We are grateful to W. Herr for critical reading of the manuscript. We are indebted to Marilyn Goodwin and Rita Duffy for their skill in preparing this manuscript. We thank P. Hinton and D. Chao for excellent technical assistance, J. Duffy, D. Greene, and M. Okler for excellent art and photographic work, and Diane Biedermann for careful animal work.

This work was supported by Public Health Service grant CA-13106 from the National Institutes of Health. M.P.Q. is a recipient of an NIH postdoctoral fellowship (F32CA97676) and funds from an institutional grant from the American Cancer Society (ACSIN153).

LITERATURE CITED

1. **Bellet, A. J. D., P. Li, E. T. David, E. J. Mackey, A. W. Braithwaite, and J. R. Cutt.** 1985. Control functions of adenovirus transformation region E1A products in rat and human cells. Mol. Cell. Biol. **5**:1933–1939.
2. **Burglin, T. R., and E. M. DeRobertis.** 1987. The nuclear migration signal of Xenopus laevis nucleoplasmin. EMBO J. **6**: 2617–2625.
3. **Carlock, L. R., and N. C. Jones.** 1981. Transformation-defective mutant of adenovirus type 5 containing a single altered E1A mRNA species. J. Virol. **40**:657–664.
4. **Dingwall, C., S. V. Sharnick, and R. A. Laskey.** 1982. A polypeptide domain that specifies migration of nucleoplasmin into the nucleus. Cell **30**:449–458.
5. **Gallimore, P. H., P. A. Sharp, and J. Sambrook.** 1974. Viral DNA in transformed cells. III. A study of the sequences of adenovirus 2 DNA in nine lines of transformed rat cells using specific fragments of the viral genome. J. Mol. Biol. **8**:49–72.
6. **Gordon, P. B., G. Conn, and V. B. Hatches.** 1985. Glycosaminoglycan production in cultures of early and late passage human endothelial cells: the influence of an anionic endothelial cell growth factor and the extracellular matrix. J. Cell Physiol. **125**: 596–607.
7. **Goustin, A. S., E. B. Leof, G. D. Shipley, and H. L. Moses.** Growth factors and cancer. Cancer Res. **46**:1015–1029.
8. **Graham, F. L., P. J. Abrahams, C. Mulder, H. L. Heijneker, S. O. Warnaar, F. A. J. deVries, W. Fiers, and A. J. Van der Eb.** 1974. Studies on in vitro transformation by DNA and DNA fragments of human adenovirus and simian virus 40. Cold Spring Harbor Symp. Quant. Biol. **39**:637–650.
9. **Haley, K. P., G. Overhauser, L. E. Babiss, H. S. Ginsberg, and N. C. Jones.** 1984. Transformation properties of type 5 adenovirus mutants that differentially express the E1A gene products. Proc. Natl. Acad. Sci. USA **81**:5734–5738.
10. **Harlow, E., B. Franza, and C. Schley.** 1985. Monoclonal antibodies specific for adenovirus early region 1A proteins: extensive heterogeneity in early region 1A products. J. Virol. **5**:533–546.
11. **Heaney, M. L., J. Pierce, and J. T. Parsons.** 1986. Site-directed mutagenesis of the *gag-myc* gene of avian myelocytomatosis virus 29: biological activity and intracellular localization of structurally altered proteins. J. Virol. **60**:167–176.
12. **Heldin, C.-H., and B. Westermark.** 1984. Growth factors: mechanism of action and relation to oncogenes. Cell **37**:9–20.
13. **Houweling, A., P. J. van der Elsen, and A. J. Van der Eb.** 1980. Partial transformation of primary rat cells by the leftmost 4.5% fragment of adenovirus 5 DNA. Virology **105**:537–550.
14. **Hunter, T.** 1985. Oncogenes and growth control. Trends Biochem. Sci. **10**:275–280.
15. **Hunter, T., and J. A. Cooper.** 1985. Protein tyrosine kinases. Annu. Rev. Biochem. **54**:897–930.
16. **Jones, N., and T. Shenk.** 1979. Isolation of adenovirus type 5 host range deletion mutants defective for transformation of rat embryo cells. Cell **17**:683–689.
17. **Kalderon, D., B. L. Roberts, W. D. Richardson, and A. E. Smith.** 1984. A short amino acid sequence able to specify nuclear location. Cell **39**:499–509.
18. **Lillie, J. W., M. Green, and M. R. Green.** 1986. An adenovirus E1a protein region required for transformation and transcriptional repression. Cell **46**:1043–1051.
19. **Lillie, J. W., P. M. Loewenstein, M. R. Green, and M. Green.** 1987. Functional domains of adenovirus type 5 E1a proteins. Cell **50**:1091–1100.
20. **Lyons, R. H., B. Q. Ferguson, and M. Rosenberg.** 1987. Pentapeptide nuclear localization signal in adenovirus E1a. Mol. Cell. Biol. **7**:2451–2456.
21. **Montell, C., G. Courtois, C. Eng, and A. Berk.** 1984. Complete transformation by adenovirus type 2 requires both E1A proteins. Cell **36**:951–961.
22. **Moran, E., T. Grodzicker, R. J. Roberts, M. Mathews, and B. Zerler.** 1986. Lytic and transforming functions of individual products of the adenovirus E1A gene. J. Virol. **57**:765–775.
23. **Moran, E., and M. B. Mathews.** 1987. Multiple functional domains in the adenovirus E1A gene. Cell **48**:177–178.
24. **Moran, E., B. Zerler, T. Harrison, and M. B. Mathews.** 1986. Identification of separate domains in the adenovirus E1A gene for immortalization activity and the activation of virus early genes. Mol. Cell. Biol. **6**:3470–3480.
25. **Orly, J., and G. Sato.** 1979. Fibronectin mediates cytokinases and growth of rat follicular cells in serum-free medium. Cell **17**: 295–305.
26. **Quinlan, M. P., and T. Grodzicker.** 1987. Adenovirus E1A 12S protein induces DNA synthesis and proliferation in primary epithelial cells in both the presence and absence of serum. J. Virol. **61**:673–682.
27. **Quinlan, M. P., N. Sullivan, and T. Grodzicker.** 1987. Growth factor(s) produced during infection with an adenovirus variant stimulates proliferation of nonestablished epithelial cells. Proc. Natl. Acad. Sci. USA **84**:3283–3287.
28. **Richardson, W. D., B. L. Roberts, and A. E. Smith.** 1986. Nuclear location signals in polyoma virus large T. Cell **44**:77–85.
29. **Roberts, B. L., W. D. Richardson, and A. E. Smith.** 1987. The effect of protein context on nuclear location signal function. Cell **50**:465–475.
30. **Ruley, H. E.** 1983. Adenovirus early region 1A enables viral and cellular transforming genes to transform primary cells in culture. Nature (London) **304**:602–606.
31. **Schneider, J. F., F. Fisher, C. R. Goding, and N. C. Jones.** 1987. Mutational analysis of the adenovirus E1a gene: the role of transcriptional regulation in transformation. EMBO J. **6**:2053–2060.
32. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
33. **Spindler, K. R., C. Y. Eng, and A. J. Berk.** 1985. An adenovirus early region 1A protein is required for maximal viral DNA replication in growth-arrested human cells. J. Virol. **53**:742–750.
34. **Sporn, M. B., and A. B. Roberts.** 1985. Autocrine growth factors and cancer. Nature (London) **313**:745–747.
35. **Stabel, S., P. Argos, and L. Philipson.** 1985. The release of growth arrest by microinjection of adenovirus E1A DNA. EMBO J. **4**:2329–2336.
36. **Stephens, C., and E. Harlow.** 1987. Differential splicing yields novel adenovirus 5 E1A mRNAs that encode 30 kd and 35 kd proteins. EMBO J. **6**:2027–2035.
37. **Stone, J., T. deLange, G. Ramsay, E. Jakobovits, J. M. Bishop, H. Varmus, and W. Lee.** 1987. Definition of regions in human c-*myc* that are involved in transformation and nuclear localization. Mol. Cell. Biol. **7**:1697–1709.
38. **Stow, N. D.** 1981. Cloning of a DNA fragment from the left-hand terminus of the adenovirus type 2 genome and its use in site-directed mutagnesis. J. Virol. **37**:171–180.
39. **Subramanian, T., M. Kuppuswamy, R. J. Nasr, and G. Chinnadurai.** 1988. An N-terminal region of adenovirus E1A essential for cell transformation and induction of an epithelial cell growth factor. Oncogene **2**:105–112.
40. **Tanabe, T., T. Nukada, Y. Nishikawa, K. Sugimoto, H. Suzuki, H. Takahashi, M. Noda, T. Haga, A. Ichiyama, K. Kangawa, N. Minamino, H. Matsuo, and S. Numa.** 1985. Primary structure of the α-subunit of transducin and its relationship to *ras* proteins. Nature (London) **315**:242–245.
41. **Taub, M.** 1984. Growth of primary and established kidney cell cultures in serum-free media, p. 3–24. *In* D. W. Barnes, D. A. Sirbasku, and G. H. Sato (ed.), Methods for serum-free culture of epithelial and fibroblastic cells. Alan R. Liss, Inc., New York.
42. **Tooze, J. (ed.).** 1981. DNA tumor viruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
43. **van den Elsen, P. J., A. Houweling, and A. Van der Eb.** 1983. Morphological transformation of human adenovirus is determined to a large extent by gene products of region E1A. Virology **131**:242–246.
44. **van Ormondt, H., J. Maat, and R. Dijkema.** 1980. Comparison of nucleotide sequences of the early E1a regions for subgroups A, B and C of human adenoviruses. Gene **12**:63–76.
45. **Wakelam, M. O., S. A. Davies, M. D. Houslay, I. McKay, C. J.**

Marshall, and A. Hall. 1986. Normal $p21^{N\text{-}ras}$ couples bombesin and other growth factor receptors to inositol phosphate production. Nature (London) **323**:173–176.

46. **Whittaker, J. L., P. J. Byrd, R. J. A. Grand, and P. H. Gallimore.** 1984. The isolation and characterisation of four adenovirus type 12-transformed human embryo kidney cell lines. Mol. Cell. Biol. **4**:110–116.
47. **Whyte, P., H. E. Ruley, and E. Harlow.** 1988. Two regions of the adenovirus early region 1A proteins are required for transformation. J. Virol. **62**:257–265.
48. **Wright, T. C., T. V. Johnstone, J. J. Costello, and M. J. Karnovsky.** 1985. Inhibition of rat cervical epithelial cell growth by heparin and its reversal by EGF. J. Cell. Physiol. **125**:499–506.
49. **Zerler, B., R. J. Roberts, M. B. Mathews, and E. Moran.** 1987. Different functional domains of the adenovirus E1A gene are involved in regulation of host cell cycle products. Mol. Cell. Biol. **7**:821–829.