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Growth factor(s) produced during infection with an adenovirus variant stimulates proliferation of nonestablished epithelial cells

(*E1A* genes)

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ABSTRACT **Infection of primary baby rat kidney cells with an adenovirus variant that encodes only the 12S gene of the E1A region, adenovirus type 5 (Ad5) 12S, results in the production of a growth factor that stimulates primary epithelial cells to proliferate. Increased epithelial cell DNA synthesis and proliferation is detectable between 24 and 36 hr after the addition of conditioned medium from Ad5 12S infected cells and not from cells infected with an E1A deletion mutant virus, Ad5 dl312. This mitogenic factor(s) is effective in the absence of serum and can override the inhibitory effect of serum on primary epithelial cells. Furthermore, there is a requirement for the continued presence of the growth factor(s) in the Ad5 12S conditioned medium to maintain epithelial cell proliferation, and the conditioned medium can maintain these cells in a proliferative state for at least 6 wk. The stimulatory activity in Ad5 12S conditioned medium is associated with large molecular weight complexes, from which it can be released by 4 M NaCl. Several characteristics of the growth factor(s) indicate that it is a unique mitogen for epithelial cells.**

The growth of normal animal cells is, in part, controlled by polypeptide growth factors that exert their effect(s) by way of plasma membrane receptor(s) (for review, see ref. 1). In contrast, many transformed cells have a decreased growth factor requirement and are able to proliferate in the absence of exogenous growth factors. This may be due to alterations in growth factor receptors, to the autocrine production of growth factors by transformed cells, or to constitutive alterations in growth factor-controlled processes (2, 3). Much recent evidence has linked growth factors and oncogenes (for review, see refs. 1, 4, and 5).

The growth of normal cells *in vitro* requires the correct culture conditions and, although the growth of fibroblasts and established epithelial cells may proceed in the presence of serum, many primary epithelial cells are, in fact, inhibited by serum components (6). Consequently, the study of epithelial cell transformation *in vitro* has proved difficult. However, human and rodent primary epithelial cells can be transformed using the DNA tumor virus adenovirus (7).

Adenoviruses contain two early transcription units, E1A and E1B, that are required for the complete transformation of primary cells (7, 8). The products of the E1A region can immortalize primary cells (9, 10) and cooperate with other viral genes and cellular oncogenes to transform primary rat cells (11). Expression of E1A proteins leads to the induction of cellular DNA synthesis and cell cycle progression (12–14). E1A gene products have been shown to regulate transcription of other adenoviral early genes as well as some cellular genes (15–18). At early times after infection and in transformed cells, two E1A transcripts, designated the 13S and 12S messages, 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 amino acids in the 289 amino acid protein. The 12S gene product is required to produce virus in growth-arrested permissive cells (19, 20) but is not responsible for the activation of other adenoviral promoters that is required for a productive infection in human cells. The 13S and the 12S gene products can immortalize primary baby rat kidney (BRK) cells and cooperate with Ha-*ras* to fully transform them (10).

We have shown that a variant of adenovirus, adenovirus type 5 (Ad5) 12S, which contains a cDNA copy of the 12S message (21) in place of the normal E1A region, induces cellular DNA synthesis and epithelial cell proliferation in BRK cells in the absence of serum (14). We have now found that Ad5 12S infected BRK cells produce a growth factor(s) that induces quiescent primary epithelial cells to synthesize DNA and proliferate. Some of the characteristics of this factor(s) are presented.

MATERIALS AND METHODS

Cells and Viruses. Primary BRK cells were prepared (11) and maintained as described (14) from 2- or 6-day-old Fisher rats (Taconic Farms, Germantown, NY). All viral stocks were propagated on 293 cells. Viral infections and titrations were performed as described (21). The viruses P111R2125 and 12SE1B$^-$ are 12S viruses in which there are mutations in the genes that encode the M_r 19,000 protein or the M_r 19,000 and M_r 55,000 proteins, respectively, and were provided by E. White (Cold Spring Harbor Laboratory).

Conditioned medium was harvested after 2, 3, or 4 days as indicated, filtered through a 0.2-μm filter (Nalgene), and stored at 4°C. To determine the kinetics of factor(s) production, the media were removed, saved, and replaced with fresh media at 12-hr intervals. When conditioned K1 medium and Dulbecco's modified Eagle's medium (DMEM) without serum were used, they were supplemented with an equal volume of the appropriate fresh medium before testing on BRK cells. Conditioned medium was replaced twice a week with fresh conditioned medium. Treatments of conditioned media were as described (22).

[^{3}H]Thymidine Labeling and Emulsion Autoradiography. Cultures were incubated with 5 μCi (1 Ci = 37 GBq) of [^{3}H]thymidine per ml of conditioned medium generated in DMEM or K1 medium for the time periods indicated and processed as described (14). Rat kidney epithelial cells were prepared from the nephrons of kidneys of 1-wk-old Fisher rats. Cells were grown for 5 days in K1 medium, trypsinized, and replated at 5×10^5 cells per 35-mm dish in K1 medium. After 1 day, the medium was replaced with medium conditioned by BRK cells infected with Ad5 12S or Ad5 dl312, to which was added 25 μCi of [^{3}H]thymidine. The cultures were incubated for 4 days. Cells were washed twice with phos-

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Abbreviations: BRK, baby rat kidney; Ad5, adenovirus type 5; EGF, epidermal growth factor; TGF-α, transforming growth factor type α.

phate-buffered saline [8 mM Na_2HPO_4/1.5 mM KH_2PO_4/2.5 mM KCl/140 mM NaCl (PBS)], fixed with methanol, −20°C, for 30 sec, and exposed to emulsion (Kodak NTB2) for 30 hr at 4°C. They were developed in one-half strength D19 (Kodak) and photographed using a 32× lens and Kodak technical Pan 2415 film.

Growth Curve Analysis. Nephrons were prepared from the kidneys of 7-day-old Fisher rats and plated in K1 medium. At 4 days after plating, cells were trypsinized and replated in K1 medium at a density of 40,000 cells per 35-mm dish. The following day, the media were replaced with various conditioned or normal media. Five days later, some cultures that had been maintained in 12S conditioned medium were changed into dl312 conditioned medium. Duplicate samples were analyzed for cell number by counting in a Coulter Counter (Coulter).

RESULTS

Cell Proliferation Induced by Conditioned Medium from Adenovirus 12S Infected Cells. Primary BRK cultures isolated from 2-day-old rats and plated in DMEM with 5% fetal calf serum consist of a mixture of fibroblasts and epithelial cells. Because fetal calf serum optimizes fibroblast and not epithelial cell growth, the epithelial cell population decreases until after 5–7 days only a monolayer of fibroblasts remains. However, infection with an Ad5 12S virus induces the epithelial cell population to undergo DNA synthesis and proliferation, even in the absence of serum (14). This raised the possibility that infected BRK cells produce a growth factor(s) that induces cellular DNA synthesis and subsequent cell division. To investigate this, primary BRK cells were infected with the Ad5 12S virus. At 3 days after infection the conditioned medium was collected from infected cells and filtered to remove cells and debris. The conditioned medium was added to new uninfected BRK cells 2–3 days after plating. Epithelial cell proliferation was first detectable as increased DNA synthesis and cell number between 24 and 36 hr after the addition of conditioned medium (data not shown). By 3–4 days, extensive cell growth was observed (Fig. 1). These cells are epithelial with respect to their morphology, poor plating efficiency, and expression of cytokeratins using epithelial cell-specific anticytokeratin antibody (ref. 14; data not shown). Conditioned media obtained from cells infected with a virus having an E1A deletion, Ad5 dl312 (23), or from mock-infected cells failed to induce cellular proliferation when added to BRK cultures (Fig. 1).

Conditioned media from Ad5 12S infected cells generated in the absence of serum was also able to induce proliferation when added to BRK cells. These media were generated in two ways. BRK cells were maintained and infected with the 12S virus in DMEM with fetal calf serum and changed into DMEM without serum for 2 days between 1 and 5 days after infection. After the 2-day period, medium was collected and analyzed for growth-stimulating potential. Alternatively, BRK cells were either plated and infected in K1 medium, a serum-free, hormonally defined medium developed for kidney epithelial cells (6), or were plated in DMEM with serum and subsequently reincubated in K1 medium after infection. 12S conditioned media were collected and supplemented with an equal volume of the respective fresh media before addition to BRK cells. Both of these types of 12S conditioned media stimulate epithelial cell proliferation (Fig. 1 and data not shown). The rapid proliferation of the epithelial cells and the decrease in the fibroblast population upon addition of conditioned medium from Ad5 12S infected BRK cells suggests the presence of an epithelial cell proliferation factor(s).

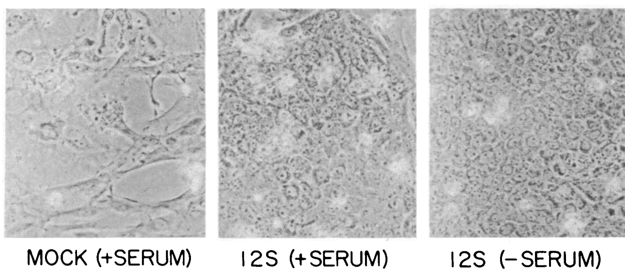

FIG. 1. Ad5 12S conditioned medium generated in the absence of serum induces epithelial cell proliferation. Primary BRK cells were incubated with conditioned medium generated by BRK cells infected with Ad5 12S or mock-infected and maintained in DMEM in the absence (−) or presence (+) of fetal calf serum. Photomicrographs were taken 3 days after the addition of conditioned medium. These photographs were taken of a representative field from a tissue culture dish. (×10.)

Epithelial cell proliferation could be maintained for at least 6 wk in the presence of fresh 12S conditioned medium generated in serum (data not shown). In addition, these cells retained their epithelial cell morphology, expression of cytokeratins, and other characteristic protein markers (Michael Lambert, M.P.Q., N.S., and T.G., unpublished data). We have, at present, no direct evidence whether the factor(s) responsible for epithelial cell proliferation is secreted into the medium or is released due to cell lysis. Conditioned medium collected at various times after infection was tested for its ability to stimulate BRK cell proliferation, as described above. High levels of stimulatory activity were first detected in media harvested at 24–36 hr after infection and continued to be produced up to 6 days after infection (data not shown). No activity was detectable in media harvested in similar ways from Ad5 dl312 infected cells. Cellular extracts made from 12S infected BRK cells at 24 hr up to 6 days after infection were also able to induce BRK epithelial cell proliferation when added to fresh medium with serum. It should be noted that integration of adenoviral DNA into chromosomal DNA is very inefficient and that about 6 days after infection the vast majority of viral DNA is lost from infected cultures. At this time, cells begin to be rapidly lost from Ad5 12S infected cultures (14). It is therefore possible that the concentration of stimulatory factor(s) produced by 12S infected cultures drops below the level that can be detected in our bioassay. Alternatively, the expression of the factor(s) may be altered.

Cellular DNA Synthesis Induced by 12S Conditioned Medium. The total intracellular levels as well as the amount of $[^3H]$thymidine incorporated into DNA were substantially greater (at least 5-fold) in BRK cells receiving conditioned medium from 12S infected cultures than from mock- or dl312-infected cultures (Fig. 2). The 12S conditioned medium harvested from cells 4 days after infection could be diluted 1:20 and yield a similar response, with respect to observable cell proliferation and $[^3H]$thymidine incorporation (data not shown).

The percentage of the epithelial cell population that was synthesizing cellular DNA was also determined. To obtain pure populations of epithelial cells, BRK cells were plated in K1 medium (see *Materials and Methods*). The following day, the medium was replaced with Ad5 12S or dl312 conditioned medium containing $[^3H]$thymidine. After 4 days cells were processed for emulsion autoradiography. There was at least a 10-fold greater number of positive nuclei in cultures that had received 12S conditioned media than those that had received dl312 conditioned media (Fig. 3). Furthermore, a greater cell density was clearly achieved in those cultures receiving 12S conditioned media. This is particularly obvious in those cultures receiving 12S conditioned media generated in DMEM with fetal calf serum. That 12S conditioned medium generated in DMEM with fetal calf serum induces epithelial

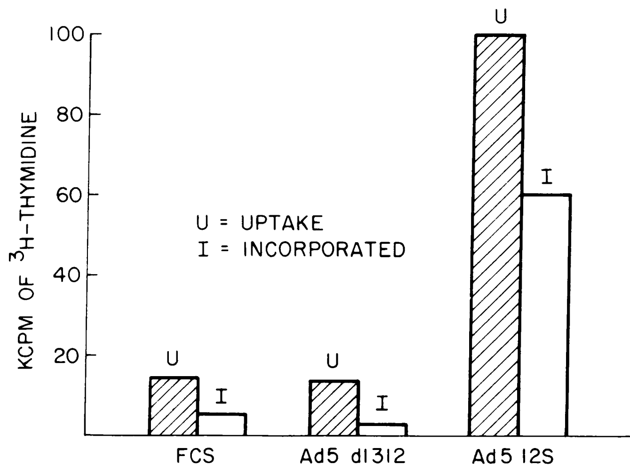

FIG. 2. Increased [^{3}H]thymidine levels in BRK cells incubated with Ad5 12S conditioned medium. Primary BRK cells were incubated with the indicated conditioned medium containing [^{3}H]thymidine for a 24-hr period. The cells were lysed and the levels of [^{3}H]thymidine were determined. FCS, fetal calf serum.

cell proliferation implies that the growth factor(s) generated by 12S infected BRK cells allows the inhibitory effects of serum (6) to be overridden.

The Effect of Conditioned Medium Is Reversible. Pure populations of BRK epithelial cells grown in 12S conditioned K1 medium grow more rapidly than cells grown in dl312 conditioned medium or fresh K1 medium (Figs. 3 and 4). To determine whether 12S conditioned medium was necessary for continued cell proliferation, 12S conditioned medium was replaced with dl312 conditioned medium. This replacement results in the cessation of growth within 24 hr and the subsequent growth rate resembles that of cells that had been maintained in dl312 conditioned medium throughout the experiment (Fig. 4). In fact, within 5 days, the epithelial cells are lost from the population, and the cultures resemble those that had been maintained in mock-conditioned media (data not shown). These results indicate that the continued presence of the growth factor(s) in 12S conditioned medium is required to maintain epithelial cell proliferation. This situation is unlike that seen in infected cells, where replacement of the conditioned medium with fresh DMEM or K1 medium does not result in a cessation of proliferation (14).

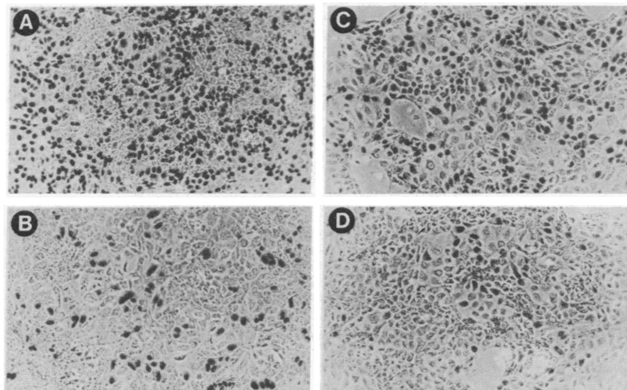

FIG. 3. Epithelial cell DNA synthesis and proliferation in the presence of Ad5 12S conditioned medium. Primary BRK epithelial cells were prepared from nephrons. Twenty-five microcuries of [^{3}H]thymidine was added to conditioned media harvested from BRK cells that had been infected with Ad5 12S (*A* and *C*) or Ad5 dl312 (*B* and *D*) and maintained for 2 days in DMEM with fetal calf serum (*A* and *B*) or K1 medium (*C* and *D*). After 4 days the cultures were processed for emulsion autoradiography. (×10.)

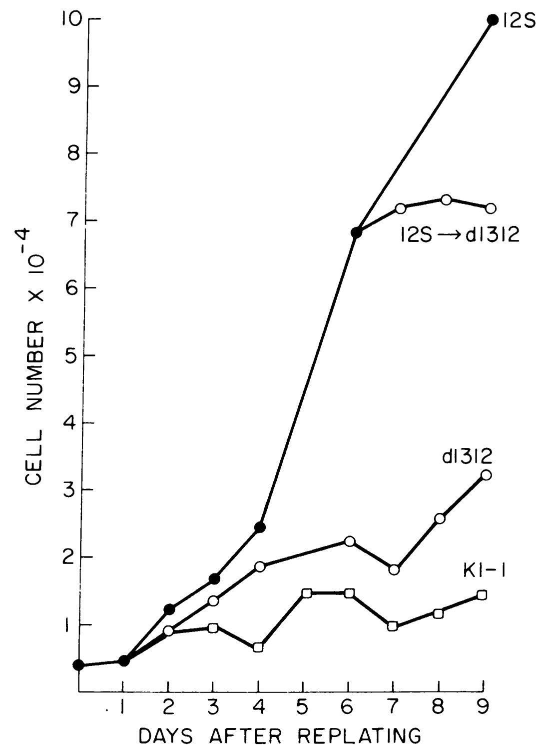

FIG. 4. Epithelial cell proliferation requires the continued presence of 12S conditioned medium. Primary BRK cells were plated in the presence of K1 medium. The following day sets of cultures were changed into the conditioned medium indicated or K1 medium. After 5 days some cultures that had been maintained in 12S conditioned medium were changed into dl312 conditioned medium. Duplicate samples were analyzed for cell number by counting in a Coulter Counter.

No permanently transformed foci appeared in cultures transfected with the Ha-*ras* gene in the presence of 12S conditioned medium (data not shown). Thus, the presence of a proliferation factor(s) does not effect transformation by Ha-*ras*, suggesting that the induction of a growth factor(s) is not the sole function of the E1A 12S product. It cannot be ruled out that the concentration of the factor(s) in the conditioned medium from infected cells is sufficient to induce cellular DNA synthesis and proliferation but insufficient to produce additional responses.

Conditioned media from cell lines containing integrated E1A regions and expressing E1A gene products were negative for the induction of epithelial cell proliferation (data not shown). These cell lines included cells of human, mouse, and rat origin and of epithelial and fibroblastic nature. Thus, the constitutive expression of the 12S gene need not, *per se*, result in the production of high levels of stimulatory factor(s) in the culture medium (see *Discussion*). However, upon superinfection with the 12S virus, these cells transiently produce detectable levels of the growth factor(s).

Activity in Conditioned Medium Is Not Due to Residual Virus Particles. The residual virus titers in Ad5 12S conditioned medium produced a multiplicity of infection (moi) of 0.01–0.001. A general proliferative response was not detected in BRK cultures infected with the 12S virus at a moi of <0.5–1.0 (14). In addition, no viral DNA was detected in low molecular weight DNA extracted from BRK cells treated with 12S conditioned medium, although reconstruction experiments showed that viral DNA would have been detected in cells infected with a moi of 0.05–0.1. No viral proteins (E1A, the early M_r 72,000 DNA binding protein or virion proteins) could be detected in immunoprecipitates from cells treated with 12S conditioned medium (data not shown).

We have shown that the hybrid adenovirus Ad5.SV4 that contains the coding sequences for the early region of simian virus 40 (SV40) in place of adenovirus E1 region (24) also induces BRK cellular proliferation in the absence of serum (14). The SV40 early region, unlike E1A, is able to cause full morphological transformation of these cells. Within 3 days after infection, the Ad5.SV4 infected cells, unlike the Ad5 12S infected cells, fail to stain with anticytokeratin monoclonal antibodies (14). Ad5.SV4 infected cells are also able to grow in soft agar, whereas Ad5 12S infected cells cannot (14). The titers of residual virus in conditioned medium from Ad5.SV4 infected BRK cells are similar to those obtained from 12S conditioned medium. However, the addition of Ad5.SV4 conditioned medium to new BRK cells induces neither cellular DNA synthesis nor proliferation (data not shown; see *Discussion*).

Characteristics of Stimulatory Factor(s) Present in Ad5 12S Conditioned Medium. To characterize the stimulatory factor(s) in 12S conditioned medium, several physical properties were determined and the results are summarized in Table 1. Stimulatory activity is abolished by trypsin treatment of conditioned medium, indicating that the factor(s) is a protein(s). The sensitivity of the 12S virus-induced growth factor(s) to acid and heat treatments distinguishes it from growth factors such as epidermal growth factor (EGF) or transforming growth factor type α (TGF-α), which are able to elicit growth responses in certain epithelial cells (22, 25). This suggests that the 12S virus is inducing a unique growth factor. To confirm this, the ability of 12S conditioned media to compete with EGF for receptor binding was determined. No competition with ^{125}I-labeled EGF for binding to normal rat kidney 49F cells was observed at concentrations of 12S conditioned medium that are sufficient to induce the proliferation of epithelial cells, and, furthermore, the addition of EGF to media did not result in epithelial cell proliferation (data not shown). Together, these data indicate that the factor(s) in 12S conditioned medium does not use the EGF receptor to mediate the response and that EGF does not affect BRK epithelial cells.

Table 1. Analysis of the properties of 12S conditioned medium

Treatment of conditioned medium	Stimulation of cell proliferation
None	+
Incubation	
Trypsin (50 μg/ml), 37°C, 2 hr	−
Trypsin pretreated with soybean trypsin inhibitor (100 μg/ml), 37°C, 2 hr	+
Soybean trypsin inhibitor, 37°C, 2 hr	+
Heat	
43°C, 30 min	+
50°C, 30 min	−
Dialysis against 0.1% acetic acid, 4°C, 16 hr, lyophilization, resuspension in DMEM + fetal calf serum	−
Lyophilization, resuspension in DMEM + fetal calf serum	+
Storage	
4°C, 6 wk	+
4°C, 8 wk	−
Centrifugation, 100,000 × g	
Supernatant fraction	−
Pellet fraction	+
100,000 × g pellet + 4 M NaCl, recentrifugation	
Supernatant	+
Pellet	−

Ad5 12S conditioned medium generated in DMEM with 5% fetal calf serum was treated as indicated. Subsequently, media were recombined with fresh DMEM with fetal calf serum and added to primary BRK cultures 2 days after plating to assay for the presence of high levels (+) or absence (−) of growth stimulatory factor(s).

The stimulatory activity in 12S conditioned media seems to be associated with high molecular weight complexes and is contained in the pellet fraction when conditioned medium is centrifuged at 100,000 × g at 4°C. However, when the pellet fraction is reconstituted in DMEM with fetal calf serum, treated with increasing salt concentrations (up to 4 M NaCl), and recentrifuged, the activity is released into the supernatant fraction (Table 1).

DISCUSSION

We have shown that primary BRK cells, infected with an adenovirus that contains a cDNA copy of the E1A 12S mRNA in place of the normal E1A region, produce a growth factor(s) that induces synthesis of cellular DNA and proliferation of primary BRK epithelial cells. Thus, these epithelial cells, which normally die rapidly in culture, not only survive but proliferate for several weeks in the presence of conditioned medium isolated from Ad5 12S infected cells. Medium from mock-infected cells or cells infected with an adenovirus mutant that lacks the E1A gene fail to produce stimulatory factors.

The growth factor(s) is probably induced by the E1A 12S protein and not by other adenoviral gene products. First, the 12S protein does not transactivate other viral genes upon infection of BRK cells or even permissive human cells (14, 21). Second, adenovirus mutants that carry the *E1A* 12S gene along with mutations in the E1B region, the other region known to be required for full transformation of primary cells, are able to induce the production of growth factor(s) in BRK cells (unpublished observations). Whether the 13S gene product of the E1A region has this property is difficult to determine because it transactivates adenoviral gene expression, which results in cell death.

There is no direct evidence as to whether or not the activity found in the 12S conditioned medium is the 12S protein itself. It has been shown that vaccinia virus codes for a protein that has a segment homologous to EGF and TGF-α and that vaccinia virus infected cells release a growth factor with mitogenic activity (26). However, we have assayed conditioned medium for the E1A 12S protein by immunoprecipitation analysis with negative results. Furthermore, addition of E1A monoclonal antibodies to the 12S conditioned medium does not reduce its activity.

The production of the growth factor(s) present in 12S conditioned medium may be an early event in the immortalization/transformation process. Preliminary studies suggest that viruses carrying E1A 12S mutations that result in the inability to induce growth factor production are also unable to effect epithelial cell immortalization (unpublished observations). However, it should be noted that E1A 12S protein may provide additional functions necessary for transformation. This idea is supported by the inability of 12S conditioned medium to complement the Ha-*ras* gene to fully transform BRK cells. The growth factor(s) in 12S conditioned medium may not be required for the maintenance of fully transformed cells. No stimulatory activity on BRK cells was detected in conditioned medium from adenoviral transformed BRK cells that constitutively express the E1A 12S protein. Transformed epithelial cells may undergo several changes and may lose many of their original epithelial cell characteristics such that they no longer produce the same factor(s) that they did initially. BRK cells infected with Ad5.SV4 very rapidly lose some epithelial cell characteristics (14). Most transformed epithelial cell lines replicate very well in the presence of serum, even though the primary cells do not (6). It is, however, possible that adenovirus transformed BRK cells

produce the stimulatory factor(s) at a level too low to be detected in the bioassay used. In this regard, it should be noted that the levels of the E1A proteins are much higher in infected cells than in transformed cells (unpublished observations). It is not known whether continued high levels of E1A expression are toxic to cells and if transformed cells are selected that have lower levels of E1A expression and thus lower levels of growth factor(s).

The production of growth factor(s) could also serve the lytic pathway of adenovirus. Adenovirus normally infects differentiated epithelial cells, which are growth arrested (27). The ability to induce the production of growth factor(s) would enable infected epithelial cells to enter a proliferative phase and thus support viral DNA replication. The production of a growth factor by the infected cells would also stimulate the surrounding uninfected cells to grow and thus be able to support a secondary infection.

EGF and TGF-α seemed possible candidates for the 12S induced growth factor due to their ability to affect epithelial cells. However, with respect to heat stability, resistance to acetic acid, and lack of binding to the EGF receptor, at least a component of the 12S induced factor(s) seems quite distinct from TGF-α and EGF (22, 25). Furthermore, addition of EGF to medium failed to stimulate BRK cell growth or survival. Although several growth factors from a variety of tissues have been identified that possess characteristics similar to those of EGF and TGFs, there are several growth factors that do not (28, 29), including some that are specific for epithelial cells (30, 31).

The 12S induced growth factor sediments with large molecular weight complexes. The 12S induced factor could be bound to high molecular weight carrier proteins, as has been shown for other important mitogens, such as the insulin-like growth factors (31). The components of the extracellular matrix (ECM) modulate the growth responses of epithelial cells (32) and may be important in regulating proliferation in normal and pathologic states. The ECM is composed of high molecular weight proteins and glycosaminoglycans. Epithelial cells normally rest on a basement membrane composed of collagen types IV and V, fibronectin, laminin, and proteoglycans (33) and attach to factors provided by the serum present in routine culture medium or to factors they secrete themselves (34). Some mitogens and growth factors bind to ECM proteoglycans (35). It is possible that the 12S induced growth factor interacts with extracellular components and thus behaves like a large molecule. The ability of the 12S growth factor to be released from large molecular weight complexes by high salt treatment could reflect an ionic interaction between the growth factor and extracellular molecules. For the purpose of understanding the nature and role of the 12S induced growth factor, further work is necessary to purify the growth factor(s) and to identify the molecules with which it interacts.

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