

## Plasma Membrane Receptors for Ecotropic Murine Retroviruses Require a Limiting Accessory Factor

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**A retroviral vector was used to express various amounts of the receptor (ecoR) for ecotropic host range murine retroviruses on naturally barren hamster, mink, and human cells. These cells and murine cells were then incubated for 2 h with dilutions of a helper-free ecotropic retrovirus that encodes human growth hormone, and the number of infected cells was later determined by growth hormone-specific immunofluorescence. For all cells under the conditions of these studies, virus adsorption was the limiting step of infection and the cellular capacities for infection were unsaturated either at cell surfaces or at intracellular sites. Thus, infections occurred at low multiplicities of infection per cell and were directly proportional to virus and cell concentrations, and only a small percentage (ca. 5%) of the infectious virions became adsorbed from the medium during the 2-h incubations. Although increasing the adsorption by raising virus or cell concentrations results in more infections in the cultures, increasing adsorption by raising the number of ecoR above a low threshold had no effect on infections. Thus, cells with a low number of ecoR were infected as efficiently as highly adsorbing cells that contained many times more ecoR. To reconcile these results, we conclude that only a small, set number of cell surface ecoR can be functional for infection and that all excess ecoR can only bind virus into an unsalvageable pool. Therefore, retroviral receptors on single cells are functionally diverse. Our results suggest that activity of ecoR in infection requires a limiting second cellular component.**

Cell surface receptors perform critical functions in retroviral diseases. These functions include binding viral envelope glycoproteins to mediate specific virus attachment onto susceptible cells (10, 16, 32, 36). In addition, receptor blockade occurs in interference to superinfection (3, 4, 10, 11, 19, 51, 55, 56). By this means, a cell harboring one retrovirus excludes additional viruses of the same host range class. Interactions between viral envelope glycoproteins and host cell receptors can also cause specific pathogenic changes, including mitogenesis and cytotoxicity (7, 14, 18, 21, 22, 28, 30, 31, 35, 37, 42, 44, 45, 47-49, 53). Such pathogenesis has been implicated in important retroviral diseases, including human and feline AIDS (7, 22, 30, 31, 42, 49), leukemias (14, 21, 27, 28, 35, 47), lymphomas (37), anemias (45, 48), hemangioma (44), and neural degeneration (53).

Despite their importance, little is understood about interactions between retroviral envelope glycoproteins and cell surface receptors or about the mechanisms of virus penetration into cells. The receptor for the human immunodeficiency virus is the CD4 glycoprotein (36, 46). However, it is uncertain whether infection involves fusion of virion membranes with endosomal or cell surface membranes (2, 33, 41, 50). Some cells that lack CD4 can be infected by the human immunodeficiency virus (5, 8, 29). Expressing CD4 in murine cells and some human cells does not confer susceptibility to infection (2, 6, 32), perhaps because of an inhibitor in the resistant cells (2, 54). Alternatively, it has been speculated that human immunodeficiency virus penetration may require a second receptor component that is present in most human cells but not in murine cells (2, 6, 32).

Recently, complementary DNAs (cDNAs) that encode cell surface receptors for mouse ecotropic retroviruses (1,

25) and for gibbon ape leukemia virus (39) were isolated. Although these receptors appear to be unrelated, they both may be glycoproteins with multiple membrane-spanning regions (1, 39). The ecotropic receptor (ecoR) occurs on cells of mice and rats but not on cells of other species. Genetic analysis has suggested that ecoR may be essential for the viability of cultured murine fibroblasts (16). In this study, we have developed efficient methods to express ecoR cDNA in nonmurine cells. We used these cells to study the role of ecoR in virus binding and infection. Our results suggest that a cellular component in addition to ecoR is required for infection.

### MATERIALS AND METHODS

**Cells and viruses.** Swiss mouse NIH 3T3 fibroblasts, mink lung CCL64 fibroblasts, Chinese hamster ovary cells (CHO), and human osteogenic sarcoma cells (Ostk<sup>-</sup>) were from American Type Culture Collection (Rockville, Md.).  $\psi$ -2 ecotropic packaging cells (34) and PA-12 amphotropic packaging cells (38) were from R. C. Mulligan (Massachusetts Institute of Technology, Cambridge) and A. D. Miller (Fred Hutchinson Cancer Center, Seattle, Wash.), respectively. CHO cells were grown in alpha minimum essential medium supplemented with 10% fetal bovine serum. Other cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

Helper-free ecotropic host range viruses from  $\psi$ -2 cells that encoded the neomycin phosphotransferase gene (MSV-neo) and the human growth hormone (pSFF-hGH) have been described elsewhere (9, 26, 27). Virus preparations were harvested by placing fresh culture medium on half-confluent monolayers of virus-producing cell lines for 16 to 24 h, removing the medium, and filtering it through a 0.2- $\mu$ m-pore-size filter. Cells ( $10^5$ ) in 25-cm<sup>2</sup> flasks were infected by

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incubating them with 1 ml of virus-containing medium for 2 h at 37°C in the presence of Polybrene (8 µg/ml) (4).

**Plasmid construction.** The eukaryotic expression clone pJET that encodes *ecoR* was generously provided by J. M. Cunningham (Brigham and Women's Hospital, Boston, Mass.). The 2.3-kbp *Bam*HI-*Eco*RI fragment containing *ecoR* cDNA was removed from pJET and cloned into the retroviral expression vector pSFF (4) to form pSFF-*ecoR* (see Fig. 1).

**Retroviral vector expression.** pSFF-*ecoR* (10 µg) was transfected as a calcium phosphate precipitate (15, 27) into 25-cm<sup>2</sup> culture dishes that contained a 1:1 mixture of ψ-2 and PA-12 cells at a total cell concentration of  $2 \times 10^5$  cells per dish. At 72 h after transfection, the cells were split into 12 minicultures of 100 cells per well in a 96-well (0.32-cm<sup>2</sup>) plate. The cells were then grown and assayed for *ecoR* expression by RNA slot blotting (4). After maximal expression of *ecoR* in the cocultures, ψ-2 and PA-12 cells were isolated by limiting dilution cloning as described previously (26). These cell clones synthesized *ecoR* and produced helper-free virus that encodes *ecoR*. Expression of *ecoR* in nonmurine cells was accomplished by infection with the amphotropic host range virus released from the PA-12 cells or alternatively by transfection with pJET or pSFF-*ecoR*.

**Assays for ecotropic gp70 binding to cell surface *ecoR*.** Cells ( $10^5$ ) were plated onto coverslips in 9.5-cm<sup>2</sup> dishes 1 day before analysis. gp70 that had been isolated from ecotropic Friend murine leukemia virus (2 µg/ml) (16, 52) was added to the medium overlying cell cultures for 2 h at 37°C. Cell monolayers were rinsed and then sequentially incubated for 1 h at 37°C with goat antiserum made to gp70 (12) and then with fluorescein-conjugated rabbit anti-goat antibody (Zymed Laboratories, Burlingame, Calif.) as described previously (20). After the cells had been rinsed three times with culture medium for 10 min per rinse, cultures were fixed with cold methanol and mounted for fluorescence microscopy. Alternatively, for quantitative analysis of cell surface immunofluorescence, the labeled cells were released from the monolayers by using 8 mM EDTA in 0.9% NaCl, pH 8.0 (13), and then counted with a hemacytometer. After addition of 0.1% sodium dodecyl sulfate in sterile water, the cell lysates were assayed for fluorescence in a fluorometer (Perkin-Elmer LS-3) at an excitation wavelength of 495 nm and an emission wavelength 525 nm.

For immunoelectron microscopy,  $2 \times 10^4$  cells were plated onto 2-cm<sup>2</sup> chamber slides (Nunc Inc., Naperville, Ill.) for 16 to 24 h. The gp70 and goat antiserum to gp70 were sequentially incubated with the cells at 37°C as described above for 2- and 1-h periods. Then the cultures were incubated for 1 h at 37°C with 1:4-diluted gold (5-nm diameter)-conjugated rabbit anti-goat antiserum (Amersham, Arlington Heights, Ill.). Cells were then washed twice for 5 min per wash with cold serum-free Dulbecco's modified Eagle's medium and twice with 0.1 M sodium cacodylate buffer (pH 7.4). Cells were then fixed with a 1:1 mixture of 1.5% glutaraldehyde and 1.5% paraformaldehyde at 4°C for 20 min, rinsed with 0.1 M sodium cacodylate buffer, postfixed in 1% osmium tetroxide, dehydrated in a graded series of ethanol dilutions, and then embedded in Spurr's Epoxy (Electronmicroscopy Sciences, Fort Washington, Pa.).

**Quantitative analysis of ecotropic-virus infection.** Quantitative analyses of ecotropic-virus infections was done by using the helper-free virus that encodes human growth hormone (hGH) as described previously (26). Briefly,  $10^5$  cells that had been infected 48 h earlier with dilutions of the helper-free virus were plated on coverslips in 9.5-cm<sup>2</sup> dishes 1 day

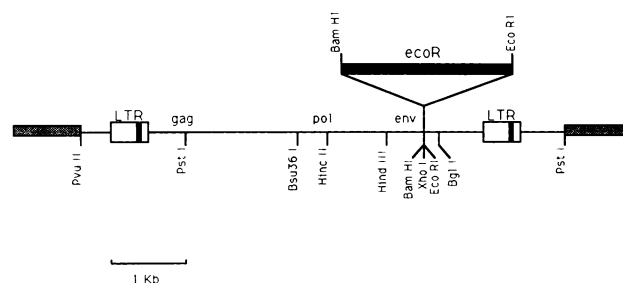


FIG. 1. Construction of pSFF-*ecoR*. A 2.3-kbp *Bam*HI-*Eco*RI fragment containing the *ecoR* cDNA coding region was inserted into the *Bam*HI and *Eco*RI sites of the retroviral expression vector pSFF to form pSFF-*ecoR*. The stippled bars represent pSP64 vector sequences. LTR, long terminal repeat.

before analysis. Cells were fixed with 3.7% formaldehyde in phosphate-buffered saline (140 mM NaCl, 2.7 mM KCl, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, 8.1 mM Na<sub>2</sub>HPO<sub>4</sub>) for 20 min at room temperature, and the coverslips were then incubated in 0.2% Triton X-100 in phosphate-buffered saline for 10 min. Dilutions of rabbit antiserum to hGH (donated by the National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, Md.) (1:2,000) and of fluorescein-conjugated goat anti-rabbit immunoglobulin (Tago Immunologicals, Burlingame, Calif.) (1:150) were sequentially incubated with the cells for 1 h at 37°C. After being washed with Dulbecco's modified Eagle's medium three times and phosphate-buffered saline once, the cells were mounted for immunofluorescence microscopy. The fraction of fluorescent cells was determined by analyzing at least 10 to 15 microscopic fields that contained a total of at least 200 fluorescent cells. Multiplicity of infection was calculated from the binomial distribution as  $-\log P/0.44$ , where  $P$  is the fraction of nonfluorescent cells (26).

## RESULTS

### Retroviral vector for *ecoR* expression in nonmurine cells.

The retroviral expression vector pSFF-*ecoR* (Fig. 1) was transfected into 1:1 cocultures of ψ-2 and PA-12 retroviral packaging cells in conditions optimal for ping-pong amplification (4, 26). In this method, the helper-free retrovirions released from either type of packaging cell are cross-infectious for the other. This results in an efficient back-and-forth process of infection whereby the cells acquire multiple copies of the provirus and efficiently express the encoded protein. Figure 2 shows a typical slot blot analysis of RNA synthesized in such cocultures. Several of the amplified cocultures contained large amounts of *ecoR*-specific RNA (e.g., slots 2, 5, 6, and 9) compared with cultures of normal mouse cells (slot 1).

ψ-2 and PA-12 cells, which were isolated from these cocultures by limiting dilution cloning (26), released substantial amounts of helper-free virions that encode *ecoR*. The *ecoR*-encoding virus with an amphotropic host range was then used to infect different nonmurine cell lines. The resulting nonmurine cells then became highly susceptible to infection by helper-free ecotropic host range MSV-neo virions. As shown in Fig. 3, the cells infected by the latter virions grew as colonies in the presence of the selection compound G418. In contrast, the control nonmurine cell lines were completely resistant to infection by MSV-neo virions. The nonmurine cells that synthesized *ecoR* also

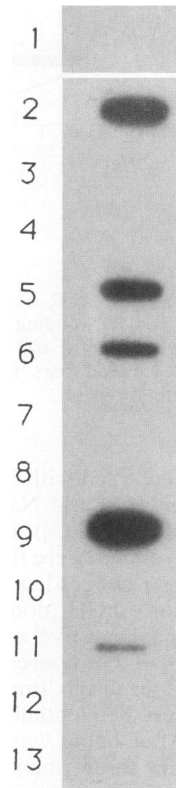


FIG. 2. RNA slot blotting of  $\psi$ -2-PA-12 coculture expressing *ecoR* cDNA. RNA extracted from individual cocultures (20  $\mu$ g) was blotted onto nitrocellulose and hybridized with the  $^{32}$ P-labeled nick-translated *Bam*HI-*Eco*RI *ecoR* cDNA fragment. Slot 1, RNA extracted from control coculture; slots 2 to 13, RNA from 12 cocultures transfected with pSFF-*ecoR*.

were able to bind gp70 that had been isolated from an ecotropic retrovirus (see below). Indeed, we used the immunofluorescence microscopy assay for gp70 binding (see Materials and Methods) to quantitatively determine the titers of the amphotropic virions that encode *ecoR*. By this criterion,

we estimated that our preparation of these virions had a titer on mink cells of  $10^4$ /ml for a 2-h adsorption time at 37°C.

The nonmurine cells that grew in the presence of G418 (Fig. 3) were isolated as pure clones. As indicated by RNA slot blotting, they all synthesized *ecoR*-specific RNA (data not shown). Using these methods, we were therefore able to isolate CCL64 mink and *Ostk*<sup>-</sup> human cell clones that produced substantial amounts of *ecoR*. Cell clones that stably express lower quantities of *ecoR* were obtained by transfecting *ecoR* expression vectors directly into nonmurine cells. Because hamster cells lack both ecotropic and amphotropic receptors (17, 40, 43), they were first transfected with an *ecoR* expression vector. The resulting cell clones that weakly expressed *ecoR* were then infected with a helper-free virus from  $\psi$ -2 cells that encodes this receptor. By these methods, we obtained cell clones that lack helper virus contamination and that stably express different quantities of *ecoR*. The properties of these cell clones are described below.

**Specific gp70 binding to cells with *ecoR*.** Albritton et al. (1) found that human cells stably transfected with pJET could be infected by ecotropic retroviruses. However, they did not describe evidence that the putative *ecoR* could bind gp70 that had been isolated from an ecotropic retrovirus. Previous workers reported that such  $^{125}$ I-labeled gp70 bound specifically to mouse cells, although the iodination substantially inactivated the receptor-binding activity of the glycoprotein (10). We found (16, 20) that unmodified gp70 is much more stable and that it binds to *ecoR* in a relatively specific manner without significant background. The cells with bound gp70 can then be labeled by immunological methods (16, 20). Figure 4 shows a typical immunofluorescence analysis of ecotropic gp70 binding to viable murine and nonmurine cell lines. In this example, mink cells with *ecoR* (CEN cells) bind gp70 extensively (panel C), whereas control mink cells are completely unlabeled (panel B). As with murine cells (panel A), the *ecoR* on nonmurine cells is clustered into patches. This clustering is caused by antibody-dependent aggregation of *ecoR* in the membranes. Receptors are not clustered when the labeling is done at 0°C or when the cells are fixed before reaction with gp70 (20). In all of these respects, results with human, hamster, and mink cell clones were identical.

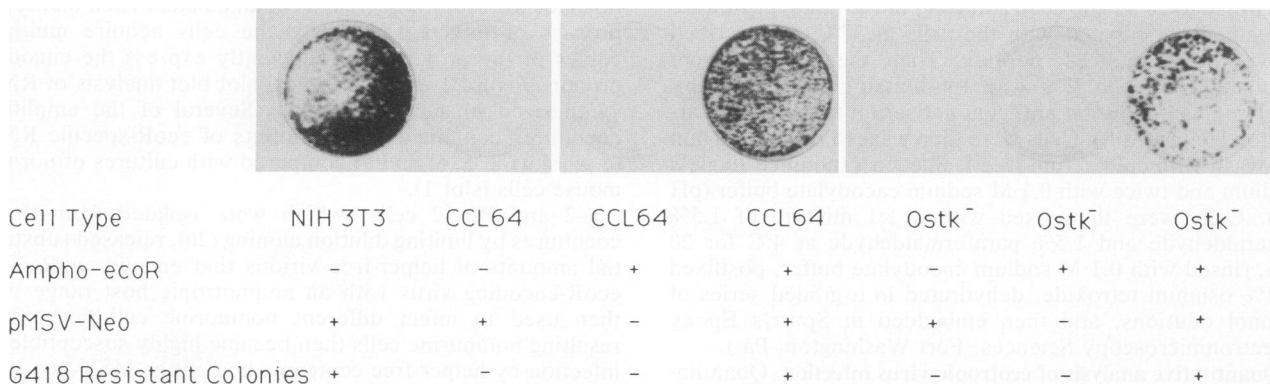


FIG. 3. G418-resistant colonies from mouse NIH 3T3, mink CCL64, and human *Ostk*<sup>-</sup> cells infected with pMSV-neo virus. Cells (NIH 3T3, CCL64, and *Ostk*<sup>-</sup>) were plated at  $10^5$ /100-mm dish. Where indicated, cultures were exposed to amphotropic virions encoding *ecoR* (Ampho-*ecoR*) 1 day later and/or to pMSV-neo virus at 3 days after plating. G418-resistant colonies appeared on the dishes after selection for 8 to 12 days. G418 concentrations used for selection were 500, 1,000, and 350  $\mu$ g/ml for NIH 3T3, CCL64, and *Ostk*<sup>-</sup> cells, respectively. G418-resistant colonies were stained with methylene blue.

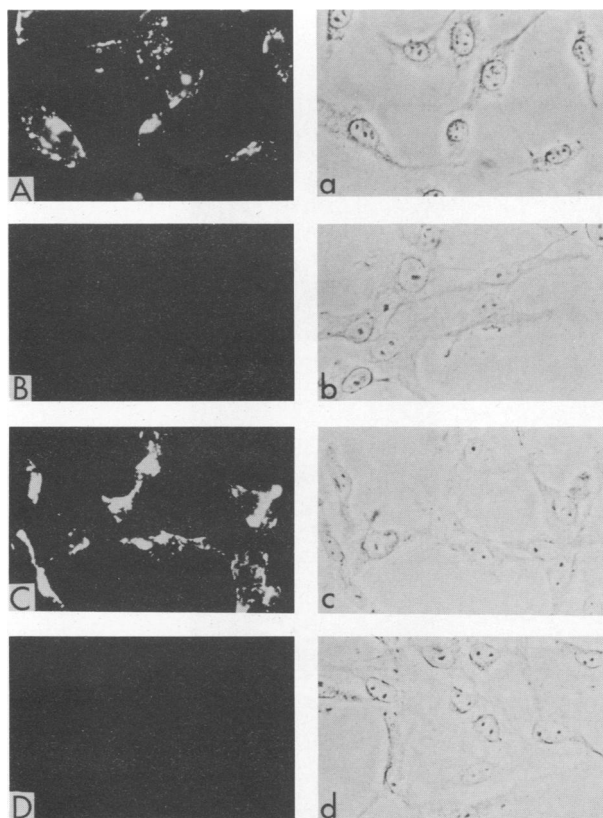


FIG. 4. Immunofluorescence analysis of gp70 binding to murine and mink cells. Cells were incubated sequentially with gp70, goat antibody to gp70, and fluorescein-conjugated rabbit anti-goat antibody as described in Materials and Methods. Each microscopic field is shown in fluorescence (A, B, C, and D) and phase-contrast (a, b, c, and d). (A and a) Murine NIH 3T3 cells; (B and b) mink CCL64 cells; (C and c) CEN cells (mink CCL64 cells with ecoR); (D and d) CEN cells incubated with all reagents except gp70.

Previously, we demonstrated that murine cells were specifically and efficiently killed when they were incubated with ecotropic gp70 and subsequently an antiserum to gp70 in the presence of complement (16). This procedure also caused specific killing of nonmurine cells that expressed recombinant ecoR (results not shown).

Figure 5 shows an immunoelectron microscopic analysis of gp70-ecoR complexes with gold-labeled secondary antiserum. The gp70-ecoR in the plasma membrane are clustered into patches that appear thicker than adjacent unlabeled regions (panels B, E, and G). Panel F shows a coated pit that contains gp70-ecoR complexes, but these were extremely rare. Even after unbound gold-labeled antiserum was removed and the viable cells were cultured for an additional 2 h at 37°C, the label remained almost exclusively on the plasma membranes. Thus, almost no label was evident in intracellular regions. We were unable to detect any significant background labeling of cells that lack ecoR (panel D) or of cells incubated with all reagents except gp70 (panels A and C). These results support previous evidence that gp70-ecoR complexes remain accessible to extracellular reagents for many hours (16, 20). Either these gp70-ecoR complexes are in a substantially stagnant cell surface pool or they cycle between endosomes and cell surfaces and become trapped in surface aggregates following addition of the antiserum to

gp70 (20). Because our immunological labeling protocols involve sequential 1- or 2-h incubations of viable cells with different reagents (gp70, antibody to gp70, and then conjugated secondary antiserum), rapidly and irreversibly endocytosed gp70-ecoR complexes would not be labeled. Therefore, these results do not exclude the possibility that a fraction of the gp70-ecoR complexes might be rapidly and irreversibly endocytosed (see Discussion).

**Basic characteristics of infections by ecotropic retroviruses.** To quantitatively analyze the susceptibilities of different cell lines to infection, we incubated cell cultures under standard conditions for 2 h with several dilutions of an ecotropic helper-free retrovirus that encodes hGH. We subsequently measured the fraction of cells that contained hGH by a sensitive hGH immunofluorescence assay (26). From this information we were able to calculate the multiplicities of infection and therefore the total number of infections that occurred in the cell cultures (see Materials and Methods). Cells that lacked ecoR could not be infected to a detectable extent and therefore will not be further described.

Figure 6 shows typical results based on infection of different cell lines that contain ecoR. Within experimental error, the infections were always directly proportional to virus concentrations, indicating that the cellular capacities for infection are unsaturated in the conditions of our studies. Because extracellular virus concentrations could influence infection only at the step of binding to cell surface receptors, these simple results suggest that adsorbing more virus onto receptors will result in higher levels of infection. In addition, the relative susceptibilities of these cells to infection were in the order mouse > hamster > mink > human. As shown below, these differences in susceptibility occurred regardless of the quantities of ecoR on the cell surfaces.

Only a small percentage of infectious hGH virions become adsorbed onto cells from the medium during the 2-h incubation used for these assays. For example, as reported in Table 1, 1 ml of virus-containing medium was first incubated at 37°C for 2 h either in an empty flask (blank) or in a flask that contained  $10^5$  murine or nonmurine cells that contained ecoR. Subsequently, these media were removed onto fresh cultures of  $10^5$  NIH 3T3 cells for an additional 2 h, and the multiplicities of infection in these NIH 3T3 cultures were then determined. The medium that had been preadsorbed onto NIH 3T3 cells contained only 3 to 4% fewer infectious virions than the virus-containing medium that had been incubated in the empty flask. Similarly, nonmurine cells with ecoR adsorbed only small fractions of the infectious virus. The results of this and other independent studies using ecoR-containing cell lines indicate that only approximately 5% of infectious hGH virions become adsorbed onto cell cultures during the 2-h incubations at 37°C. Thus, the true numbers of infectious hGH titers must be many times higher (approximately 20 times) than the titers estimated by standard 2-h adsorption assays. Low-efficiency adsorption also occurs when other ecotropic retroviruses are used. For example, similar results were obtained when a preparation of ecotropic murine leukemia virus was assayed sequentially on two S+L- cell cultures (19a).

The number of infections that occur in cell cultures incubated with a preparation of ecotropic hGH virus is also directly proportional within experimental error to the number of cells plated into the 25-cm<sup>2</sup> flask (Fig. 7). Thus, the multiplicities of infection were independent of the number of cells in the culture. This is compatible with the results in Table 1, which indicate that virus concentrations are not substantially reduced because of virus incubation with cells.

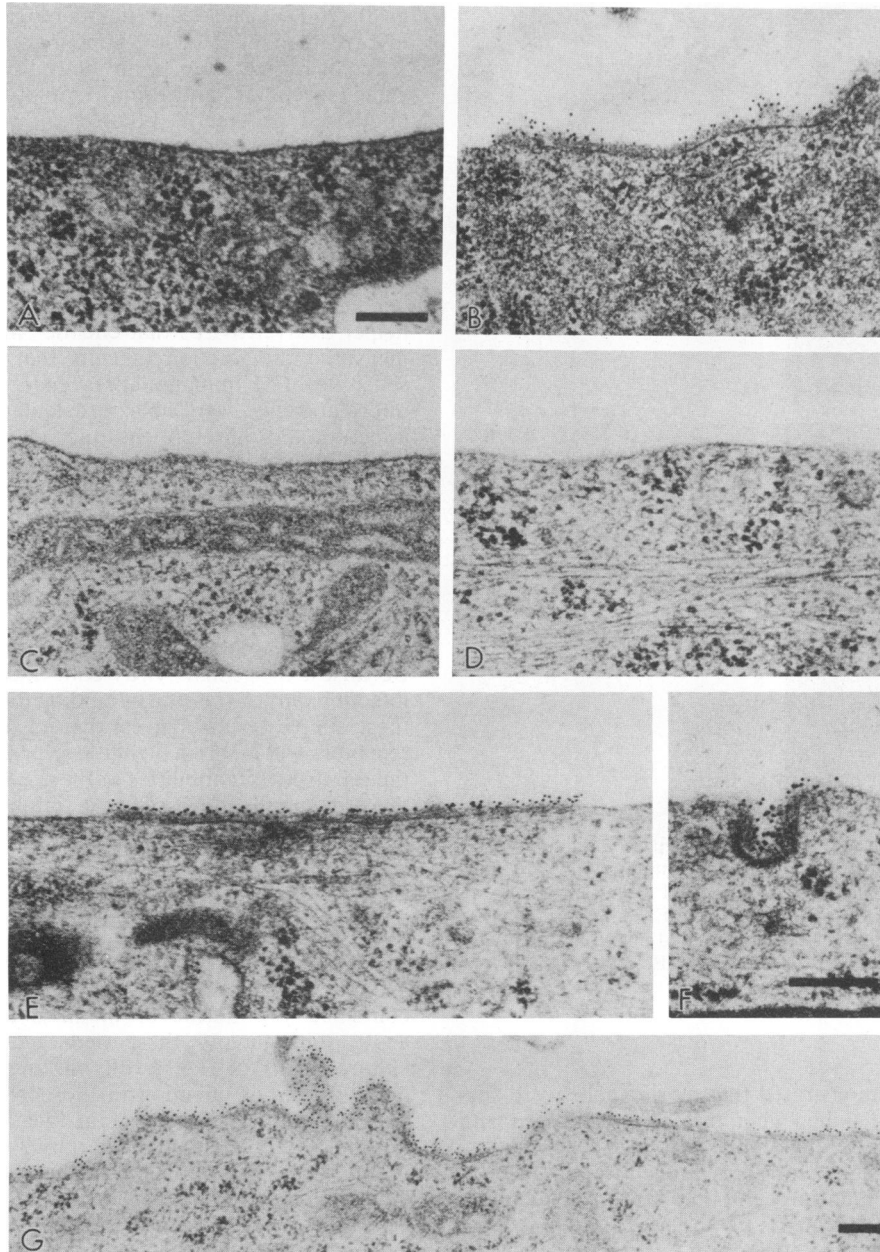


FIG. 5. Immunoelectron microscopic analysis of gp70-ecoR complexes. Cells were incubated at 37°C with or without gp70 for 2 h, with goat antibody to gp70 for 1 h, and with 5-nm-gold-labeled rabbit anti-goat antibody for 1 h. After being washed and fixed (see Materials and Methods), samples were analyzed by electron microscopy. (A) Murine NIH 3T3 fibroblasts incubated without gp70. No labeling is seen. (B) NIH 3T3 cells incubated with gp70. A patch of surface labeling is shown. (C) CEN cells incubated without gp70. These are mink CCL64 cells that contain ecoR. No labeling is seen. (D) Mink CCL64 cells incubated with gp70. These cells lack ecoR and are unlabeled. (E, F, and G) CEN cells incubated with gp70. These cells have ecoR in surface patches that are labeled. All bars are 200 nm.

Considered together, our results suggest that the number of hGH virus infections that occur in a cell culture is directly proportional to the virus and cell concentrations and that only a small percentage of the infectious virus becomes adsorbed onto cells during 2-h incubations at 37°C.

**Cells have limited amounts of functional ecoR.** As described above, we isolated clones of hamster and human cells that stably express different quantities of cell surface ecoR. The stability of expression within each cell clone and the differ-

ences between clones were clearly evident by immunofluorescence microscopy (Fig. 8). Moreover, the fluorescence emission differences between clones were also evident when they were quantitatively measured by fluorimetry. These measurements were always performed in the presence of saturating quantities of gp70 and of the primary and secondary antisera.

Table 2 shows results of a typical experiment in which we used fluorimetry to measure gp70 binding to the cell clones.

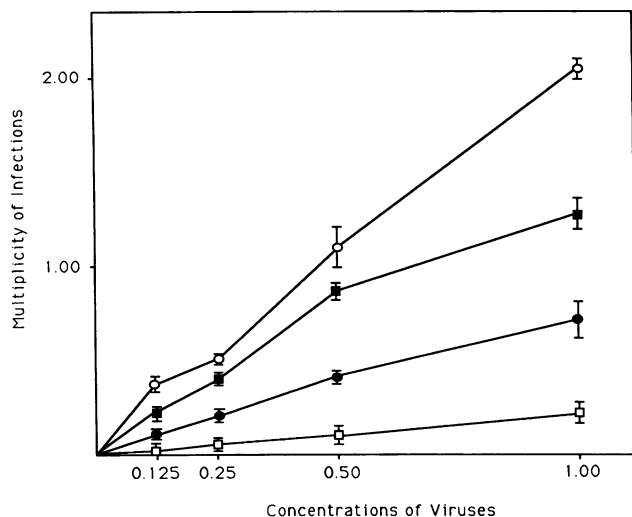


FIG. 6. Effect of ecotropic hGH virus concentration on infection of cells from different species. Different cell lines that contain *ecoR* were infected with ecotropic hGH virus at various viral concentrations. The undiluted virus preparation was defined as having the concentration 1.0. Its titer on NIH 3T3 cells was  $2 \times 10^5$ /ml. Cells which expressed hGH were detected by growth hormone-specific immunofluorescence. Multiplicities of infections were calculated as described in Materials and Methods and are plotted against viral concentrations. The multiplicities of infection are shown as means  $\pm$  standard errors of the means from three sets of cell countings. Symbols: ○, murine NIH 3T3 cells; ■, hamster CER-18-C11 cells; ●, mink CEN cells; □, human OstEN cells.

One clone of human osteogenic sarcoma cells (ostER-C17) expresses fewer gp70-binding sites than NIH 3T3 cells, whereas another (ostEN) expresses several times more. The hamster cell clones that contain *ecoR* differ from each other by approximately 10-fold in their capacities to bind gp70.

TABLE 1. Adsorption of virions onto mouse and hamster cells that express *ecoR*

Cell types for incubation <sup>a</sup>	Multiplicity of infection <sup>b</sup>	Specific virion adsorption (%) <sup>c</sup>
Blank/NIH 3T3	0.89	0
NIH 3T3/NIH 3T3	0.86	3.4
CER-18-C11/NIH 3T3	0.81	9.0
CER-D-C110/NIH 3T3	0.84	5.6

<sup>a</sup> A large volume of culture medium (Dulbecco's modified Eagle's medium with 10% fetal bovine serum) containing a diluted sample of helper-free ecotropic hGH virus was prepared. The titer on NIH 3T3 cells was  $2 \times 10^5$ /ml. Aliquots of this medium (1 ml) were consecutively placed for 2 h at 37°C into 25-cm<sup>2</sup> flasks that contained  $10^5$  cells per flask of the cell types shown. Each sample of medium was incubated first with the cell culture on the left of the slash and then with the cell culture on the right. Each cell pair was analyzed in duplicate.

<sup>b</sup> NIH 3T3 cells that had been infected with ecotropic hGH virus during the second incubations were analyzed for hGH-specific immunofluorescence. From these results, the multiplicities of infection were determined (see Materials and Methods). The values shown are the average of the two measurements. The virus-containing culture medium that had been incubated first with cells for 2 h contained almost as much virus as the sample that had been incubated first in an empty (blank) flask.

<sup>c</sup> Adsorption during the first incubation. These values are clearly only approximate, because they are based on the small differences between the multiplicities of infection in column 2. They suggest that only a small percentage of virus is adsorbed onto cell cultures under the conditions of these experiments.

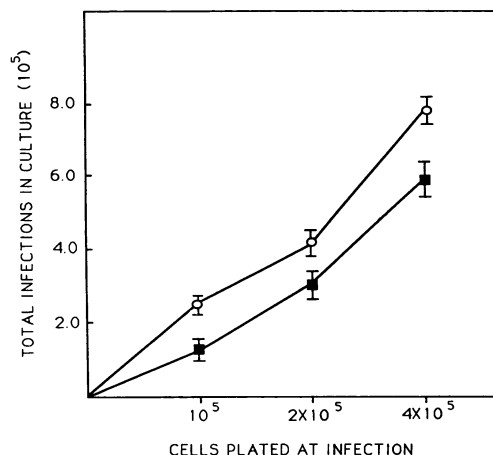


FIG. 7. Total virus infections in cell cultures as a function of the number of target cells. Different numbers of cells were plated in 25-cm<sup>2</sup> flasks for infection with a single concentration of helper-free ecotropic hGH virus (Fig. 6). Each flask contained a 1-ml aliquot of the virus-containing medium. The virus titer measured on NIH 3T3 cells was  $2 \times 10^5$ /ml. Multiplicities of infection were measured later. The total number of infections in each culture was calculated as the multiplicity of infection times the number of cells at the time of infection. Within experimental error, multiplicities of infection were independent of cell number, and the total number of infections was therefore proportional to the number of cells in the cultures. The total numbers of infections are shown as means  $\pm$  standard errors of the means from three sets of cell countings. Symbols: ○, murine NIH 3T3 cells; ■, hamster CER-18-C11 cells (these have *ecoR*).

Similar binding differences were obtained when we used whole ecotropic virions rather than purified gp70 as the ligand.

Table 2 also shows the results of hGH virus infections of these cell clones. Consistent with the results described in Fig. 6, the murine NIH 3T3 cells are approximately 7 to 8, 3, and 1.5 times more susceptible to infections than are human, mink (CEN), and hamster cells, respectively. Strikingly, however, the susceptibility to infection of the different human cell clones and hamster cell clones that express *ecoR* are independent of their quantities of cell surface *ecoR*. This same result was reproducibly obtained in two independent experiments. However, the nonmurine cells that lack *ecoR* were completely resistant to infection. Therefore, although a low threshold amount of *ecoR* must be essential for infection, additional increases in *ecoR* clearly have no effect on the efficiencies of infection.

## DISCUSSION

**Function of *ecoR* in nonmurine cells.** By all criteria used in this study, the *ecoR* cDNA isolated by Albritton et al. (1) encodes authentic *ecoR* that are indistinguishable from those that occur naturally on murine cells. Most important, the recombinant *ecoR* binds ecotropic gp70 specifically, and it mediates ecotropic virus infections of nonmurine cells. Although human Ostk<sup>-</sup> cells (Fig. 6) or human EJ bladder carcinoma cells (1) that express *ecoR* are infected only 10 to 15% as efficiently as murine fibroblasts, this difference cannot be ascribed to a deficiency of the recombinant *ecoR*, because hamster cells with this *ecoR* are infected almost as efficiently as murine cells (Fig. 6 and Table 2).

**Under the conditions of these experiments, infections were limited by viral adsorption onto cell surface *ecoR*.** At the low

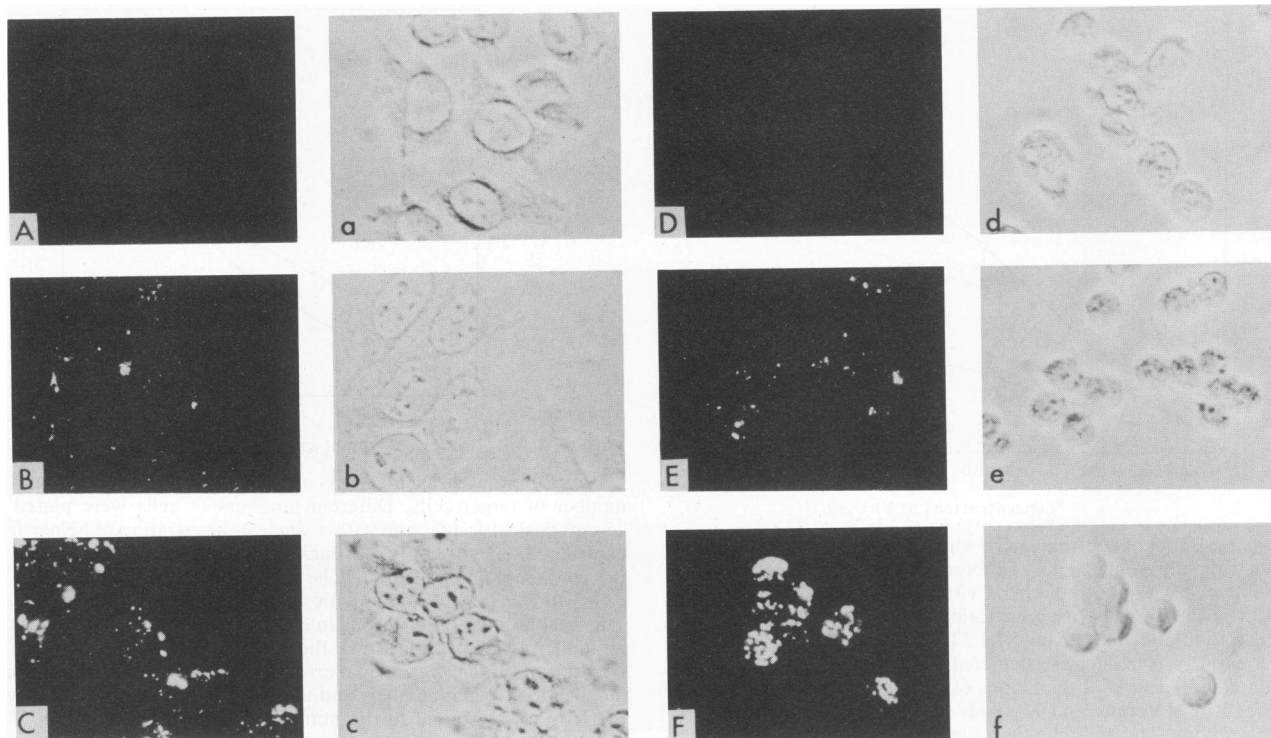


FIG. 8. Immunofluorescence microscopy of gp70 binding to human and Chinese hamster ovary cells that contain different quantities of *ecoR*. Each microscopic field is shown in fluorescence (A, B, C, D, E, and F) and phase-contrast (a, b, c, d, e, and f). Cells were incubated with gp70, then with antibody to gp70, and then with fluorescein-conjugated secondary antibody (see Materials and Methods). (A and a) Human *Ostk*<sup>-</sup> cells; (B and b) OstER-C17 (derivatives of *Ostk*<sup>-</sup> containing low levels of *ecoR*); (C and c) OstEN (derivatives of *Ostk*<sup>-</sup> containing high levels of *ecoR*); (D and d) CHO cells; (E and e) CER-18-C11 (derivatives of CHO containing low levels of *ecoR*); (F and f) CER-D-C19 (derivatives of CHO containing high levels of *ecoR*).

concentrations of ecotropic retroviruses that were used to infect proliferating cultured cells under the conditions of our experiments, adsorption of virus was clearly the limiting step in infection. Thus, during 2-h incubations of a helper-free ecotropic hGH virus with cell cultures in standard conditions, only a small percentage (ca. 5%) of the infectious virions were adsorbed (Table 1). Furthermore, the infections were directly proportional to the concentrations of virus in the media (Fig. 6), suggesting that the capacity of the cells to be infected was unsaturated. Because the only way for extracellular virus concentrations to influence infection occurs at the adsorption step, we conclude that increased adsorption onto cells causes a proportional increase in infection. The fact that infections were also directly proportional to cell concentrations (Fig. 7) provides additional evidence that the limiting step of infection is the interaction of virus with cells to produce adsorbed virus.

**Only a portion of cell surface *ecoR* are functional for infection.** In apparent contrast to the fact that infections were limited by virus adsorption onto the cells (see above), the infections did not correlate with the abilities of the cells to adsorb virus. As shown in Table 2, cells of a given type with a small number of *ecoR* were infected as efficiently as cells that contained abundant *ecoR* (in Table 2, compare OstER-C17 with OstEN or CER-18-C11 with CER-D-C110 and CER-D-C19).

On the basis of these results, we conclude that cells with widely different total amounts of *ecoR* must contain the same limited amount of *ecoR* that is functional for infection.

Thus, we infer that OstER-C17 and OstEN contain the same amount of functional *ecoR*. Similarly, the hamster cell clones CER-18-C11, CER-D-C110, and CER-D-C19 must contain the same amount of functional *ecoR*. Therefore, *ecoR* above a low threshold amount would be nonfunctional and would have no influence on infection. According to this idea, raising the concentration of an infectious virus would by mass-action principles enhance its adsorption onto functional as well as nonfunctional receptors, and this would result in a proportional increase in the multiplicity of infection (Fig. 6). In contrast, adding *ecoR* to cells in an amount above the threshold quantity would result only in more nonfunctional receptors. Although the resulting cells would bind virus or gp70 more extensively, this additional bound virus would not result in infection.

In addition, our results imply the following. First, we infer that *ecoR* that is nonfunctional for infection must occur on normal mouse cells rather than solely on nonmouse cells that contain *ecoR*. Otherwise, one would have to conclude that the *ecoR* on mouse NIH 3T3 cells functions less efficiently than the receptors on CER-18-C11 hamster cells. The latter cells contain only approximately 35% as much *ecoR* as NIH 3T3 cells, although they are infected approximately 65% as efficiently. BM3C3 hamster fibroblasts, which are an E36 hamster × mouse hybrid cell line containing a fragment of mouse chromosome 5 (23, 24), also contain relatively little *ecoR*, although they are infected approximately 60% as efficiently as NIH 3T3 cells (data not shown). Second, the results in Table 2 suggest that virus that attaches onto

TABLE 2. Measurement of gp70 binding and ecotropic-virus infection using cells that express different amounts of ecoR

Cell type <sup>a</sup>	gp70 specific fluorescence <sup>b</sup>	Multiplicity of infection <sup>c</sup>
NIH 3T3	61.8 ± 13.1	3.07 ± 0.20
Ostk <sup>-</sup>	0.1 ± 8.7	0
OstER-C17	45.9 ± 10.6	0.36 ± 0.01
OstEN	141.8 ± 10.4	0.43 ± 0.03
CHO	-9.2 ± 7.3	0
CER-18-C11	22.7 ± 5.1	1.95 ± 0.09
CER-D-C110	98.0 ± 4.4	1.92 ± 0.19
CER-D-C19	226.9 ± 3.8	1.92 ± 0.09
CCL64	-10.0 ± 14.0	0
CEN <sup>d</sup>	569.5 ± 44.8	1.06 ± 0.13

<sup>a</sup> NIH 3T3, Ostk<sup>-</sup>, CHO, and CCL64 cell lines are described in Materials and Methods. The pJET vector and the pSFF-ecoR retroviral vector which was used to make helper-free ecotropic and amphotropic retrovirus encoding ecoR are described in Materials and Methods. OstEN cells were derived by infection of Ostk<sup>-</sup> cells with helper-free amphotropic virus encoding ecoR. OstER-C17 cells were derived by transfection of Ostk<sup>-</sup> cells with pJET. CER-18-C11 cells were derived by transfection of CHO cells with pJET. CER-D-C19 and CER-D-C110 cells were derived from CER-18-C11 cells by infection of the latter with helper-free ecotropic virus encoding ecoR. CEN cells were derived by infection of CCL64 cells with helper-free amphotropic virus encoding ecoR.

<sup>b</sup> Fluorescence was measured in a fluorospectrophotometer. Cells were incubated with or without gp70, then with antibody to gp70, and then with fluorescein-conjugated secondary anti-immunoglobulin antibody as described in Materials and Methods. The gp70 binding specific fluorescence was obtained by measuring the fluorescence value of 10<sup>5</sup> cells incubated with gp70 and subtracting the fluorescence value of 10<sup>5</sup> cells incubated without gp70. Values represent means ± standard errors of the means from four readings.

<sup>c</sup> Multiplicity of infection was determined by infecting cells with ecotropic hGH virus as described in Materials and Methods and later measuring the fraction of infected cells by hGH-specific immunofluorescence microscopy as described in Materials and Methods. Values represent means ± standard errors of the means from three counts. The virus preparation used for infection had a titer on NIH 3T3 cells of 3 × 10<sup>5</sup>/ml.

<sup>d</sup> Despite several efforts, we were unable to isolate CCL64 mink cells transfected with pJET that expressed lower amounts of ecoR. Therefore, only this single clone of infected mink cells was analyzed. It expresses a relatively high level of ecoR.

nonfunctional ecoR must be essentially unsalvageable, perhaps because the ecoR in these complexes remains permanently inactive or perhaps because the nonfunctional ecoR rapidly inactivates attached virus. If the virus that bound to nonfunctional ecoR could be salvaged after adsorption and transfer of cells to fresh medium, we would expect that cells with the most ecoR would be infected most efficiently. Clearly, however, this is not the case (Table 2). Third, the fact that each type of cell seems to contain a set amount of potentially functional ecoR (Table 2) strongly suggests that there is a limiting second cellular component that occurs in cells from different species and is required for ecoR to be active in facilitating virus infection. After enough ecoR to saturate this accessory factor is made, additional ecoR constitutes a nonfunctioning pool of spare receptors.

What is the nature of the putative second cellular component? Possibly, it is a second receptor subunit that occurs in limited supply. Alternatively, it could be a limiting enzyme that modifies a portion of the ecoR. It appears from our results that the excess ecoR which is nonfunctional in infections turns over only slowly on cell surfaces and remains accessible to primary and conjugated secondary antisera for many hours (see Materials and Methods and Fig. 4, 5, and 8). If virus or gp70 that binds to the functional class of ecoR was rapidly and irreversibly endocytosed, it would escape detection by our labeling protocols. Therefore, we

emphasize that our labeling protocols and metabolic studies have provided information only about the inactive class of ecoR.

Our results also suggest that cell lines have inherent differences in susceptibility to ecotropic-virus infections and that these differences are independent of the amounts of ecoR in their membranes (Fig. 8 and Table 2). Murine NIH 3T3 fibroblasts were most susceptible to ecotropic-virus infection. Different hamster cell lines with ecoR were all approximately 60 to 70% as susceptible to infection as NIH 3T3 cells. Human osteogenic sarcoma cells with ecoR are only approximately 10% as susceptible to infection as NIH 3T3 cells. Similarly, it was reported that human bladder carcinoma cells with ecoR were only 10% as susceptible as NIH 3T3 cells (1). Accordingly, the susceptibilities of cells from different species appear to correspond with the evolutionary distance of the species from mice and to occur in the sequence mouse > hamster > mink > human. Additional analysis will be required to determine whether these apparent differences between species are caused by restrictions in adsorption or penetration or whether they occur at a subsequent step of infection.

On the basis of the considerations and definitions given above, we therefore propose that functional ecoR are formed by interaction between the gp70-binding ecoR protein and an accessory factor that occurs in limiting quantities in cells from different species. If verified by further studies, this result would have major implications for understanding the mechanisms of retroviral infections. For example, infections might be blocked not only by interfering with ecoR but also by interfering with the accessory factor. The molecular and metabolic differences between the active and inactive classes of ecoR would also provide critical insights into the mechanism of retroviral infection of cells. Finally, it is possible that the same or a related accessory factor(s) is required for activities of other retroviral receptors. Indeed, it has been speculated that a need for an accessory factor could explain why mouse cells and some human cells that express recombinant human CD4 cannot be infected by human immunodeficiency virus (2, 6, 32). If the accessory proteins of mouse cells interact only nonfunctionally with human CD4, the dominant inhibitory effect of the mouse environment (2, 54) could be explained. Additional studies that include direct investigations of retroviral receptor proteins will be required to test these possibilities.

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