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Transformation of Differentiated Rat Hepatocytes with Adenovirus and Adenovirus DNA

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Primary cultures of hepatocytes isolated by collagenase perfusion of adult rats were transformed by infection with adenovirus type 5 or transfection with adenovirus DNA. Total virion DNA or recombinant plasmid DNA containing the adenovirus E1A and E1B genes transformed hepatocytes at comparable frequencies. No foci of replicating hepatocytes were detected after transfection with a plasmid containing the E1A gene alone. The frequency of transformation by the adenovirus E1A and E1B genes was dependent on the composition of the culture medium. Transformation occurred at a low frequency when the transfected hepatocytes were maintained in a chemically defined medium (CDM), but the frequency was enhanced 8- to 10-fold when the cells were maintained in (i) serum-supplemented medium or (ii) CDM supplemented with epidermal growth factor. Cell lines derived from the adenovirus-transformed colonies of hepatocytes expressed adenovirus E1A and E1B RNAs. When hepatocytes were maintained in CDM supplemented with dimethyl sulfoxide and transfected with plasmids containing the E1A and E1B genes, it was possible to derive cell lines that retained the ability to express several liver-specific genes, including albumin, transferrin, hemopexin, and the third component of complement. The amount of albumin secreted per cell varied from 1 to 5 pg per cell per 24 h, and in one cell line it was below detectable levels by passage 9. Adenovirus-transformed hepatocytes were not tumorigenic when inoculated subcutaneously into neonatal syngeneic rats. We conclude that the adenovirus E1A and E1B genes are capable of transforming adult rat hepatocytes, a differentiated epithelial cell type.

Adenoviruses have been used advantageously for studying tumor induction and transformation of cells in culture (3, 62). Cells transformed by adenoviruses retain the leftmost 14% of the adenovirus genome (20, 55), which contains the two adenovirus early region 1 (E1) genes E1A and E1B. Restriction fragments of the adenovirus genome containing these genes represent the minimal amount of genetic information needed to fully transform baby rat kidney (BRK) cell cultures (24). Adenoviruses have been classified as oncogenic or nononcogenic on the basis of their ability to form tumors in infected hamsters (29). Several adenovirus species transform primary cultures of rodent cells; however, tumorigenicity of the transformed cells varies. Rat cells transformed by E1 genes of the oncogenic group A adenovirus type 12 (Ad12) are tumorigenic when transplanted into syngeneic hosts (2, 17), whereas cells transformed by nononcogenic group C adenoviruses Ad2 and Ad5 vary in tumorigenicity (19, 44). The E1A gene plays a particularly important role in transformation because E1A alone immortalizes primary cultures of rat cells (17, 28) and cooperates with E1B or activated cellular or viral oncogenes in cotransfection assays to transform primary rat cells to a fully transformed, neoplastic phenotype (18, 52, 66). The ability of the E1A gene to cooperate with activated cellular oncogenes to induce a tumorigenic phenotype suggests that adenovirus is a useful model system for studying the molecular changes that cause tumor initiation and progression.

Viral transformation of cells in culture has been used extensively to examine the mechanisms of carcinogenesis. More recently, DNA transfection has made it possible to study in vitro transformation by introducing specific oncogenic virus and cellular DNA sequences into different cell types (24). Many transfection studies have been done to define the genes or DNA sequences necessary for transformation of NIH 3T3 cells (37, 42, 46, 57). Although NIH 3T3 cells were initially mouse embryo cells, they were adapted to grow indefinitely in monolayer culture and during this process apparently acquired some of the modifications that occur during transformation of a primary cell. Subsequent studies demonstrated that although transfection with a single oncogene is sufficient for transformation of NIH 3T3 cells, cooperation between two oncogenes is necessary for transformation of primary cells (39, 43, 48, 52, 61). The realization that transformation of primary cells by DNA involved multiple factors or multiple steps provides support for the concept of multistage progression in cancer (13) postulated from studies of chemically induced mouse skin tumors in vivo (7, 15, 26, 27) and chemically induced transformation of cells in culture (36). Although primary cells by definition do not form stable cell lines, many primary cell types replicate. We have concentrated on using primary hepatocytes, cells that do not replicate in culture, to determine what oncogenic information is required for immortalization or transformation of a nonreplicating epithelial differentiated cell type.

Most tumors that occur in humans are carcinomas that originate from differentiated adult epithelial cells or stem cells. Therefore, it is particularly appropriate to use differentiated epithelial cells as a model system in the study of the molecular changes that contribute to transformation in vitro. Model systems for studying transformation of differentiated epithelial cells with defined genetic information are limited (9, 10, 47, 49, 65), and no system for studying adenovirus transformation in primary cultures of adult differentiated epithelial cells has been described. Most transformation studies with adenovirus have been done in BRK cells (17, 23, 24, 28, 52, 63, 66), although transformation assays have been done in human embryonic retinoblasts (8, 18), rat embryo brain cells (16, 17), and liver epithelial cell lines (44, 45).

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Our previous studies have shown that primary cultures of hepatocytes isolated from adult rats by collagenase perfusion can be immortalized by infection with simian virus 40 (SV40) (31) or by transfection with SV40 DNA (64). These SV40-immortalized hepatocytes were subpassaged to form stable cell lines that retained the ability to express a number of liver-specific genes (63a). Two advantages of the hepatocyte system developed by transfection with SV40 DNA are that it can be extended (i) to examine the transformation of hepatocytes by transfection with other DNA sequences and (ii) to study the effect of this process on the expression of differentiated functions. We therefore initiated a study to transform hepatocytes with adenovirus to test the potential of specific genetic information that is different from SV40 in its potential to transform a hepatocyte.

The primary objectives of this study were to (i) determine whether adenovirus can transform an adult differentiated epithelial cell; (ii) define the adenovirus genetic information required for transformation; and (iii) determine the effect of transformation by adenovirus on the expression of liver-specific genes. We report that transformation of hepatocytes was accomplished by transfection with E1A and E1B genes and that the transformed cells retained the ability to express several liver-specific functions. Development of the system made it possible to compare the effects of adenovirus transformation with those of SV40 on the expression of tissue-specific functions by hepatocytes.

MATERIALS AND METHODS

Preparation of hepatocyte cultures. Hepatocytes were isolated from adult male Fischer F344 rats (180 to 200 g) by in situ perfusion with collagenase as described previously (4) and modified (14, 30). Serum-supplemented medium (SSM) was L-15 medium containing 5% fetal bovine serum and chemically defined medium (CDM) was RPCD (64). RPCD supplemented with culture-grade epidermal growth factor (EGF; 25 ng/ml of medium; Collaborative Research, Inc., Bedford, Mass.) was designated CDM+EGF, and CDM supplemented with EGF and dimethyl sulfoxide (DMSO, 2%; Sigma Chemical Co., St. Louis, Mo.) was designated CDM+EGF+DMSO. Isolated hepatocytes were washed three times with SSM or CDM and plated at a density of 10^6 cells per culture on 60-mm plastic cell culture dishes coated with rat tail collagen (12). Cultures were fed fresh SSM or CDM 5 to 8 h after plating and subsequently were fed SSM, CDM, CDM+EGF, or CDM+EGF+DMSO every 3 days.

The 293 cell line, an adenovirus-transformed human embryonic kidney cell line (22), was obtained from David Spector (The Pennsylvania State University College of Medicine, Hershey, Pa.) and grown in Dulbecco modified Eagle medium containing 10% fetal bovine serum, penicillin (150 U/ml), and streptomycin and kanamycin (100 μg/ml each).

Adenovirus infection. Monolayers of hepatocytes were infected with 0.01 to 1.0 PFU of Ad5 (kindly provided by David Spector) within 24 h of plating. All cultures were infected in a volume of 1.0 ml of virus suspension. After adsorption of virus for 3 h at 37°C, fresh medium was added.

Transfection of hepatocytes. Hepatocytes were maintained in culture for 18 to 24 h before they were transfected by using the calcium phosphate procedure (23) as modified previously (64). After 4 h, the transfection mixture was removed, the cells were washed twice in L-15 medium, and the transfected cultures were maintained for 24 h in SSM. At this time, cultures were refed CDM, CDM+EGF, or CDM+EGF+DMSO.

Recombinant plasmids. The recombinant plasmid pXC1 contains nucleotides 1 to 1338 (Ad2) and 1342 to 5792 (Ad5) of group C adenovirus DNA inserted between the *Pst*I and *Eco*RI sites of pBR322 (Fig. 1). Nucleotide 1338 is the *Xba*I site of Ad2, and nucleotide 1342 is the *Xba*I site of Ad5; the difference in nucleotide numbers of the two *Xba*I sites reflects base-pair differences in the two serotypes and does not influence the production of functional E1A proteins. Nucleotide 1 of the adenovirus DNA was joined to the *Pst*I site of pBR322 by using oligo(dG) · oligo(dC) homopolymers. Nucleotide 5792 of the adenovirus sequences was joined to the *Eco*RI site of pBR322 by using an *Eco*RI linker (59). The recombinant plasmid pXC2 contains nucleotides 1 to 448 and 1349 to 5792 of adenovirus DNA and was generated by substituting a DNA fragment from the *dl*312 genome which lacks the 449 to 1348 E1A sequences (34) for a corresponding wild-type fragment in pXC1 (S. Bondopadhyay and D. Spector, unpublished data). The E1A deletion in pXC2, as in *dl*312 DNA, removes both control and coding sequences so that no E1A RNA or proteins are produced (35, 56). The recombinant plasmid pXC3 contains nucleotides 1 to 2810 and 3328 to 5792 of adenovirus DNA (Spector, unpublished data), and pXC4 contains nucleotides 1 to 1770 and 3328 to 5792 of adenovirus DNA (V. Dias-Ferrao and D. Spector, unpublished data). The recombinant plasmids pXC1, pXC2, pXC3, and pXC4 were kindly provided by D. Spector. Plasmid pLA8 (52) contains nucleotides 1 to 3322 and was provided by Earl Ruley (Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, Mass.). Plasmid pAd12RICC2 (6) contains Ad12 E1A and E1B genes and was provided by René Bernards (Massachusetts Institute of Technology and Whitehead Institute for Biomedical Research, Cambridge, Mass.). Plasmid pBRWT2 contains the entire SV40 genome inserted into the *Eco*RI site of pBR322 and was provided by Mary J. Tevethia (The Pennsylvania State University College of Medicine, Hershey, Pa.). Plasmids used as probes for adenovirus RNA in Northern (RNA blot) hybridization

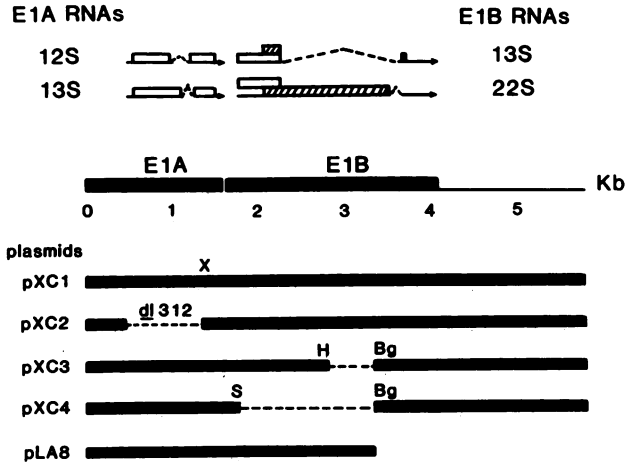

FIG. 1. Physical map of E1 of Ad2 and Ad5 showing spliced mRNA species and the structures of E1-containing plasmids used in this study. The positions of pertinent restriction endonuclease cleavage sites are indicated by the following abbreviations: Bg, *Bgl*II; H, *Hin*dIII; S, *Sac*I; and X, *Xba*I. The E1A RNAs include 9S, 12S, and 13S species; and the E1B RNAs include 9S, 13S, 14.5S, 14S, and 22S species. The E1A 12S and 13S and E1B 13S and 22S RNAs are shown because only these products have been identified in transformed cells. Kb, Kilobase.

studies included pHpaE (adenovirus nucleotides 1 to 1572), pE1b-E (adenovirus nucleotides 1572 to 2810), and pE1b-F (adenovirus nucleotides 2810 to 3943) and were provided by David Spector (58).

Rocket immunoelectrophoresis. The amount of rat albumin secreted into the culture medium was measured by rocket immunoelectrophoresis as described previously (40). Rat albumin (fraction V; Sigma) was diluted in the appropriate culture medium and used as a standard.

Immuno-overlay technique. The procedure for localizing albumin-secreting cells by immuno-overlay has been described (31, 53, 64). Briefly, cultures were covered with a mixture of 1% agarose in CDM supplemented with the purified immunoglobulin G fraction of goat anti-rat albumin (1:64 dilution; Cooper Biomedical, Inc., Malvern, Pa.). The immuno-overlay was removed from the culture after 24 h, washed three times with borate-buffered saline (24 h total), and incubated for 24 h with a 1:64 dilution of rabbit anti-goat immunoglobulin G (Cooper Biomedical). The immuno-overlay was washed three times, placed on a glass slide, dried at 60°C, and stained with Coomassie blue to visualize albumin-secreting colonies.

Immunoprecipitation. Plasma proteins secreted by adenovirus-transformed hepatocyte cell lines were radioactively labeled as described previously (21, 33) except that labeling was for 6 h. Cells were labeled at approximately 70% confluence. Immunoprecipitation was performed by reacting labeled medium with specific antisera followed by adsorption to staphylococcal protein A (IgSORB; The Enzyme Center, Boston, Mass.) as described previously (21, 32). Specific antisera were directed against rat albumin, the third component of complement, transferrin (Cooper Biomedical), hemopexin (John Taylor, Gladstone Foundation Laboratories for Cardiovascular Disease, San Francisco, Calif.), rat serum plasma proteins, and purified alpha-fetoprotein (Warren Liao, M. D. Anderson Hospital and Tumor Institute, Houston, Tex.). Proteins contained in the immune complexes were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (38) on 10 to 18% gradient gels, followed by fluorography (5). Molecular weights of specific polypeptides were determined with ^{14}C-labeled protein standards (New England Nuclear Corp., Boston, Mass.).

Northern blot analysis of adenovirus RNA. RNA was isolated from cell lines by lysis in guanidine thiocyanate (11), followed by centrifugation through cesium chloride (41). The RNA pellet was suspended and further purified by two extractions with phenol-chloroform-isoamyl alcohol (25:24:1). The RNA was then precipitated with ethanol and stored at −20°C. RNA was suspended just before use, and the RNA concentration was determined spectrophotometrically. For Northern blot hybridizations, RNA was loaded into each gel lane and electrophoretically separated on 1.4% agarose gels buffered in 20 mM MOPS (3-[*N*-morpholino] propanesulfonic acid; pH 7.0), 5 mM sodium acetate, 1 mM EDTA, and 3.7% formaldehyde. Before electrophoresis, the RNA was denatured in gel buffer containing 50% formamide. Electrophoretically separated RNA was transferred by blotting onto nitrocellulose filters which were baked at 80°C in a vacuum and prehybridized at 46°C for 48 h. The prehybridization buffer contained 50% deionized formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% bovine serum albumin, 0.1% polyvinylpyrrolidone, 0.1% Ficoll (Pharmacia Fine Chemicals, Piscataway, N.J.), 50 mM NaH_2PO_4 (pH 6.5), 0.1% sodium dodecyl sulfate, and 0.5 mg of sonicated salmon sperm DNA per ml. Plasmid DNA was nick translated by the procedure of Rigby et al. (50) and denatured in hybridization buffer (50% deionized formamide, 5× SSC, 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, 0.02% Ficoll, 20 mM NaH_2PO_4 [pH 6.5], 0.1% sodium dodecyl sulfate, 0.1 mg of sonicated salmon sperm DNA per ml, and 10% dextran sulfate). Hybridization was done at 46°C for 48 h with the addition of 1×10^6 to 5×10^6 cpm of specific probe per ml to each hybridization reaction. After hybridization, the filters were washed three times in 2× SSC at room temperature, three times in 0.1× SSC at 50°C for 30 min each, and then twice in 2× SSC at room temperature. Filters were then exposed to Kodak XAR-5 film at −70°C (Eastman Kodak Co., Rochester, N.Y.).

TABLE 1. Transformation frequency of primary rat hepatocytes infected with Ad5

MOI[a]	No. of colonies/total no. of dishes[b]	Avg no. of colonies/dish
Mock	0/8	0
0.03	3/8	0.4
0.1	11/11	1.0
0.3	5/9	0.6
1.0	0/8	0

[a] Expressed as PFU per cell. MOI, Multiplicity of infection.
[b] Each culture dish contained approximately 10^6 hepatocytes. The data represent the results from three independent experiments.

Tumor studies. Cells to be tested for tumorigenicity were harvested at subconfluent density by trypsinization. Trypsin activity was neutralized by using CDM supplemented with 5% fetal bovine serum. The cells were washed once in phosphate-buffered saline to remove serum proteins and suspended in Hanks balanced salt solution. The cell number was determined by using a hemacytometer, and cell viability was measured by trypan blue dye exclusion. Approximately 5×10^6 to 1×10^7 cells were injected subcutaneously into the flank of neonatal syngeneic Fischer F344 rats within 24 h after birth. Rats were palpated for tumors at weekly intervals, and all animals were maintained for at least 6 months.

RESULTS

Transformation of hepatocytes by adenovirus infection. When primary cultures of adult rat hepatocytes were infected with Ad5, foci of transformed cells were observed 2 to 4 months after infection. (The use of the term "transformed" to describe these cells is discussed in detail in the Discussion.) The frequency of transformation (number of colonies per 10^6 cells) was related to the multiplicity of infection (Table 1). At low multiplicity of infection (0.03 to 0.1 PFU per cell), approximately 1 focus developed per 10^6 cells and the amount of cell destruction caused by lytic adenovirus infection of the hepatocytes was limited. At higher multiplicity of infection (0.3 to 1.0 PFU per cell), the frequency of transformation was reduced and degeneration and sloughing of the entire monolayer occurred. The small foci of epithelial cells that replicated after low-multiplicity infection with adenovirus grew slowly and consisted of cells that grew on top of one another, forming dense colonies. These colonies were easily distinguished from normal hepatocytes because after 2 to 4 months, the hepatocytes had senesced and sloughed from the culture dish. Spontaneously arising foci of replicating epithelial cells in hepatocyte cultures have not been observed in a large number of independent experiments.

Transformation by transfection. Our goal was to define more specifically the adenovirus genetic information that

TABLE 2. Transformation frequency of primary rat hepatocytes[a] after transfection with adenovirus or SV40 DNA

Virus DNA	No. of colonies/total no. of dishes	Avg no. of colonies/dish
–[b]	0/31 (4)[c]	0
Ad5 DNA	42/7 (3)	6.0
pXC1[d]	162/21 (5)	7.7
pXC2/pXC4	77/27 (7)	2.9
pLA8	5/28 (4)	0.2
pXC3	1/11 (3)	0.1
pXC2	0/20 (5)	0
pXC4	0/23 (6)	0
pAd12RICC2	0/24 (5)	0
pBRWT2	162/15 (8)	10.8

[a] Hepatocytes were maintained in SSM.
[b] –, Hepatocytes were transfected with carrier DNA alone.
[c] Number of independent experiments is given in parentheses.
[d] Plasmid DNAs are defined in Materials and Methods.

was sufficient to transform hepatocytes. To accomplish this goal, we used the calcium phosphate technique to introduce adenovirus DNA into primary cultures of rat hepatocytes. Continuously growing foci of transformed hepatocytes arose after primary cultures were transfected with Ad5 DNA purified from virions (Table 2). The frequency of focus formation after transfection with Ad5 DNA was enhanced approximately 5- to 10-fold compared with that observed after virus infection. The use of the transfection procedure dramatically reduced the cell destruction caused by lytic virus infection.

To define more specifically the adenovirus genes required for transforming nonreplicating differentiated hepatocytes, the cells were transfected with plasmids containing only the adenovirus early region (E1A and E1B genes), the region previously shown to be sufficient for transformation of primary cultures of BRK cells (24). The transformation frequency by a plasmid containing the adenovirus E1A and E1B genes (pXC1) was comparable to that for the entire adenovirus genome and comparable to that obtained when hepatocytes were transfected with SV40 DNA (Table 2). Foci of epithelial cells were first visualized 2 to 4 months after transfection regardless of whether transformation occurred as a result of Ad5 infection, transfection with the entire Ad5 genome, or with pXC1. Cell lines were derived from specific foci, and cells from each cell line differed somewhat morphologically (Fig. 2).

BRK cells transfected with the Ad5 E1A gene alone form foci of continuously dividing immortalized cells (28). These cells differ in growth characteristics and morphology from BRK cells that arise after transfection with both the E1A and E1B genes. We examined whether the adenovirus E1A gene alone would immortalize rat hepatocytes. In five independent experiments, transfection of hepatocytes with a plasmid containing a functional E1A region (pXC4) did not yield foci of proliferating cells. Transfection with a plasmid containing only a functional E1B region (pXC2) did not yield foci. When similar cultures were cotransfected with both the pXC2 and pXC4 plasmids, foci of continuously dividing tightly associated cells arose, but at a frequency lower than that observed after transfection with pXC1, a plasmid encoding both E1A and E1B genes. We would have predicted that the transformation frequency for cotransfection with two separate plasmids (one carrying the E1A gene and the other carrying the E1B gene) would be lower than that for transfection with a single plasmid (encoding the E1A and E1B genes) since the cotransfection requires that both plasmid DNAs be taken up by the same cell. The ability to transform by cotransfection with the pXC2 and pXC4 plasmids was an important control in that it demonstrated that both plasmids had the potential to be biologically active, even though neither could transform when used alone.

We next examined the ability of hepatocytes to be transformed by a plasmid containing adenovirus nucleotides 1 to 3322 (pLA8). The pLA8 plasmid contains a deletion and does not have the capacity to code for an intact E1B 496R protein. Hepatocytes were transformed by transfection with the pLA8 plasmid but at low frequency (Table 2). Transformed cells in these foci grew particularly slowly, and the cells often retracted from the culture dish to form dense aggregates that were released into the culture medium. However, these transformed cells could be subcultured to form stable cell lines (Fig. 2) that grew to low densities. When hepatocytes were transfected with a second plasmid containing a larger deletion in E1B nucleotides encoding the carboxy terminus of the 496R protein (pXC3), transformation also occurred at low frequency (Table 2). No transformation was observed after transfection with pAd12RICC2, a plasmid which contains the E1 region of Ad12. It was not surprising that transformation was not accomplished after transfection with Ad12 E1 sequences, since it has been previously demonstrated in other systems that the frequency of transformation by Ad12 is less than that by Ad5 (62).

Effect of medium composition on transformation frequency. We have shown previously that the frequency at which primary rat hepatocytes are immortalized by SV40 DNA depends on the type of medium in which the hepatocytes are maintained (64). The use of SSM or supplementation of the CDM with EGF increased the frequency of immortalization by SV40 DNA. The studies on transformation of hepatocytes by adenovirus virion DNA and plasmids containing adenovirus DNA sequences reported in Table 2 were done using hepatocytes plated in SSM. To determine whether transformation by adenovirus was similarly affected by the composition of the medium, hepatocytes were transfected with pXC1, pLA8, or SV40 DNA. The transfected cultures were maintained in SSM, CDM, CDM+EGF, or CDM+EGF+DMSO. If hepatocytes transfected with pXC1 (the intact E1 region of adenovirus) were maintained in CDM supplemented with EGF, the frequency of transformation was similar to that observed in SSM (Table 3) and the colonies were first visualized 6 to 8 weeks after transfection. Hepatocytes in CDM were transformed at lower frequencies, and colonies were not visualized until 3 to 4 months after transfection. The effect of medium composition on transformation frequencies of hepatocytes transfected with a plasmid containing an intact E1 region was similar to that observed in hepatocytes transfected with SV40 DNA (64). Very different results were obtained when hepatocytes were transfected with pLA8 (a plasmid containing an intact E1A region and a deletion in the E1B region) in that the frequency of transformation was low regardless of the culture medium (Table 3).

Expression of adenovirus RNA. Individual foci of adenovirus-transformed hepatocytes were picked from several cultures and were subpassaged repeatedly to form cell lines. Cell lines were derived from cultures transformed by infection with Ad5 or transfected with a variety of adenovirus DNAs, including virion DNA, pXC1, pXC3, and pLA8 DNAs, and cotransfected with pXC2 and pXC4 DNAs. RNA was extracted from seven of these cell lines and analyzed by Northern blot hybridization (Fig. 3). The adenovirus-transformed 293 cell line was used as a positive

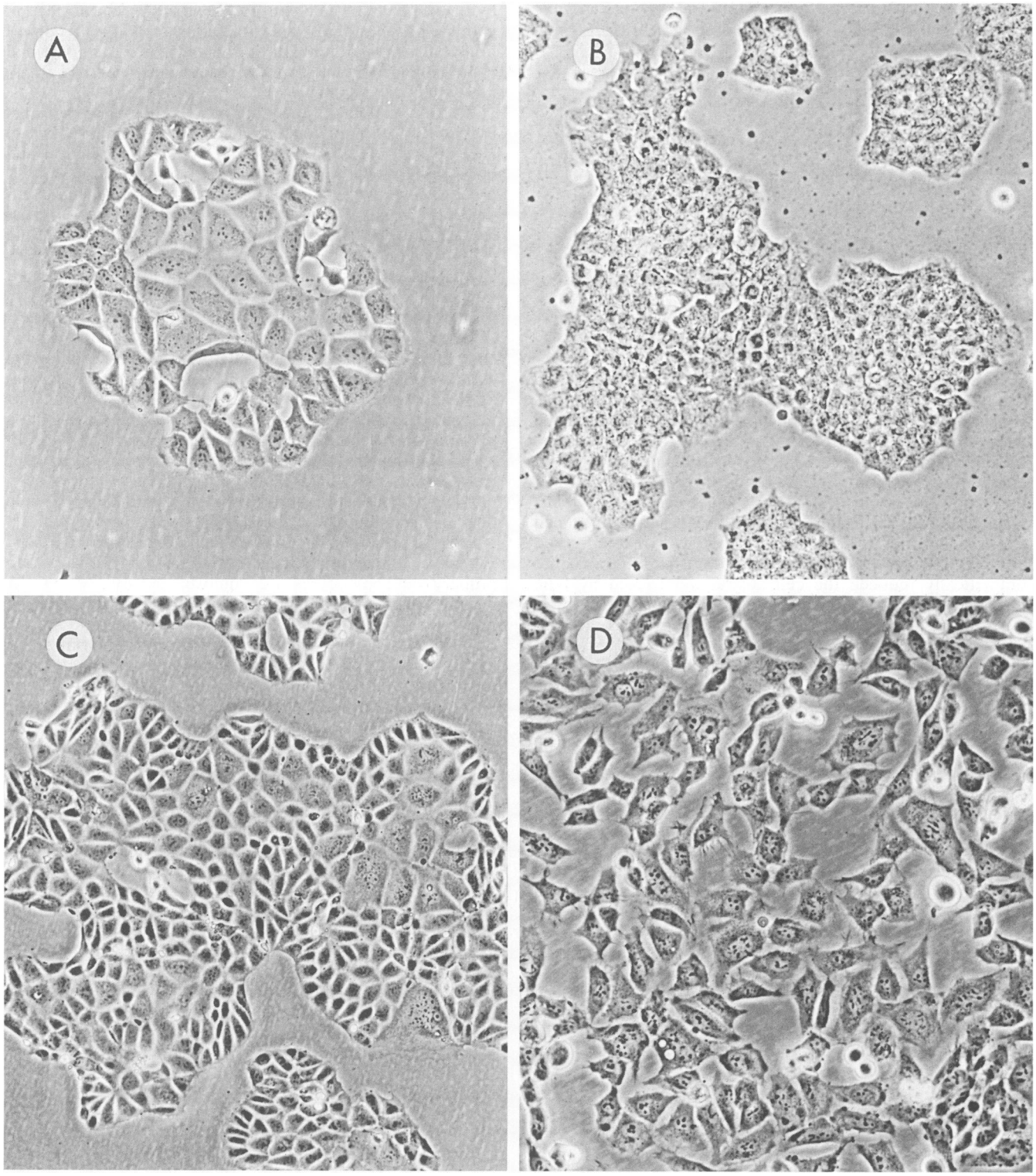

FIG. 2. Morphology of hepatocyte cell lines derived from colonies of adenovirus-transformed hepatocytes. (A) Ad5-1 cells, derived by infection of hepatocytes with Ad5; (B) AdPX4 cells, derived by transfection with pXC1; (C) AdCO2 cells, derived by cotransfection with plasmids pXC2 and pXC4; and (D) AdP1 cells derived by transfection with pLA8.

control for expression of E1A and E1B RNA. The SV40-immortalized hepatocyte cell line CWSV8 (63a) which expresses SV40 RNA sequences was used as a negative control. When RNA from CWSV8 cells was hybridized to a radioactively labeled E1A probe, a faint hybridization band migrating at approximately 28S was observed. This 28S band is a background band, which most likely represents probe trapping and was also observed when hybridization to RNA

TABLE 3. Effect of medium supplementation on the transformation frequency of primary rat hepatocytes

Medium	Transfected DNA		
	pXC1	pBRWT2	pLA8
SSM	7.7 (162/21)[a]	10.8 (162/15)	0.2 (5/28)
CDM	1.0 (14/14)	1.6 (52/32)	0.2 (2/13)
CDM + EGF	4.9 (117/24)	8.5 (356/42)	0.2 (3/20)
CDM + EGF + DMSO	1.7 (68/40)	7.6 (267/35)	0 (0/20)

[a] Values represent average number of colonies per dish. Numbers in parentheses indicate total number of colonies per total number of cultures examined. All values represent the sum of data from at least four independent experiments.

from adenovirus-transformed hepatocyte cell lines and 293 cells was done. In the lane containing RNA from the control 293 cells (Fig. 3A, lane 1) the intense band in the autoradiograph represents hybridization to E1A 12 and 13S RNAs migrating together to form a wide band. This 12 to 13S RNA was expressed by all the adenovirus-transformed hepatocyte cell lines and not by CWSV8. High-molecular-weight RNAs which hybridized to the radioactively labeled E1A probe also were observed and may represent RNAs that read through to the E1B gene or to host cell sequences. Hepatocytes transformed by pLA8, a plasmid that does not have the coding sequences for a complete 496R protein, expressed authentic size class E1A RNAs as well as higher-molecular-weight RNAs (Fig. 3A, lane 5).

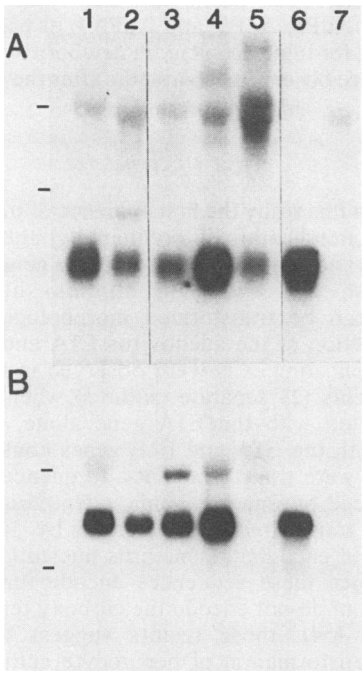

FIG. 3. Expression of adenovirus E1A and E1B RNA in adenovirus-transformed hepatocyte cell lines. Total cellular RNA was isolated from each of the cell lines and was hybridized to ^{32}P-labeled plasmid DNAs containing adenovirus E1A (A) or E1B (B) genes. RNAs were isolated from lanes: 1, 293 cells; 2, AdPX3 cells, derived by transfection with pXC1; 3, AdCO1 cells, derived by cotransfection with plasmids pXC2 and pXC4; 4, AdCO2 cells, derived by cotransfection with plasmids pXC2 and pXC4; 5, AdP4 cells, derived by transfection with pLA8; 6, Ad5-6 cells, derived by infection with Ad5; and 7, CWSV8 cells, derived by transfection with pBRWT2, a plasmid containing the SV40 genome. The 293 cells were used as a positive control, and the CWSV8 cells were used as a negative control. Markers indicate the positions of the 28S and 18S RNA bands.

When RNA from control 293 cells was hybridized to a radioactively labeled E1B probe, an intense band migrating at 22S was observed and represented hybridization to the 22S E1B RNA. A faint band migrating at approximately 28S was also seen and most likely represented background hybridization (Fig. 3B, lane 1). Cell lines transformed by transfection with DNA containing intact E1A and E1B regions expressed the 22S E1B RNA (Fig. 3B, lanes 1 to 4, 6), whereas one cell line (AdP4) transformed by DNA containing adenovirus nucleotides 1 to 3322 (an intact E1A region and an E1B region containing a deletion) did not express the 22S E1B RNA. These results indicate that transformation can be maintained in AdP4 cells without expression of the 22S E1B RNA. However, as will be demonstrated below, AdP4 cells did not express albumin, and although they are of liver origin they may not be of hepatocyte origin.

Expression of albumin. The next goal was to determine the effect of transformation of hepatocytes by adenovirus on the expression of liver-specific genes. We initially concentrated on the liver-specific gene albumin. We had previously determined that a small percentage (17 to 20%) of the colonies that arose after transfection of hepatocytes with SV40 DNA contained cells that secreted albumin if the hepatocytes were maintained in CDM or CDM+EGF (64). This percentage increased to 50% if the hepatocytes were maintained in CDM+EGF+DMSO. When hepatocytes were transfected with adenovirus DNA and maintained in CDM or CDM+EGF, none of the colonies continued to secrete albumin (data not shown). However, when hepatocytes transfected with adenovirus DNA were maintained in CDM+EGF+DMSO, a small portion of the colonies (16%) contained cells that continued to secrete albumin (Table 4). These colonies characteristically were small, grew slowly, and were first visualized 3 to 4 months after transfection.

Derivation of albumin-producing adenovirus-transformed hepatocyte cell lines. Albumin-producing cells present in colonies that arise after transfection of hepatocytes with SV40 DNA can be successfully subcultured to yield SV40-immortalized hepatocyte cell lines (63a). Eleven albumin-producing SV40-immortalized hepatocyte cell lines were developed that continued to produce albumin (at levels as high as 30 to 40 pg per cell per 24 h) for at least 30 passages and expressed numerous other liver-specific genes. The same procedures used to isolate SV40-immortalized hepatocyte cell lines were used to derive cell lines from foci of adenovirus-transformed hepatocytes. Ten colonies of adenovirus-transformed hepatocytes were subcultured by mechanically disrupting the original colonies and plating the aggregates of cells on collagen-coated culture plates. When the secondary cultures were analyzed for albumin produc-

TABLE 4. Percentage of albumin-secreting colonies after transformation of primary rat hepatocytes with adenovirus or SV40 DNA

Plasmid DNA	No. of albumin-secreting colonies/ total no. of colonies[a]	% Colonies secreting albumin
pXC1	10/61 (8)[b]	16
pBRWT2	59/131 (7)	45

[a] Hepatocytes were maintained in CDM + EGF + DMSO, and the albumin-secreting colonies were identified by immuno-overlay.
[b] Number of independent experiments is shown in parentheses.

tion by immuno-overlay, only two of the original colonies yielded cultures containing cells that continued to secrete albumin. Cells in the other eight cultures continued to proliferate, but no albumin secretion was detected. The two secondary cultures of albumin-secreting transformed hepatocytes contained many colonies, and all were positive for albumin production as determined by immuno-overlay. When several colonies were dispersed, replaced in culture, and analyzed for albumin production, all of the tertiary colonies that arose were positive. These cultures were trypsinized and replated to establish two albumin-secreting adenovirus-transformed hepatocyte cell lines (designated AdPX3 and AdPX4). At early passage, these cell lines secreted albumin at levels from 1 to 4 pg per cell per 24 h. The amount of albumin secreted per cell for AdPX3 and AdPX4 was less than that produced by primary hepatocytes in culture or by many of the SV40-immortalized hepatocyte cell lines. It is of interest that the only two albumin-secreting cell lines that could be established came from cells transfected with pXC1.

To examine whether the albumin-secreting AdPX3 and AdPX4 cell lines expressed other liver-specific genes, cells were radioactively labeled with [^{35}S]methionine and radioactively labeled plasma proteins secreted into the culture medium were immunoprecipitated by using specific antisera. Both cell lines secreted additional plasma proteins including transferrin, hemopexin, and the third component of complement (Fig. 4). These proteins migrated to the same positions as those secreted by cultures of freshly isolated normal rat hepatocytes. Neither of the two adenovirus-transformed cell lines secreted alpha-fetoprotein, an oncodevelopmental protein expressed in regenerating liver and often during the process of hepatocarcinogenesis.

When the AdPX3 cells were analyzed for albumin production with continued passage in culture, the amount of albumin secreted per cell declined slowly but steadily during the first nine subpassages until it was no longer detectable by rocket immunoelectrophoresis (less than 1 pg per cell per 24 h) (Table 5). Although the cells lost the ability to secrete albumin with passage, the morphology of the cells did not change. When AdPX4 cells were analyzed for albumin production with continued passage in culture, the amount of albumin secreted per cell declined steadily but was still detectable by passage 13.

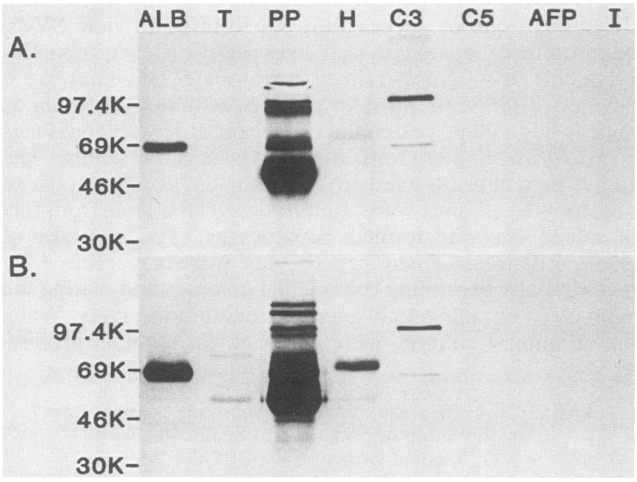

FIG. 4. Plasma proteins secreted by albumin-secreting adenovirus-transformed hepatocyte cell lines. The cell lines AdPX3 (A) and AdPX4 (B) were radioactively labeled with [^{35}S]methionine, and plasma proteins were immunoprecipitated from the culture medium by using antibodies to albumin (ALB), transferrin (T), a mixture of purified plasma proteins (PP), hemopexin (H), the third (C3) and fifth (C5) complement components, alpha-fetoprotein (AFP), and rat immunoglobulin (I). Marks at the left of the gels indicate the positions of the molecular weight markers. K, Kilodaltons.

TABLE 5. Albumin secretion by adenovirus-transformed hepatocytes

Adenovirus	Passage no.	Albumin secretion (pg/cell per 24 h)
AdPX3	3	1.5
	5	0.5
	6	0.4
	7	0.1
	8	0.05
	9	0
	10	0
AdPX4	3	3.2
	4	3.1
	5	1.0
	6	1.4
	7	1.3
	13	1.0

Tumorigenicity. The adenovirus-transformed cell lines, Ad5-1, AdP1, AdP2, AdP5, and AdPX4, at passages below 10 were tested for tumorigenicity in newborn syngeneic rats. No tumors were observed at 6 months after the animals were injected.

DISCUSSION

We report in this study the first evidence of transformation of an adult nonreplicating differentiated epithelial cell type by adenovirus and define which adenovirus genes are needed to accomplish this aim. Our studies show that rat hepatocytes can be transformed morphologically by the cooperative action of the adenovirus E1A and E1B genes. No foci of replicating cells were observed in five independent experiments (23 separate cultures) when hepatocytes were transfected with the E1A gene alone. Hepatocytes transfected with the E1A and E1B genes contained on the same plasmid were transformed at a frequency comparable to that produced by intact genomic adenovirus DNA. The frequency of transformation decreased by 10- to 50-fold when a plasmid encoding adenovirus nucleotides 1 to 3322 was used. Since these sequences encode functional E1A polypeptides but do not encode the carboxy terminus of the 496R protein (54), these results suggest that efficient adenovirus transformation of hepatocyte cultures requires intact coding sequences for the 496R protein. In addition, we observed that the enhanced frequency of transformation by adenovirus of hepatocytes maintained in SSM or CDM+EGF instead of CDM was not seen when a plasmid that did not encode an intact 496R protein was used. These findings indicate that transformation of primary hepatocytes by adenovirus is different from what has been reported previously for other cell types. For example, transfection of primary BRK cells with E1A sequences alone immortalized BRK cells (17, 28). The immortalized BRK cells differed from BRK cells transformed by virus in that they had a fibroblastic appearance and grew to low densities. Similar results have been obtained for human embryonic reti-

noblasts except that the frequency of immortalization was lower than for BRK cells and successfully establishing an E1A-immortalized human embryonic retinoblast cell line was more difficult (17). The effect of cooperation between the E1A and E1B genes has been shown; when BRK cells were transfected with E1A and E1B, the transformation frequency was higher than that seen for E1A alone and the transformants were morphologically different from E1A transformants and the same as cells transformed by virus (28). It has been previously shown that transfection of BRK cells with DNA containing intact E1A sequences and truncated E1B sequences similar to the constructs used in this report resulted in a transformation frequency only slightly lower than when transfection was done with an intact E1 region (51). Evidence from a number of experiments suggests that at least two E1B proteins are involved in the transformation process (for a review, see reference 1); one of the most recent studies clearly demonstrated that expression of proteins from both E1B reading frames is necessary for DNA-mediated transformation of CREF cells (1).

The terms immortalization and transformation need clarification. In our previous studies involving transfection of hepatocytes, we defined the epithelial cells that replicate in hepatocyte cultures after transfection with SV40 DNA as immortalized because these cells could be established into cell lines but were not tumorigenic when inoculated into newborn syngeneic rats. SV40-immortalized hepatocytes underwent changes with time in culture such that they became tumorigenic at later passages, and we designated these later passage cells SV40-transformed hepatocytes (unpublished data). Our preference would have been to refer to cells that replicate in hepatocyte cultures after transfection with adenovirus DNA as adenovirus-immortalized hepatocytes. Instead, we have designated cells that replicate in hepatocyte cultures after transfection with adenovirus DNA as transformed for two reasons. First, it was not possible to use "immortalize," because this term in adenovirus systems relates to the events that result after introduction of only E1A DNA and hepatocytes did not develop into replicating epithelial cells unless the cells received both E1A and E1B sequences. Second, we did not want to use the term "fully transformed," because replicating hepatocytes containing adenovirus information, at least at low passage, did not produce tumors when inoculated into newborn syngeneic animals.

We report in this paper that although hepatocytes cultured in CDM or CDM+EGF and transfected with adenovirus DNA (genomic or the E1 region) yielded colonies of epithelial cells, these cells did not produce albumin. We have previously shown that under identical conditions, transfection with SV40 DNA does yield albumin-producing cells (64). Supplementation of the medium with DMSO increased the frequency of obtaining albumin-producing colonies after transfection with SV40 DNA, but it was not necessary to use DMSO to obtain albumin-producing colonies. In contrast, to derive albumin-producing adenovirus-transformed cells, it was necessary to maintain the transfected hepatocytes in CDM+EGF+DMSO. These results are most likely related to (i) the effect of the culture medium composition on the length of time that primary hepatocytes continue to secrete albumin and (ii) the time required for immortalization by SV40 compared with adenovirus. When hepatocytes are cultured in CDM or CDM+EGF, the ability to secrete detectable levels of albumin is lost by 2 to 4 weeks. In contrast, when hepatocytes are fed CDM+EGF+DMSO, albumin secretion remains high for at least 60 days and in some cultures for longer than a year. In CDM+EGF, transformation by SV40 DNA was accomplished as early as 3 weeks after plating, whereas adenovirus transformation required at least 6 weeks. It is likely that failure to produce albumin-producing adenovirus-transformed hepatocytes in CDM+EGF is caused by the fact that hepatocytes in this medium lose the ability to secrete albumin before replication of the transformed cells begins.

Our data suggest that adenovirus functions have different effects than SV40 functions on the expression of tissue-specific genes in transformed hepatocytes. Although albumin-producing adenovirus-transformed colonies arose when hepatocytes transfected with adenovirus were maintained in CDM+EGF+DMSO and cell lines were generated from these colonies, the cells differed from SV40-immortalized hepatocytes in that they produced lower levels of albumin. SV40-immortalized hepatocyte cell lines secreted albumin at levels similar to freshly isolated hepatocytes placed in culture and up to 40-fold greater than the adenovirus-transformed hepatocyte cell lines. SV40-immortalized hepatocyte cell lines retained the ability to secrete albumin for at least 30 to 40 subpassages (unpublished data). The explanation for this difference in albumin gene expression between SV40-immortalized and adenovirus-transformed hepatocytes is unknown. If the goal is to generate established cell lines that express differentiated functions at levels like those found in liver and that can be used for molecular studies on differentiation and expression of tissue-specific functions, the hepatocytes should be transfected with SV40 and not adenovirus genetic information.

Of 61 adenovirus-transformed colonies that arose in transfected hepatocyte cultures and were examined by immuno-overlay, 10 retained the ability to secrete albumin, a plasma protein produced by normal hepatocytes. Two adenovirus-transformed cell lines were established from these colonies, and these cell lines continued to secrete albumin and additional plasma proteins including hemopexin, transferrin, and the third component of complement. These results are important for two reasons. First, the ability of these adenovirus-transformed cells to express albumin and other liver-specific genes and failure to express alpha-fetoprotein indicate that these cells arose from differentiated hepatocytes. Second, adenovirus transformation of hepatocytes yielded cells that expressed E1A RNA and the tissue-specific genes albumin, hemopexin, transferrin, and the third component of complement, suggesting that expression of the E1A gene does not eliminate expression of these tissue-specific genes in hepatocytes. (We did not look specifically for E1A proteins in these cells.) This second finding is of interest since adenovirus E1A gene products are known to modify the expression of certain tissue-specific genes. For example, E1A proteins inhibit transcription of the insulin gene in pancreatic beta cell lines (60) and repress the synthesis of immunoglobulin heavy chain genes in mouse plasmacytoma cell lines (25).

We have shown in this study that the hepatocyte system previously developed to study immortalization or transformation of an epithelial differentiated cell type is not limited to SV40 and can be extended to adenovirus genetic information. We also have demonstrated that this system can be used to learn more about adenovirus transformation. Specifically, we have shown that adenovirus can transform a differentiated epithelial cell, that this process can be accomplished by using cells in a CDM to yield transformed cells that grow in a CDM, and that both E1A and E1B functions are needed to accomplish this process.

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