JOURNAL OF VIROLOGY, Jan. 1997, p. 412–418 Vol. 71, No. 1
0022-538X/97/$04.00+0
Copyright © 1997, American Society for Microbiology

A Single Locus on Human Chromosome 21 Directs the Expression of a Receptor for Adenovirus Type 2 in Mouse A9 Cells

GREGORY A. MAYR AND PAUL FREIMUTH*

Biology Department, Brookhaven National Laboratory, Upton, New York 11973

Received 12 July 1996/Accepted 16 September 1996

The receptors on human cells which mediate adsorption of adenoviruses have not been identified. We found that murine A9 cells and Chinese hamster ovary (CHO) cells failed to bind significant levels of radiolabeled adenovirus type 2 (Ad2) virions but that derivatives of these cells carrying human chromosome 21 exhibited high levels of virus binding that was specific for the viral fiber protein. G418-resistant A9 cell transformants expressing Ad2 receptors were detected at a frequency of about 10^{-4} following cotransfection with high-molecular-weight DNAs from mouse cells containing human chromosome 21 and plasmid DNA containing a neomycin resistance gene. The Ad2 receptors on the transformed A9 cells were similar to those on human cells with respect to their concentration on the cell membrane, their affinity for the viral fiber protein, and their ability to direct virus into cells along a pathway leading to delivery of the viral DNA genome into the cell nucleus. Furthermore, identical human DNA fragments were present in three independent mouse cell transformants expressing Ad2 receptors, supporting the conclusion that these human DNA fragments correspond to a gene or locus on chromosome 21 that directs the expression of Ad2 receptors in these cells.

Adenoviruses are nonenveloped DNA viruses which cause ophthalmic, respiratory, gastrointestinal, and urinary tract infections in humans. More than 40 human adenovirus serotypes have been isolated, and these have been divided into six subgroups (A to E) based on nucleic acid homology, oncogenic potential, and the biological properties of their protein components. The cell type distribution of the receptors which mediate virus adsorption is likely to be a major determinant of virus tropism; however, receptors have not been identified for any adenovirus serotype. Virus-binding studies suggest that serotypes within a subgroup share receptors, while distantly related viruses (e.g., adenovirus type 3 [Ad3] and Ad5 from subgroups B and C, respectively) bind different receptors (6, 24, 28, 37). However, serotypes Ad2 and Ad12 (from subgroups C and A, respectively) compete for the same receptor (1). Thus, the tropism of human adenoviruses is governed by at least two distinct receptors.

Studies with the prototypic Ad2 and Ad5 viruses showed that the ligand for the adenovirus receptor resides in the distal tip of the viral fiber protein (15, 22, 26, 35, 42), a rod-shaped molecule which projects radially from each of the 12 vertices of the icosahedral capsid (32). Subsequent to virus adsorption, Ad2 and several other serotypes bind to αv-containing integrins through an Arg-Gly-Asp (RGD) sequence in the viral penton base (2, 28), a pentameric protein that forms the capsid vertices and anchors the fiber protein to the vertex (32). Recruitment of integrins into the initial virus-receptor complex enhances the internalization of virus. Ad2 mutants lacking the penton base RGD sequence are internalized at a rate markedly slower than that of the wild-type virus (2, 10), and inhibition of αv integrin function or expression decreases the susceptibility of host cells to infection (1, 3, 40). The proximity of the RGD and fiber ligands at each capsid vertex suggests that contact between integrins and the fiber receptor could occur, possibly generating a signal that regulates the endocytosis of virus-receptor complexes. Although integrins are not essential for infection of cultured cells, the striking conservation of the penton base RGD sequence implies that their role is important in vivo. Consequently, the cell type distribution of αv integrins may also determine virus tropism.

Identification of the receptors on host cells that recognize adenovirus fibers would make possible future investigation of the relationship between virus tropism and receptor distribution and of the mechanism of integrin-mediated virus internalization. In addition, the identification of receptors used by different virus serotypes would indicate the range of cell type-specific gene delivery that is possible with natural fiber-receptor interactions and thus would be of value in the development of adenoviruses as vectors for gene therapy. Earlier attempts to identify receptors by using fiber-affinity chromatography (14, 38), ligand overlay assays (6, 7), or phage display (17) led to the isolation of several fiber-binding proteins, although it has not been determined whether any of these correspond to functional virus receptors. Furthermore, only about 10^4 receptors are present on typical cultured human cell lines (10, 34, 40), which has made it difficult to obtain receptor proteins in sufficient quantities for partial sequence analysis. In view of the obstacles to a direct biochemical approach to identify adenovirus receptors, we conducted experiments to evaluate gene transfer as an alternate approach. Here we report that receptors for the Ad2 fiber protein are not expressed at detectable levels on the murine A9 cell line in a radiolabeled virus-binding assay but that A9-21 cells, an A9 derivative containing human chromosome 21, have a fiber-specific virus-binding capacity comparable to that of human A549 or HeLa cells. Furthermore, receptor function was transferred into A9 cells by transfection with high-molecular-weight A9-21 DNA at a frequency expected for transfer of a single gene. Independent transformants expressing the Ad2 receptor contain identical restriction fragments of human DNA, supporting the conclusion that these sequences are functionally related to receptor expression in the transformed cells.

* Corresponding author. Mailing address: Biology Department, Brookhaven National Laboratory, Upton, NY 11973. Phone: (516) 344-3350. Fax: (516) 344-3407. E-mail: freimuth@bnl.gov.

TABLE 1. ^{3}H-labeled Ad2 virus binding by A9 clones containing single human chromosomes

Cell line[a]	Selection	Ad2 binding[g]
A9-1[b]	G418	−
A9-2[b]	G418	−
A9-3[b]	G418	−
A9-4[c]	G418	ND
A9-5[b]	G418	−
A9-6[b]	G418	−
A9-7[b]	G418	−
A9-8[b]	G418	−
A9-9[b]	G418	−
A9-10[b]	G418	−
A9-11[b]	G418	−
A9-12[b]	G418	−
A9-13[d]	Hygromycin B	−
A9-14[d]	Hygromycin B	−
A9-15[b]	G418	−
A9-16[e]	G418	−
A9-17[f]	G418	−
A9-18[b]	G418	ND
A9-19[b]	G418	−
A9-20[b]	G418	−
A9-21[d]	Hygromycin B	+
A9-22[d]	Hygromycin B	−

[a] The single human chromosome in each A9 clone is indicated by the number following the hyphen.
[b] Isolated by Koi et al. (21).
[c] Isolated by Ning et al. (33).
[d] Isolated by Cuthbert et al. (5).
[e] Isolated by A. Phillips and B. E. Weissman (35a).
[f] MCH313C4 cells isolated by Goyette et al. (13).
[g] −, no binding; +, binding; ND, not determined.

MATERIALS AND METHODS

Cells and virus. A comprehensive panel of mouse A9 clones containing single human chromosomes tagged with drug resistance genes (Table 1) was obtained from C. Barrett (National Institute of Environmental Health Services, Research Triangle Park, N.C.). These cells were grown in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum and either 400 μg of G418 (Gibco) per ml or 100 U of hygromycin B (Calbiochem) per ml as indicated in Table 1 to maintain the human chromosomes. Chinese hamster ovary cells (CHO-K1 [ATCC CCL 61]) and E7b cells, a CHO-K1 clone containing human chromosome 21 (31), were obtained from D. Patterson (Eleanor Roosevelt Institute, Denver, Colo.). E7b cells were grown in purine-free Ham's F12D medium containing 5% dialyzed fetal bovine serum to maintain selection for 21q. CHO-K1 cells were grown in complete Ham's F12 medium containing 5% fetal bovine serum. Mouse A9 cells (ATCC CCL 1.4) and human A549 cells (ATCC CCL 185) were grown in DMEM containing 10% fetal bovine serum. The method used to prepare [^{3}H]thymidine-labeled Ad2 virus was described previously (1). Radiolabeled virus-binding assays were conducted either on cells in suspension as described previously (1) or on adherent cells in 24-well dishes (Costar). In all cases, the data reported represent the averages of triplicate determinations. Construction of Ad2RAE, an Ad2 mutant in which the Arg-Gly-Asp (RGD) sequence in the viral penton base that mediates binding of wild-type Ad2 to αv integrins was inactivated by conversion to Arg-Ala-Glu (RAE), was described previously (2).

Transfection. A9-21 cells were scraped into phosphate-buffered saline (PBS), centrifuged, resuspended in 0.15 M NaCl–0.1 M EDTA (4.5 ml per 10-cm-diameter dish of cells) and processed by the method of Jeffreys and Flavell (18) for extraction of high-molecular-weight DNA. pMAMneo DNA (ClonTech) was extracted from *Escherichia coli* cells by alkaline lysis and then banded once in cesium chloride. It was necessary to shear the A9-21 DNA prior to the formation of calcium phosphate precipitates to avoid macroscopic aggregation of the DNA. Sixteen micrograms of A9-21 DNA and 4 μg of pMAMneo DNA in 0.8 ml of water were mixed with 0.2 ml of 1.25 M $CaCl_2$ and then gently drawn into a tuberculin syringe and expelled three times through a 22-gauge needle. The DNA solution was then added drop by drop to 1 ml of 2× HEPES-buffered saline (280 mM NaCl, 50 mM HEPES, 1.5 mM Na_2HPO_4; pH 7.05) while air was constantly bubbling into the mixture. After the mixture was allowed to stand for 20 min at room temperature, 1 ml of DNA precipitate was added to each 10-cm-diameter dish of A9 recipient cells.

Ten 10-cm-diameter dishes were seeded with a 20-fold dilution of A9 cells from one confluent 10-cm-diameter plate 24 h before application of the calcium phosphate DNA precipitate. The culture medium was changed 16 h after transfection, and the cultures were further incubated for 24 h, at which time the cells were trypsinized and replated into 30 10-cm-diameter dishes in medium containing 0.8 mg of G418 (Gibco) per ml. The medium was replaced with fresh medium containing 0.8 mg of G418 per ml 24 h later and then once every 48 h. Approximately 1,000 G418-resistant colonies grew out on each plate, and colonies were sufficiently large for rosette screening 11 days after onset of G418 selection.

Rosette assay. Viral penton base-fiber complexes (pentons) were purified from extracts of HeLa S3 cells infected with Ad2RAE virus (2) by anion-exchange and hydroxylapatite chromatography as described previously (4) and were then dialyzed against 0.9% NaCl (wt/vol) and adjusted to an A_{280} of 1. One milliliter of sheep blood in citrate buffer (Tom Morris, Reisterstown, Md.) was washed three times with 15 ml of 0.9% NaCl, and the buffy coat was removed. Ad2RAE pentons were coupled to the sheep erythrocytes by the procedure described by Goding (12). Briefly, 0.2 ml of a 50% suspension of erythrocytes in 0.9% NaCl was mixed with 0.1 ml of RAE pentons and 0.6 ml of 0.27 M piperazine buffer (pH 6.5) in a 15-ml conical polypropylene tube. A 0.4-ml portion of 0.01% $CrCl_3$ was then added drop by drop while the solution was vortexed. After the mixture was allowed to stand for 3 min at room temperature, the cells were washed twice with 15 ml of PBS. The final cell pellet was suspended in 50 ml of PBS containing 5% fetal bovine serum. To screen transformed colonies, the medium was removed and replaced with 7 ml of the penton-erythrocyte suspension. After incubation for 30 to 60 min at room temperature, the erythrocyte suspension was recovered for reuse and the plates were washed with PBS. Colonies that retained erythrocytes were drawn into a 200-μl pipet tip during observation at 40× magnification with an inverted microscope. The colonies were transferred to wells in a 96-well plate for initial outgrowth and were then seeded at a low density in 10-cm-diameter plates to isolate rosette-positive subclones.

Fiber-binding assay. Viral fiber protein was purified from Ad2-infected HeLa cells by anion-exchange and hydroxylapatite chromatography essentially as described previously (4). Ten microliters of a 2.7-mg/ml solution of fiber protein in 10 mM sodium phosphate buffer (pH 8.2) was added to 1 mCi of ^{125}I-labeled Boulton-Hunter reagent (Dupont NEN) and incubated on ice for 3 h. The solution was then desalted by chromatography on a column (0.7 by 7 cm) of Bio-Gel P6DG (Bio-Rad) conditioned in PBS containing 1% gelatin. The specific activity of the ^{125}I-labeled fiber protein, determined by scintillation counting, was 1.9×10^4 cpm/ng. Binding isotherms were conducted on confluent cell monolayers in 24-well cluster plates (Costar). Cells were incubated for 2.5 h at 25°C in 0.2 ml of HEPES-buffered DMEM (pH 7.2) per well containing 0.4 mg of bovine serum albumin per ml and various amounts of ^{125}I-labeled fiber. Wells were then washed five times with PBS, cells were lysed with 0.3 N NaOH, and radioactivity in lysates was measured by scintillation counting. Nonspecific binding was determined in the presence of a 100-fold molar excess of unlabeled fiber protein. Each datum point is the mean ± standard deviation of triplicate samples.

DNA cloning and analysis. Genomic DNA was prepared as previously described (18). For Southern blot analysis, DNA was electrophoresed in 0.8% agarose gels (5 μg per lane) and then transferred to Nytran+ nylon membranes (Schleicher and Schuell). DNA fragments used for hybridization probes were purified from low-melting-point agarose gels and labeled with ^{32}P by using a random-primer DNA labeling kit (Gibco BRL). Blots were washed at 37°C in 2× SSC (1× SSC is 0.15 NaCl plus 0.015 M sodium citrate)–1% sodium dodecyl sulfate (SDS) and 0.5× SSC–1% SDS and then washed once at 65°C in 0.3× SSC–0.1% SDS.

To study viral DNA synthesis, duplicate cell monolayers in 33-mm-diameter dishes were infected with 100 virions per cell of CsCl-purified Ad2 or Ad2RAE virus. Low-molecular-weight DNA was extracted from the cells immediately after adsorption or 2 days later by the procedure of Hirt (16). DNAs were digested with *Bam*HI and analyzed by Southern blotting as described above, using ^{32}P-labeled Ad2 viral DNA as a hybridization probe.

A library of *Eco*RI fragments of T14 genomic DNA was constructed in the λZAP cloning vector (Stratagene). Dephosphorylated vector DNA (1.5 μg) and *Eco*RI-digested T14 DNA (2 μg) were ligated overnight with 1,000 U of T4 DNA ligase (New England BioLabs) and then added to lambda packaging extract (Promega). *E. coli* BB4 cells (Stratagene) were infected with the recombinant phage, and plaque lifts were screened with ^{32}P-labeled BLUR-8 (20), a cloned human *Alu* repeat, to identify phage containing human DNA inserts. A unique sequence probe derived from one such phage was used to screen a commercial HeLa cell genomic DNA library (Stratagene). Plaque lifts and hybridizations were performed by standard procedures (27).

RESULTS

Mouse A9 cells carrying human chromosome 21 express Ad2 receptors. In preliminary experiments, we found that monolayers of mouse L929 cells failed to bind radiolabeled Ad2 virions (data not shown), indicating either that these cells do not express receptors capable of binding the Ad2 fiber protein or that the concentration of Ad2 receptors on these cells was below the limit of detection of this assay. This result

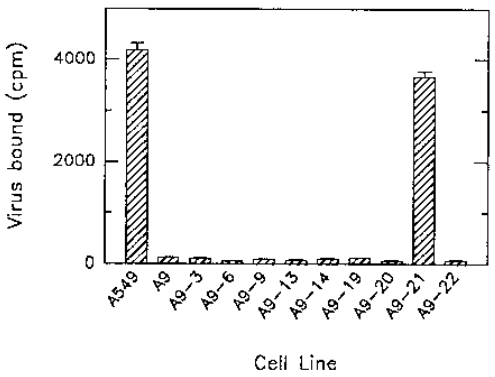

FIG. 1. Binding of radiolabeled Ad2 virus by mouse-human hybrid cells. Confluent monolayers of human A549 cells, mouse A9 cells, and A9 cell clones containing single human chromosomes (see Table 1) were incubated in 24-well plates with 0.2-ml portions of binding buffer (Hanks' balanced salt solution containing 5% fetal bovine serum) containing 1,000 particles per cell of [^{3}H]thymidine-labeled Ad2 virus for 1 h at room temperature. Wells were then rinsed three times with PBS, cells were lysed with 0.2 ml of 0.3 M NaOH per well, and radioactivity in lysates was measured by liquid scintillation counting. Each value shown is the mean ± standard deviation of triplicate samples.

suggested that the Ad2 receptor might be cloned by gene transfer, using mouse cells as recipients for exogenous human DNA coding for the receptor. Two factors are critical for the success of this approach. First, the target gene must be transferred at a detectable frequency. Typical transfer frequencies for single genes after transfection of cells with high-molecular-weight DNA in calcium phosphate precipitates are about 1:10,000 (23, 29, 41, 45). Given the difficulty in preparing DNA fragments larger than about 200 kb, the target gene or genes must be small enough to reside entirely on a single DNA fragment of this size. Second, the gene product must be expressed by recipient cells in functional form and at detectable concentrations.

To investigate whether mouse cells can express specific receptors for Ad2 virus, a panel of hybrid cells containing single human chromosomes in a mouse A9 cell background (Table 1) was screened for binding radiolabeled Ad2 virus. A9 cells are a subclone of L929 cells. Only A9-21 cells, which contain human chromosome 21, demonstrated significant levels of binding (Fig. 1). Incubation of A9-21 cells with purified Ad2 fiber protein blocked subsequent binding of virus (Fig. 2), indicating that the virus receptors on A9-21 cells, like those on human cells, bind virus specifically through the viral fiber subunit. These results indicate that A9 cells can express specific Ad2 receptors and therefore are suitable recipient cells for exogenous human DNA coding for the Ad2 receptor.

The failure of all other clones that we tested in the hybrid cell panel to bind Ad2 virus suggested that one or more genetic elements on human chromosome 21 direct the expression of Ad2 receptors in A9 cells but did not exclude the possibility that a low frequency of random A9 clones expresses an endogenous murine homolog of the Ad2 receptor. To further investigate whether expression of Ad2 receptors in rodent cells is correlated with the presence of human chromosome 21, virus-binding assays were also conducted on E7b cells, which contain chromosome 21 in a Chinese hamster ovary (CHO) cell background. As shown in Fig. 2, E7b cells also exhibited Ad2 binding that was specific for the viral fiber protein, while the parental CHO cells did not bind virus above background levels. Therefore, expression of Ad2 receptors in A9 and CHO cells is strongly correlated with the presence of one or more genes on human chromosome 21.

DNA-mediated transfer of Ad2 receptors into mouse cells. To determine if Ad2 receptors could be genetically transferred into A9 cells by DNA transfection, it was necessary to first develop a screening procedure capable of detecting expression of fiber-specific receptors in colonies of transformed cells. In situ rosetting has been used in other systems to identify cells transformed to express novel cell surface receptors (23, 30). In this procedure, antibodies or ligands specific for the target receptor are coupled to the surfaces of intact erythrocytes. Colonies of transformants expressing the target receptor are then identified by their ability to bind the modified erythrocytes. Attempts to couple the isolated Ad2 fiber protein directly to sheep erythrocytes with chromic chloride (12) resulted in agglutination of the erythrocytes for reasons that are not understood. However, native complexes of the viral fiber and penton base (pentons) were readily coupled without aggregating the cells. These results suggest that pentons are coupled to erythrocytes through the penton base subunit, orienting the fiber component favorably for binding to Ad2 receptors on target cells. To avoid binding of the penton-modified erythrocytes (P-RBCs) to integrins on target cells through the RGD sequence of wild-type pentons, we used pentons from an Ad2 mutant virus, Ad2RAE, whose penton base RGD ligand was inactivated by conversion to Arg-Ala-Glu (2). In pilot studies, we observed that the P-RBCs bound to human A549 cells and to A9-21 hybrid cells, but not to mouse A9 cells. Furthermore, incubation of A549 and A9-21 cells with fiber protein inhibited the subsequent binding of P-RBCs, indicating that the binding was specific for the viral fiber protein.

A9 cells were then cotransfected with a mixture of high-molecular-weight DNA from A9-21 cells and plasmid DNA containing a neomycin resistance gene (pMAMneo). Transformants were selected for resistance to G418. When G418-resistant colonies reached about 1 mm in diameter, they were incubated with a suspension of P-RBCs. Colonies that retained P-RBCs after washing were detected at a frequency of about 1:30,000. Rosette-positive subclones were isolated, expanded in culture, and then screened for expression of Ad2 receptors

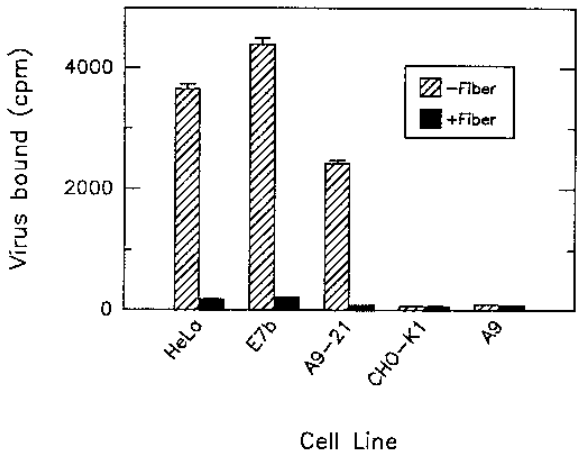

FIG. 2. Ad2 receptors on hybrid cells bind the viral fiber subunit. Confluent monolayers of hybrid cells containing human chromosome 21 (E7b and A9-21), human A549 cells, CHO-K1 cells, or mouse A9 cells were incubated in 24-well plates with 0.2-ml portions of binding buffer containing Ad2 fiber protein (10 μg/ml) or in binding buffer alone for 30 min at room temperature prior to addition of 1,000 particles per cell of [^{3}H]thymidine-labeled Ad2 virions. After incubation for an additional 30 min at room temperature, the monolayers were washed and cell-associated radioactivity measured by liquid scintillation counting. Binding without fiber (−Fiber) and with fiber (+Fiber) is shown. Each value shown is the mean ± standard deviation of triplicate samples.

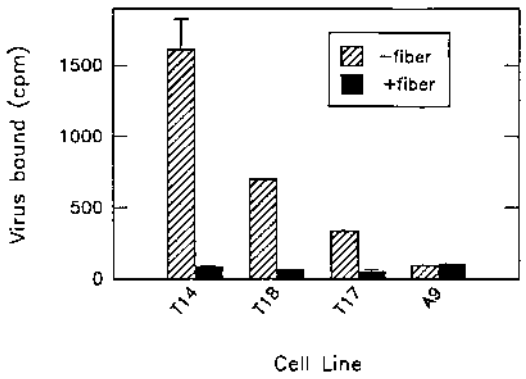

FIG. 3. Specificities of Ad2 receptors on A9 cell transformants. The binding of [^{3}H]thymidine-labeled Ad2 virions by confluent monolayers of transformed cells (T14, T17, and T18) or the parental A9 cells, and inhibition of virus binding by Ad2 fiber protein were determined exactly as described in the legend to Fig. 2.

in the radiolabeled virus-binding assay. Two primary transformants, T14 and T17, were isolated from independent transfections. In addition, a secondary transformant, T18, was isolated following transfection of A9 cells with T14 genomic DNA and pMAMneo. Significant levels of Ad2-binding activity were present in all three transformed cell lines, and incubation of the cells with purified Ad2 fiber protein blocked the subsequent binding of virus (Fig. 3), indicating that these cells expressed fiber-specific Ad2 receptors.

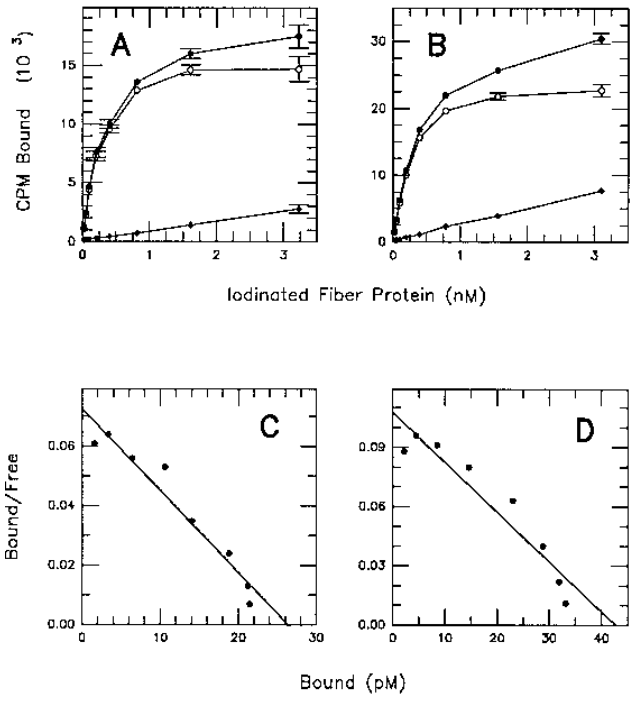

FIG. 4. Concentrations and affinities of the Ad2 receptors on A549 and T14 cells. (A and B) Isotherms of ^{125}I-labeled Ad2 fiber protein binding by adherent A549 (A) and T14 (B) cells. Total binding (●), nonspecific binding (◆), and specific binding (○) are shown. (C and D) Scatchard plots of the data in panels A and B, respectively. The data were fitted to straight lines with correlation coefficients of 0.95. The apparent K_ds, determined from the slopes, were 0.37 nM (C) and 0.40 nM (D), and the numbers of receptor sites per cell, determined from the x intercepts, were 9,600 (C) and 10,400 (D).

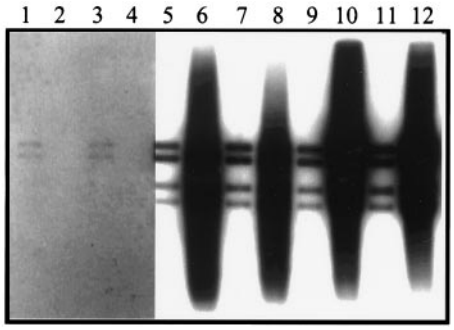

FIG. 5. Expression of specific receptors is required for Ad2 infection of A9 cells. Duplicate monolayers of A9, T14, and A549 cells were incubated with wild-type Ad2 or the penton base RGD mutant Ad2RAE (100 particles per cell), and low-molecular-weight DNA was extracted from the infected cultures either immediately (odd-numbered lanes) or after incubation for 48 h at 37°C (even-numbered lanes). Equal volumes of the recovered DNAs were digested with *Bam*HI and analyzed by Southern blotting with ^{32}P-labeled Ad2 DNA, as described in Materials and Methods. DNAs extracted from A9 cells, T14 cells, and A549 cells are shown in lanes 1 to 4, 5 to 8, and 9 to 12, respectively, where the first pair of samples in each set are from cells infected with wild-type Ad2, and the second pair of samples are from cells infected with Ad2RAE. The autoradiogram of lanes 1 to 4 was developed after a 4-day exposure to compensate for the relatively small amount of virus that initially bound to A9 cells. The autoradiogram of lanes 5 to 12 was developed after a 16-h exposure.

The Ad2 receptor on T14 cells has functional characteristics of the human Ad2 receptor. To further characterize the Ad2 receptors on the mouse cell transformants, the equilibrium binding of ^{125}I-labeled Ad2 fiber protein by T14 cells, the transformant with the highest virus-binding activity, was compared to that of human A549 cells. Scatchard plots of the data (Fig. 4) showed that the concentrations of Ad2 receptors on both cells were similar (about 10,000 molecules per cell) and that the Ad2 receptors on both cell types had the same affinity for the viral fiber protein (K_ds = 0.4 nM). These results are consistent with the hypothesis that the Ad2 receptors on T14 and A549 cells are identical molecules.

To determine if the Ad2 receptors on T14 cells can direct virus into cells along a pathway leading to productive infection, we investigated whether viral DNA is synthesized in T14 cells following adsorption of virus. Duplicate monolayers of A9, T14, or A549 cells were infected with Ad2RAE or wild-type Ad2, and low-molecular-weight DNA was then extracted from the cells either immediately or after incubation of the cultures for 2 days at 37°C. The DNA was digested with *Bam*HI and analyzed on a Southern blot with a viral DNA probe. A long exposure of the autoradiogram showed that a small amount of virus initially attached to A9 cells, but no viral DNA associated with the cells was detected 48 h later (Fig. 5, lanes 1 to 4). The binding of wild-type Ad2 and Ad2RAE by A9 cells was equivalent, indicating that virus adsorption did not occur through an integrin-dependent pathway. A much greater quantity of viral DNA was initially associated with T14 and A549 cells (Fig. 5, lanes 5 to 12), which was readily detected on a short exposure of the blot. In striking contrast to A9 cells, T14 cells supported extensive viral DNA replication (lanes 5 to 8), as did A549 cells (lanes 9 to 12). These results indicate that the receptors on T14 cells support the internalization of bound virus and that once internalized, the virus can enter the cell cytoplasm and deliver the viral DNA genome to the cell nucleus. However, despite the ability of T14 cells to support extensive viral DNA synthesis, virus titers in lysates of Ad2-infected T14 cells did not significantly increase over residual eclipse phase levels, in good

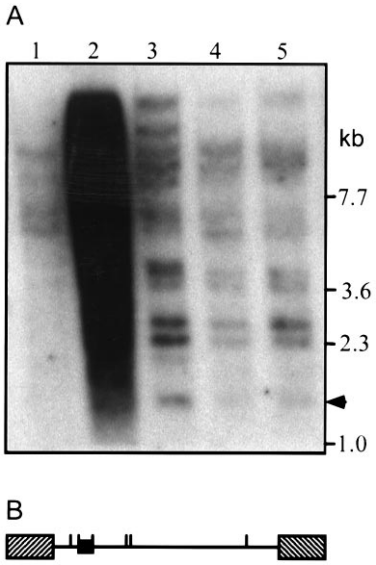

FIG. 6. Human DNA contents in A9 cell transformants expressing specific receptors for Ad2. (A) Samples (5 μg) of genomic DNAs from A9 cells (lane 1), A9-21 cells (lane 2), or the A9 cell transformants T14, T17, and T18 (lanes 3 to 5, respectively) were digested with *Eco*RI and analyzed by Southern blotting with ^{32}P-labeled BLUR-8, a cloned human Alu repeat probe (20), as described in Materials and Methods. The arrowhead indicates a 1.2-kb *Eco*RI fragment present in all three transformants which was cloned from a T14 genomic DNA library. (B) Diagram of a clone isolated from a HeLa cell genomic DNA library which contains the 1.2-kb *Eco*RI fragment indicated in panel A (solid box). The line represents the 19.5-kb HeLa DNA insert, and the hatched boxes represent the phage arms. Tick marks above the line show the positions of *Eco*RI sites in the HeLa DNA.

agreement with earlier reports that mouse and hamster cells are nonpermissive hosts for human adenoviruses (9, 25, 46).

Transformants expressing Ad2 receptors contain identical fragments of human DNA. If the expression of Ad2 receptors on the transformed cells is directed by a transfected gene derived from human chromosome 21, then these cells should contain identical fragments of human DNA corresponding to that gene. To examine their human DNA content, DNAs from the transformants, parental A9 cells, and A9-21 cells were analyzed on Southern blots with a human Alu repeat probe (Fig. 6A). At the stringency of hybridization used in this experiment, the Alu probe cross-hybridized to four DNA fragments longer than >5 kb in a sample of A9 DNA containing a large molar excess of pMAMneo plasmid (lane 1). An identical pattern of Alu-reactive fragments was also detected in A9 DNA without added pMAMneo (not shown), indicating that these fragments are derived from the mouse genome and not from pMAMneo. The Alu probe hybridized to a continuum of fragments in the A9-21 DNA sample (lane 2), which was expected since the ~300-bp Alu element is repeated in human genomic DNA once every 5 kb on average (20). In addition to the large fragments seen in A9 DNA, all three A9 cell transformants contained a similar set of small, 1- to 5-kb *Eco*RI fragments that hybridized with the Alu repeat probe (lanes 3 to 5), suggesting that identical fragments of human DNA are present in these cells.

To further characterize the human DNA contained in the transformed cells, a library of T14 *Eco*RI fragments constructed in the λZAP vector was screened with the Alu repeat probe, and a clone containing a 1.2-kb *Eco*RI fragment which corresponded in size to that of the smallest *Eco*RI fragment observed in transformant DNA on Southern blots (Fig. 6A, arrowhead) was isolated. Partial DNA sequence analysis revealed an Alu repeat with a primate-specific sequence motif (19) located at one end of the 1.2-kb *Eco*RI fragment. Aside from the Alu element, the sequence of this fragment had no significant matches to any sequences in the GenBank database. A probe derived from the end of the 1.2-kb *Eco*RI fragment opposite the Alu repeat was used to screen a HeLa cell genomic DNA library. One phage with a 19.5-kb insert containing the 1.2-kb Alu-positive *Eco*RI fragment was isolated (Fig. 6B). To determine if the HeLa cell DNA fragment cloned in this phage corresponds to human DNA sequences in the transformed cells, a Southern blot of genomic DNAs from the transformants and control cells was screened with a unique sequence probe derived from this clone. The probe, shown in Fig. 7B, is contained entirely within a single *Hin*dIII fragment of HeLa DNA and is bisected by an internal *Xba*I site. As predicted from the map, the probe hybridized to a single *Hin*dIII fragment and two *Xba*I fragments in human A549 DNA (Fig. 7A, lanes 1 and 6). Importantly, the probe hybridized to an identical pattern of fragments in DNAs from the T14, T17, and T18 transformants (Fig. 7A, lanes 2 to 4 and 7 to 9) but did not hybridize to any *Hin*dIII or *Xba*I fragments of mouse A9 DNA (lanes 5 and 10). Together, these results strongly support the conclusion that all three transformants contain human DNA derived from the same small region of chromosome 21. Given that DNA-mediated transfer of any single gene in the background of an entire mammalian genome occurs at a frequency of about 1/10,000 transformants (41), then it would be improbable that the human DNA fragment identified in Fig. 7A could be found in three independent transformants unless this DNA fragment was functionally related to expression of Ad2 receptors in these cells.

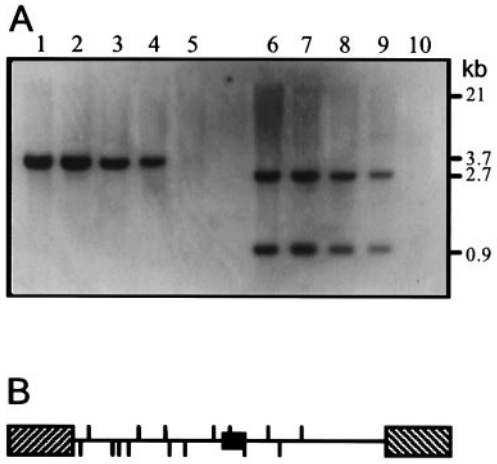

FIG. 7. Identical fragments of human DNA in the A9 cell transformants expressing specific receptors for Ad2. (A) Samples (5 μg) of genomic DNAs from A549 cells (lanes 1 and 6), the three A9 transformants T14, T17, and T18 (lanes 2 to 4 and 7 to 9, respectively) and A9 cells (lanes 5 and 10) were digested with *Hin*dIII (lanes 1 to 5) or *Xba*I (lanes 6 to 10) and analyzed by Southern blotting with a ^{32}P-labeled DNA fragment derived from the same 19.5-kb HeLa genomic DNA clone described in the legend to Fig. 6B. (B) Diagram of the 19.5-kb HeLa genomic DNA clone (also shown in Fig. 6B). The positions of *Xba*I sites and *Hin*dIII sites in the HeLa DNA insert are indicated by tick marks above and below the line, respectively. The solid box shows the position of an *Eco*RV-*Hin*dIII fragment which does not contain repetitive elements and was used to probe the blot shown in panel A.

DISCUSSION

In this study, we evaluated gene transfer as an approach to identification and molecular cloning of the human receptor for Ad2 virus. These experiments were prompted by our initial observation that mouse L929 cells did not bind detectable amounts of purified, radiolabeled Ad2 virions. Consistent with these results, all clones that we tested in an A9-human hybrid cell mapping panel, except for A9-21 cells, also failed to bind radiolabeled Ad2 (Table 1), as did CHO-K1 cells (Fig. 2). Our results are in good agreement with those reported by other groups showing that mouse 3T3 cells failed to bind radiolabeled Ad2 virus (36) and that CHO cells failed to bind Ad5 fiber protein (37). These results support the conclusion that mouse A9 cells do not express fiber-specific receptors for Ad2 virus and therefore are appropriate recipient cells for expression of exogenous human DNA encoding the Ad2 receptor.

Our observation that Ad2 virus is specifically bound by A9-21 and E7b cells (Fig. 2) suggests that the Ad2 receptor is encoded by one or more genes on human chromosome 21 and that the human Ad2 receptor protein is expressed in functional form on A9-21 and E7b cells. There are, however, alternative interpretations of these data. For example, although the majority of A9 clones do not express an Ad2 receptor (Table 1), expression of an endogenous receptor may occur at a low frequency, and the correlation with the presence of human chromosome 21 may be a coincidence. This possibility seems unlikely, considering that specific Ad2 receptors were also expressed on several CHO cell derivatives which contained fragments of human chromosome 21 (unpublished observations). Furthermore, screening of A9 transformants with the in situ penton-rosette assay indicated that, if an endogenous receptor gene exists, its expression in random A9 subclones occurs at a frequency of $<10^{-4}$. Thus, it is likely that one or more genes on human chromosome 21 are functionally related to the expression of Ad2 receptors in A9-21 cells. However, we cannot exclude the possibility that expression of an endogenous receptor gene is induced by sequences on human chromosome 21 or that chromosome 21 encodes a factor that modifies an existing mouse protein, enabling it to bind Ad2 virus.

The hypothesis that the Ad2 receptor on A9-21 cells is encoded or induced by a single gene or locus on human chromosome 21 is strongly supported by the results of our transfection experiments. A9 cell transformants expressing Ad2 receptors were detected at a frequency within the range expected for transfer of a single gene (about 1:30,000) following transfection with high-molecular-weight DNA from A9-21 cells or from the primary transformant T14. These transformants contain a similar set of *Eco*RI fragments that hybridize to a human *Alu* repeat probe on Southern blots (Fig. 6) and contain identical restriction fragments that hybridize to a unique sequence probe derived from a HeLa cell genomic DNA clone (Fig. 7). It is unlikely that three random, independent clones contain the same transfected DNA fragments, unless these fragments were functionally related to Ad2 receptor expression in the transformed cells. In addition, the observations that Ad2 receptors on T14 and A549 cells bound fiber protein with the same affinity and were expressed at similar concentrations on the cell membrane (Fig. 4) indicates either that both cell lines express the human Ad2 receptor or that the properties of receptor specificity, affinity, and expression level are conserved between the mouse and human Ad2 receptor proteins. The variation in the virus-binding capacities of different transformants (Fig. 3) could result from differences in the copy numbers of the transfected gene or from different levels of receptor gene expression or different receptor mRNA half-lives in these cells. We have recently isolated additional secondary transformants derived from T14 DNA and found that some of these clones have significantly higher virus-binding capacities than T14 cells (unpublished data), supporting the hypothesis that the level of receptor expression varies with the site of integration of the transfected DNA in the recipient cell genome. Experiments to identify candidate Ad2 receptor mRNA and cDNA clones by hybridization to human DNA fragments cloned from the A9 cell transformants are in progress. Several other virus receptors and cell surface proteins have been cloned by similar approaches (23, 30, 39, 45).

A number of earlier reports documented abortive infection of various rodent cells by Ad2 and other group C adenoviruses. Limited synthesis of early viral gene products and viral DNA was observed in mouse 3T3 cell cultures infected with Ad2, although <50% of cells in the cultures synthesized levels of early viral gene products detectable by immunofluorescence despite infection of the cultures at high multiplicity (9). Ad2 DNA synthesis also was readily detected in subconfluent primary cell cultures derived from C57/BL mouse embryos (46) and in CHO cells (25). In addition, group C adenoviruses can abortively infect mouse hepatocytes in vivo following intravenous injection of virus (8, 44), and abortive infection of mouse lung and other tissues in vivo with group C adenoviruses has also been well documented (11, 43). In contrast to these results, we were unable to detect viral DNA synthesis in mouse A9 cells infected with Ad2 virus, but we found that viral DNA synthesis was extensive in T14 cells (Fig. 5). It is likely, therefore, that A9 cells are also capable of replicating Ad2 DNA and that virus bound nonspecifically to A9 cells is unable to deliver the viral DNA genome to the cell nucleus, which could be the result if A9 cells either fail to internalize virus or route the virus to an inappropriate intracellular compartment. It remains to be determined whether expression of fiber-specific receptors is required for the susceptibility of mouse cells to infection by adenovirus or whether alternate pathways for virus attachment and entry exist in vivo that may be retained by some but not all cultured mouse cells.

There is substantial evidence that, although integrins promote the internalization of virus by host cells, they are not essential for Ad2 infection of cultured human cells (2, 40). The mechanism of integrin-mediated uptake of virus by human cells has not been determined; however, simultaneous engagement of the fiber and penton base ligands on the virus capsid by their respective receptors would juxtapose the integrin and fiber receptor, possibly resulting in the formation of a complex. It would be interesting to determine whether such an interaction, if it exists, generates a signal that regulates the internalization of virus. Ad2 mutants lacking functional penton base RGD sequences are internalized by cultured human cells at a rate markedly slower than that of the wild-type virus (2, 10). A comparison of the kinetics of wild-type and RGD mutant virus infection of T14 cells would indicate whether integrins are also involved in virus uptake by these cells. Integrin-mediated uptake of Ad2 by T14 cells would imply that, if the formation of a receptor-integrin complex generates a signal that regulates endocytosis of virus, then this signal may be generated when the receptor and integrin components are derived from different species. Recently we found that internalization of RGD-negative Ad2 mutants by A549 cells occurs at a rate which is dependent on the concentration of fiber receptors on the cell membrane (10), suggesting that virus can be internalized by an integrin-independent pathway that requires the interaction of each virus particle with multiple fiber receptors. Thus, if the human fiber receptor and mouse integrin cannot interact to generate a signal, Ad2 virus might enter cells by this alternate,

integrin-independent mechanism. Molecular cloning of the Ad2 receptor will provide a powerful approach to understanding the molecular mechanism of adenovirus entry into human cells.

ACKNOWLEDGMENTS

We thank Carl Anderson for many helpful discussions throughout this project, Lois Annab and Carl Barrett at NIEHS and David Patterson at the Eleanor Roosevelt Institute for providing cell lines, and Sydell Lamb for assistance with cell culture.

This work was supported by a grant (to P.F.) from the U.S. Department of Energy Office of Health and Environmental Research.

REFERENCES

1. **Bai, M., L. Campisi, and P. Freimuth.** 1994. Vitronectin receptor antibodies inhibit infection of HeLa and A549 cells by adenovirus type 12 but not by adenovirus type 2. J. Virol. **68:**5925–5932.
2. **Bai, M., B. Harfe, and P. Freimuth.** 1993. Mutations that alter an Arg-Gly-Asp (RGD) sequence in the adenovirus type 2 penton base protein abolish its cell-rounding activity and delay virus reproduction in flat cells. J. Virol. **67:**5198–5205.
3. **Belin, M.-T., and P. Boulanger.** 1993. Involvement of cellular adhesion sequences in the attachment of adenovirus to the HeLa cell surface. J. Gen. Virol. **74:**1485–1497.
4. **Boulanger, P. A., and F. Puvion.** 1973. Large-scale preparation of soluble adenovirus hexon, penton and fiber antigens in highly purified form. Eur. J. Biochem. **39:**37–42.
5. **Cuthbert, A. P., D. A. Trott, R. M. Ekong, S. Jezzard, N. L. England, M. Themis, C. M. Todd, and R. F. Newbold.** 1995. Construction and characterization of a highly stable human:rodent monochromosomal hybrid panel for genetic complementation and genome mapping studies. Cytogenet. Cell Genet. **71:**68–76.
6. **Defer, C., M.-T. Belin, M.-L. Caillet-Boudin, and P. Boulanger.** 1990. Human adenovirus-host cell interactions: comparative study with members of subgroups B and C. J. Virol. **64:**3661–3673.
7. **Di Guilmi, M. A., A. Barge, P. Kitts, E. Gout, and J. Chroboczek.** 1995. Human adenovirus serotype 3 (Ad3) and the Ad3 fiber protein bind to a 130-kDa membrane protein on HeLa cells. Virus Res. **38:**71–81.
8. **Duncan, S. J., F. C. A. Gordon, D. W. Gregory, J. L. McPhie, R. Postlethwaite, R. White, and H. N. A. Willcox.** 1978. Infection of mouse liver by human adenovirus type 5. J. Gen. Virol. **40:**45–61.
9. **Eggerding, F. A., and W. C. Pierce.** 1986. Molecular biology of adenovirus type 2 semipermissive infections. I. Viral growth and expression of viral replicative functions during restricted adenovirus infection. Virology **148:**97–113.
10. **Freimuth, P.** 1996. A human cell line selected for resistance to adenovirus infection has reduced levels of the virus receptor. J. Virol. **70:**4081–4085.
11. **Ginsberg, H. S., L. L. Moldawer, P. B. Sehgal, M. Redington, P. L. Kilian, R. M. Chanock, and G. A. Prince.** 1991. A mouse model for investigating the molecular pathogenesis of adenovirus pneumonia. Proc. Natl. Acad. Sci. USA **88:**1651–1655.
12. **Goding, J. W.** 1976. The chromic chloride method of coupling antigens to erythrocytes: definition of some important parameters. J. Immunol. Methods **10:**61–66.
13. **Goyette, M. C., K. Cho, C. L. Fasching, D. B. Levy, K. W. Kinzler, C. Paraskeva, B. Vogelstein, and E. J. Stanbridge.** 1992. Progression of colorectal cancer is associated with multiple tumor suppressor gene defects but inhibition of tumorigenicity is accomplished by correction of any single defect via chromosome transfer. Mol. Cell. Biol. **12:**1387–1395.
14. **Hennache, B., and P. Boulanger.** 1977. Biochemical study of KB-cell receptor for adenovirus. Biochem. J. **166:**237–247.
15. **Henry, L. J., D. Xia, M. E. Wilke, J. Deisenhofer, and R. D. Gerard.** 1994. Characterization of the knob domain of the adenovirus type 5 fiber protein expressed in *Escherichia coli*. J. Virol. **68:**5239–5246.
16. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. **26:**365–369.
17. **Hong, S. S., and P. Boulanger.** 1995. Protein ligands of the human adenovirus type 2 outer capsid identified by biopanning of a phage-displayed peptide library on separate domains of wild-type and mutant penton capsomers. EMBO J. **14:**4714–4727.
18. **Jeffreys, A. J., and R. A. Flavell.** 1977. A physical map of the DNA regions flanking the rabbit β-globin gene. Cell **12:**429–439.
19. **Jelinek, W. R., and C. W. Schmid.** 1982. Repetitive sequences in eukaryotic DNA and their expression. Annu. Rev. Biochem. **51:**813–844.
20. **Jelinek, W. R., T. P. Toomey, L. Leinwand, C. H. Duncan, P. A. Biro, P. V. Choudary, S. M. Weissman, C. M. Rubin, C. M. Houck, P. L. Deininger, and C. W. Schmid.** 1980. Ubiquitous, interspersed repeated sequences in mammalian genomes. Proc. Natl. Acad. Sci. USA **77:**1398–1402.
21. **Koi, M., M. Shimizu, H. Morita, H. Yamada, and M. Oshima.** 1989. Construction of mouse A9 clones containing a single human chromosome tagged with neomycin-resistance gene via microcell fusion. Jpn. J. Cancer Res. **80:**413–418.
22. **Levine, A. J., and H. S. Ginsberg.** 1967. Mechanism by which fiber antigen inhibits multiplication of type 5 adenovirus. J. Virol. **1:**747–757.
23. **Littman, D. R., Y. Thomas, P. J. Maddon, L. Chess, and R. Axel.** 1985. The isolation and sequence of the gene encoding T8: a molecule defining functional classes of T lymphocytes. Cell **40:**237–246.
24. **Lonberg-Holm, K., R. L. Crowell, and L. Philipson.** 1976. Unrelated animal viruses share receptors. Nature (London) **259:**679–681.
25. **Longiaru, M., and M. S. Horwitz.** 1981. Chinese hamster ovary cells replicate adenovirus deoxyribonucleic acid. Mol. Cell. Biol. **1:**208–215.
26. **Louis, N., P. Fender, A. Barge, P. Kitts, and J. Chroboczek.** 1994. Cell-binding domain of adenovirus serotype 2 fiber. J. Virol. **68:**4104–4106.
27. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
28. **Mathias, P., T. Wickham, M. Moore, and G. Nemerow.** 1994. Multiple adenovirus serotypes use αv integrins for infection. J. Virol. **68:**6811–6814.
29. **Mendelsohn, C., B. Johnson, K. A. Lionetti, P. Nobis, E. Wimmer, and V. R. Racaniello.** 1986. Transformation of a human poliovirus receptor gene into mouse cells. Proc. Natl. Acad. Sci. USA **83:**7845–7849.
30. **Mendelsohn, C. L., E. Wimmer, and V. R. Racaniello.** 1989. Cellular receptor for poliovirus: molecular cloning, nucleotide sequence, and expression of a new member of the immunoglobulin superfamily. Cell **56:**855–865.
31. **Moore, E. E., C. Jones, F.-T. Kao, and D. C. Oates.** 1977. Synteny between glycinamide ribonucleotide synthetase and superoxide dismutase. Am. J. Hum. Genet. **29:**389–396.
32. **Nermut, M. V.** 1984. The architecture of adenoviruses, p. 5–34. *In* H. S. Ginsberg (ed.), The adenoviruses. Plenum Press, New York.
33. **Ning, Y., J. L. Weber, A. M. Killary, D. H. Ledbetter, J. R. Smith, and O. M. Pereira-Smith.** 1991. Genetic analysis of indefinite division in human cells: evidence for a cell senescence-related gene(s) on human chromosome 4. Proc. Natl. Acad. Sci. USA **88:**5635–5639.
34. **Persson, R., C. Wohlfart, U. Svensson, and E. Everitt.** 1985. Virus-receptor interaction in the adenovirus system. Characterization of the positive cooperative binding of virions on HeLa cells. J. Virol. **54:**92–97.
35. **Philipson, L., K. Lonberg-Holm, and U. Pettersson.** 1968. Virus-receptor interaction in an adenovirus system. J. Virol. **2:**1064–1075.

35a. **Phillips, A., and B. E. Weissman.** Unpublished data.

36. **Seth, P., M. Rosenfeld, J. Higginbotham, and R. G. Crystal.** 1994. Mechanism of enhancement of DNA expression consequent to cointernalization of a replication-deficient adenovirus and unmodified plasmid DNA. J. Virol. **68:**933–940.
37. **Stevenson, S. C., M. Rollence, B. White, L. Weaver, and A. McClelland.** 1995. Human adenovirus serotypes 3 and 5 bind to two different cellular receptors via the fiber head domain. J. Virol. **69:**2850–2857.
38. **Svensson, U., R. Persson, and E. Everitt.** 1981. Virus-receptor interaction in the adenovirus system. I. Identification of virion attachment proteins of the HeLa cell plasma membrane. J. Virol. **38:**70–81.
39. **Uze, G., G. Lutfalla, and I. Gresser.** 1990. Genetic transfer of a functional human interferon α receptor into mouse cells: cloning and expression of its cDNA. Cell **60:**225–234.
40. **Wickham, T. J., P. Mathias, D. A. Cheresh, and G. R. Nemerow.** 1993. Integrins αvβ3 and αvβ5 promote adenovirus internalization but not virus attachment. Cell **73:**309–319.
41. **Wigler, M., A. Pellicer, S. Silverstein, and R. Axel.** 1978. Biochemical transfer of single-copy eucaryotic genes using total cellular DNA as donor. Cell **14:**723–731.
42. **Xia, D., L. J. Henry, R. D. Gerrard, and J. Deisenhofer.** 1994. Crystal-structure of the receptor-binding domain of adenovirus type-5 fiber protein at 1.7-angstrom resolution. Structure **2:**1259–1270.
43. **Yang, Y., Q. Li, H. C. Ertl, and J. M. Wilson.** 1995. Cellular and humoral immune responses to viral antigens create barriers to lung-directed gene therapy with recombinant adenoviruses. J. Virol. **69:**2004–2015.
44. **Yang, Y., F. A. Nunes, K. Berencsi, E. E. Furth, E. Gönczöl, and J. M. Wilson.** 1994. Cellular immunity to viral antigens limits E1-deleted adenoviruses for gene therapy. Proc. Natl. Acad. Sci. USA **91:**4407–4411.
45. **Young, J. A. T., P. Bates, and H. E. Varmus.** 1993. Isolation of a chicken gene that confers susceptibility to infection by subgroup A avian leukosis and sarcoma viruses. J. Virol. **67:**1811–1816.
46. **Younghusband, H. B., C. Tyndall, and A. J. D. Bellet.** 1979. Replication and interaction of virus DNA and cellular DNA in mouse cells infected by a human adenovirus. J. Gen. Virol. **45:**455–467.