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A Retrovirus Expressing the 12S Adenoviral E1A Gene Product Can Immortalize Epithelial Cells from a Broad Range of Rat Tissues

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An epithelial cell-transforming virus could be of great use, both in the culture of epithelial cell lines and in the study of carcinogenesis. Since the adenoviral E1A gene has been shown to partially transform some epithelial cells from primary rat cell cultures, we constructed retrovirus vectors containing either the 12S or 13S E1A cDNA sequences to facilitate the transfer of these genes into a variety of primary cell types. The 12S E1A virus induced proliferation and immortalization of epithelial cells in rat kidney, liver, heart, pancreas, and thyroid primary cultures. In the two cases tested, heart and liver cultures, E1A-immortalized cells were nontumorigenic, but could be completely transformed by subsequent introduction of the *ras* oncogene. To our surprise, the 13S virus had a greatly reduced immortalization potential. We discuss these data in light of the model of Spindler et al. (K. R. Spindler, C. Y. Eng, and A.-J. Berk, J. Virol. 53:742–750, 1985), in which the 12S E1A protein is required for the complete induction of the cellular DNA replication machinery in the quiescent human epithelial cells in which adenoviruses normally replicate.

The culture of normal epithelial cells from primary tissues has been a difficult task, often requiring the precise definition of a hormonally supplemented, serum-free medium for each cell type (3, 31). Unlike fibroblasts, many epithelial cell types will not grow in high concentrations of serum, perhaps owing to the presence of one or more inhibitory factors (2, 12, 20). Although some oncogenic viruses have been useful for the transformation of specific differentiated cell types in vitro, such as the transformation of pre-B cells by Abelson virus (34), no viruses have been consistently successful in the transformation of diverse epithelial cells (33).

The human adenoviruses have been shown to transform a few epithelial cell types, allowing their continuous growth in serum-supplemented medium (7, 25, 35). However, adenovirus transformation of many rodent tissues, and most human tissues tested, is extremely inefficient. It is likely that the inefficiency of adenovirus transformation results from a variety of factors such as (i) the lytic properties of adenovirus in many cell types (ii) the low frequency with which adenoviral DNA integrates into the host chromosome, and (iii) a possible tissue specificity of expression and/or function of the adenoviral transforming proteins.

To examine the propensity for epithelial cell transformation of one of the adenoviral transforming genes in a variety of primary cells, without the constraints of adenovirus infection, and in the absence of other adenoviral gene products, we have constructed and describe here the properties of retrovirus vectors containing either the 12S or 13S early-region 1A (E1A) sequences from adenovirus type 2-adenovirus type 5 hybrid cDNAs.

MATERIALS AND METHODS

Construction of plasmids and viruses. Adenovirus 2-adenovirus 5 hybrid E1A 12S and 13S cDNA sequences (23), spanning the region from a *Hin*dIII linker at approximately nucleotide 492 to the *Hpa*I site at nucleotide 1571, were inserted at the *Bam*HI site of the retroviral vector DOL (16) after the addition of *Bam*HI linkers. The resulting vector DNAs, rv12S and rv13S, were transfected into ψ2 cells (19) by $CaPO_4$ precipitation. Transiently expressed viral particles were harvested from the medium 24 h posttransfection to infect fresh ψ2 cells, since it has been shown that higher viral titers are produced by ψ2 cells when retroviral genomes are introduced by infection as opposed to transfection (14). Fresh ψ2 cells, treated for 19 h with 0.2 μg of tunicamycin per ml to make them permissive for reinfection (28), were infected with the transiently harvested virus. All retroviral infections were performed by incubating cells with 1 ml (60-mm dish) or 2 ml (100-mm dish) of virus stock in the presence of 8 μg of Polybrene (Aldrich) per ml for 2.5 h at 37°C with occasional shaking. At the end of this period, 4 or 8 ml of medium (Dulbecco modified Eagle medium [DME] containing 10% fetal calf serum) was added back. G418-resistant ($G418^r$) clones obtained by selection for 2 weeks in the presence of 1 mg of G418 (GIBCO Laboratories) per ml were expanded and used throughout these experiments as a source of rv12S and rv13S virus.

Primary cell culture. Kidney, heart, liver, pancreas, thyroid, adrenal, and brain tissues were obtained from 2-day-old Fischer rats (F3444; Taconic Farms). With the exception of brain, organs were finely minced and then digested at 37°C for 30 min with stirring in 0.25 mg of collagenase per ml–2.5 mg of dispase per ml in phosphate-buffered saline (PBS). Following the dispase-collagenase treatment liver epithelial cells were purified from total liver cells by two rounds of low-speed centrifugation (2,000 rpm for 20 s and 1,000 rpm for 16 s in a Sorvall RT6000 centrifuge) and a final step in which cells were suspended and the heavier epithelial cells were allowed to settle to the bottom of a tube for 10 min. Brain tissue was minced and then triturated until a single-cell suspension was obtained. Cells were plated at a density of 5×10^5 per 60-mm plate in DME–5% fetal calf serum.

Growth in soft agar was tested by adding cells to 3 ml of medium (DME, 5% fetal calf serum, 0.5% agarose) at 45°C and pipetting the mixture onto a prehardened 3-ml layer of medium containing 0.5% agarose in a 60-mm culture dish.

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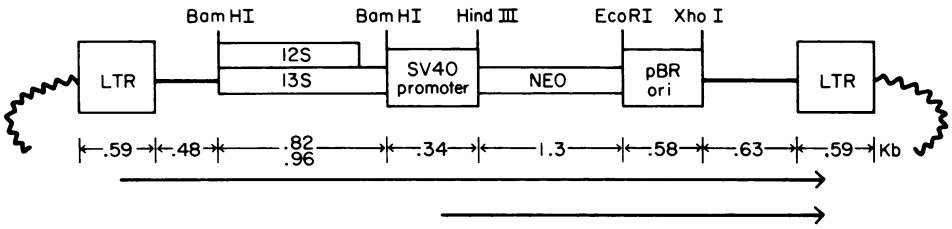


FIG. 1. Structure of retrovirus vectors encoding the 12S and 13S adenovirus E1A sequences. Adenoviral 12S and 13S E1A cDNA sequences (see Materials and Methods) were inserted in the retroviral vector DOL (16). This vector, which has had the normal retroviral splice signals removed, was designed to express two transcripts. The E1A sequences are expressed from a transcript initiated from the long terminal repeat (LTR) promoter, and the neomycin phosphotransferase (NEO) gene is initiated downstream from the simian virus 40 (SV40) promoter. The large arrow indicates sequences which are transmitted as viral RNA, with the sizes, in kilobases, of fragments making up the vector shown below. The small arrow indicates the subgenomic mRNA transcript encoding the neomycin phosphotransferase gene, and wavy lines indicate plasmid backbone sequences.

Plates were fed 2 ml of medium at 1 and 2 weeks and scored for growth at 3 weeks.

Tumorigenicity assays were conducted by trypsinizing cells, rinsing them once in PBS, and injecting 10^6 cells subcutaneously into 14-day-old syngeneic rats. The T24 *ras* oncogene was added to cells already immortalized by E1A by infecting the cells with a recombinant retrovirus containing the *ras* gene (kindly provided by S. Compiere).

Immunoprecipitation. Equal numbers of cells (5×10^6) were labeled for 3 h with 400 μCi of [^{35}S]methionine (>1,000 Ci/mmol; Amersham Corp.) in 100-mm plates containing 2 ml of DME without methionine. The monolayers were washed once with PBS and lysed with 1.0 ml of 3D RIPA buffer (PBS containing 1% Triton X-100, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate, 0.1% bovine serum albumin, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride) for 30 min on ice. Equal trichloroacetic acid-precipitable counts were immunoprecipitated with a mixture of four anti-E1A monoclonal antibodies (M1, M29, M37, and M73) as described previously (10). A monoclonal antibody directed against T antigen (PAb 419) (9) was used for control immunoprecipitations. Samples were electrophoresed on 10% polyacrylamide gels.

Immunofluorescence. Since it was difficult to grow some E1A-immortalized cells on glass cover slips, all immunofluorescence experiments were carried out with plastic culture dishes. Cells were rinsed twice with PBS, treated for 1 min with methanol at room temperature, and then rinsed once with distilled water and once with PBS. They were then incubated for 1 h at 37°C with the primary antibody, rinsed three times with PBS, and then incubated for 1 h with rhodamine-conjugated goat anti-mouse immunoglobulins (Cappel Laboratories). After additional washing, Gelvatol (Monsanto) and a cover slip were then applied to view the cells directly on each plastic plate.

Monoclonal antibody LE41, which reacts specifically against mammalian cytokeratin 8, was kindly provided by E. B. Lane (17). Additional monoclonal antibodies against vimentin and desmin were obtained from Amersham.

RESULTS

Properties of rv12S and rv13S, retroviruses encoding the 12S and 13S adenoviral E1A sequences. Two vectors, rv12S and rv13S, were constructed to each express two transcripts, an E1A transcript initiated in the viral long terminal repeat and a shorter transcript encoding the neomycin phosphotransferase gene, allowing selection of virus-infected cells by using the antibiotic G418. The structures of the viruses (Fig. 1) are identical except the E1A sequence, encoding alternatively the 12S or 13S E1A mRNA, inserted at a *Bam*HI site. ψ2 cell lines capable of expressing high titers of rv12S or rv13S were constructed and used as a source of virus for all the experiments described below. The titers of these stocks varied from 10^4 to 10^5 viruses per ml as assayed by their ability to confer G418 resistance on NIH 3T3 cells (data not shown).

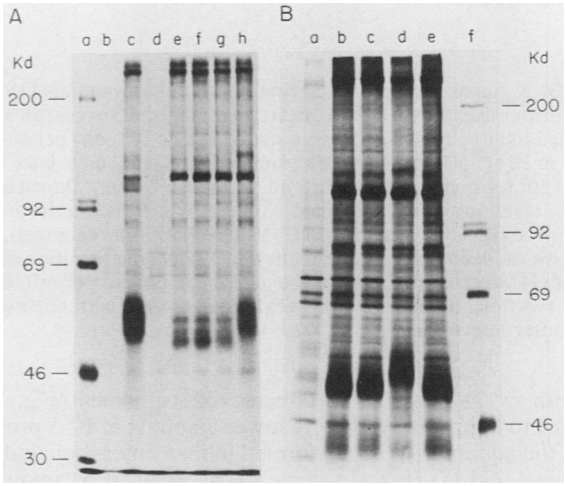


FIG. 2. Immunoprecipitation of 12S- and 13S-encoded protein in rv12S- and rv13S-infected ψ2 and 3T3 cells. After infection with rv12S or rv13S and G418 selection (1 mg/ml for 2 weeks), individual ψ2 clones and populations of NIH 3T3 cells (>1,000 clones) were expanded. Cells were labeled with [^{35}S]methionine, and equal numbers of trichloroacetic acid-precipitable counts were used for immunoprecipitation with a mixture of anti-E1A monoclonal antibodies (M1, M29, M37, and M73) to ensure complete recognition of 12S and 13S protein. Markers, present in lane a (panel A and lane f (panel B) are a mixture of ^{14}C-methylated proteins (Amersham) containing myosin (200 kilodaltons), phosphorylase *b* (92.5 kilodaltons), bovine serum albumin (69 kilodaltons), ovalbumin (46 kilodaltons), and carbonic anhydrase (30 kilodaltons). (A) E1A proteins in three rv12S-infected ψ2 lines (lanes e, f, and g), one rv13S-infected ψ2 line (lane h), and 293 cells (lane c). As a control, lysates from one rv12S-infected ψ2 clone (lane d) and from 293 cells (lane b) were immunoprecipitated with monoclonal antibody PAb 419. (B) E1A proteins expressed in populations of G418^r NIH 3T3 cells infected with stocks of rv12S (lanes b, c, and e) or rv13S (lane d) virus. As a control, the population in lane b was also immunoprecipitated with monoclonal antibody PAb 419 (lane a).

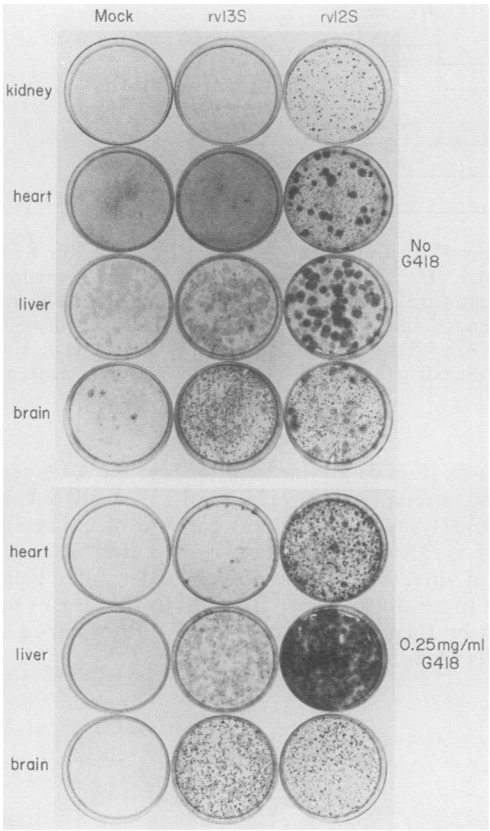


FIG. 3. Infection of diverse primary cell cultures with rv13S and 12S retroviruses. Total kidney, heart, and brain cell suspensions and purified liver epithelial cells were plated at 5×10^5 cells per 60-mm plate in DME–5% fetal bovine serum and infected 2 days later with 10^4 to 10^5 retroviral particles in 1 ml. In the experiment shown here, mock infections were performed with medium from uninfected ψ2 cells. In experiments involving G418 selection (bottom panel), the drug was added 4 days after infection to a final concentration of 0.5 mg of G418 per ml (0.25 mg of the active component per ml). Cells were fed fresh medium twice weekly and stained with Giemsa 14 days after infection.

Both rv12S- and rv13S-infected ψ2 cell lines (Fig. 2A, lanes e to h) produced slightly lower amounts of E1A protein than the adenovirus 5-transformed human embryonic kidney cell line, 293 (7) (Fig. 2A, lane c), as assayed by immunoprecipitation. Although E1A protein levels varied somewhat among ψ2 cell lines, when populations of infected G418^r NIH 3T3 cells were analyzed, equal levels of 12S (Fig. 2B, lanes b, c, and e) and 13S (Fig. 2B, lane d) proteins were seen, suggesting that on average, the two viruses express similar levels of the different E1A proteins. A number of additional proteins seen in these immunoprecipitations, such as the prominent bands at approximately 100 and 300 kilodaltons, migrate at the same size as a set of proteins previously described as specific E1A binding proteins (11, 38).

Induction by rv12S, but not rv13S, of cellular proliferation in primary cell cultures from a wide variety of organs. Total primary cells from 2-day-old rat heart, kidney, pancreas, thyroid, adrenal, and brain tissues were plated at 5×10^5 cells per 60-mm dish in DME–5% fetal calf serum and infected 2 days later with 1 ml of rv12S or rv13S virus stock or mock infected. Liver epithelial cells were partially purified from total liver (see Materials and Methods) and then plated and infected in the same manner. Cells were fixed and stained with Giemsa 2 weeks after infection. rv12S virus induced rapid proliferation of cells in every culture (Fig. 3, top), including pancreas (see Fig. 4), adrenal, and thyroid (not shown) cells. In contrast, although rv13S induced some slight proliferation of liver epithelial cells, induction of proliferation equivalent to that induced by rv12S was seen only in brain cell cultures. No induction of proliferation was detected after rv13S infection of kidney, heart, pancreas, thyroid, or adrenal cells. Mock infection with either medium from ψ2 cells (Fig. 3) or medium containing the DOL virus with no E1A insert (data not shown) failed to induce cell proliferation.

To demonstrate infection with rv13S in tissues in which proliferation was not observed, the experiment described above was repeated; however, primary cells were cultured in medium containing G418, beginning 4 days after infection (Fig. 3, bottom). After 10 days of G418 selection, the plates were stained with Giemsa. As expected, similar numbers of dense foci appeared on both rv13S- and rv12S-infected brain cells. In contrast, in the liver epithelial cell cultures, similar numbers of G418^r colonies appeared after infection with either virus, but the cells proliferated to high density only after infection with rv12S virus. In heart cell cultures, large numbers of extremely sparse colonies appeared after rv13S infection and G418 selection and were difficult to observe by Giemsa staining. Kidney cultures (not shown) were generally unable to survive G418 selection, although G418^r rv12S and rv13S colonies appeared at low frequency in one experiment. No mock-infected cells remained after 10 days of G418 selection.

G418 selection of rv13S-infected heart and liver cells demonstrated that these cells were genuinely infected with rv13S virus. Nevertheless, the inability of 13S-encoded protein to induce proliferation could have resulted from instability of the protein in these cells. Because these cells did not proliferate well, we had difficulty examining E1A expression in them by immunoprecipitation. Both rv13S- and rv12S-infected G418^r heart and liver cells, however, contained readily detectable levels of E1A, properly localized to the cell nucleus, as demonstrated by immunofluorescence with a mixture of monoclonal antibodies which recognize both 12S and 13S protein (data not shown).

All rv12S-induced foci gave rise to cells which had a greatly increased plating efficiency, following trypsinization, relative to their uninfected counterparts. In a typical experiment, cells were grown for 2 weeks after infection and then stained with Giemsa (Fig. 4A). A duplicate set of plates was trypsinized, and one-fifth of the cells were passaged onto fresh culture dishes. At 2 weeks later, the plates were fixed and stained with Giemsa (Fig. 4B). As can be seen, the ability of rv13S to increase plating efficiency was limited. We were unable, for example, to passage rv13S-infected heart, kidney, or pancreas cells, and even the increased plating efficiency of rv13S-infected liver epithelial cells was seen only in about 50% of the more than 10 repetitions of the experiment. In contrast, after infection of primary brain cell cultures, the only case in which rv13S induced dense foci, the rv13S-infected cells could be passaged as easily as their rv12S-infected counterparts.

Proliferation and immortalization of epithelial cells by rv12S. Immunofluorescence studies with a panel of monoclonal antibodies directed against intermediate filaments demonstrated that prior to infection, the primary cultures described in this report contained a wide variety of cell types. For example, heart cell cultures contained vimentin-positive, cytokeratin-positive, and desmin-positive cells. Nevertheless, by morphology and growth characteristics,

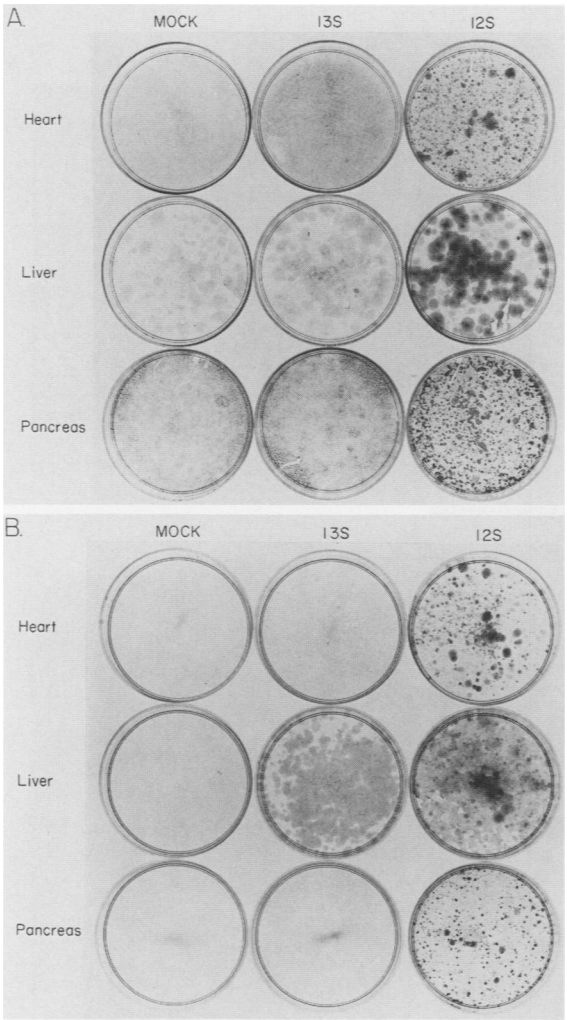


FIG. 4. Trypsinization and serial passage of rv12S- and rv13S-infected cells. Heart, liver, and pancreas cells were plated and infected as described in Materials and Methods. (A) Set of plates stained with Giemsa 2 weeks after infection. (B) Identical set of plates, trypsinized 2 weeks after infection, diluted 1:5, replated, and allowed to grow for an additional 2 weeks prior to Giemsa staining.

only one or at most two types of foci appeared after infection in any culture. Except for foci induced in brain cell cultures, most foci in the other primary cultures described here had epithelial morphologies. The two colonies shown in Fig. 5A, column 1, for example, are representative of the small and large foci (left and right colony, respectively) induced by rv12S infection of heart cells (Fig. 3). Similarly, trypsinization and serial passage of rv12S-infected cultures yielded cells with an epithelial morphology, such as the clonal line of rv12S-infected pancreas cells shown in Fig. 5A, column 2.

An exception to this general pattern was found with rv12S- and rv13S-induced brain cell foci (Fig. 5A, column 3; left and right panels, respectively). These cells had a typical fibroblastic morphology.

Immunofluorescence studies with a monoclonal antibody, LE41 (17), specific to mammalian cytokeratin 8, demonstrated that cells in most rv12S-induced foci were of epithelial origin. For example, the panel in Fig. 5B, column 1, shows staining of a colony of heart cells induced to proliferate by infection with rv12S and passaged for several months. Curiously, we often found that only cells near the peripheries of the colonies showed extensive cytokeratin networks. This was also found to be the case with rv12S-immortalized cells that had been cloned by limiting dilution and cultured for several months (Fig. 5B, column 2). The only cells which did not stain positively with LE41 were the cells derived from rv12S or rv13S infection of brain cell cultures. Additionally, no filamentous staining was seen when this antibody was used to stain NIH 3T3 cells.

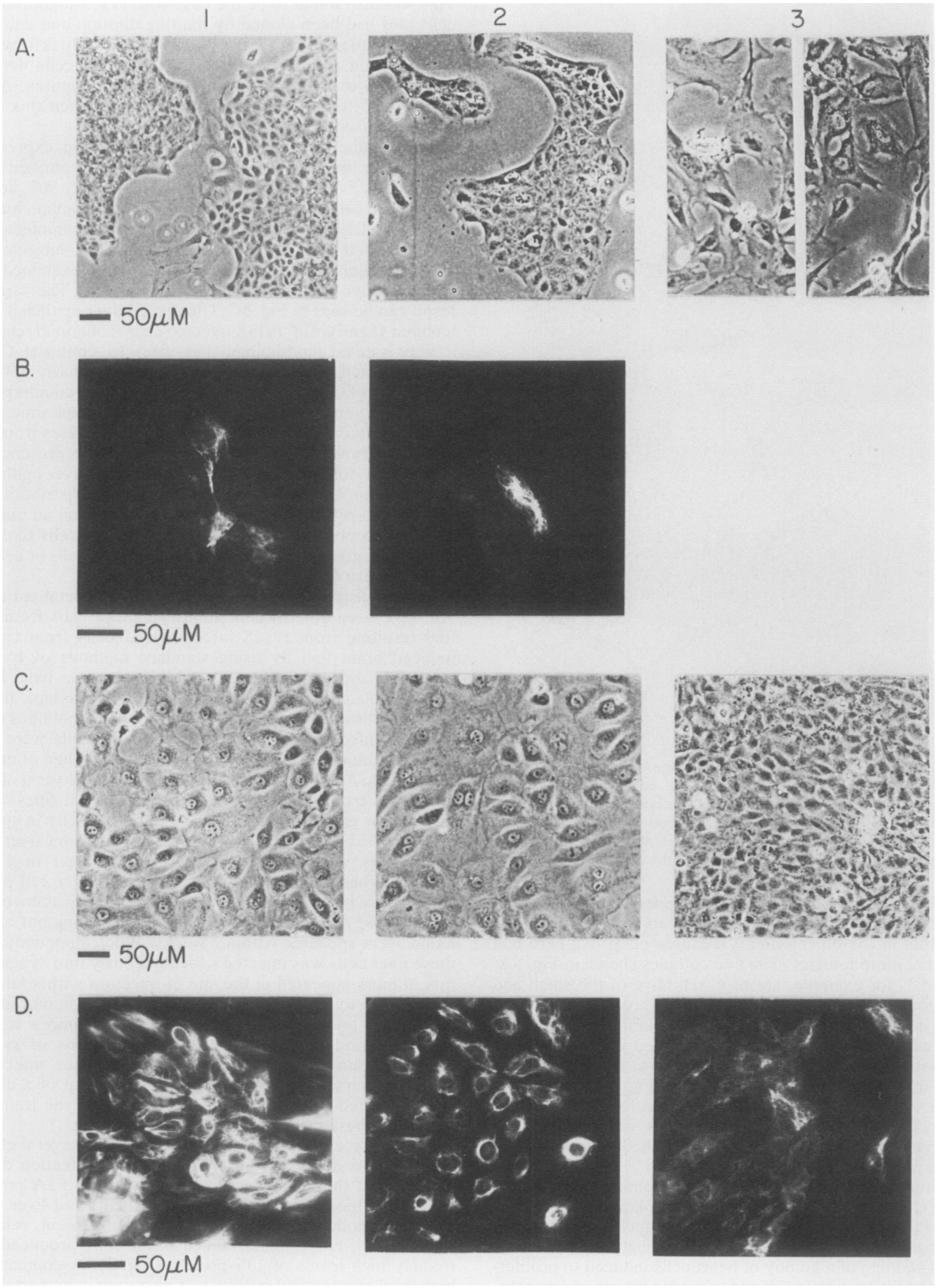
To examine the phenomenon of cytokeratin expression during E1A immortalization in more detail, we purified non-hepatocytic liver epithelial cells to approximately 90% homogeneity as determined by morphology and immunofluorescence with LE41 antibody. These cells were left uninfected or were infected with rv13S or rv12S and then subjected to G418 selection. After 2 weeks, colonies were examined and representative examples were photographed. The striking result can be seen in Fig. 5C. Uninfected liver epithelial cells (column 1) and G418^r rv13S-infected cells (column 2) retained a very similar morphology and size. In contrast, G418^r rv12S-infected cells (column 3) proliferated to nearly 10 times the density of their uninfected or rv13S-infected counterparts, resulting in very small cells containing little cytoplasmic area.

Immunofluorescent staining with LE41 of plates from the same experiment (Fig. 5D) demonstrated the presence of extensive cytokeratin networks in uninfected liver epithelial cells (column 1), a less extensive but uniform distribution of filaments in rv13S-infected cells (column 2), and an uneven distribution of cytokeratins in rv12S-infected cells (column 3), with the greatest staining found in isolated cells or cells at the periphery of a colony.

Growth properties of rv12S- and rv13S-immortalized cells. We have been able to continuously culture cells from any foci resulting from rv12S infection and cells from rv13S-induced brain foci by using standard methods of trypsin treatment and serial passage. Additionally, we have been able to isolate clonal lines of these cells by passage at low dilution followed by cylinder cloning. Two clonal lines each of rv12S-infected heart and liver epithelial cells were cultured continuously for 10 months without any sign of crisis.

After 2 to 3 months of culture, heart (rv12S), liver (rv12S), and brain (rv13S) cell populations and clonal lines were assayed for growth in soft agar and tumorigenicity in syngeneic rats Table 1). The rv12S-infected heart and liver cell populations were unable to grow in soft agar or cause tumors. When rv12S-immortalized heart or liver cell populations were infected with a recombinant retrovirus encoding an activated *ras* gene (Fig. 6), large foci growing out of the monolayers appeared within 2 weeks. When a population of these liver cells was injected subcutaneously into syngeneic rats, tumors appeared at the site of injection with a latency period of 6 to 12 weeks. Surprisingly, rv13S-immortalized brain cells grew in soft agar and caused tumors with a latency period of 6 to 12 weeks. Populations of rv13S-infected brain cells, harvested 2 weeks after infection, caused tumors with a frequency similar to that of a clonal rv13S-infected brain cell line, suggesting that the transformation of these cells requires no second event.

Immunoprecipitation of E1A proteins from several clonal lines of cells derived from rv12S or rv13S infection documented that these lines continued to express E1A protein (Fig. 7). Although rv12S-immortalized heart and liver cells produced moderate levels of 12S-encoded protein, relative to 293 cells, rv13S-immortalized brain cells produced extremely high levels of 13S protein (Fig. 7). A comparably high level of retroviral mRNA was also seen in immortalized brain cells (data not shown).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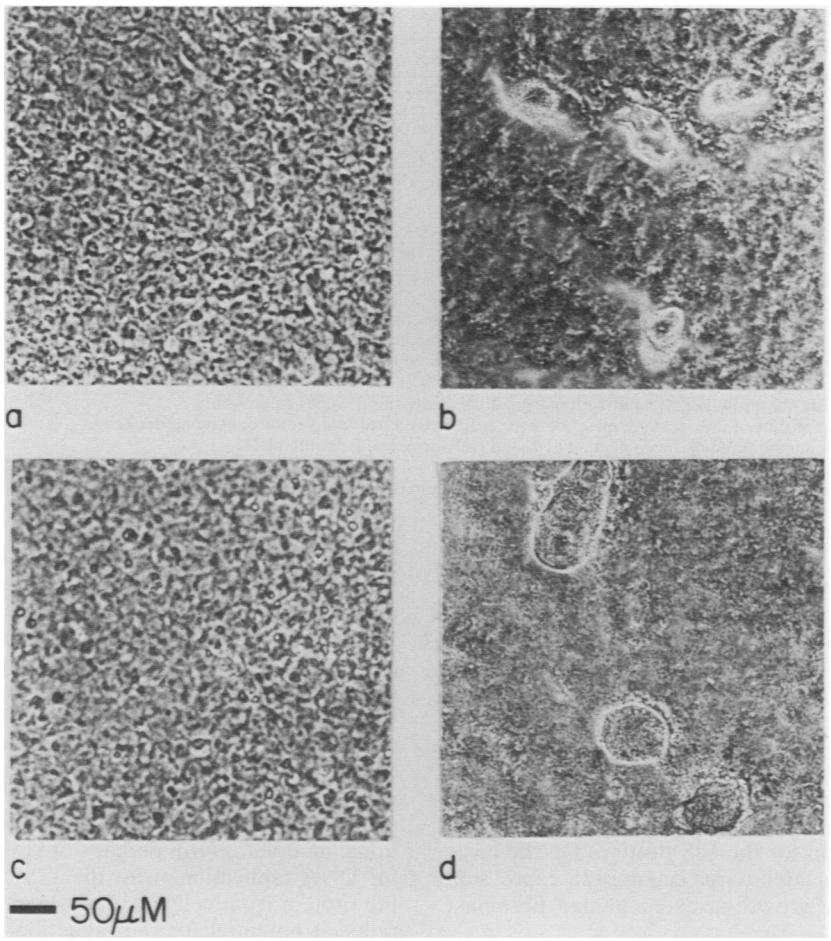

FIG. 6. Growth properties of rv12S- and rv13S-infected cells. Heart (a and b) and liver (c and d) epithelial cells infected by rv12S virus were maintained in culture for 8 weeks by routine trypsinization and serial passage. Cells were left uninfected (panels a and c) or were infected (panels b and d) with a recombinant retrovirus containing the T24 *ras* gene. Cells were photographed 3 weeks after infection.

DISCUSSION

More potent immortalization function in 12S E1A protein than in its 13S counterpart. The two E1A mRNAs result from differential splicing of the same primary transcript, using different splice donors joined in frame to the same splice acceptor (26). Consequently, the proteins differ only in the presence of an additional internal 46 amino acids present in the 13S-encoded product. Although the ability to transcriptionally *trans*-activate other adenoviral early genes maps primarily to the 13S protein (22), other phenotypes of the E1A proteins, including the ability to induce cell proliferation and partial transformation of primary rodent cells (6, 8, 13, 21, 23, 27, 37) and the ability, under some circumstances, to repress enhancer-mediated transcription (4, 36), have been found in both proteins. Hence, the dramatic difference reported here in the immortalization properties of rv12S and rv13S, two otherwise isogenic viruses expressing similar levels of the 12S and 13S E1A proteins, was an unexpected result.

Previous reports on the immortalization potential of the 12S and 13S E1A proteins have relied on DNA transfection or adenoviral infection to transfer these genes into primary cells (8, 18, 23, 30, 37). A wide variation in the level of expression of E1A could be achieved by these methods, along with the expression of additional adenoviral proteins by the latter method. Although adenovirus mutants expressing only the 12S protein have been found to have a higher

FIG. 5. Morphology of some rv12S- and rv13S-infected cells. Cells were photographed directly on plastic dishes with a light microscope or with a fluorescence microscope after staining with anti-cytokeratin monoclonal antibody LE41. (A) Phase-contrast micrographs of the small dense and large diffuse foci (Fig. 3) resulting from rv12S infection of heart cells (column 1), a clonal line of rv12S-infected pancreas cells cultured for 3 months (column 2), and colonies resulting from rv12S (column 3, left panel) or rv13S (column 3, right panel) infection of brain cells. (B) Fluorescence micrographs of rv12S-infected heart cells (column 1) and a clonal line of rv12S-infected pancreas cells (column 2) after staining with LE41 antibody. In each case, the cells had been serially passaged for 3 months. (C) Liver epithelial cells left uninfected (column 1) or colonies resulting from infection with rv13S (column 2) or rv12S (column 3) following 10 days of G418 selection. All three samples were photographed 14 days after plating with identical magnification and photographic enlargement. (D) Fluorescence micrographs of uninfected (column 1), G418^r rv13S-infected (column 2), or G418^r rv12S-infected (column 3) liver epithelial cells stained with antibody LE41, 14 days after plating as described above.

TABLE 1. Growth properties of E1A-immortalized cells

Property	Expression in following cells:			
	Liver (12S infected)[a]	Heart (12S infected)[a]	Brain (13S infected)[b]	Harvey NRK[c]
Growth in soft agar	−	−	+[d]	+
Focus formation after *ras*[e] addition	+	+	−[f]	ND[g]
Tumorigenicity in syngeneic rats	−	−	+ (6/7, 6–12 wks)[h]	+ (2/2, 6 wks)[h]
Tumorigenicity in syngeneic rats after *ras* addition[e]	+ (2/2, 6–12 wks)[h]	ND	ND	ND

[a] Heart and liver cell populations had been cultured for 8 to 12 weeks before injection.
[b] A clonal line of rv13S-infected brain cells, cultured for 12 weeks, was injected into 3 animals; populations of brain cells harvested 2 weeks after infection with rv13S were used for the remaining injections. The one tumor-free animal resulted from the latter experiment.
[c] Positive control.
[d] These cells had a limited ability to grow in soft agar, forming small colonies after 3 weeks of growth.
[e] The T24 *ras* oncogene was transduced into cells by infection with a defective retroviral vector containing the gene.
[f] After the addition of *ras*, the medium rapidly became acidified, and cells detached from the plate.
[g] ND, Not done.
[h] Numbers indicate the ratio of animals with tumors to the total number of animals injected followed by the time required for the visible appearance of tumors.

focus-forming potential than mutants expressing only the 13S protein, this has generally been attributed to cytotoxicity resulting from 13S *trans*-activation of additional adenoviral genes (8, 18, 23, 37). The data presented here show that in the absence of other adenoviral genes and under conditions in which similar, moderate, levels of 12S and 13S protein are expressed, the 12S protein has a greater ability to induce cell proliferation and subsequent immortalization of primary epithelial cells.

The conservation of the 12S polypeptide in the A, B, and C subgroups of human adenoviruses implies that there is an important unique function for the 12S protein. On the basis of the observation that an adenovirus lacking 12S expression replicated less well in a growth-arrested human fibroblast line than the wild-type virus did and induced lower levels of cellular DNA synthesis in a G_0-arrested nonpermissive rodent cell line, Spindler et al. (32) have proposed a role for the 12S protein in viral replication in quiescent cells. The greater potential of the 12S protein to induce proliferation in primary epithelial cells provides additional support for the model in which the 12S protein may be required for full activation of the cellular DNA replication machinery in the quiescent epithelial cells of the throat, lung, and gut in which adenoviruses normally replicate. This model would not rule out the possibility that the 12S protein is also primarily a transcriptional modulator (18), perhaps of key cellular genes required for DNA replication, with the 13S domain acting to divert the protein from cellular to viral gene *trans*-activation. The reduced potential for the induction of proliferation by the 13S protein could perhaps be overcome by overexpression of the protein or by expression of additional oncogenes. This is supported by our observation that in the one tissue in which rv13S has an immortalization potential equal to that of rv12S, the 13S protein was expressed at extremely high levels.

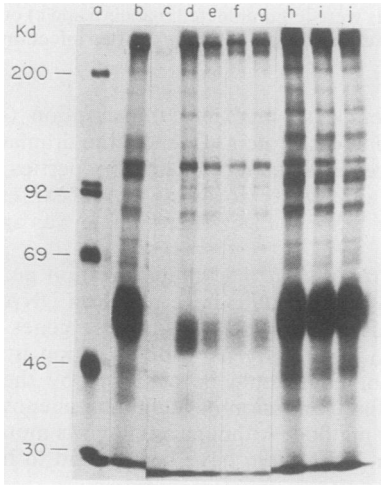

FIG. 7. Protein expression of E1A-immortalized cells. Heart, liver, and brain cells infected with rv12S or rv13S were cultured for 2 to 3 months by trypsinization and serial passage. Clonal lines were obtained by passage at low density and isolation by using cloning cylinders. Cells were labeled with [^{35}S]methionine, and equal numbers of trichloroacetic acid-precipitable counts were used for immunoprecipitation with a mixture of anti-E1A monoclonal antibodies (M1, M29, M37, and M73), or PAb 419 as a control. Lanes: b, 293 cells; d, rv12S-infected heart cells; e, rv12S-infected liver cells; f, g, rv12S-infected liver cell lines; h, i, j, rv13S-infected brain cell lines; c, lysate from a rv12S-infected liver cell population immunoprecipitated with PAb 419; a, marker proteins.

Immortalization of a wide variety of epithelial cells by rv12S. Previously, the E1A proteins have successfully been introduced into only a limited number of primary cell types by transfection or adenoviral infection. In this report we show that retroviruses can be used to introduce E1A into a wide variety of rat primary cell cultures and that the 12S-encoded protein in particular will immortalize cells from nearly every tissue. Most of the cells immortalized by rv12S are derived from simple epithelia, as demonstrated by immunofluorescent staining with LE41, a monoclonal antibody directed against cytokeratin 8, an intermediate filament present in most simple epithelia and absent from stratified epithelia, such as is found in skin, and from endothelial cells. The reduction in filamentous staining with LE41 after rv12S infection may be an artifact of the staining procedure, with the extreme density of the cells making it difficult to visualize cytokeratin networks. Alternatively, the extreme compaction of the cytoplasm of rv12S-immortalized cells may also entail a reduction of cytoskelatal filaments in these cells.

The data presented here do not convincingly demonstrate an epithelial specificity of immortalization by E1A, however, tempting though this may be, given the observation that adenoviruses replicate in various epithelia in vivo. Clearly, adenoviruses and the adenoviral transforming proteins can be used to transform or immortalize a variety of cell types in

addition to epithelial cells, including fibroblasts, and retinoblasts (1, 5, 24). Many transformed cells containing E1A have an epithelioid morphology, and Roberts et al. (29) have shown, using retroviral vectors similar to those described here, that the 12S E1A product in particular induces this morphology in NIH 3T3 cells. However, under the culture conditions described here, with a low serum concentration of 5% acting to retard fibroblast growth, infection with the rv12S retrovirus caused predominantly the epithelial cells from a number of rat tissues and purified epithelial cells from the liver to undergo rapid proliferation and eventual immortalization. This suggests a use for these viruses in the study of carcinogenesis and in the establishment of epithelial cell lines. Determining whether these lines will retain much of a differentiated phenotype, given the dramatic morphological consequences of 12S protein expression, requires further experiments. Preliminary data relevant to this question show that rv12S-immortalized cells from the human thyroid epithelium retain a number of differentiated properties specific to thyrocytes (R. D. Cone, M. Platzer, L. A. Piccinini, M. Jaramillo, Y. Gluzman, and T. F. Davies, submitted for publication).

Primary cells completely transformed by high-level E1A expression. Although the 12S E1A protein, expressed at moderate levels, greatly increased the density to which primary epithelial cells could grow, it did not transform the cells to a tumorigenic phenotype nor allow them to grow in soft agar. An unidentified rv13S-transformed cell type from brain cell cultures was able to grow in soft agar, caused tumors in syngeneic rats, and was found to contain very high levels of 13S protein. Although we have not yet analyzed rv12S-immortalized brain cells in detail, rv12S- and rv13S-induced brain cell foci were indistinguishable in their growth rate and morphology. If the rv12S-infected brain cells also contain high levels of E1A protein and are tumorigenic, a model which would fit the results obtained thus far would be that the higher levels of E1A protein result directly from unusually high levels of retroviral mRNA in these cells. We do not yet know whether retroviral mRNA levels in immortalized brain cells are actually higher than normal or only appear so in relation to levels in other rv12S- or rv13S-infected cells, in which E1A protein may be repressing transcription from the enhancer-driven retroviral promoter. In either case, the quantity of protein present surpasses the threshold of 13S E1A protein required for induction of proliferation, a threshold which is higher for the 13S species than for the 12S species, with the attendant result that rv12S and rv13S have equal focus-forming potential in this cell type.

It has been shown previously that transfection of the adenovirus type 5 E1A gene into NIH 3T3 cells may transform them to a tumorigenic state (15). The level of E1A protein expressed in these cells was not reported. Our results suggest that at very high levels of expression, the E1A protein can completely transform a primary cell to a tumorigenic cell.

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LITERATURE CITED

1. **Albert, D. M., A. S. Rabson, and A. J. Dalton.** 1968. *In vitro* neoplastic transformation of ureal and retinal tissues by oncogenic DNA viruses. Invest. Ophthalmol. **7**:357–365.
2. **Assoian, R. K., A. Kamoriya, C. A. Meyers, D. M. Miller, and M. B. Sporn.** 1983. Transforming growth factor β in human platelets. J. Biol. Chem. **258**:7155–7160.
3. **Barnes, D. W., D. A. Sirbasku, and G. H. Sato.** 1984. Cell culture methods for molecular and cell biology, vol. 3, p. 1–125. Alan R. Liss, Inc., New York.
4. **Borrelli, E., R. Hen, and P. Chambon.** 1984. Adenovirus-2 E1A products repress enhancer-induced stimulation of transcription. Nature (London) **312**:608–612.
5. **Byrd, P. J., K. W. Brown, and P. H. Gallimore.** 1982. Malignant transformation of human embryo retinoblasts by cloned adenovirus 12 DNA. Nature (London) **298**:69–71.
6. **Gallimore, P. H., P. J. Byrd, J. L. Wittaker, and R. J. A. Grand.** 1985. The properties of rat cells transformed by DNA plasmids containing adenovirus type 12 DNA or specific fragments of the E1 region: a comparison of transforming frequencies. Cancer Res. **45**:2670–2680.
7. **Graham, F. L., J. Smiley, W. C. Russell, and R. Nairn.** 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J. Gen. Virol. **36**:59–72.
8. **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.
9. **Harlow, E., L. V. Crawford, D. C. Pimm, and N. M. Williamson.** 1981. Monoclonal antibodies specific for simian virus tumor antigens. J. Virol. **39**:861–869.
10. **Harlow, E., R. Franza, and C. Schley.** 1985. Monoclonal antibodies specific for adenovirus early region 1A proteins: extensive heterogeneity in early region 1A products. J. Virol. **55**: 533–546.
11. **Harlow, E., P. Whyte, B. R. Franza, and C. Schley.** 1986. Association of adenovirus early region 1A proteins with cellular polypeptides. Mol. Cell. Biol. **6**:1579–1589.
12. **Holley, R. W., P. Bohlen, R. Fava, J. H. Baldwin, G. Kleeman, and R. Armour.** 1980. Purification of kidney epithelial cell growth inhibitors. Proc. Natl. Acad. Sci. USA **77**:5889–5992.
13. **Hurwitz, D. R., and G. Chinnadurai.** 1985. Evidence that a second tumor antigen coded by adenovirus early region E1A is required for efficient cell transformation. Proc. Natl. Acad. Sci. USA **82**:163–167.
14. **Hwang, L. S., and E. Gilboa.** 1984. Expression of genes introduced into cells by retroviral infection is more efficient than that of genes introduced into cells by DNA transfection. J. Virol. **50**:417–424.
15. **Kelekar, A., and M. D. Cole.** 1986. Tumorigenicity of fibroblast lines expressing the adenovirus E1A, cellular p. 53, or hormonal c-*myc* genes. Mol. Cell. Biol. **6**:7–14.
16. **Korman, A., J. D. Frantz, J. Strominger, and R. C. Mulligan.** 1987. Expression of human class II major histocompatibility complex antigens using retrovirus vectors. Proc. Natl. Acad. Sci. USA **84**:2150–2154.
17. **Lane, E. B.** 1982. Monoclonal antibodies provide specific intramolecular markers for the study of epithelial tonofilament organization. J. Cell Biol. **92**:665–673.
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. **Mann, R., R. C. Mulligan, and D. Baltimore.** 1983. Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. Cell **33**:153–159.
20. **McMahon, J. B., J. G. Farrelly, and P. T. Iype.** 1982. Purification and properties of a rat liver protein that specifically inhibits the proliferation of nonmalignant epithelial cells from rat liver.

Proc. Natl. Acad. Sci. USA **79**:456–460.
21. **Montell, C., G. Curtois, C. Eng, and A. Berk.** 1984. Complete transformation by adenovirus 2 requires both E1A proteins. Cell **36**:951–961.
22. **Montell, C., E. F. Fisher, M. H. Caruthers, and A. J. Berk.** 1982. Resolving the functions of overlapping viral genes by site specific mutagenesis at a mRNA splice site. Nature (London) **295**:380–384.
23. **Moran, E., T. Grodzicker, R. J. Roberts, M. B. Mathews, and B. Zerler.** 1986. The lytic and transforming functions of individual products of the adenovirus E1A gene. J. Virol. **57**:765–775.
24. **Mukai, N., S. S. Kalter, L. B. Cummins, V. A. Matthews, T. Nishida, and T. Nakajima.** 1980. Retinal tumour induced in the baboon by human adenovirus 12. Science **210**:1023–1025.
25. **Paraskeva, C., and P. H. Gallimore.** 1980. Tumorigenicity and *in vitro* characteristics of rat liver epithelial cells and their adenovirus-transformed derivatives. Int. J. Cancer **25**:631–639.
26. **Perricaudet, M., G. Akusjarvi, A. Vortanen, and U. Pettersson.** 1979. Structure of 2 spliced mRNAs from the transforming region of human subgroup C adenoviruses. Nature (London) **281**:694–696.
27. **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.
28. **Rein, A., A. M. Schultz, J. P. Bader, and R. H. Bassin.** 1982. Inhibitors of glycosylation reverse retroviral interference. Virology **119**:185–192.
29. **Roberts, B. E., J. S. Miller, D. Kimelman, C. L. Cepko, I. R. Lemischka, and R. C. Mulligan.** 1985. Individual adenovirus type 5 early region 1A gene products elicit distinct alterations of cellular morphology and gene expression. J. Virol. **56**:404–413.
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. **Sato, G. H., A. B. Pardee, and D. A. Sirbasku.** 1982. Growth of cells in hormonally defined media, p. 541–615. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
32. **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.
33. **Teich, N., J. Wyke, T. Mak, A. Bernstein, and W. Hardy.** 1984. Pathogenesis of retrovirus-induced disease, p. 845–923. *In* R. Weiss, N. Teich, H. Varmus, and J. Coffin (ed.), RNA tumor viruses, vol. I. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
34. **Teich, N. M., and T. M. Dexter.** 1978. Effects of murine leukemia virus infection on differentiation of hematopoietic cells *in vitro*. Cold Spring Harbor Conf. Cell Proliferation **5**:657–670.
35. **van der Eb, A. J., H. van Ormondt, P. I. Schrier, J. H. Lupker, H. Jochemsen, P. J. van den Elsen, J. de Leys, J. Maat, C. P. van Beveren, R. Dijkema, and A. de Waard.** 1979. Structure and function of the transforming genes of human adenoviruses and SV40. Cold Spring Harbor Symp. Quant. Biol. **44**:383–398.
36. **Velcich, A., and E. Ziff.** 1985. Adenovirus E1A proteins repress transcription from the SV40 promoter. Cell **40**:705–716.
37. **Winberg, G., and T. Shenk.** 1984. Dissection of overlapping functions within the adenovirus type 5 E1A gene. EMBO J. **3**:1907–1912.
38. **Yee, S., and P. E. Branton.** 1985. Detection of cellular proteins associated with human adenovirus type 5 early region 1A polypeptides. Virology **147**:142–153.