Adenovirus E1A 12S Protein Induces DNA Synthesis and Proliferation in Primary Epithelial Cells in Both the Presence and Absence of Serum

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Infection of primary baby rat kidney (BRK) cells with an adenovirus that carries an E1A 12S cDNA in place of the normal E1A region (adenovirus 5 [Ad5] 12S) resulted in the induction of cellular DNA synthesis and proliferation of the epithelial cells in the population, even in the absence of serum. Increased cellular DNA synthesis was first detectable by 12 h after infection and was maintained at a 10- to 20-fold higher level than in mock-infected cells. By 5 days after infection there was a 10-fold-greater number of 12S virus-infected BRK cells. These infected BRK cells retained many of their normal epithelial cell characteristics and were not transformed. The expression of the E1A 12S protein(s) occurred early after infection. There was no induction of adenoviral gene expression or viral DNA replication in these cells. The early effects of a fully transforming gene product(s) were also examined. The Ad5-simian virus 40 hybrid virus, Ad5.SVR4, in which the early region of simian virus 40 has replaced the E1 region of Ad5, was used to infect BRK cells. The kinetics of expression of the T antigens were similar to those of the 12S polypeptides. Infection with Ad5.SV4 also resulted in the induction of cellular DNA synthesis and cell proliferation at levels similar to those observed with the 12S virus. However, infection with Ad5.SVR4 resulted in cells that had lost some of their epithelial cell characteristics and were fully transformed. Thus, although the early cellular events induced by the two genes were similar, they did not yield the same final cellular phenotype.

The E1A region of adenovirus encodes products important in both the lytic cycle of the virus and oncogenic transformation, although the biochemical functions of E1A proteins are not fully understood. Expression of the E1A region is required for efficient transcription of viral early genes (3, 4, 21, 27, 40) and also increases the transcription of several cellular genes either resident within the genome or carried on plasmids or viral vectors (14, 16, 28, 32, 41, 54, 62). Although no direct interaction between DNA and E1A protein(s) has been demonstrated, sequences that are required for transactivation by E1A have been identified for several different promoters (16, 26, 65). The E1A region is required for an efficient productive adenovirus infection of quiescent permissive cells and for the stimulation of cellular DNA synthesis (1, 35, 52, 53). The induction of G₁-arrested cells to progress into the S phase after infection by adenovirus is also a function of the E1A region (7, 8, 10, 43, 49, 55, 56, 58, 66). The cell cycle progression of growing cells is also altered by the expression of the E1A region (2, 38, 39).

Together with the E1B region, the E1A region is capable of complete transformation of primary rat cells (13, 15). In collaboration with other viral and cellular oncogenes, E1A is capable of transforming primary cells (47). E1A expresses activities that extend the growth potential of primary cells, resulting in cell lines that are capable of growing in vitro as established cell lines that show morphological alterations (24, 46, 67).

Two E1A mRNAs and their corresponding proteins are produced at early times after productive infection and in transformed cells. A 12S mRNA and a 13S mRNA are generated by differential splicing and encode proteins that are, respectively, 243 and 289 amino acids in length. Since

the splicing process maintains the same reading frame in both RNAs, the 289-amino-acid protein differs only in the presence of an additional internal 46-amino-acid stretch (4, 11, 29, 42).

The 12S and 13S proteins act with equal efficiency in conjunction with the products of the polyomavirus middle T antigen or T24 *ras* genes to produce transformed BRK cells which are tumorigenic in nude mice (67). Several independent studies have shown that the 12S protein has only a subset of the activities responsible for affecting transcription of viral and cellular genes (5, 9, 12, 17, 20, 25, 30, 35-37, 44, 57, 64, 67). The 13S protein is capable of transactivating viral early genes. The 12S protein expressed from an intact viral genome does not transactivate other adenoviral genes (17, 35, 37). Whether the 12S protein possesses any transactivating activity remains controversial and may depend upon the experimental system used. The 12S and 13S proteins both seem to stimulate DNA synthesis when microinjected into growth-arrested 3T3 cells (53). The 12S protein stimulates viral replication in quiescent cells (35, 52), suggesting that the 12S gene product may play a role in stimulating cell proliferation responses.

A virus that carries an adenovirus 2 (Ad2) E1A 12S cDNA in place of the normal E1A region is defective for transcriptional activation of viral early genes and thus replicates inefficiently in permissive human HeLa cells (37). Because the E1A 12S cDNA virus can be used to efficiently infect all of the cells in a culture and because it is not cytotoxic, we used it to study the early effects of the 12S gene product on primary baby rat kidney (BRK) cells. We found that the 12S gene product is able to stimulate cellular DNA synthesis as well as cellular proliferation. In addition, the 12S gene product induces the proliferation of the epithelial cell population, even in the absence of serum.

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MATERIALS AND METHODS

Cells and viruses. Cultures of BRK cells were prepared from 2-day-old rats (Fisher F344; Taconic Farms) as described previously (47). They were plated on 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) or on K1 medium (59). K1 medium was prepared by combining equal volumes of medium F12 (GIBCO) and DMEM that was supplemented with hydrocortisone (40 ng/ml), insulin (5 µg/ml), prostaglandin E₁ (250 ng/ml), selenium (10⁻⁸ M), transferrin (5 µg/ml), and triiodothyronine (4 ng/ml).

The construction of the 12S cDNA virus used in these studies has been described previously (37). The virus Ad5.SVR4 is an Ad5-simian virus 40 (SV40) recombinant virus in which the region of Ad5 between the *PvuII* and *BglII* sites (map units 1.3 to 9.4) is deleted and replaced by the *HpaII*-*BamHI* (nucleotides 346 to 2533) fragment containing the early region of SV40 (63; K. Van Doren, Ph.D. thesis, State University of New York at Stony Brook, Stony Brook, 1985). The viruses P111R2125 and 12SE1B⁻ are 12S viruses in which there are mutations in the 19K protein or 19K and 55K protein genes, respectively, and were provided by E. White (Cold Spring Harbor Laboratory). Viral stocks were propagated on 293 cells. Viral infections and titrations were performed as previously described (37). Micrographs were taken at 10× on a Nikon microscope with Tri-X Pan film, 400 ASA (Eastman Kodak Co.). Cells in culture dishes were fixed with methanol and stained with 2% Giemsa.

[³H]thymidine labeling. Cultures were incubated with 2 to 5 µCi of [³H]thymidine (20 Ci/mmol; Amersham Corp.) per ml of DMEM plus FCS or of K1 medium for various times. The medium was removed, and the cells were washed three times with phosphate-buffered saline (PBS) at 4°C, lysed with 0.3 N NaOH, and scraped into a tube. The lysates were neutralized by the addition of Tris hydrochloride (pH 7.6) to 0.15 M and an equal volume of 0.3 N HCl. Aliquots were assayed for total of incorporated counts by trichloroacetic acid precipitation and liquid scintillation counting.

Immunoprecipitation and electrophoresis. BRK cells in 60-mm culture dishes were labeled for 1 h with 100 µCi of L-[³⁵S]methionine (>1,000 Ci/mmol; New England Nuclear Corp.) in 1 ml of DMEM lacking methionine. At the end of the labeling period, the cells were placed on ice and washed with PBS at 4°C. The cells were lysed with 0.5 ml of 50 mM Tris hydrochloride, (pH 7.6)–1% Nonidet P-40–10 µM leupeptin (Boehringer Mannheim Biochemicals) and centrifuged for 15 min at 4°C in an Eppendorf centrifuge. The extracts were precleared with nonimmune serum and protein A-bearing *Staphylococcus aureus* Cowan 1. The immunoprecipitations were performed with anti-E1A (M73) or anti-T antigen (PAb 419) monoclonal antibodies obtained from E. Harlow (18, 19) or anti-E2 72K DNA-binding protein rabbit antiserum obtained from B. Stillman (Cold Spring Harbor Laboratory). The immune complexes were precipitated with protein A-Sepharose CL4B (Pharmacia, Inc.). The pellets were washed, suspended in sample buffer, boiled for 5 min, and separated on a 10% sodium dodecyl sulfate–polyacrylamide gel. The gels were fluorographed with 22% 2,5-diphenyloxazole (PPO) in dimethyl sulfoxide and exposed to SB-5 X-ray film (Kodak).

Indirect immunofluorescence. BRK cells were grown on 12-mm cover slips placed in tissue culture dishes and were fixed by incubation in methanol for 30 s at –20°C. They were rinsed in PBS and incubated with the antikeratin monoclonal

antibody LE61 (31) (diluted 1/10 with PBS) for 30 min at 37°C in a humidified chamber. The cover slips were washed three times with PBS at 22°C and incubated with goat anti-mouse immunoglobulin conjugated with rhodamine (Cooper Biomedical, Inc.) (diluted 1/50 with PBS) for 30 min at 37°C in a humidified chamber. The cover slips were again washed three times with PBS and once with water and mounted with glycerol-gelatin (Sigma Chemical Co.). Photographs were taken at 630× with Tri-X Pan film on a Leitz microscope with an epifluorescent light source.

Southern analysis. Viral DNA was extracted from infected BRK cells by the procedure of Hirt (23). A total of 20% of the DNA extracted from each dish was digested with *Sall* (New England BioLabs, Inc.) according to the supplier's specifications, loaded on an 0.8% agarose gel, and subjected to electrophoresis. Adenoviral sequences were detected by transfer of the DNA to nitrocellulose by the method of Southern (50), hybridization to nick-translated *dI309* DNA (33), and autoradiography on XAR-5 X-ray film (Kodak).

RESULTS

Induction of epithelial cell proliferation by the 12S cDNA virus. To examine the effects of the Ad2 12S gene product in primary cells, we made use of a virus (hereafter referred to as the 12S virus) that carries an Ad2 12S cDNA in place of the normal E1A region (37). Because of the method of construction, the 12S virus carries Ad2 sequences from the left end of the genome to the *XbaI* site (base pair 1336) located at the distal end of the E1A region. The rest of the viral genome is composed of Ad5 sequences derived from the nondefective virus Ad5 *dI309* (27).

Primary BRK cultures isolated from 2-day-old rats and plated in DMEM plus 5% FCS consisted of a mixture of fibroblasts and epithelial cells (Fig. 1). Because the presence of serum optimized fibroblast and not epithelial cell growth, the epithelial cell population decreased rapidly until only a sparse, nonproliferating monolayer of fibroblasts remained (Fig. 1). However, if these cultures were infected 2 to 3 days postplating with the 12S virus at a multiplicity of infection (MOI) of 10, there was rapid cellular proliferation (Fig. 1 and 2). Microscopic examination of these infected cultures revealed that the epithelial cell subpopulation exhibited a striking proliferative response (Fig. 1). In mock- or Ad5 *dI312* (which contains a large E1A deletion [27])-infected cultures, the epithelial cells decreased in number and disappeared by about 5 to 6 days postplating (unpublished results; see Fig. 1 and 2). Epithelial cell proliferation was first detectable in the 12S virus-infected cells between 24 and 36 h postinfection. Initially, the epithelial cells formed discretely delineated islands, with tightly packed cells. By about 3 days postinfection, the islands enlarged and covered most of the surface of the dish (Fig. 2), obscuring the fibroblasts. By 5 days postinfection, there was a 10-fold-greater number of cells in cultures infected with the 12S virus than in those infected with Ad5 *dI312* (data not shown). These rapidly growing areas thus prevented the observation of any effect on the fibroblasts in the population. By about 8 to 10 days postinfection, large numbers of cells detached from the dish, although epithelial cell growth continued. Many of the cells that detached from the dish early after infection continued to proliferate in suspension for 3 to 4 days (data not shown). By 2 to 3 weeks postinfection, discrete foci of cells were found, presumably representing those cells that had stably integrated adenoviral DNA into their genomes (see below and reference 37).

FIG. 1. Proliferation of 12S virus- and Ad5.SVR4-infected BRK epithelial cells in the absence and presence of serum. Primary BRK cells from 2-day-old rats were infected 2 days postplating with the viruses indicated at the left and maintained in the medium indicated at the top. The micrographs were taken at 36 h postinfection (hpi) or 12 days postinfection (dpi). DME = DMEM. KI-1 = K1. Ad5.SV4 = Ad5.SVR4.

The 12S virus-infected cells retained their epithelial cell morphology (Fig. 1). They were difficult to trypsinize and exhibited a poor plating efficiency. However, if 12S virus- but not mock- or *dl312*-infected cells were trypsinized and replated on their respective dishes, epithelial cells did attach. Cytokeratins are those intermediate filament proteins which are specifically expressed by epithelial cells (for a review, see reference 34). Therefore, the expression of cytokeratins by 12S virus-infected BRK cells was examined by indirect immunofluorescence with a monoclonal antibody, LE61, which recognizes cytokeratins from most soft or simple nonsquamous internal epithelial cells (31). Cells within the enlarging epithelial cell islands demonstrated immunofluorescence staining typical of cytokeratin filaments (Fig. 3).

The degree and rapidity of the proliferative response was dependent upon the MOI used (data not shown). When cells were infected with the 12S virus at an MOI of less than 1 PFU per cell, a general proliferative response was not seen, although some foci appeared after several weeks. In general, maximum cellular proliferation was obtained after infecting cells with the 12S virus at an MOI of 5 to 20. At an MOI of 50 to 100, there was extensive cellular proliferation, but some cytotoxic effects were also observed. These were probably due to some expression of E1A-dependent adenoviral genes. Such multiplicity-dependent leakiness of E1A mutants has been well documented (45, 48).

Infection of BRK cells with wild-type virus or a virus that carries an E1A 13S cDNA (13S virus [37]) resulted in extensive cell death (Fig. 2) starting at 24 to 36 h postinfection.

FIG. 2. Proliferation of primary BRK cells infected with an E1A 12S cDNA virus. BRK cells were infected with the designated virus at 2 days postplating. At 3 days postinfection, the cells were fixed with methanol and stained with 2% Giemsa.

tion. This result was probably due to an increased level(s) of viral gene products, such as the 72 K DNA-binding protein, that are cytotoxic. As expected, small numbers of transformed foci could be obtained after infection of cells with wild-type virus at a low MOI (<0.01 PFU per cell) (data not shown; see reference 37 and references therein). 12S viruses in which deletions within the E1B region inactivate the 19K protein or both the 19K and 55K protein genes were con-

structed (see Materials and Methods). Infection of primary BRK cells with these viruses induced extensive cell proliferation, suggesting that E1B gene products play no role in this response (data not shown). Together, these data indicate that the stimulus for primary epithelial cell proliferation after infection with the 12S virus is the 12S protein itself (see below). Cell lines were derived from 12S virus-infected BRK cells which integrated the 12S gene and continued to express the 12S protein (data not shown).

Induction of cellular DNA synthesis in 12S virus-infected cells. To examine levels of cellular DNA synthesis, we incubated cultures of primary BRK cells infected with the 12S virus or Ad5 dl312 with [3 H]thymidine at different times postinfection and for increasing times up to 24 h. Figure 4A illustrates the results obtained when cells were infected and pulse-labeled for 1 h at 4-h intervals over a 24-h time period. The induction of cellular DNA synthesis by the 12S virus was a very early event: by 12 h postinfection there was already evidence of increased incorporation of [3 H]thymidine into DNA. No increased cellular DNA synthesis was observed in dl312-infected cells. The intracellular levels (data not shown) and the amount of incorporated [3 H]thymidine were always greater in 12S virus-infected cultures than in dl312-infected cultures (Fig. 4). Cultures were also labeled for 24-h intervals every day for 1 week postinfection. A 20- to 40-fold increase in [3 H]thymidine incorporation in 12S virus-infected cultures as compared with dl312-infected cultures was observed at all times examined (Fig. 4 and data not shown). No viral DNA replication was detected (see below). Thus, the increase in DNA synthesis was due to cellular DNA replication.

Although the presence of the 12S gene product induced cellular DNA synthesis and cellular proliferation, it did not enable the cells to grow in soft agar (data not shown).

Absence of viral DNA synthesis in 12S virus-infected BRK cells. To examine the levels of adenoviral DNA in infected cells, we infected BRK cells with the 12S virus or Ad5 dl312; at different days postinfection, extracted low-molecular-weight (Hirt) DNA and analyzed by Southern hybridization to a nick-translated probe of genomic adenoviral DNA. As expected, the levels of adenoviral DNA were similar in both 12S virus- and dl312-infected cells (Fig. 5). The levels of adenoviral DNA in the Hirt supernatants from both 12S

FIG. 3. Expression of cytokeratin(s) by 12S virus-infected BRK cells. Primary BRK cells infected with the 12S virus were fixed 3 days postinfection and processed for indirect immunofluorescence with the antikeratin monoclonal antibody LE61 (31) as described in Materials and Methods. Panels A and B represent two different fields.

FIG. 4. Induction of cellular DNA synthesis in BRK cells infected with 12S virus and Ad5.SVR4. At 2 days postplating, BRK cells were infected with 12S virus (○); Ad5 *dl312* (▲), or Ad5.SVR4 (●). They were incubated with [³H]thymidine for 1 h at 4-h intervals postinfection (A) or for 24-h intervals each day postinfection (B). At the end of the labeling period the cells were lysed, and the levels of [³H]thymidine incorporation were determined. (B) Levels of [³H]thymidine incorporation at 1 and 6 days postinfection. Ad5.SV4 = Ad5.SVR4.

FIG. 5. Southern analysis of adenoviral sequences in infected BRK cells. BRK cells were infected with 12S virus or Ad5 *dl312*. At the days postinfection (p.i.) indicated at the top, low-molecular-weight Hirt DNA was extracted and digested with *SalI*, which cuts Ad5 DNA three times and produces fragments of 19.5, 9.4, 6.8, and 0.3 kilobases (the last fragment has migrated off the gel). DNA from 20% of a 60-mm culture dish was loaded in each lane. The marker lane (M) contains purified genomic Ad5 *dl309* DNA cut with *SalI* and is equivalent to 2×10^4 copies per genome equivalent. The DNA was transferred to nitrocellulose and hybridized to ³²P nick-translated Ad5 *dl309* DNA. The exposure time was 2 h, resulting in the overexposure of the marker lane.

virus- and *dl312*-infected cells decreased every day postinfection. Thus, the observed incorporation of [³H]thymidine was due to cellular DNA synthesis and not viral DNA replication. By about 2 weeks postinfection, very few cells remained in mock- or *dl312*-infected cultures, while foci of immortalized cells were present in the 12S virus-infected cultures. Examination of chromosomal DNA isolated from these cultures demonstrated hybridization to adenoviral DNA, presumably representing the stable integration of adenoviral sequences into the genomes of these cells (data not shown).

Adenoviral gene expression in infected BRK cells. In permissive human cells, the 12S virus expresses the 12S mRNA and protein but is defective for the expression of other early viral mRNAs and proteins (37). These results suggest that the induction of cellular proliferation in primary BRK cells is due, directly or indirectly, to the activity of the 12S protein itself. To examine the levels of expression of early viral proteins, we infected BRK cells with the 12S virus, wild-type Ad5 *dl309*, or Ad5 *dl312* or mock infected them. At different times postinfection, the cells were metabolically labeled with [³⁵S]methionine for 1 h. Labeled cell extracts were immunoprecipitated with antibodies directed against E1A proteins or the 72K DNA-binding protein encoded by the E2 region. In wild-type-infected BRK cells, both 12S and 13S gene products were immunoprecipitated from cell lysates by a monoclonal antibody, M73, which recognizes both 12S and 13S polypeptides (19). The levels of expression of 12S and 13S proteins were similar (Fig. 6D).

In cultures infected with the 12S virus, as expected, only the 12S-specific protein was detected. The amount of 12S

FIG. 6. Expression of E1A proteins in infected BRK cells. Primary BRK cells were infected with the indicated viruses. They were incubated with [35 S]methionine for 1 h until the hour postinfection (hpi) indicated at the top of each lane. The cells were lysed, and immunoprecipitations were done with anti-E1A monoclonal antibody M73 (19). The immunoprecipitates were analyzed on 10% sodium dodecyl sulfate–polyacrylamide gels, which were fluorographed. The arrowheads indicate the position of actin migration in these gels. The positions of the 13S (○) and 12S (●) polypeptides are indicated. Mr(kD), Molecular masses (in kilodaltons) of 14 C-labeled marker proteins.

protein immunoprecipitated from 12S virus-infected cells was two- to threefold lower than the amount of 12S protein detected in wild-type-infected cells (Fig. 6). The 12S polypeptide could be detected at 6 h postinfection, and maximal synthesis was observed between 12 and 18 h postinfection. It should be noted that maximal E1A protein concentrations in infected human cells occur between 14 and 18 h postinfection (51). Northern analysis of cytoplasmic RNA isolated from 12S virus-infected cells showed that the E1A 12S but not the 13S RNA could be detected between 6 and 24 h postinfection (data not shown).

The amount of 72K protein that was immunoprecipitated

from 12S virus-infected BRK cells was about 50-fold lower and was detectable much later than in wild-type-infected BRK cells (Fig. 7). The amount of 72K protein detected in 12S virus-infected cells resembled that found in *d1312*-infected cells (data not shown). Thus, the 12S protein did not increase the expression of the E2 72K protein gene. These results again suggest that the induction of cellular DNA synthesis and epithelial cell proliferation observed after infection with the 12S virus are due to the 12S gene product(s).

Effect of SV40 T antigen in BRK cells. Because the 12S virus does not fully transform primary rat cells, we wanted

to compare the effects observed with the 12S gene product with those observed with a transforming gene product. The early region of SV40 can fully transform primary rat cells (see reference 61 and references therein). The Ad5-SV40 hybrid virus Ad5.SVR4 contains the SV40 early region in place of the Ad5 E1 region (63; Van Doren, Ph.D. thesis), and the large- and small-T-antigen sequences are expressed from the SV40 early promoter. The adenoviral sequences of Ad5.SVR4 correspond to those found in the Ad5 12S virus. Adenoviral gene expression in Ad5.SVR4-infected BRK cells resembled that observed in 12S virus-infected BRK cells (data not shown). Thus, the SV40 gene products do not activate adenoviral gene expression. Therefore, we can readily compare the effects of the 12S gene product and the early region of SV40 in primary BRK cells.

BRK cells were infected with Ad5.SVR4 or the 12S virus and metabolically labeled with [35 S]methionine at different times postinfection. Labeled cell extracts were immunoprecipitated with monoclonal antibody PAb 419, which recognizes large T antigen (18), or with monoclonal antibody M73 (19). The kinetics of expression of T antigen were similar to that of the E1A 12S protein (Fig. 8). T antigen could be detected by 8 h postinfection, by which time the E1A 12S protein could also be detected. Maximal amounts of both T antigen and the E1A 12S protein were detected at about 18 h postinfection.

BRK cells infected with Ad5.SVR4 showed increased levels of cellular DNA synthesis that were similar to those found in 12S virus-infected cells (Fig. 4). As expected, viral DNA synthesis did not occur (data not shown). The BRK cells also proliferated in response to T antigen (Fig. 1). However, Ad5.SVR4-infected BRK cells, unlike those infected with the 12S virus, exhibited a very high plating efficiency. At very early times postinfection, clones of Ad5.SVR4-infected BRK cells could be expanded into mass

FIG. 7. Levels of E2 72K DNA-binding protein in infected BRK cells. BRK cells were infected with 12S virus or Ad5 d1309. They were labeled with [35 S]methionine for 1 h until the hour post infection (hpi) indicated at the top of each lane. Immunoprecipitations were done with anti-E2 72K DNA-binding protein rabbit antiserum (B. Stillman, Cold Spring Harbor Laboratory). The immunoprecipitates were analyzed on a 10% sodium dodecyl sulfate-polyacrylamide gel, which was fluorographed. Mr(kD), Molecular masses (in kilodaltons) of 14 C-labeled marker proteins.

FIG. 8. Similar levels of viral gene expression in infected BRK cells grown in DMEM or K1 medium. BRK cells were infected with the indicated viruses and maintained in DMEM plus FCS or K1 medium thereafter. The cells were labeled with [35 S]methionine for 1 h until the hour postinfection (hpi) indicated at the top of each lane. The cells were lysed, and immunoprecipitations were done with the anti-E1A monoclonal antibody M73 (19) for 12S virus-infected cells or the anti-large-T-antigen monoclonal antibody PAb 419 (18) for Ad5.SVR4-infected cells. The positions of the 12S and large-T (LT) polypeptides are indicated. The migrations (in kilodaltons) of 14 C-labeled marker proteins are indicated in lane Mr. DME = DMEM. K1-1 = K1. Ad5.SV4 = Ad5.SVR4.

cultures. These cells also grew well in soft agar (data not shown). These characteristics probably reflected their completely transformed character and the loss of some epithelial cell characteristics. Furthermore, although Ad5.SVR4-infected BRK cells exhibited cytokeatin filaments at early times postinfection, by 3 days postinfection there was evidence of disarranged cytokeatins (data not shown). Thus, both the E1A 12S protein and SV40 T antigens induce cellular DNA synthesis and epithelial cell proliferation.

Proliferation of infected BRK cells in the absence of serum. Both 12S virus- and Ad5.SVR4-infected BRK cultures showed extensive proliferation of the epithelial cell popula-

tion under conditions which are not supportive of epithelial cell growth and are, in fact, inhibitory, i.e., the presence of serum. We examined the ability of these cells to grow in a serum-free, hormonally defined medium, K1 (see Materials and Methods), which was developed to optimize kidney epithelial cell growth and is inhibitory to fibroblast growth (59, 60). The only growth factor in K1 medium is insulin. When BRK cells were initially plated in K1 medium, no fibroblasts grew, but the life-span of the epithelial cells was still only 4 to 5 days. Thus, although K1 medium enriches the epithelial cell population, it cannot extend their life-span in cultures. The same was true when BRK cells were plated in DMEM plus 5% FCS and transferred to K1 medium 2 to 3 days postplating.

Cells were plated in DMEM plus 5% FCS and infected 2 to 3 days later with the 12S virus, Ad5 *dl*312, or Ad5.SVR4 or mock infected. Thereafter, they were maintained in K1 medium. The epithelial cells in the 12S virus- and Ad5.SVR4-infected cultures proliferated as well in K1 medium as in DMEM plus 5% FCS (Fig. 2). At the same time, there was a rapid decline in the number of fibroblasts. This decline was expected, since this medium does not support fibroblast growth. No epithelial cell growth was observed in Ad5 *dl*312- or mock-infected cultures (Fig. 2). BRK cells infected with the 12S virus or Ad5.SVR4 synthesized cellular DNA at similar rates in K1 medium and DMEM (data not shown). To determine whether infected BRK cells could grow in DMEM in the absence of serum, we plated BRK cells on DMEM plus 5% FCS. Subsequent to infection, they were transferred to DMEM without serum for 2 days. During that time period, the epithelial cells continued to proliferate in the 12S virus-infected cultures (data not shown). Cultures could not be maintained for extended time periods in the absence of serum. The number of fibroblasts decreased rapidly in the absence of serum (data not shown). Thus, the 12S protein and the SV40 T antigen can induce cellular DNA synthesis and cell proliferation even in the absence of serum.

It should be noted that the levels of adenoviral gene expression in the 12S virus- and Ad5.SVR4-infected cultures maintained in K1 medium or DMEM plus FCS were similar. The expression of T antigen and 12S protein were similar under both culture medium conditions (Fig. 8). While the level of expression of the E2 72K protein was slightly lower in K1 medium, viral DNA failed to replicate in either medium (data not shown).

DISCUSSION

Primary BRK epithelial cells in culture rapidly cease growth and die, even in the presence of serum. We have shown that infection with an adenovirus that only produces the E1A 12S protein induces these cells to replicate their DNA and proliferate in the presence or absence of serum. These results are consistent with the observations of Spindler et al. (52) that a mutant adenovirus that produces the 12S but not the 13S protein can induce cellular DNA synthesis in a growth-arrested rat fibroblast cell line.

Although 12S virus-infected BRK cells enter a continuous proliferation state, they are not transformed. They exhibit a poor plating efficiency and do not grow in soft agar. They retain their epithelial cell phenotype at least with respect to morphology, ability to grow in K1 medium, low plating efficiency, and expression of cytokeratins. Thus, the 12S gene product of adenovirus could be used to prolong the life-span in culture of primary epithelial cells that maintain at least some of their normal characteristics.

The ability of the 12S gene product to induce cellular DNA synthesis and epithelial cell proliferation may serve both the lytic and transformation pathways of adenovirus. Adenovirus normally infects terminally differentiated, noncycling epithelial cells that are arrested in G₀ (61, 66). To undergo replication, the virus makes use of the DNA synthetic machinery of the host. It has been shown that the 12S gene product is necessary for maximal viral replication in quiescent permissive cells (35, 52) but not in growing permissive cells (36). This may be the reason for the conservation of the 12S gene product in all of the adenovirus serotypes (61).

The presence of the 12S gene product enhances the transformation frequency of adenovirus (17, 35). Furthermore, a round of cell division seems to be required to establish cellular transformation (61). The 12S gene product of the E1A region induces the cellular DNA synthesis observed in adenovirus-infected semipermissive primary fibroblast cells and serum-starved rat cell lines (52). These data and the data reported here suggest that the presence of the 12S gene product overcomes quiescence and shifts cells into a proliferative state, either directly or indirectly.

The 12S protein itself seems to be responsible for the induction of epithelial cells to enter the S phase and divide. Viral gene expression in 12S virus-infected BRK cells is similar to that in Hela cells (37); thus, other viral early genes are poorly expressed, and adenoviral DNA replication does not occur. Therefore, the induction of cellular DNA synthesis does not require the expression of other adenoviral genes. Although both the E1A and E1B regions are needed for transformation by adenovirus (13, 15), the absence of E1B expression does not inhibit or alter the effect of the 12S protein on primary epithelial cells. Whether the 13S gene product can effect the same response(s) is difficult to determine because of its ability to activate viral gene expression, which leads to cytotoxicity. However, it was previously demonstrated that an adenovirus (Ad5 *pm*975) that expresses only the 13S-encoded 289-amino-acid protein induced much less cellular DNA synthesis in a serum-starved rat cell line than either an adenovirus (Ad5 *dl*1500) that expresses only the 12S-encoded 243-amino-acid protein or wild-type virus (52).

The mitogenic effects of the 12S protein are probably not due to the E1A transcription transactivation activity, because 12S protein does not exhibit this phenotype (17, 24, 36, 37, 51). However, it is possible that the 12S protein alters the expression of genes involved in cell cycle progression. Both E1A proteins have been reported to repress the activity of certain viral and cellular enhancers (5, 6, 22, 64). Cell cycle progression may be inhibited by certain cellular gene products. Perhaps repression of their expression by E1A proteins releases cells from growth arrest.

Both adenovirus and SV40 can transform BRK cells. The early region of SV40 is able to fully transform these cells, while the 12S protein cannot. Cells expressing large T antigen after infection with Ad5.SVR4 lost some of their epithelial cell characteristics, were easily passaged, and were able to grow in soft agar. Cells infected with the 12S virus were not able to do so. Thus, the T antigen provides additional functions to those specified by the 12S protein. Both of the genes induced cellular DNA synthesis and proliferation in the absence of serum, suggesting that these are characteristics important for the immortalization of BRK cells. Whether the common phenotypes induced by the genes are produced by similar mechanisms remains to be determined.

The serum independence and uncontrolled proliferation

induced by the E1A 12S gene product in BRK cells are also important characteristics of transformed cells. These properties could be induced by several different mechanisms. First, for example, the 12S virus-infected cells themselves could produce a growth factor(s) which provides the stimulus for proliferation in an autocrine fashion. Second, because growth factors mediate their effect via cell surface receptors, the cellular receptors could be altered to behave as if they were always stimulated. Finally, the series of intracellular events that normally lead to the initiation of DNA synthesis following growth factor stimulation could be constitutively active. Preliminary data suggest that the production of a growth factor by BRK cells infected with the 12S virus is at least one component of the transformation pathway of the 12S protein.

The majority of human tumors are carcinomas. Most in vitro transformation experiments have been done with fibroblasts, although malignant tumors of nonepithelial cells account for only 10 to 20% of human neoplasms. The 12S gene could be used to immortalize primary epithelial cells that could serve as a model system(s) in the study of carcinogenesis.

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