ECOTROPIC MURINE LEUKEMIA VIRUS RECEPTOR: VIRAL INFECTION AND CATIONIC AMINO ACID TRANSPORT

by

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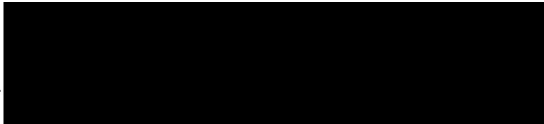

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Abstract

The early steps in retroviral infection involve the binding of viral envelope glycoprotein to specific cell surface receptors. Murine leukemia viruses (MuLVs) have been classified into ecotropic, amphotropic, xenotropic and dualtropic host-range/interference groups. These MuLV class differences are determined by their viral envelope glycoproteins that bind to specific corresponding receptors on surfaces of susceptible cells. Thus, ecotropic MuLVs can only infect mouse and rat cells, but not cells of other species. The complementary DNA (cDNA) encoding the receptor (ecoR) for ecotropic MuLV has been molecularly cloned. Studies in this dissertation have focused on functional characterization of ecoR and ecoR mediated viral infection, identification of ecoR as a cationic amino acid transporter, and studies of the effects of viral infections on the transport activity of ecoR. To study ecoR mediated viral infection, various amounts of ecoR were expressed in cells of different species including human, mink and hamster. 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 expressed on the cell surfaces above a low threshold had no effect on infection. These results implied that only a small, set number of cell surface ecoR were functional for infection and that additional ecoR constitute a pool that can mediate adsorption of virus but not infection. This suggests that the activity of ecoR in infection requires a limiting second cellular component. To investigate the normal physiological function of ecoR, ecoR was expressed in Xenopus laevis oocytes by injecting RNA transcribed from the cloned cDNA. These injected oocytes specifically bound envelope glycoprotein (gp70)

from an ecotropic host-range MuLV. A stereoselective, saturable, sodium independent uptake of lysine, arginine and ornithine was detected in the injected oocytes by both electrophysiological and chemical methods . The properties of ecoR correspond to those of system y^+, a previously characterized transport system for cationic amino acids. Therefore, ecoR was identified as the basic amino acid transporter, system y^+. Mink cells that express a high level of ecoR were used to study the effects of gp70 and viral infections on the transport activity of ecoR. It was found that chronic infections only partially (50-70%) down-modulate the transport activity of ecoR/y^+ transporter, despite a complete (3-4 orders of magnitude) interference to superinfection. This suggests that viral interference does not result from a complete depletion of viral receptors from the surfaces of infected cells. Moreover, extracellular gp70 adsorbed onto ecoR/y^+ at 37°C partially inhibits cationic amino acid uptake but not outflow, suggesting that the virus binding site on ecoR is in a mobile region that changes conformation during the amino acid transport cycle. Finally, it was shown by site-directed mutagenesis that two potential N-linked glycosylations on ecoR are not required for gp70 binding, viral infection, or basic amino acid transport. Elimination of glycosylation site 2 renders ecoR fully interactive with virus.

host cell plasma membranes. The cell and its progeny usually remain viable and permanently infected.

3. Classification of Retroviruses

Retroviruses have been divided into three subfamilies. The largest subfamily of retroviruses is the oncoviruses. Viruses in this group can be further classified into two groups. One of these groups includes viruses that have classical oncogenes in their genomes. Such viral oncogenes are derived from normal cellular genes called protooncogenes. RSV belongs to this category and its *src* gene has a counterpart in the normal cellular genome. The other group includes viruses such as spleen focus forming virus (SFFV), murine leukemia viruses (MuLVs), or feline leukemia viruses that lack such oncogenes and contain only retroviral-specific nucleic acid sequences. Members of the oncovirus subfamily are usually grouped according to the host species from which they have been isolated. For instances, murine leukemia viruses, avian sarcoma viruses, and feline leukemia viruses were isolated from mice, chicken, and cats respectively. Oncoviruses are also grouped based on the morphology of their virions (i.e. type A, B, C, and D particles). Mouse mammary tumor virus is a B-type oncovirus, whereas murine leukemia viruses and feline leukemia viruses are C-type viruses.

Human immunodeficiency viruses (HIV) belong to the second subfamily of retroviruses, lentiviruses. Viruses from this subfamily induce progressive diseases with extremely long latent periods. Other features of lentiviruses that are different from oncoviruses include the complexity of their genomes (2) and the remarkable sequence heterogeneity of their *env* glycoproteins (3). Animals infected with lentiviruses usually contain numerous viral "quasispecies" with different *env* glycoproteins.

Spumaviruses are the third subfamily of retroviruses. Spumaviruses have been isolated principally as contaminants of primary tissue culture cells. These viruses derive their name from the characteristic "foamy" degeneration they induce in cultured cells. They may induce persistent infections, but they have not yet been implicated in any disease.

II. Viral Entry

1. Envelope glycoprotein

Binding of viral envelope glycoproteins onto specific cell surface receptors initiates infection. The envelope glycoprotein of a specific virus is usually named according to its molecular weight. Thus, the receptor-binding subunit of the envelope glycoprotein of murine leukemia virus is gp70, and the integral membrane subunit is p12E. These subunits derive from a common precurssor, gPr90, that is encoded by the viral *env* gene. gPr90 is first cleaved intracellularly to form gp70 plus an intermediate protein p15E that is later partially cleaved in virions at its carboxyl terminus to form p12E plus a small peptide of 16 amino acids (4, 5). In case of HIV, the product of the *env* gene is synthesized as a precussor (gp160) that is cleaved to form the receptor binding glycoprotein (gp120) and the transmembrane glycoprotein (gp41). The transmembrane subunits of retroviral envelope glycoproteins are believed to catalyze membrane fusion (6, 7).

2. Viral Receptors

Viruses exploit cell surface molecules as their receptors for cell entry. In many cases, these molecules are proteins. For example, rhinoviruses (common cold viruses) enter cells by attaching to the cell adhesion molecule ICAM-1 (8); Epsten-Barr virus infects lymphocyte by means of the C3d complement receptor (9); and transmissible gastroenteritis virus (TGEV), an enteropathogenic coronavirus, uses aminopeptidase N as its receptor to infect

enterocytes (10, 11). Other viruses (e.g., orthomyxoviruses such as influenza A virus or paramyxoviruses such as Sendai virus , mumps or measles viruses) apparently use carbohydrates on glycolipids and/or glycoproteins as their receptors (12).

For retroviruses, four cell surface receptors have been identified and molecularly cloned . CD4, a glycoprotein involved in T cell activation and regulation, is the receptor for HIV(13, 14, 15). The receptor for ecotropic MuLV (16) is a major transporter for basic amino acids (17, Manuscript#2 in this thesis). The receptor for both gibbon ape leukemia virus (GALV) (18) and feline leukemia virus subgroup B (FeLV-B) (19) is a molecule homologous to a yeast phosphate transporter (20). The receptor for avian leukosis and sarcoma viruses subgroup A (ALSV-A) has sequence homology to the low density lipoprotein receptor (H. Varmus, pers. comm.). Interestingly, the molecular structures and the physiological functions of these identified retroviral receptors are distinct and diverse.

3. Fusion and Endocytosis

The mechanisms of viral entry into cells after binding to surface receptors are not clearly understood. It is believed that a membrane fusion event occurs between the viral envelope and a membrane of the host cell and that this results in transfer of the viral core into the cytoplasm. Polycations, such as polybrene or DEAE-Dextran, can enhance the rate of adsorption of retroviral particles, probably by decreasing negative charges on the cell surface and thus reducing electrostatic repulsion between the cell and negatively charged virions (21, 22). Fungizone (Amphotericin B), an anti-fungal natural product, also can influence this membrane fusion process by an unknown mechanism (23).

Fusion can happen either at the cell surface or in low pH endosomes after receptor-mediated endocytosis of virus particles. Several viruses have been well-characterized in this respect. Sendai virus, a paramyxovirus, undergoes a pH-independent fusion that occurs at cell surfaces (24, 25). On the contrary, orthomyxovirus (influenza virus), causes a pH-dependent fusion after the virus particle is internalized into endosomal vesicles (26, 27, 28). Apparently, low pH causes the receptor-binding subunit of the influenza A hemagglutinin to fall away, thereby enabling the transmembrane subunit to insert its amino terminus into the endosomal membrane (29, 30). Semliki Forest virus (an arbovirus) and vesicular stomatitis virus (a rhabdovirus) also fuse with membranes only at low pH (27, 31). Relatively, little is known about the mechanisms of entry for retroviruses, in part because only a small proportion of the viral particles (c.a., 0.1%) successfully infect the cells. Consequently, the pathways followed by the majority of virus particles may be irrelevant to the actual pathway of infection (32).

Ultrastructural studies show that retroviral particles can be taken up by coated pits into endosome (33). Receptor-mediated endocytosis has also been reported for human T-cell leukemia virus type-1 (HTLV-1) (34) and for HIV (35, 36, 37). Mouse mammary tumor virus (MMTV) probably also enters cells by endocytosis (38). However, other evidence implies that some retroviruses including HIV may enter cells via fusion at plasma membranes. Ultrastructural observations show fusion of HIV-1 virions with plasma membranes (39, 40). Infection of cells by HIV-1 and by Rous sarcoma virus subgroup A are unaffected by raising endosomal pH (41), suggesting that these virus enter cells by a pH-independent fusion that occurs at cell surfaces. It has also been shown that HIV infection can be mediated by CD4 mutants that are defective in endocytosis (42). Evidence also suggests that ecotropic MuLVs

may enter cells by fusion at plasma membranes but this is uncertain (23, 41, 43, 44).

4. Accessory Factors in addition to Viral Receptors

Some evidence has suggested that retroviral entry may require secondary factor(s) in addition to the virus-binding receptors. For instance, murine cells (15) and human glioma cells (45) transfected with human CD4 are not permissive to cell fusion and entry by HIV-1. A wide range of mammalian cells expressing human CD4 bind gp120 but do not permit HIV-1 infection, membrane fusion, or infection of vesicular stomatitis virus(VSV) that has been pseudotyped with an HIV-1 envelope (46). However, HIV pseudotyped with MuLV envelopes that have wide host-range can enter and replicate in non-human cells (45, 47, 48). Thus the block to HIV-1 infection is at the cell surface and does not affect virus adsorption.

III. Viral Receptors and Pathogenesis

Retroviruses cause many diseases including tumors, immunodeficiencies, anemias, bone and joint diseases and neuropathies (49, 50, 51, 52, 53). The pathogenesis of retroviral diseases is complex and involve different mechanisms for different viruses. Retroviruses with oncogenes induce tumors (54, 55). In other cases, insertion of the viral genome into a host chromosome inactivates or activates the cellular genes that are adjacent to the inserted viral genome (56). For instance, avian leukosis virus lacks an oncogene and causes slowly developing bursal lymphomas by proviral integration adjacent to the cellular *myc* protooncogene (57).

The pathogenic effects of some retroviruses have been mapped to their *env* genes. Viruses in this category include feline leukemia and immuunodeficiency viruses (58, 59), avian subgroup F leukosis viruses (60), Friend and Rauscher spleen focus forming viruses and murine leukemia

viruses (53, 61). The *env* genes of HIV-1 (62) and HTLV-1 (49) also have been implicated in their pathogenesis. These viruses can cause leukemias, lymphomas, anemias, neural degenerations, immunodeficiencies and other illnesses in mice, birds, cats and humans. In these cases, it is believed that the interaction of viral envelope glycoprotein with a host protein causes the abnormalities associated with these viruses. For instance, it has been shown that the envelope glycoprotein gp55 of Friend spleen focus forming virus interacts with the erythropoietin receptor (63) and stimulates erythroblast proliferation in the absence of the natural ligand erythropoietin (64).

Several retroviruses including HIV cause cytopathic effects (CPE) that involve their *env* glycoproteins. Such CPE may involve both formation of syncytia and killing of single cells. The natural function of CD4 on T-lymphocytes is to associate with MHC II molecules on antigen-presenting cells (65) and to mediate immune responses by interacting with T-cell receptors. Binding of gp120 to CD4 down-regulates CD4 and CD4-associated p56lck and thereby suppresses T cell functions (66). A HIV-encoded protein Vpu also causes rapid intracellular degradation of CD4 (67). Both types of CPE, syncytium induction and individual cell killing, have been observed in HIV infection and both types of CPE probably involve the HIV *env* glycoproteins (68, 69, 70)

IV. Murine Leukemia Viruses (MuLVs)

1. Discovery

MuLVs provide a genetically well-defined model to study virus-host interactions. The first strain of MuLV was discovered when Gross successfully transmitted leukemia by inoculating neonatal C3H mice with extracts of Ak thymic leukemia cells (71). Studies of different MuLV isolates, however, have

suggested that they have mainly evolved as endogenously inherited genes rather than as horizontally transmitted infectious agents (53).

2. Classification, Host-range, and Viral Interference

MuLVs have been classified into five host-range/interference groups, i.e. ecotropic, xenotropic, amphotropic, dualtropic (also called polytropic) and 10A1. These class differences are determined by the viral envelope glycoprotein "knobs" that bind to their specific corresponding receptors on the surfaces of susceptible cells. Thus, ecotropic MuLVs can infect mouse and rat cells but not cells from other species. Xenotropic MuLVs can only infect non-murine species while amphotropic, dualtropic and 10A1 have a broad host-range that includes both murine and nonmurine species (72, 73). Interestingly, hamster cells are resistant to all the groups of MuLV except 10A1 (74).

Similar to other retroviruses, entry of MuLV is initiated by binding of viral envelope glycoprotein (gp70) to its cell surface receptor. Cells infected with one group of MuLV become resistant to the superinfection by all viruses from the same host-range group. This phenomenon, viral interference, occurs only within the same group of viruses. For example, cells infected with ecotropic virus become resistant to superinfection by ecotropic viruses but remain susceptible to xenotropic, amphotropic or dualtropic viruses. Interference happens at the level of viral entry since pseudotyped viruses (virus with same genome but different envelope) can overcome the interference. The mechanism of viral interference is not yet understood. It is believed that the functional virus receptor sites are lost from the infected cell by either of two mechanisms. The first model is that the receptor sites are blocked by the viral gp70 that is expressed on the surfaces of infected cells (75, 76, 77). The second model is that gp70 down-regulates cell surface receptor expression. For

example, in the case of avian reticuloendotheliosis virus, the newly-synthesized viral *env* glycoprotein forms a complex with newly synthesized receptors in the endoplasmic reticulum, thus preventing receptor processing to cell surfaces (78). A similar mechanism accounts at least in part for CD4 down-modulation in HIV-infected cells (79, 80, 81, 82).

Studies of chimeras constructed between *env* genes of different host-range classes of MuLVs, have suggested that receptor specificities are determined by amino terminal domains of gp70 (83, 84). In addition, a fragment containing the N-terminal region of Friend MuLV gp70 has been shown to be capable of binding to ecotropic receptors on NIH 3T3 fibroblasts. (85). However, in some other cases such as Mo-MCF gp70, more than one region of gp70 appears to be important for binding to cell surface receptors (86).

3. Pathogenesis

MuLVs, like other retroviruses, have been implicated in diseases. For instance, one strain of Friend MuLV isolate causes progressive erythroleukemia and severe anemia when it is injected into newborn susceptible mice (87). Dualtropic MuLVs (MCF viruses) produce cytopathic foci following infection of mink cells in vitro, and have been implicated in many other murine leukemias and lymphomas (53, 88, 89). The ecotropic MuLV is mainly inherited as an endogenous virus and is generally not pathogenic. However, a strain of ecotropic MuLV isolated from wild mice can induce lower limb paralysis in neonatal susceptible inbred mice (90, 91). An *env* mutant of Moloney ecotropic MuLV causes central nervous system degeneration (92, 93). Other ecotropic MuLV *env* genes appear to cause hemolytic anemia (94).

V. Receptors for Ecotropic MuLVs

1. Genetics

The genetic basis for the specificity of retrovirus receptors was first studied in chickens using classic Mendelian methods (95, 96, 97). Receptor alleles were identified at particular loci that govern susceptibility and resistance. In the mammalian species that have been studied, genetic polymorphism of receptor loci seems to be more limited. Consequently, analysis of interspecies somatic-cell hybrids have been used to map the receptor genes to particular chromosomes. By this means, the receptor genes for ecotropic, amphotropic, and dualtropic MuLVs on murine cells have been assigned to chromosomes 5, 8, and 1, respectively (98, 99, 100, 101, 102, 103, 104)

2. Biochemistry

The ecotropic MuLV envelope glycoprotein gp70 has been purified from virions and shown to bind onto mouse cells but not onto cells of other mammalian species (105). Studies based upon cross-linking (106), gel filtration chromatography (107), affinity chromatography (108) or immunoprecipitation (109), have identified several putative gp70 binding proteins ranging in molecular weights from 10,000 to 190,000. The relationship of these isolated proteins to each other or to the receptor for MuLV have not been elucidated.

3. Molecular Cloning

A putative receptor gene (ecoR) for ecotropic MuLV was molecularly cloned in 1989 (16). The strategy used in cloning this gene is involved transfection of mouse genomic DNA into nonpermissive human cells, selection of the transfected cells that were susceptible to infection by ecotropic recombinant retroviruses containing drug resistance genes, and subsequent identification of the putative receptor clone from a genomic library by hybridization with a highly repetitive mouse-specific DNA sequence. The genomic clone was then used to isolate a corresponding ecoR cDNA. As expected, it mapped to mouse chromosome 5 (110).

The putative ecoR gene encodes a protein of 622 amino acids with 12-14 potential trans-membrane domains. There are two potential N-linked glycosylation sites in the third extracellular loop of the putative receptor. ecoR is highly homologous to Tea, a gene that might be involved in T-cell early activation. Tea was isolated from a T-cell lymphoma (111). The Tea cDNA isolated by MacLeod et. al. was truncated at the 5' end and was not a full length clone.

The human homologue of ecoR has been isolated from a T-cell line by Yoshimoto et. al.(112). The human cDNA is highly homologous to the murine counterpart (87.6% amino acid identity) and encodes a protein of 629 amino acids. The genomic gene, mapped to human chromosome 13q12-q14 (113), was also isolated and consists of 11 exons and 10 introns.

4. Physiological Function

While the gene that encodes a putative receptor for ecotropic MuLV was isolated before I began my thesis work, the normal physiological function of this gene remained unknown. Several lines of evidence suggested that ecoR might encode a transporter molecule. First, the molecular structure of the putative ecotropic receptor resembles a transporter molecule. These structural features include 12-14 putative trans-membrane domains, lack of a leader sequence at the amino terminus, and intracellular amino and carboxyl termini (16). Secondly, it has been known that the ecotropic receptor has a wide tissue distribution which suggests a ubiquitous function of this protein (114). Finally, genetic evidence implied that ecotropic receptors are probably required for survival of fibroblasts in culture (115). This evidence suggested that ecotropic receptors might encode a transporter for a specific substrate in the culture media.

Molecular cloning of the receptor for the ecotropic MuLV opened the door to study virus-host interaction at the molecular level, to identify the physiological function of the receptor, and to elucidate the pathogenesis of retrovirus-related diseases.

Thesis Rationale

This thesis is assembled with a manuscript format and composed of introduction, four manuscripts, discussion and conclusions. Results related to the project that have not been submitted for publication are included in the appendices. The focus of this project was to study and understand the step of viral entry during retrovirus infection through studies of the ecotropic murine retrovirus receptor (ecoR).

Initially, ecoR was expressed by a retrovirus vector in cells of different species such as human, mink, and hamster that naturally do not express ecoR. Expression of ecoR in these cells confers susceptibility to ecotropic viral infection. Cells expressing different amounts of ecoR were found equally susceptible to infection. These results suggest that there is a limiting accessory factor in addition to ecoR that is required for an efficient infection with ecotropic murine retrovirus (see Manuscript #1). ecoR mRNA was synthesized in vitro with SP6 RNA polymerase, and the mRNA was injected into Xenopus laevis oocytes. Expression of ecoR in oocytes was detected by specific gp70 binding. It was found that oocytes expressing ecoR had an enhanced ability to transport the cationic amino acids arginine, lysine, and ornithine. Further characterization of the transport activity of ecoR led to its identification as the widely-expressed Na^+-independent transporter for basic amino acids, generally known as system y+ (see Manuscript #2). Following this discovery, the effect of viral infection on the transport activity of ecoR/y+ was studied. It was found that chronic viral infection only partially down-modulates the cell surface expression of ecoR/y+, although the cells became completely resistant to superinfection by ecotropic viruses. When added as an impermeant ligand, gp70 slightly inhibits uptake but not outflow of arginine through ecoR/y+. This implies that gp70 binds to a

mobile region in ecoR that changes conformation during the amino acid transport cycle (see Manuscript #3). The function of the two N-linked glycosylations in the extracellular loop of ecoR was studied by site-directed mutagenesis. It was found that neither of the glycosylations on ecoR were required for efficient gp70 binding, viral infection, or amino acid transport. Interestingly, viral infection resulted in a partial down-modulation of one of the carbohydrate mutants, but in a complete down-modulation of the other mutant. These results indicate a role for N-linked glycosylation in controlling virus-host interaction. This is a novel function for protein glycosylation (see Manuscript #4).

Manuscript #1

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

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Running Title: Activation of Retroviral Receptors

Abstract

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 were later determined by growth hormone-specific immunofluorescence. For all cells in 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 per cell, were directly proportional to virus and to cell concentrations, and only a small percentage (c.a., 5%) of the infectious virions became adsorbed from the media during the 2 h incubations. Although increasing 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 its 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 (HIV) is the CD4 glycoprotein (36, 46). However, it is uncertain whether infection involves fusion of virion membranes with endosomal or with cell surface membranes (2, 33, 41, 50). Some cells that lack CD4 can be infected by HIV (5, 8, 29). Expressing CD4 in murine cells and some human cells does not confer susceptibility to infection (2, 6, 32), perhaps due to an inhibitor in the resistant cells (2, 54). Alternatively, it has been speculated that HIV 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) were isolated that encode cell surface receptors for mouse ecotropic retroviruses (1, 25) and for gibbon ape leukemia virus (39). Although these receptors appear to be unrelated, they both may be glycoproteins with multiple membrane spanning regions that

could fold to form transport channels (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 ($Ostk^-$) were from American Type Culture Collection (Rockville, MD). Ψ-2 ecotropic packaging cells (34) and PA-12 amphotropic packaging cells (38) were from R.C. Mulligan (Massachusetts Institute of Technology, Cambridge, MA) and A.D. Miller (Fred Hutchinson Cancer Center, Seattle, WA), respectively. CHO cells were grown in Alpha minimum essential medium supplemented with 10% fetal bovine serum. Other cells were maintained in Delbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

Helper-free ecotropic host-range viruses from Ψ-2 cells that encoded the neomycin phosphotransferase gene (MSV-neo) and the human growth hormone (pSFF-hGH) have been described (9, 26, 27). Virus preparations were harvested by placing fresh culture medium on half confluent monolayers of virus-producing cell lines for 16-24 h, removing the medium, and filtering it through a 0.2 um filter. Cells (10^5) in 25 cm^2 flasks were infected by incubating them with 1 ml of virus-containing medium for 2 h at 37^oC in the presence of Polybrene (8 ug/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, MA). The 2.3 kilobase pair (kbp) BamHI-EcoRI 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. Ten ug of pSFF-ecoR was transfected as a calcium phosphate precipitate (15, 27) into 25 cm^2 culture dishes that contained a 1:1 mixture of Ψ-2 and PA-12 cells at a total cell concentration of $2x10^5$ cells per dish. At 72 h after transfection, the cells were split into 12

minicocultures of 100 cells per well in a 96 well (0.32 cm^2) 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 non-murine cells was accomplished by infection with the amphotropic host-range virus released from the PA-12 cells, or alternatively by transfection with pJET or with pSFF-ecoR.

Assays for ecotropic gp70 binding to cell surface ecoR. Cells (10^5) were plated onto coverslips in 9.5 cm^2 dishes one day before analysis. Two ug/ml of gp70 that had been isolated from ecotropic Friend murine leukemia virus (16, 52) was added to the media overlaying 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, CA) as described previously (20). After rinsing the cells three times for 10 min with culture medium, 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 using 8 mM EDTA in 0.9% NaCl, pH 8.0 (13) and then counted with a hemocytometer. 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 the excitation wavelength 495 nm and the emission wavelength 525 nm.

For immunoelectronmicroscopy, $2x10^4$ cells were plated onto 2 cm^2 chamber slides (Nunc Inc., Naperville, IL) for 16-24 h. The gp70 and goat antiserum to gp70 were sequentially incubated with the cells at 37°C as described above for 2 h and 1 h periods. Then, the cultures were incubated for 1

h at 37°C with 1:4 diluted 5 nm gold-conjugated rabbit anti-goat antiserum (Amersham, U.K.). Cells were then washed twice for 5 min 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, post-fixed 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 using the helper-free virus that encodes human growth hormone 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^2 dishes one day before analysis. Cells were fixed with 3.7% formaldehyde in phosphate buffered saline (PBS) (140 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4) for 20 min at room temperature and the coverslips were then incubated in 0.2% triton X-100 in PBS for 10 min. 1:2000 dilutions of rabbit antiserum to human growth hormone (donated by the National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD) and 1:150 dilutions of fluorescein-conjugated goat anti-rabbit immunoglobulin (Tago Immunologicals, Burlingame, CA) were sequentially incubated with the cells for 1 h at 37°C. After washing with Dulbecco's modified Eagle's medium three times and PBS once, the cells were mounted for immunofluorescence microscopy. The fraction of fluorescent cells was determined by analyzing at least 10-15 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 was the fraction of nonfluorescent cells (26).

RESULTS

A retroviral vector for ecoR expression in nonmurine cells. The retroviral expression vector pSFF-ecoR (see Fig. 1) was transfected into 1:1 cocultures of Ψ–2 and PA12 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. Fig. 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, 9) compared with 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 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 titer 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 per ml for a 2 h adsorption time at 37°C.

FIG. 1. Construction of pSFF-ecoR. A 2.3 kilobase pair BamH1-EcoRI fragment containing the ecotropic receptor cDNA coding region was inserted into the BamH1 and EcoRI sites of the retroviral expression vector pSFF to form pSFF-ecoR. The stippled bars represent pSP64 vector sequences.

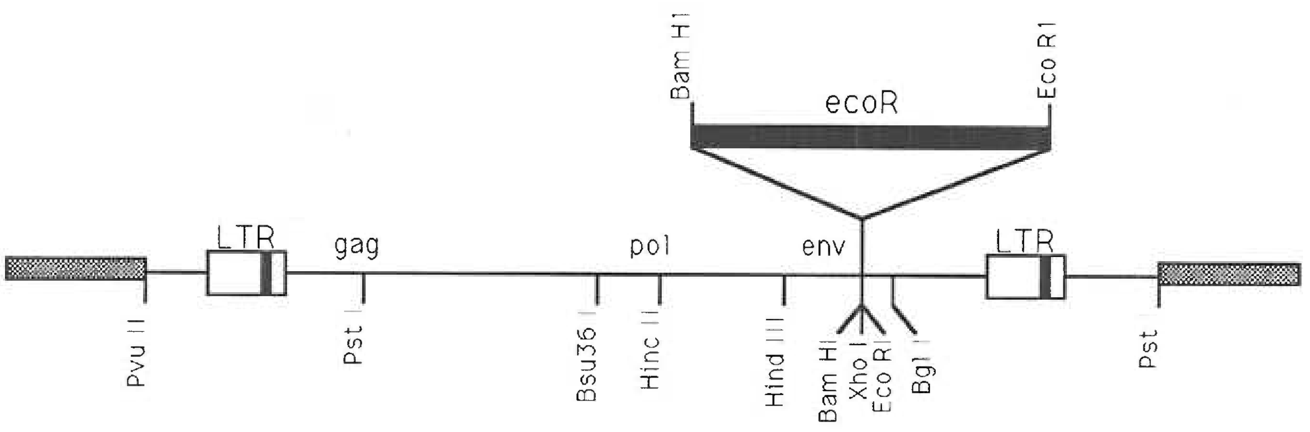
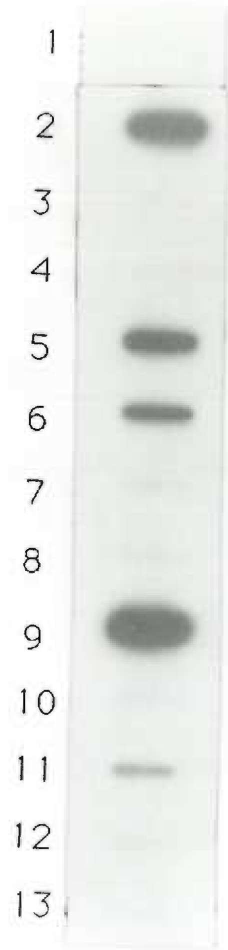

Bam H1
ecoR
Eco R1
LTR
gag
pol
env
LTR
Pvu II
Pst I
Bsu36 I
Hinc II
Hind III
Bam HI
Xho I
Eco RI
Bgl I
Pst I
1 Kb

FIG. 2. RNA slot blotting of Ψ-2/PA12 coculture expressing ecotropic receptor cDNA. Twenty ug RNA extracted from individual cocultures was blotted to nitrocellulose and was hybridized with the [^{32}P]-labeled nick translated BamH1/EcoRI ecotropic receptor cDNA fragment. Slot 1: RNA extracted from control coculture. Slot 2-13: RNA from 12 cocultures transfected with pSFF-ecoR.


1
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13

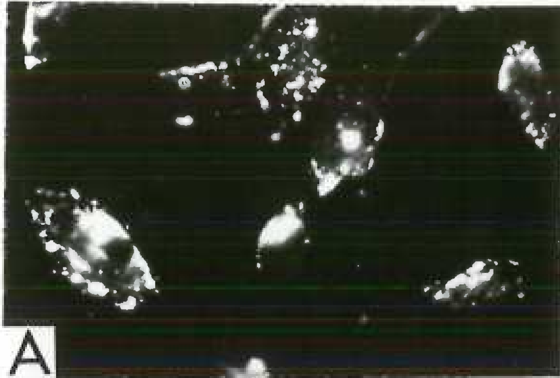
FIG. 3. G418 resistant colonies from mouse NIH/3T3, mink CCL64 and human Ostk$^-$ cells infected with pMSV-neo virus. Cells (NIH/3T3, CCL64 and Ostk$^-$) were plated at 10^5 per 100 mm dish. Where indicated, cultures were exposed to amphotropic virions encoding ecotropic receptors (ampho-ecoR) one day later and/or to pMSV-neo virus at 3 days after plating. G418 resistant colonies appeared on the dishes after selection for 8-12 days. G418 concentrations used for selection were 500 ug/ml, 1000 ug/ml and 350 ug/ml for NIH/3T3, CCL64, and Ostk$^-$ cells, respectively. G418 resistant colonies were stained with methylene blue.

Cell Type	NIH 3T3	CCL64	CCL64	CCL64	$Ostk^-$	$Ostk^-$	$Ostk^-$
Ampho-ecoR	–	–	+	+	–	+	+
pMSV-Neo	+	+	–	+	+	–	+
G418 Resistant Colonies	+	–	–	+	–	–	+

The nonmurine cells that grew in the presence of G418 (see 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$^-$ 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 Ψ-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 ecotropic receptor could bind gp70 that had been isolated from an ecotropic retrovirus. Previous workers reported that such [^{125}I]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 (frame C) whereas control mink cells are completely unlabeled (frame B). As with murine cells (frame A), the ecoR on nonmurine

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,D) and phase contrast (a,b,c,d). (A,a) Murine NIH/3T3 cells. (B,b) Mink CCL64 cells. (C,c) CEN cells. These are mink CCL64 cells with ecoR. (D,d) CEN cells incubated with all reagents except gp70.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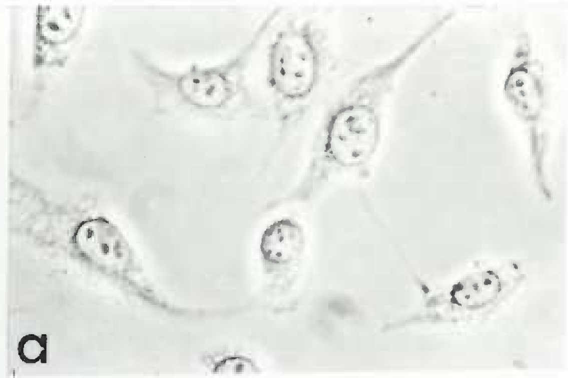
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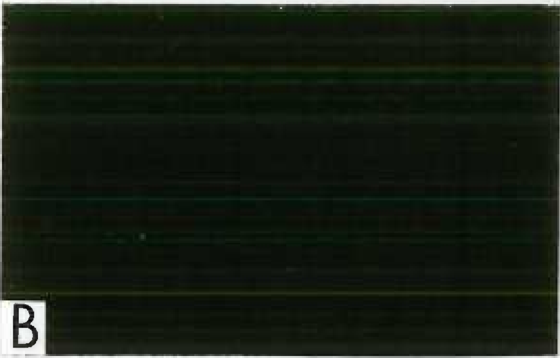
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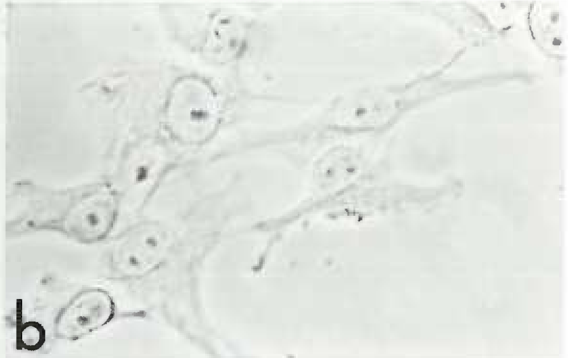
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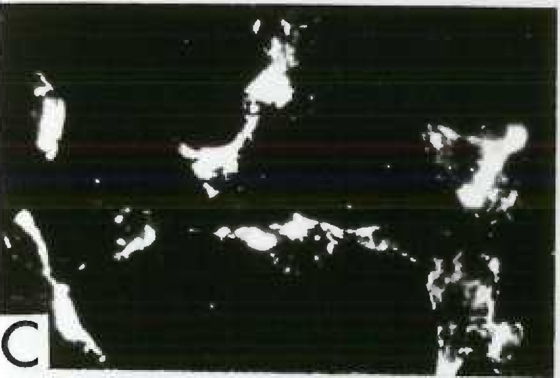
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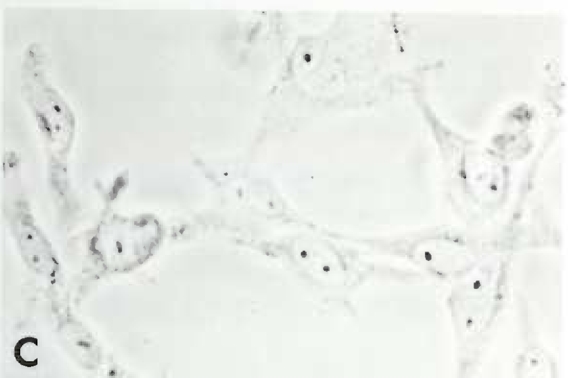
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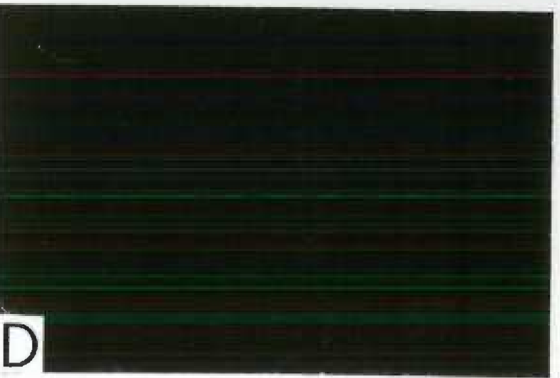
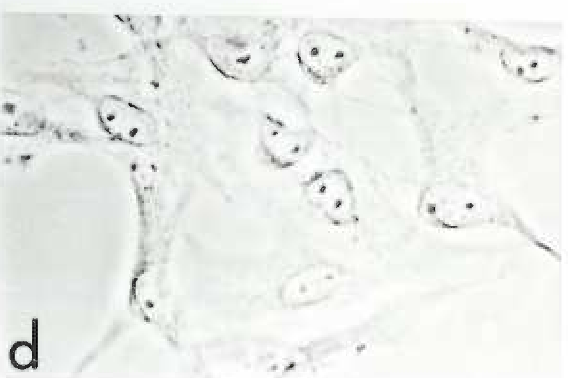

D

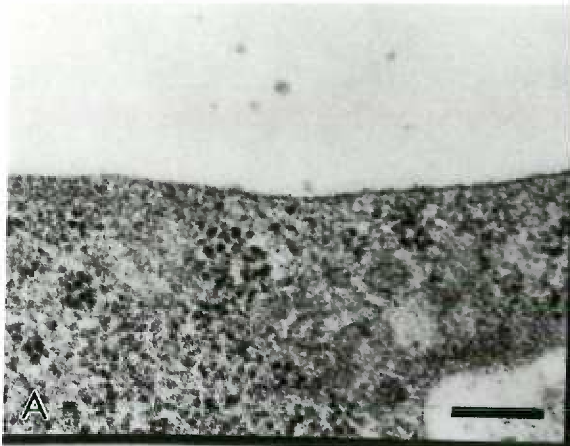

d

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 $0^{o}C$ 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.

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 immunoelectronmicroscopic analysis of gp70-ecoR complexes using gold-labeled secondary antiserum. The gp70-ecoR in the plasma membrane are clustered into patches that appear thicker than adjacent unlabeled regions (frames B, E, and G). Frame F shows a coated pit that contains gp70-ecoR complexes, but these were extremely rare. Even after removing unbound gold-labeled antiserum and culturing the viable cells for an additional two hours at $37^{o}C$, 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 (frame D) or of cells incubated with all reagents except gp70 (frames 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 into 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

FIG. 5. Immunoelectronmicroscopic analysis of gp70-ecotropic receptor complexes. Cells were incubated at 37°C with or without gp70 for 2 hr, with goat antibody to gp70 for 1 hr, and with 5 nm gold labeled rabbit anti-goat antibody for 1 hr. After washing and fixing (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 incubated with gp70. These cells lack ecoR and are unlabeled. E, F, G. CEN cells incubated with gp70. These cells have ecoR in surface patches that are labeled. All scale bars are 200 nm.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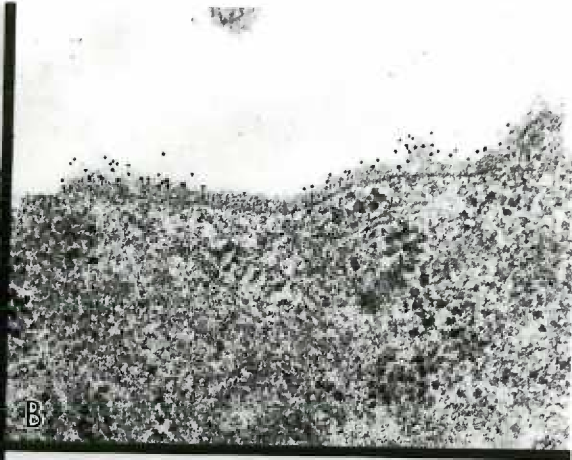
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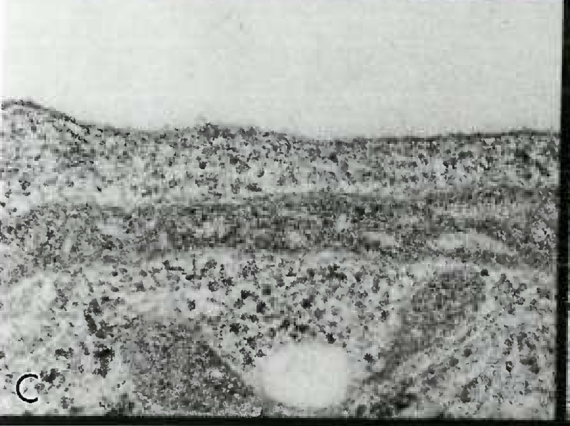
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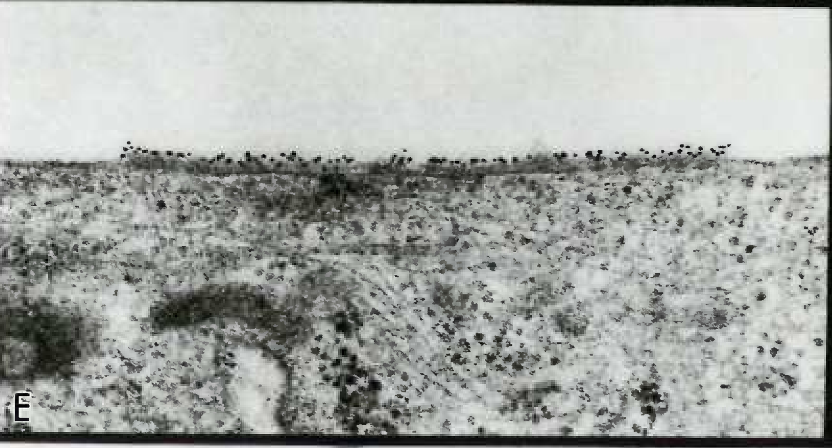
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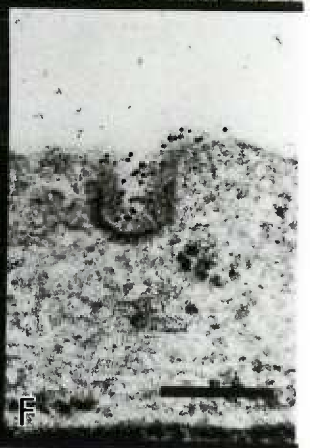

F

G

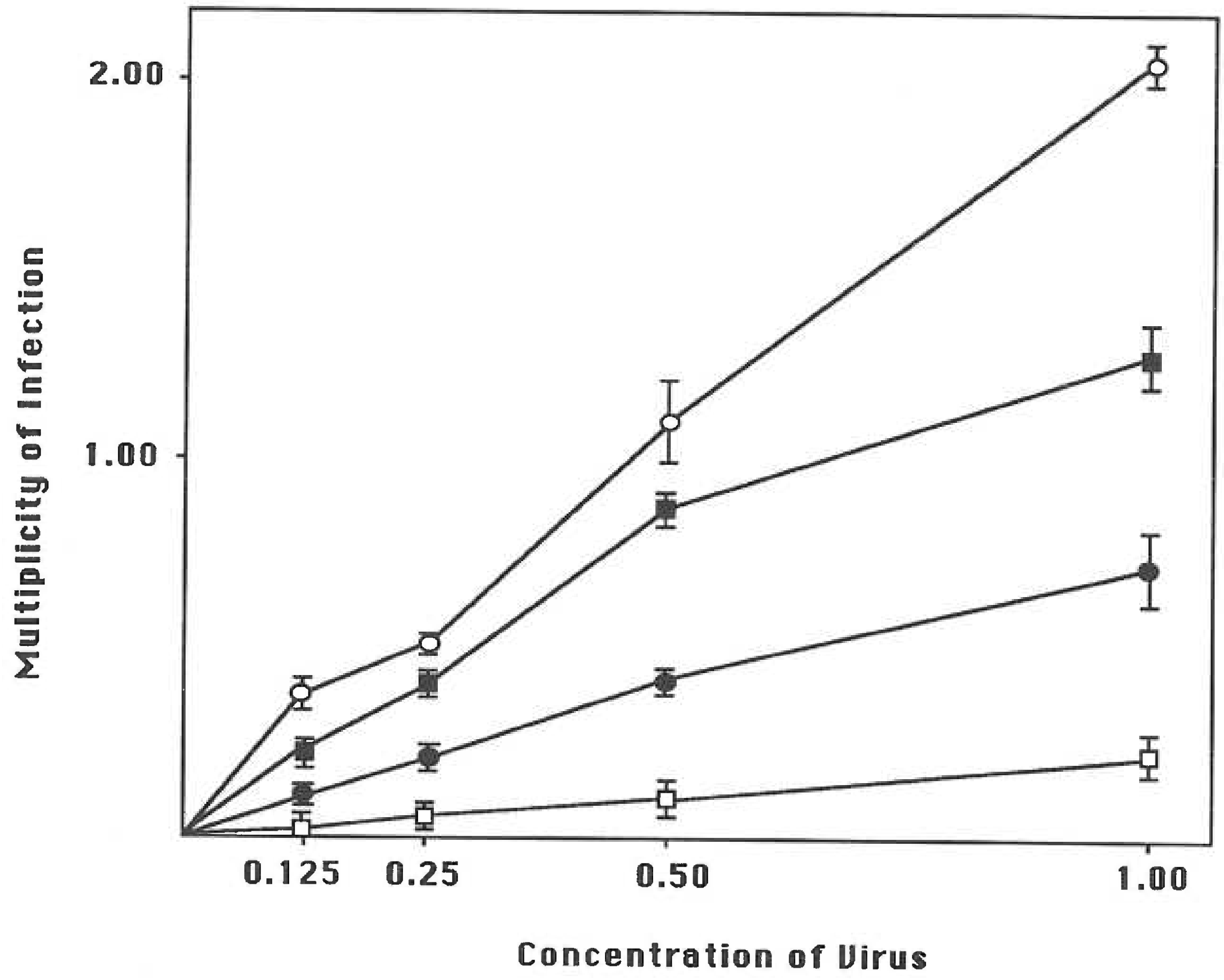
then conjugated secondary antiserum), rapidly and irreversibly endocytosed gp70-ecoR complexes would not be labeled. Therefore, these results do not exclude the possibility that a fraction of the gp70-ecoR complexes might be rapidly and irreversibly endocytosed (see Discussion).

Basic characteristics of infections by ecotropic retroviruses. To quantitatively analyze susceptibility of different cell lines to infection, we incubated cell cultures under standard conditions for 2 hr with several dilutions of an ecotropic helper-free retrovirus that encodes human growth hormone (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 the cells from the medium during the 2 h incubation periods used for these assays. For example, as reported in Table 1, 1 ml of virus-containing medium

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 2×10^5 per ml. Cells which expressed hGH were detected by growth hormone-specific immunofluorescence. Multiplicities of infections were calculated as described in the Materials and Methods and are plotted against viral concentrations. The multiplicities are shown as means ± standard error of means (SEM) from three sets of cell countings. Symbols: o, murine NIH/3T3 cells. ■, hamster CER18-Cll cells. •, mink CEN cells. □, human OstEN cells.


2.00
1.00
Multiplicity of Infection
0.125
0.25
0.50
1.00
Concentration of Virus

was first incubated at 37°C for 2 h either in an empty flask (blank) or in flasks 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 period 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 3-4% 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 using other ecotropic retroviruses. For example, similar results were obtained when a preparation of ecotropic murine leukemia virus was assayed sequentially on two S+L- cell cultures (D. Kabat, unpublished results).

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^{2} flask (see 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 due to their incubation with cells. 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 to the cell concentrations and that only a small percentage of the infectious virus becomes adsorbed onto cells during 2 h

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^2 flasks for infection with a single concentration of helper-free ecotropic hGH virus (see Fig. 6). Each flask contained a 1 ml aliquot of the virus-containing medium. The virus titer measured on NIH/3T3 cells was $2x10^5$ per ml. The multiplicities of infection were later measured. 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 were therefore proportional to the number of cells in the cultures. The total infections are shown as means $\pm$ standard error of means (SEM) from three sets of cell countings. Symbols: o, murine NIH/3T3 cells. ■, hamster CER18-Cl1 cells (these have ecoR).

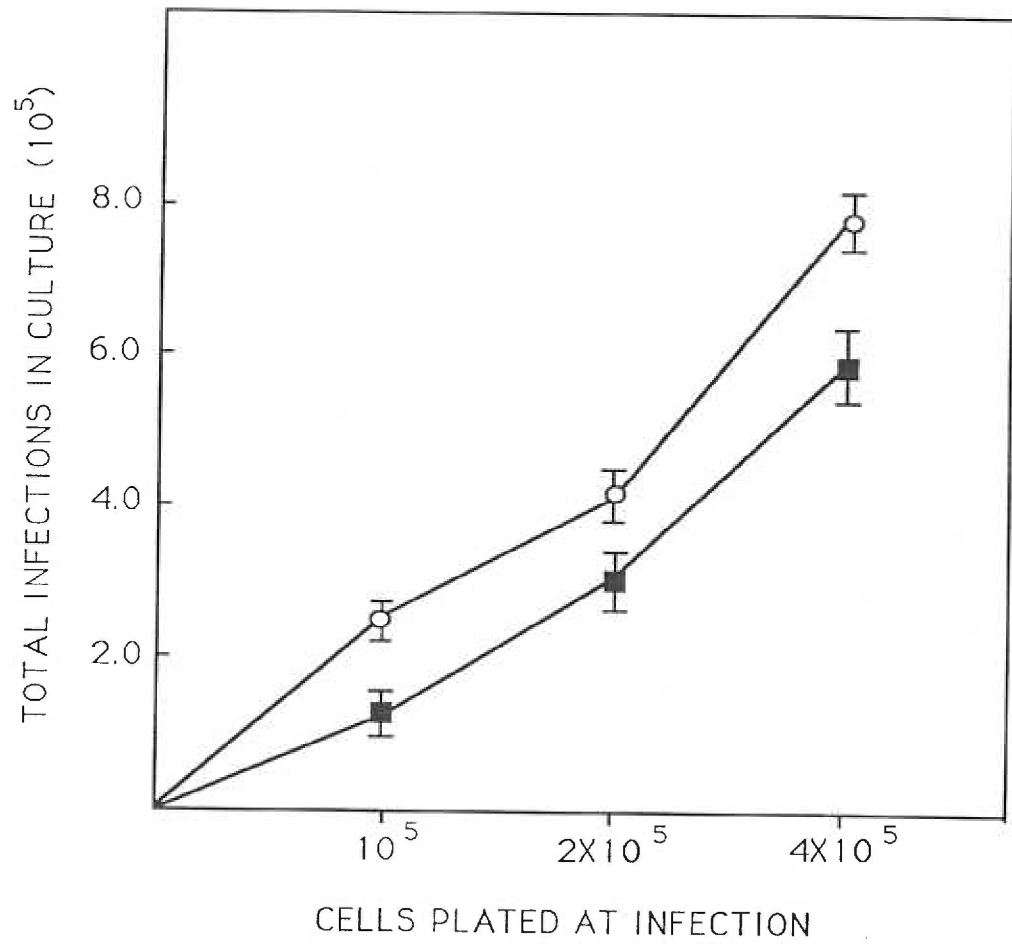

TOTAL INFECTIONS IN CULTURE (10^5)
8.0
6.0
4.0
2.0
10^5
$2X10^5$
$4X10^5$
CELLS PLATED AT INFECTION

TABLE 1. Adsorption of virions onto mouse and hamster cells that express ecotropic receptors[a]

Cell types[a]	multiplicity of infections[b]	specific virion adsorption[b] (%)
Blank/NIH 3T3	0.89	0
NIH 3T3/NIH 3T3	0.86	3.4
CER18-C11/NIH 3T3	0.81	9.0
CER-D-C110/NIH 3T3	0.84	5.6

[a]. A large volume of culture medium (Dulbecco's modified Eagle's medium with 10% fetal bovine serum) was prepared that contained a diluted sample of helper-free ecotropic hGH virus. The titer on NIH/3T3 cells was $2x10^5$ per ml. 1 ml aliquots of this medium were consecutively placed for 2 h periods at 37^oC into 25 cm^2 flasks that contained 10^5 cells per flask of the cell types shown. Each sample of medium was incubated first with the cell culture shown on the left and then with the cell culture shown on the right. For example, the medium in the first line was incubated first in an empty (blank) flask and then in a flask that contained NIH/3T3 cells. The medium used in the second line was incubated sequentially with two different flasks that contained NIH/3T3 cells. The medium in the third line was incubated first with CER-18-C11 cells (a derivative of mink CCL64 cells that contain ecoR) and then with NIH/3T3 cells. The medium in the fourth line was incubated first with CER-D-C110 cells (a derivative of mink CCL64 cells that express more ecoR) and then with NIH/3T3 cells. Each cell pair was analyzed in duplicate.

[b]. The NIH/3T3 cells that had been infected with the 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 first incubated in an empty (blank) flask. The third column, derived from data in the second column, shows the percent of infectious virus adsorbed onto cells 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.

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 differences 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. 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 approximately 10-fold in their capacities to bind gp70. 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 7-8 times, 3 times, and 1.5 times more susceptible to infections than the human, mink (CEN) and hamster cells, respectively. Strikingly, however, the susceptibility to infection of the different human cell clones that express ecoR and of the different 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.

FIG. 8. Immunofluorescent microscopy of gp70 binding to human and to Chinese hamster ovary cells that contain different quantities of ecoR. Each microscopic field is shown in fluorescence (A,B,C,D,E,F) and phase contrast (a,b,c,d,e,f). Cells were incubated with gp70 and then with antibody to gp70 and then with fluorescein-conjugated secondary antibody (see Materials and Methods). (A,a) Human Ostk$^-$ cells. (B,b) OstER-C17. These derivatives of Ostk$^-$ contain low levels of ecoR. (C,c) OstEN. These derivatives of Ostk$^-$ contain high levels of ecoR. (D,d) Hamster CHO cells. (E,e) CER18-C11. These derivatives of CHO contain low levels of ecoR. (F,f) CER-D-C19. These derivatives of CHO contain high levels of ecoR.

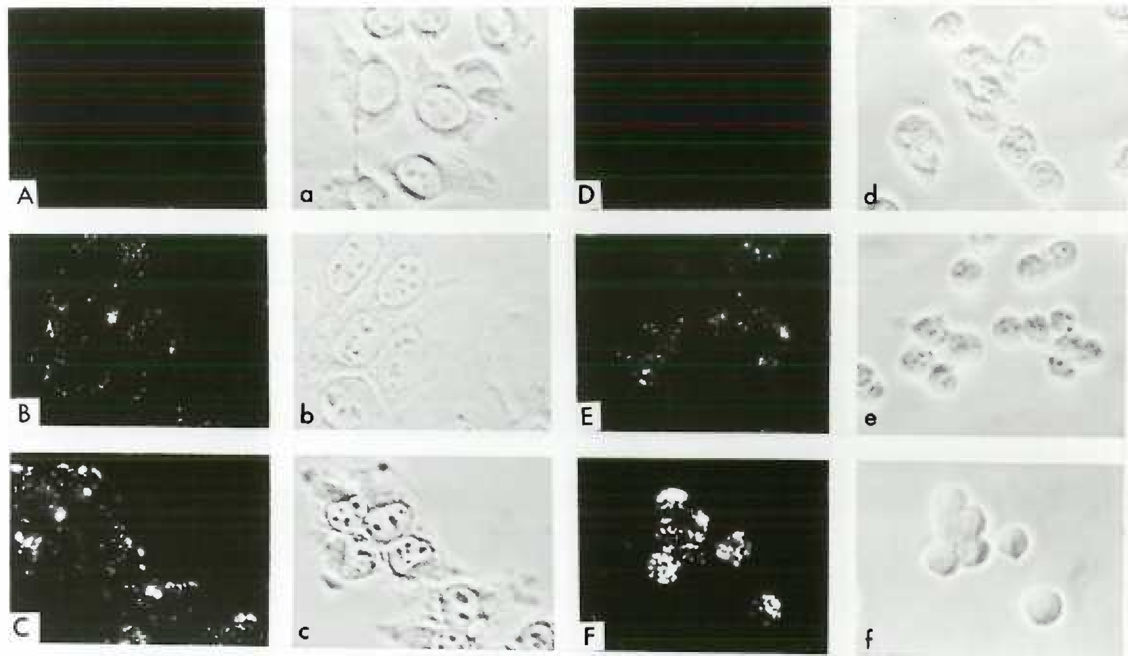

A
a
D
d
B
b
E
e
C
c
F
f

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

Cell types[a]	gp70 specific fluorescence[b]	Multiplicity of infection[c]
NIH/3T3	61.8 ± 13.1	3.07 ± 0.20
Ostk⁻	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
CER18-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[d]	569.5 ± 44.8	1.06 ± 0.13

[a]. NIH/3T3, Ostk⁻, CHO and CCL64 cell lines are described in Materials and Methods. The pJET vector and 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⁻ cells with helper-free amphotropic virus encoding ecoR. OstER-C17 cells were derived by transfection of Ostk⁻ cells with pJET. CER18-C11 cells were derived by transfection of CHO cells with pJET. CER-D-C19 and CER-D-C110 cells were derived from CER18-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.

[b]. Fluorescence was measured in a fluorospectrophotometer. Cells

were incubated either with or without gp70, and then with antibody to gp70 and with fluorescein conjugated secondary anti-immunoglobulin antibody sequentially as described in Materials and Methods. The gp70 binding specific fluorescence was obtained by measuring the fluorescence value of 10^5 cells incubated with gp70 and subtracting the fluorescence value of 10^5 cells that had been incubated without gp70. Values represent means $\pm$ standard error of means (SEM) from four readings.

[c]. Multiplicity of infection was determined by infecting cells with ecotropic hGH virus as described in Materials and Methods, and by later measuring the fraction of infected cells by hGH-specific immunofluorescence microscopy as described by Materials and Methods. Values represent mean $\pm$ SEM from three countings. The virus preparation used for infection had a titer on NIH/3T3 cells of 3×10^5 per ml.

[d]. 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.

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 importantly, the recombinant ecoR binds ecotropic gp70 specifically and it mediates ecotropic virus infections of nonmurine cells. Although human Ostk$^-$ cells (see Fig. 6) or human EJ bladder carcinoma cells (1) that express ecoR are only infected 10-15% as efficiently as murine fibroblasts, this cannot be ascribed to a deficiency of the recombinant ecoR because hamster cells with this ecoR are infected almost as efficiently as murine cells (see Fig. 6 and Table 2).

In the conditions of these experiments, infections were limited by viral adsorption onto cell surface ecoR. At the low concentrations of ecotropic retroviruses that were used to infect proliferating cultured cells in 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 (see 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 means for extracellular virus concentrations to influence infection is at the step of adsorption, 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).

Based on 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 therefore 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 onto non-functional receptors and this would result in a proportional increase in the multiplicity of infection (see Fig. 6). In contrast, adding ecoR to cells in an amount above the threshold quantity would only result in more non-functional 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 non-functional 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-Cll 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 x 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 non-functional ecoR must be essentially unsalvageable, perhaps because the ecoR in these complexes remains permanently inactive or perhaps because the non-functional ecoR rapidly inactivates attached virus. If the virus that bound to non-functional 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 (see Table 2). Third, the fact that each type of cell seems to contain a set amount of potentially functional ecoR (see 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 is made to saturate this accessory factor, additional ecoR constitutes a non-functioning 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 non-functional in infections turns over only slowly on cell surfaces and remains accessible for many hours to primary and to conjugated secondary antisera (see Materials and Methods and Figs. 4, 5 and 8). If virus or gp70 that binds to the functional class of ecoR were rapidly and irreversibly endocytosed it would escape detection by our labeling protocols. Therefore, we emphasize that our labeling protocols and metabolic studies have only provided information 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 (see 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 60-70% 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, we conclude that the susceptibilities of cells from different species appear to correspond with their evolutionary distance 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 at a subsequent step of infection.

Based on the above considerations and definitions, we therefore propose that functional ecotropic receptors 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 this 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 related accessory factor(s) are 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 HIV (2, 6, 32). If the accessory proteins of mouse cells interact only

non-functionally 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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Manuscript #2

Cell Surface Receptor for Ecotropic Murine Retroviruses Is a Basic Amino Acid Transporter

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Summary

Complementary DNA was recently isolated that encodes a cell surface receptor (ecoR) for ecotropic host-range murine retroviruses.[1] The encoded ecoR protein contains 622 amino acids and 14 hydrophobic, potential membrane-spanning sequences.[1] This receptor occurs on many or all murine cells[2] and there is genetic evidence that it may be essential for viability of cultured fibroblasts.[3] These properties suggested that ecoR might normally function as a transporter for an essential metabolite. We expressed ecoR in *Xenopus laevis* oocytes by injecting RNA transcribed from the cloned cDNA. These oocytes specifically bound the envelope glycoprotein (gp70) purified from an ecotropic murine leukemia virus (MuLV-E), and an inward current was recorded electrophysiologically when synthetic amino acid components of a culture medium were applied. The inward current resulted from a stereoselective, saturable uptake of the basic amino acids lysine, arginine and ornithine; it was sodium-independent and not substantially altered by gp70. Cysteine and homoserine were also taken up but their transport required sodium. The properties of ecoR correspond to the previously characterized y^+ amino acid transporter.[4-6] The results illustrate the subversion of a ubiquitous cell membrane protein, in this case a basic amino acid transporter, for use as a retroviral receptor.

EcoR RNA was transcribed from the cDNA and injected into *Xenopus* oocytes.[7] Expression of ecoR was detected 2 - 6 days after injection by incubating the oocytes with gp70 that had been purified from MuLV-E, followed by incubation with goat antiserum to gp70 and then with

[^{125}I]protein A.[3,8] Oocytes injected with ecoR mRNA bound substantially more gp70 than uninjected oocytes (Fig. 1a). In addition, the oocytes that had adsorbed gp70 and antibody were specifically killed by incubation with rabbit complement (Fig. 1b).[3,8]

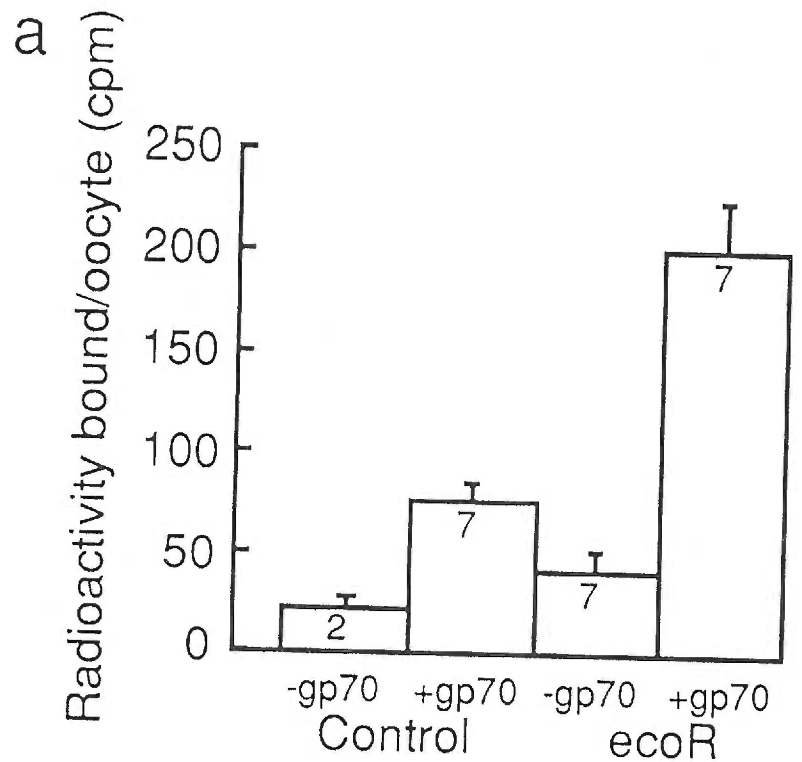
Inward currents of 50 - 100 nA were detected in injected oocytes when an amino acid mixture (MEM, GIBCO) was applied. This was found to result from a saturable, uptake of basic amino acids. Fig. 2 shows measurements made with two-electrode voltage clamp at -60 mV. Lysine induced a concentration-dependent inward current that reached a maximum at about 1 mM. Arginine, ornithine and histidine gave similar currents, but several neutral or negatively charged amino acids had no effect (Table 1). The maximal current and the K_m were estimated by fitting from hyperbolic functions (Fig. 2c) and the results are summarized in Table 1. The current became larger with hyperpolarization and smaller with depolarization, but was still inward at +20 mV. Uninjected oocytes showed little (<5 nA) or no response to lysine, arginine, ornithine, histidine, homoserine or cysteine (all at 10 mM).

System y^+ is a sodium-independent transporter for basic amino acids that has been extensively characterized.[4-6,9-11] In oocytes expressing ecoR, the current induced by lysine was also sodium-independent (Fig. 2b). One characteristic of system y^+ is that the transport of basic amino acids is inhibited by some neutral amino acids, such as homoserine, but only in the presence of sodium.[6,9-11] Homoserine itself induced an inward current in injected oocytes, but this was completely dependent on the presence of extracellular sodium (Fig. 2b). Thus, ecoR transports basic amino acids in a sodium-independent manner and some neutral amino acids by a sodium-dependent process (Table 1).

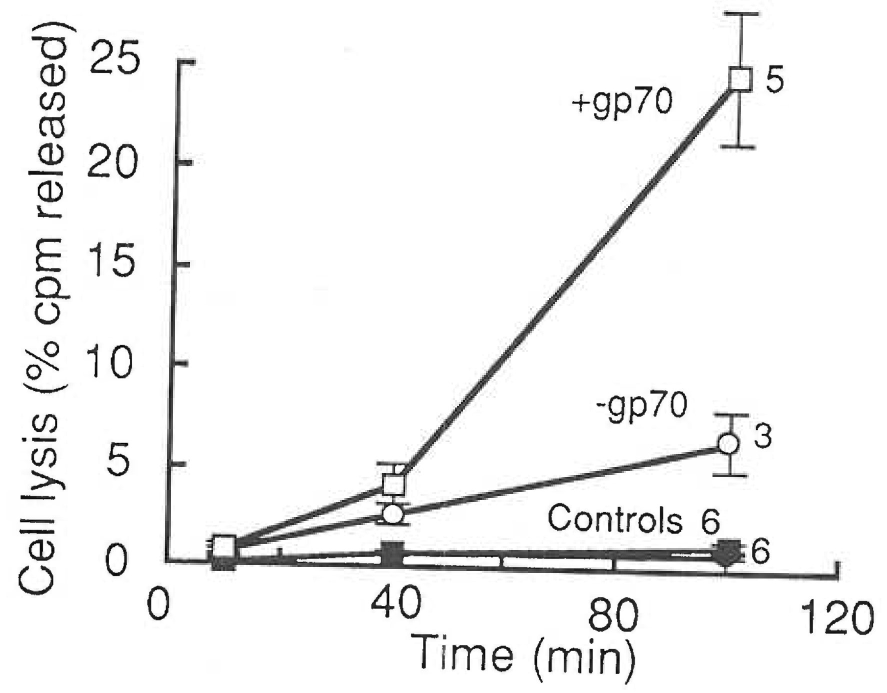
Fig. 1. Binding of gp70 and complement-induced cell lysis in *Xenopus* oocytes expressing ecoR. **a**, binding of [^{125}I]protein A to oocytes previously injected with ecoR RNA or uninjected controls. Oocytes were incubated either with (+gp70) or without (-gp70) gp70 before addition of anti-gp70 antiserum followed by [^{125}]protein A. Bars are s.e. mean. **b**, complement-induced lysis of oocytes previously injected with ecoR RNA (open symbols) compared with uninjected controls (closed symbols). Oocytes in each group were preloaded for 2 h with Tran^{35}S-label (ICN) followed by 2 h incubation with (squares) or without (circles) gp70. All oocytes were then incubated with antiserum to gp70 in the presence of rabbit complement. The total radioactivity (released by complement plus that remaining) was not different for the four groups.

METHODS: The ecoR cDNA was kindly provided by J. Cunningham. A 2.3 kb BamH1-EcoR1 fragment was removed from pJET and cloned into pGEM3 vector to form pGEM3-ecoR; this was linearized with EcoR1 for in vitro transcription. Transcription was carried out as described in ref. 7. Oocytes were injected with 5 - 10 ng ecoR RNA and maintained for 2 - 6 days as described.[7] For gp70 binding, oocytes were incubated for 2 h with purified gp70 (2 ug/ml), for 1 h with 1:200 goat anti-gp70 antiserum, and for 1 h with 5 uCi of [^{125}I]protein A in 1 ml ND-96 solution.[7] Bound radioactivity was counted for individual oocytes after three 10 min washes with 2 ml ND-96. The cytotoxicity assay was carried out by labelling three to six oocytes for 2 h with Tran-^{35}S (10 uCi/ml; ICN Biomedicals) followed by 2 h incubation in nonradioactive in ND-96, three washes in ND-96, 2 h incubation with or without gp70 (2 ug/ml) and three further washes with ND-96. Goat anti-gp70 antiserum (1:200) and rabbit complement (1:10, GIBCO) were then added Supernatant (20 ul) was taken for scintillation counting at 10, 40 and 100 min;

total radioactivity was then measured from oocytes lysed by forcing them through a small pipet tip in 0.1% sodium dodecyl sulphate.



a
Radioactivity bound/oocyte (cpm)
250
200
150
100
50
0
2
7
7
7
-gp70
+gp70
-gp70
+gp70
Control
ecoR

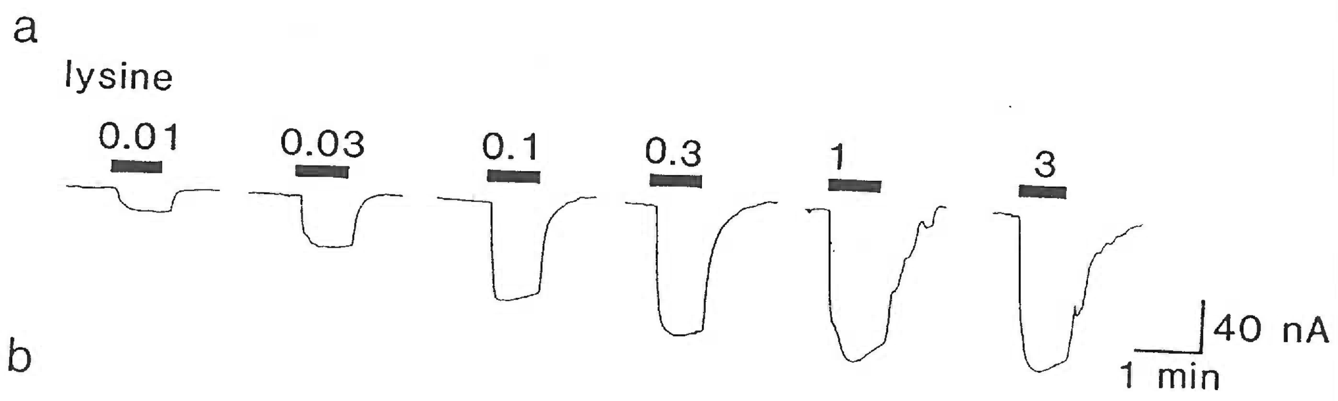


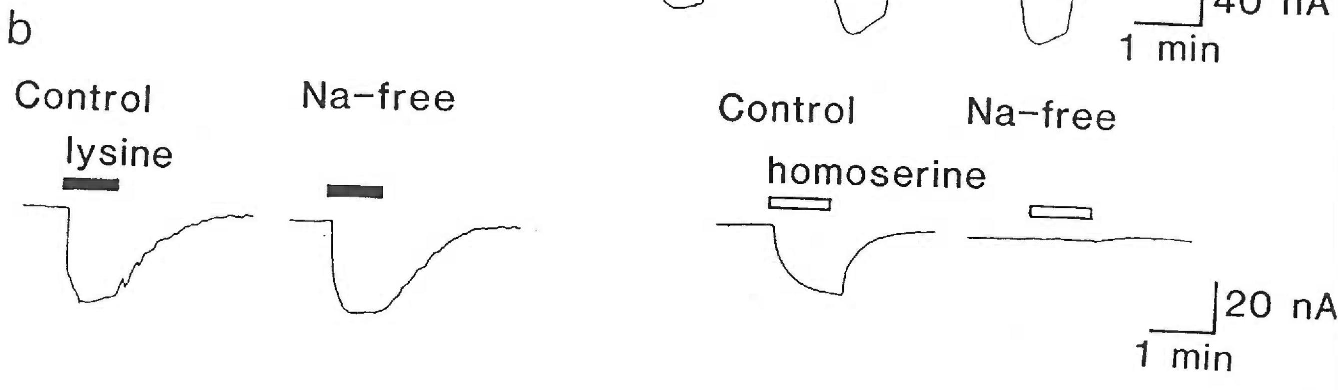
b
Cell lysis (% cpm released)
25
20
15
10
5
0
+gp70
5
-gp70
3
Controls 6
6
0
40
80
120
Time (min)

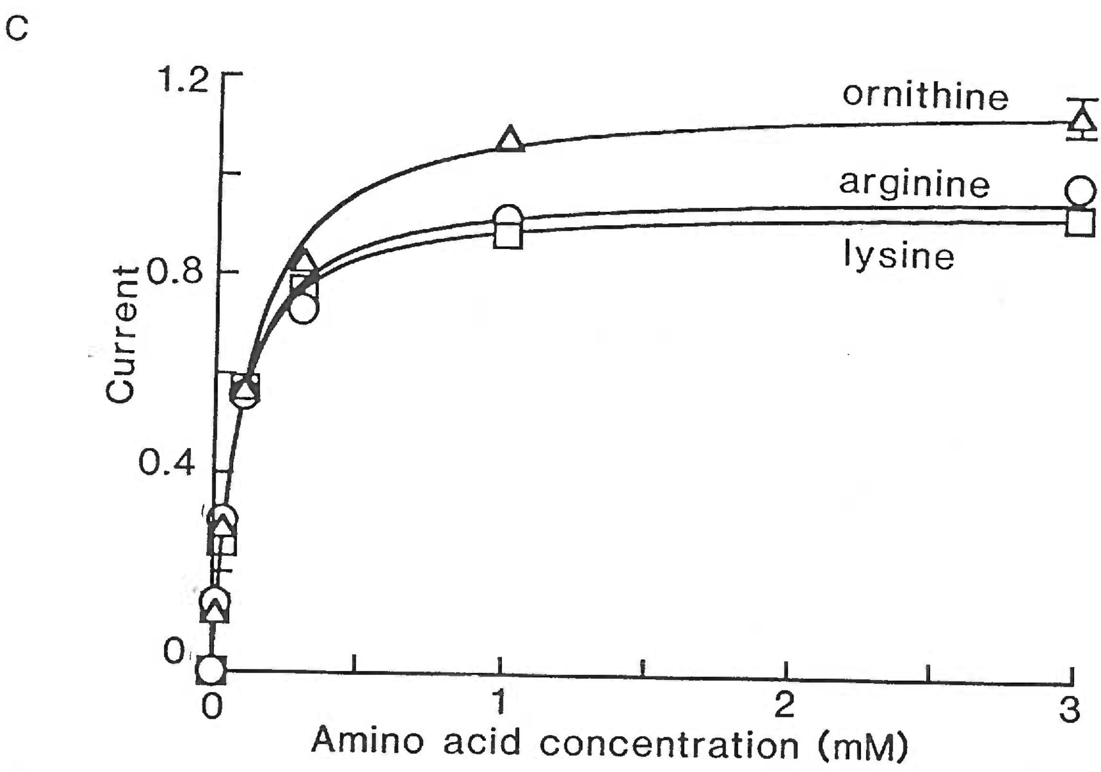
Amino acid (mM)	K_m (mM)	I_{max}
Arginine (0.01 - 10)	0.077 ± 0.002	0.98 ± 0.02
Lysine (0.01 - 3)	0.073 ± 0.008	0.95 ± 0.02
Ornithine (0.01 - 3)	0.105 ± 0.002	1.17 ± 0.03
D-arginine (0.1 - 10)	1.82 ± 0.15	1.75 ± 0.04
D-lysine (1 - 30)	21.1 ± 4.3	0.99 ± 0.15
cysteine (1 - 30)[1]	24.7 ± 2.1	1.56 ± 0.07
histidine (0.01 -1 0)[2]	1.83 ± 0.07	2.02 ± 0.06
homoserine (10)[1,3]		0.67 ± 0.04

Table 1. K_m and I_{max} determined from current measurements in the way illustrated in Fig. 2c. Amino acids are L-isomers except where stated. [1]current was absent in sodium-free solution. [2]current induced by histidine (10 mM) was reduced by 59 ± 4 % (n = 3) in sodium-free solution. [3]only one concentration applied; current is fraction of that caused by 10 mM arginine in same oocytes. Glutamine, glutamate, asparagine, aspartate, glycine, proline, and serine were each applied at 1 mM; they neither evoked a current nor blocked the action of lysine.

Fig. 2. **a**, Inward currents evoked by lysine in *Xenopus* oocyte previously injected with ecoR RNA. Lysine concentrations are indicated above each record (mM); bars indicated period of application. Holding potential was -60 mV. **b**, Current induced by lysine (1 mM) is sodium-independent. A similar result was obtained for arginine. Homoserine (10 mM; application indicated by open bars) induces a sodium-dependent inward current. Currents shown in **b** are from same oocyte; holding potential -60 mV. Sodium-free solution was substituted with equimolar Tris. **c**, Current induced by different concentrations of arginine, lysine and ornithine. Peak currents evoked by a given concentration (I) were measured. The currents were normalized to the current evoked by 10 mM arginine in the same oocyte, which ranged from 70 - 120 nA. Points are mean $\pm$ s.e. mean. Lines shown are fitted by least-squares to a function of the form $I = (I_{max} \text{ [amino acid]})/(K_m + \text{[amino acid]})$. METHODS: Two-electrode voltage-clamp recordings were made from *Xenopus* oocytes as previously described.[7] All experiments were at room temperature, and currents illustrated are in cells clamped at -60 mV. Amino acids were applied by changing the perfusing solution to one that contained the amino acid.


a
lysine
0.01
0.03
0.1
0.3
1
3
40 nA
1 min


b
Control
Na-free
lysine
Control
Na-free
homoserine
20 nA
1 min


c
1.2
0.8
0.4
0
Current
ornithine
arginine
lysine
0
1
2
3
Amino acid concentration (mM)

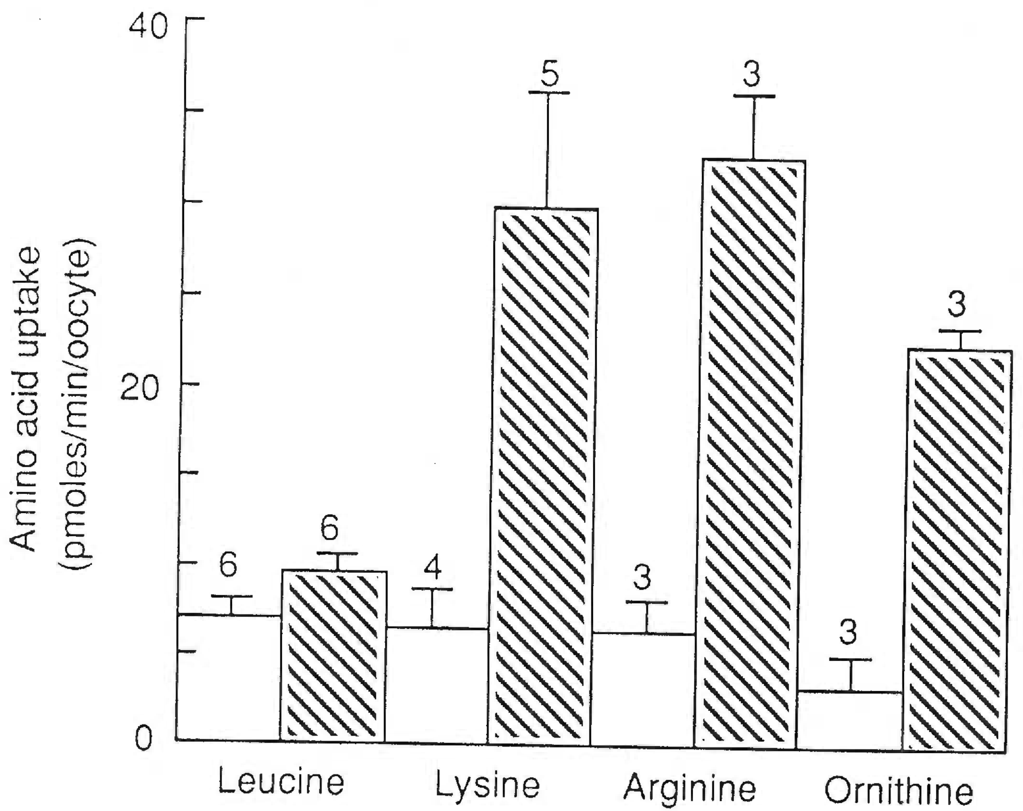
In agreement with the electrical measurements and with the known properties of system y^+ transporter, individual oocytes expressing ecoR rapidly accumulated [^{3}H]lysine, [^{3}H]arginine and [^{3}H]ornithine when these amino acids were added at 200 uM (Fig. 3). The uptake was linear within the first 3 min, and was approximately 25 - 30 pmoles/min. The uptake of [^{3}H]leucine was no greater in oocytes injected with ecoR than in control oocytes, and was comparable to the background uptake of the other amino acids in uninjected oocytes (Fig. 3). There is good agreement between the uptake measured radiochemically and that measured electrophysiologically (30 pmoles/min is corresponds to 48 nA for univalent ions) (Figs. 2 & 3).

Addition of gp70 (2 ug/ml; 4 h) or intact Moloney MuLV (20 ug/ml; 4 h) at 25 ^oC did not alter amino acid transport by ecoR. Thus, L-arginine (10 mM) evoked currents of 54.4 $\pm$ 7.8 nA in control cells, 58.0 $\pm$ 8.5 nA in gp70-treated cells and 57.2 $\pm$ 5.9 nA in virus-treated cells (five oocytes in each case from the same batch). This suggests that virus binding to ecoR might not perturb transport. However, we emphasize that this conclusion is preliminary because there are several time-and temperature-dependent stages in virus adsorption onto cells.[2,3,8,12] Further studies will also be needed to determine whether substrates for uptake, or possibly inhibitors of uptake, interfere with gp70 adsorption and/or viral infection.

Our results identify this retrovirus receptor as the sodium-independent basic amino acid uptake system generally known as y^+.[4-6,9-11] These include the agreement between the K_ms determined in the present experiments with those reported for system y^+ in several other cells, the selectivity of L-amino acids over D-amino acids, and the sodium ion dependence of homoserine transport (Table 1). Previous findings are consistent with this conclusion. Thus, both system y^+[6] and ecoR[2,23] have a ubiquitous tissue distribution

Fig. 3. Uptake of tritiated amino acids by oocytes expressing ecoR. Uptake of leucine, lysine, arginine and ornithine is shown for groups of oocytes that had been injected with ecoR RNA (filled) or uninjected controls (open). Vertical bars are s.e. mean.

METHODS: Individual oocytes were incubated for 3 min in ND-96 (1 ml) that contained 200 uM of [^{3}H] amino acid. Individual oocytes were then lysed and radioactivity was counted by liquid scintillation. L-[4,5-^{3}H]leucine and L-[4,5-^{3}H]lysine were from Amersham; L-[2,3-^{3}H]arginine and L-[2,3,-^{3}H]-ornithine were from New England Nuclear; final specific activity was 50 uCi/umole.


Amino acid uptake
(pmoles/min/oocyte)
40
20
0
6
6
4
5
3
3
3
3
Leucine
Lysine
Arginine
Ornithine

Futhermore, the predicted transmembrane topology of ecoR is homologous to other known membrane transport proteins that have 12 - 14 hydrophobic domains;[1] these include the products of the yeast genes *CAN1*[13], *CTR*[14] and *HIP1*[15], that are arginine, choline and histidine transporters, respectively Knowledge of the molecular structure of this family of transporters may lead to further understanding of inherited human diseases (such as cystinuria[16]) which result from a defective transport of basic amino acids.

These results also raise important issues pertaining to ecoR function in retrovirus infections. Interference enables a cell infected by a retrovirus to resist superinfection by any retrovirus of the same host-range class.[2,17-21] It will be important to learn whether the receptor blockade or down-regulaiton associated with interference[2,17-21] perturbs ecoR amino acid transport function. Ecotropic MuLV envelope glycoproteins have been implicated not only in leukaemogenesis[2,22-24] but in immunosuppression,[25-27] haemolytic anaemia[28] and neuronal degeneration;[29] it will be important to determine whether ecoR transport abnormalities are involved.

A cDNA was recently isolated from T-lymphoma cells that encodes a protein (*Tea*) that is highly homologous to ecoR[30] and it seems likely that this protein is also a transporter. Furthermore, a cDNA that encodes a cell surface receptor for Gibbon ape leukemia virus was recently cloned.[31] Although the expected protein product lacks primary sequence homology with ecoR, it is also a very hydrophobic protein with a similar number of membrane-spanning domains. These features suggest that this other receptor for a C-type retrovirus might also be a transport protein.

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Manuscript #3

Effects of Ecotropic Murine Retroviruses on the Dual-Function Cell Surface Receptor/Basic Amino Acid Transporter

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Running title: Retrovirus Receptor/Amino Acid Transporter

ABSTRACT

The widely-expressed Na^+-independent transporter for basic amino acids (system y^+) is the cell surface receptor (ecoR) for ecotropic host-range mouse retroviruses (MuLVs), a class of retroviruses that naturally infects only mice or rats. Accordingly, expression of mouse ecoR cDNA in mink CCL64 fibroblasts yields cells (CEN cells) that have y^+ transporter activity above the endogenous background and that bind and are infected by ecotropic MuLVs. The effect of ecotropic MuLV infection on expression of y^+ transporter was analyzed in mouse and in mink CEN fibroblasts. Chronic infection with ecotropic MuLVs caused 50-70% loss (down-modulation) of mouse y^+ transporter in plasma membranes, detected as a reduced V_{max} for uptake and outflow of L-[^{3}H]arginine with no effect on K_m's. Down-modulation was specific for mouse y^+ and did not affect other transporters or the endogenous mink y^+, suggesting that it results from specific interaction between mouse y^+ and the viral envelope glycoprotein gp70 in the infected cells. Because this partial loss of mouse y^+ from cell surfaces is insufficient to explain the complete interference to superinfection that occurs in cells chronically infected with ecotropic MuLVs, alternative explanations for interference are proposed. In contrast to the y^+ down-modulation caused by chronic infection, binding of extracellular envelope glycoprotein gp70 at 37°C resulted in noncompetitive inhibition of amino acid import by mouse y^+ but had no effect on export through this same transporter or on any transporter properties of mink y^+. The effects of gp70 on transport kinetics suggest that it slows the rate-limiting step of the amino acid import cycle, a conformational transition of the empty transporter in which the binding site moves from the inside back to the outside of the cell, and that gp70 has no effect on the rate-limiting step of the amino acid export cycle. Infected cells retain substantial y^+ activity. Moreover, the virus binding site on ecoR is in a mobile region that changes conformation during the amino acid transport cycle.

INTRODUCTION

Recent studies suggested that the cell surface receptor (ecoR) for ecotropic host-range murine leukemia viruses (MuLVs) is the widely-expressed Na^{+}-independent transporter for basic amino acids lysine, arginine and ornithine (system y^{+}) (1,2). These studies used *Xenopus laevis* oocytes that were injected with messenger RNA transcribed from cloned ecoR cDNA (3). The transport activities, detected using radioactive amino acid uptake (1,2) and electrophysiological measurements (2), corresponded with previously characterized properties of system y^{+} (4-7).

These results raise important questions pertaining to interactions between the dual functions of this "Trojan Horse" (8) ecoR/y^{+} transporter. For example, interference enables cells that synthesize a viral envelope glycoprotein to efficiently resist superinfection by any retrovirus that uses the same cell surface receptor (9). For several viruses, including human immunodeficiency virus (HIV) (10-13) and avian reticuloendotheliosis virus (14), interference involves depletion of receptors from surfaces of infected cells. Apparently, complexes of the receptor and the envelope glycoprotein form in the rough endoplasmic reticulum shortly after protein synthesis, and these complexes are degraded intracellularly. In the case of HIV, a virus-encoded protein Vpu stimulates degradation of the cell surface receptor CD4 (15). However, elimination of y^{+} transporter from plasma membranes would probably be lethal to cells that lack alternative transporters for essential basic amino acids. Conceivably, starvation for amino acids could be involved in aspects of pathogenesis that have been mapped to the envelope gene of ecotropic MuLVs, such as hemolytic anemia, immunodeficiency, and neural degeneration (16-19).

Although we previously found that adsorption of the gp70 envelope glycoprotein from an ecotropic MuLV had no effect on ecoR/y^+ transporter activity in *Xenopus* oocytes at 25°C (2), we emphasized that this result was inconclusive because gp70 binding to ecoR is highly temperature-dependent (20,21). As is the case with the envelope glycoprotein encoded by HIV (22), binding at 37°C could be qualitatively as well as quantitatively different than at 25°C.

We have addressed these issues by comparing y^+ transporter functions in uninfected and infected mammalian cells and in uninfected cells incubated with ecotropic gp70 at 37°C. We observed several inhibitory effects of ecotropic MuLV and of its *env* glycoprotein gp70 that are specific for the mouse y^+ transporter.

MATERIALS AND METHODS

Cells and viruses--Mouse embryo Balb/c 3T3 fibroblasts and mink lung CCL64 fibroblasts were from American Type Culture Collection (Rockville, MD). Ψ–2 ecotropic packaging cells line (23) and PA12 amphotropic packaging cells (24) were from R.C. Mulligan (MIT, Cambridge, MA) and A.D. Miller (Fred Hutchinson Cancer Center, Seattle, WA), respectively. CEN cells, a derivative of CCL64 cells that express ecoR on their surfaces, have been described previously (25). Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

Ecotropic Rauscher murine leukemia virus (R-MuLV) (26) and dualtropic MCF-13 virus (27) were described previously. R-MuLV pseudotyped with an amphotropic host-range envelope was obtained from PA12 cells that were infected with R-MuLV. Helper-free ecotropic host-range virus that encodes human growth hormone was described elsewhere (25,28). Virus preparations were obtained by removing medium that had been placed on half-confluent monolayers of virus-producing cell lines 16 h earlier, and by filtering it through a 0.2 um pore filter. Viral infections were done by incubating 10^5 cells in 25 cm^2 flasks with 1 ml of virus-containing medium for 2 h at 37^oC in the presence of polybrene (8 ug/ml).

Amino acid transport assays--The amino acid transport assay was based on the method initially described by Gazzola et al. (29). All washes of the monolayers and measurements of amino acid uptake were done in Earle's balanced salt solution (EBSS) (1.8 mM $CaCl_2$, 5.3 mM KCl, 0.8 mM $MgSO_4$, 117 mM NaCl, 1 mM NaH_2PO_4, 5.6 mM D-Glucose, 0.03 mM phenol red, pH 7.4) except in the experiments where choline chloride and choline hydrogen phosphate replaced sodium chloride and sodium hydrogen phosphate, respectively. Choline hydrogen phosphate was prepared by

stoichiometric titration of 250 mM choline bicarbonate with phosphoric acid followed by boiling to remove CO_2. L-[2,3,4,5-^{3}H]arginine and L-[2,3,4,5-^{3}H] proline were from Amersham (Arlington Heights, IL). 10^5 cells were plated in 2 cm^2 wells of a 24-well cluster dish two days before transport assay. Cells were washed with amino acid-free EBSS and then exposed to the radioactive substrate for 0.5 to 1 min at 37°C. L-[^{3}H]arginine in EBSS with a specific activity of 50 uCi/umole was used in uptake experiments. After incubation with radioactive solute, cells were washed rapidly twice with 2 ml of ice-cold phosphate-buffered saline (PBS) [140 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4], extracted with 200 ul of 5% trichloroacetic acid, and the soluble phase was then counted in a liquid scintillation counter. The protein concentration of each culture was measured by the Bio-Rad protein assay (Bio-Rad, Richmond, CA) after dissolving the 5% trichloroacetic acid-insoluble phase of the culture in 200 ul of 0.1 N NaOH. The rates of amino acid uptake for individual cultures were standardized to their protein concentrations and fitted by least squares to the Michaelis-Menten equation. The least-squares fitting analysis resulted in computer-derived K_m and V_{max} estimates $\pm$ standard error of the estimate. These values are indicated in the figure legends.

For measuring initial rate of outflow of an amino acid, cells plated in the 24-well dish as described above were washed once with 2 ml of amino acid-free EBSS and then incubated with 200 u1 of 1 mM L-[^{3}H]arginine (50 uCi/umole) for 1.5 h at 37°C. Cells were rapidly washed three times with 2 ml of ice-cold EBSS before 1 ml of EBSS was added to the cells at 37°C. Samples taken at different times were measured for radioactivity in a liquid scintillation spectrometer. Radioactivity measured in efflux was standardized to the protein concentration of individual cultures as described above.

Adsorption of gp70 that had been purified from ecotropic Friend MuLV

was done at a concentration of 4 ug/ml in culture medium at 37°C for 2 h, as described previously (20,21,25).

Assay for gp70 expression on the surfaces of infected cells--Cells (10^5) were plated onto coverslips in 6-well (9.5 cm^2) dishes 1 day before analysis. Cells were sequentially incubated for 1 h at 37°C with a 1:200 dilution of goat antiserum made to gp70 (30) and then for 1 h with a 1:200 dilution of fluorescein-conjugated rabbit anti-goat antibody (Zymed Laboratories, Burlingame, CA). After cells were rinsed three times with culture media and once with PBS, they were fixed with cold methanol, rinsed with PBS, and mounted for fluorescence microscopy.

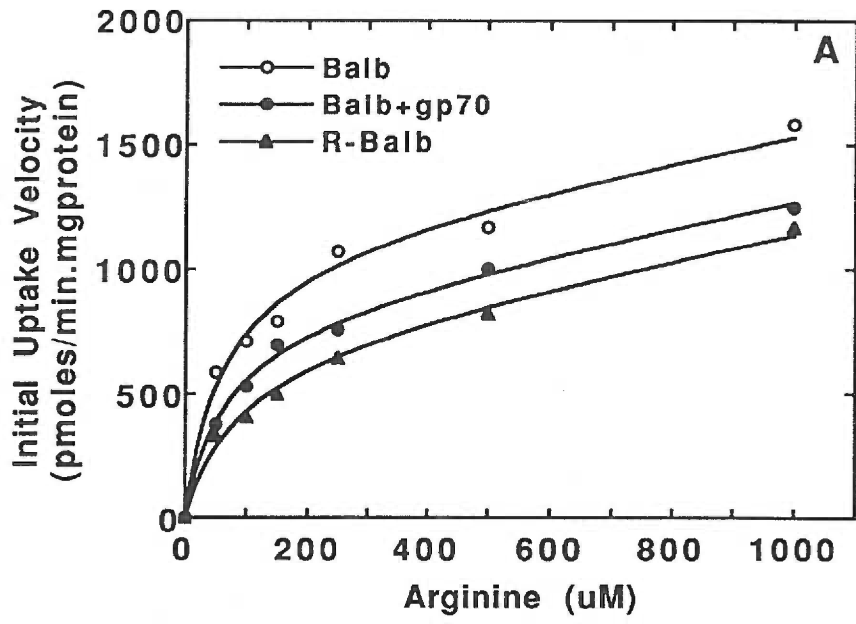
Quantitative analysis of ecotropic-virus infection--Quantitative analysis of ecotropic-virus infections by using the helper-free virus that encodes human growth hormone (hGH virus) has been described previously (25). Briefly, cells infected 48 h earlier with the helper-free hGH virus were plated on coverslips in 9.5 cm^2 dishes 1 day before analysis. Cells were fixed with 3.7% formaldehyde in PBS and permeablized with 0.2% Triton X-100 in PBS. Dilutions of rabbit antiserum to hGH (donated by the National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD) (1:3,000) and of fluorescein-conjugated goat anti-rabbit immunoglobulin (Tago Immunologicals, Burlingame, CA) (1:200) were sequentially incubated with the cells for 1 h at 37°C. After washing with media three times and PBS 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 (25,28).

RESULTS

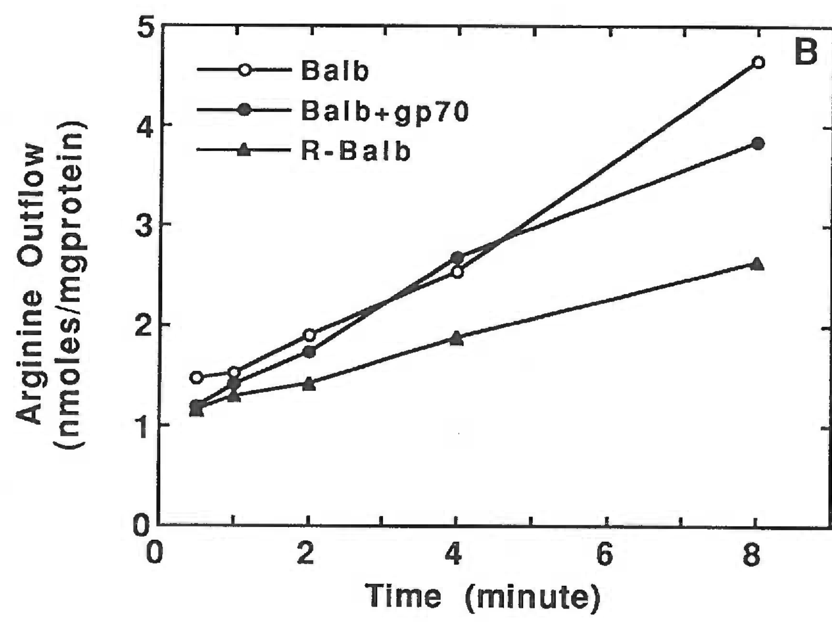
y^+ transporter activities in uninfected and infected mouse fibroblasts-- Initial studies strongly suggested that mouse Balb/c 3T3 fibroblasts contain an amino acid transporter with properties consistent with system y^+ plus a non-saturable component for L-[^{3}H]arginine uptake. For example, as illustrated in Fig. 1A, the uptake had a high affinity component with a K_m of approximately 60-100 uM as described for y^+ (1,2,4,5), and an additional non-saturable component that imparted a secondary slope to the L-[^{3}H]arginine saturation curve. Similar secondary slopes have been reported for some other cells (4-7). Furthermore, as described in Fig. 2, uptake of 200 uM of [^{3}H]arginine was Na^+-independent and was inhibited by L-lysine but not by L-proline. A unique characteristic of system y^+ is that it transports homoserine and several other neutral amino acids with a low affinity (i.e., $K_m \sim 25$ mM) by a Na^+-dependent cotransport mechanism (2,4-7). Uptake of L-[^{3}H]arginine into Balb/c 3T3 fibroblasts was partially inhibited by 10 mM homoserine in the presence of Na^+ (Fig. 2A) but not in its absence (Fig. 2B).

As shown in Fig. 1A, chronic infection of the Balb/c 3T3 fibroblasts with ecotropic MuLV (greater than 99% of the cells were infected as determined by immunofluorescence for viral gp70) or saturation binding of the ecotropic envelope glycoprotein gp70 to the cells at 37°C caused substantial but incomplete inhibitions of the y^+ component of L-[^{3}H]arginine uptake. These inhibitions were noncompetitive since they were not overcome by high concentrations of L-[^{3}H]arginine. The initial rate of efflux of L-[^{3}H]arginine from preloaded cells was also reduced substantially in infected compared with uninfected cells (Fig. 1B). However, gp70 adsorption onto the cells had no significant effect on efflux of the amino acid.

Fig. 1. Measurements of L-[^{3}H]arginine uptake and outflow in mouse Balb/c 3T3 fibroblasts. Uptake and outflow of L-[^{3}H]arginine were measured in uninfected mouse Balb/c 3T3 (Balb, o), Balb/c 3T3 infected with ecotropic Rauscher MuLV (R-Balb, ▲), and uninfected Balb/c 3T3 that contain adsorbed ecotropic gp70 (Balb+gp70, •). Panel (A) shows uptake of L-[^{3}H]arginine. The cells were washed thoroughly and then exposed to different concentrations of L-[^{3}H]arginine (50 uCi/umole) at 37°C for 1 min. After rapidly washing twice with 2 ml ice-cold PBS, the radioactive arginine accumulated in the cells was extracted with 5% TCA as described in Materials and Methods. The lines in Panel (A) were drawn by computer using least squares fitting to the equation v=Vmax[S]/(Km+[S]) + 0.48[S]. The computer-derived Km estimates were 61$\pm$ 13 uM, 66$\pm$8 uM, and 88$\pm$15 uM for the uninfected cells, uninfected cells with adsorbed gp70, and infected cells, respectively. The corresponding Vmax values for these same cells were 1110$\pm$58, 836$\pm$27, and 713$\pm$33 pmoles/min-mgprotein, respectively. Panel (B) shows outflow of L-[^{3}H]arginine from cells preloaded by incubation for 1.5 h at 37°C with 1 mM of the radioactive amino acid. For such highly preloaded cells, the initial rate of outflow should be constant (as observed) and equal to Vmax for export. Both uptake and outflow experiments were repeated once, with similar results.

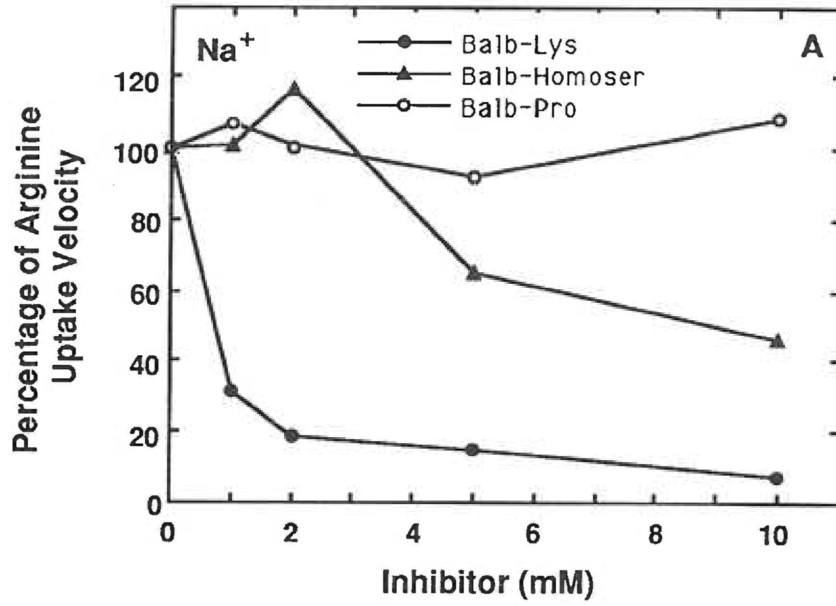


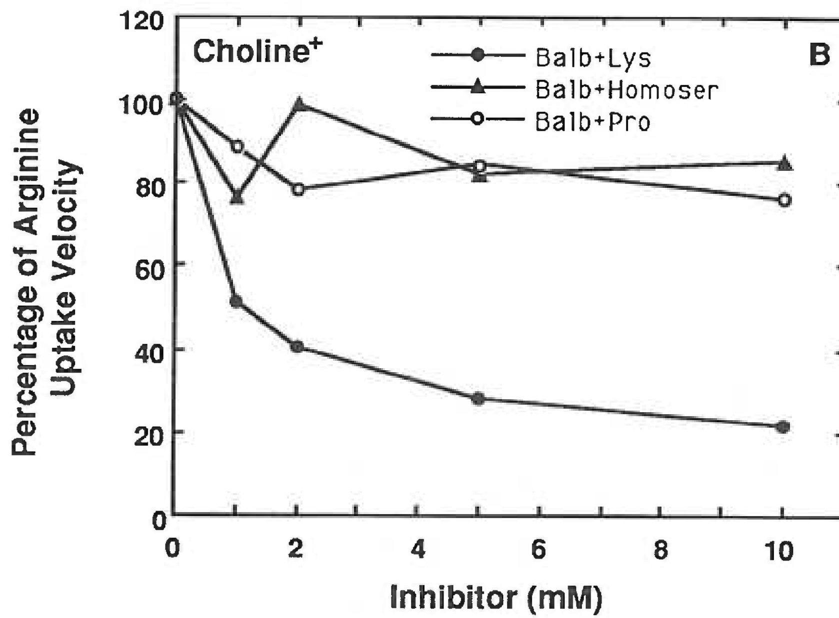
A
Balb
Balb+gp70
R-Balb
Initial Uptake Velocity (pmoles/min.mgprotein)
2000
1500
1000
500
0
0
200
400
600
800
1000
Arginine (uM)



B
Balb
Balb+gp70
R-Balb
Arginine Outflow (nmoles/mgprotein)
5
4
3
2
1
0
0
2
4
6
8
Time (minute)

Fig. 2. **inhibition of L-[^{3}H]arginine uptake into Balb c/3T3 fibroblasts by lysine, homoserine, and proline in the presence (Panel A) or absence (Panel B) of sodium.** Cells were washed and then exposed to 200 uM L-[^{3}H]arginine (50 uCi/umole) in the presence of inhibitors at different concentrations for 1 min at 37°C. In Panel B, the EBSS medium contained choline chloride and choline hydrogen phosphate instead of sodium chloride and sodium hydrogen phosphate. The data are plotted as the percentage of the uptake observed in the absence of inhibitors. The 100% values for L-[^{3}H]arginine uptake were 710 and 855 pmoles/min-mgprotein from Panel A and Panel B, respectively.

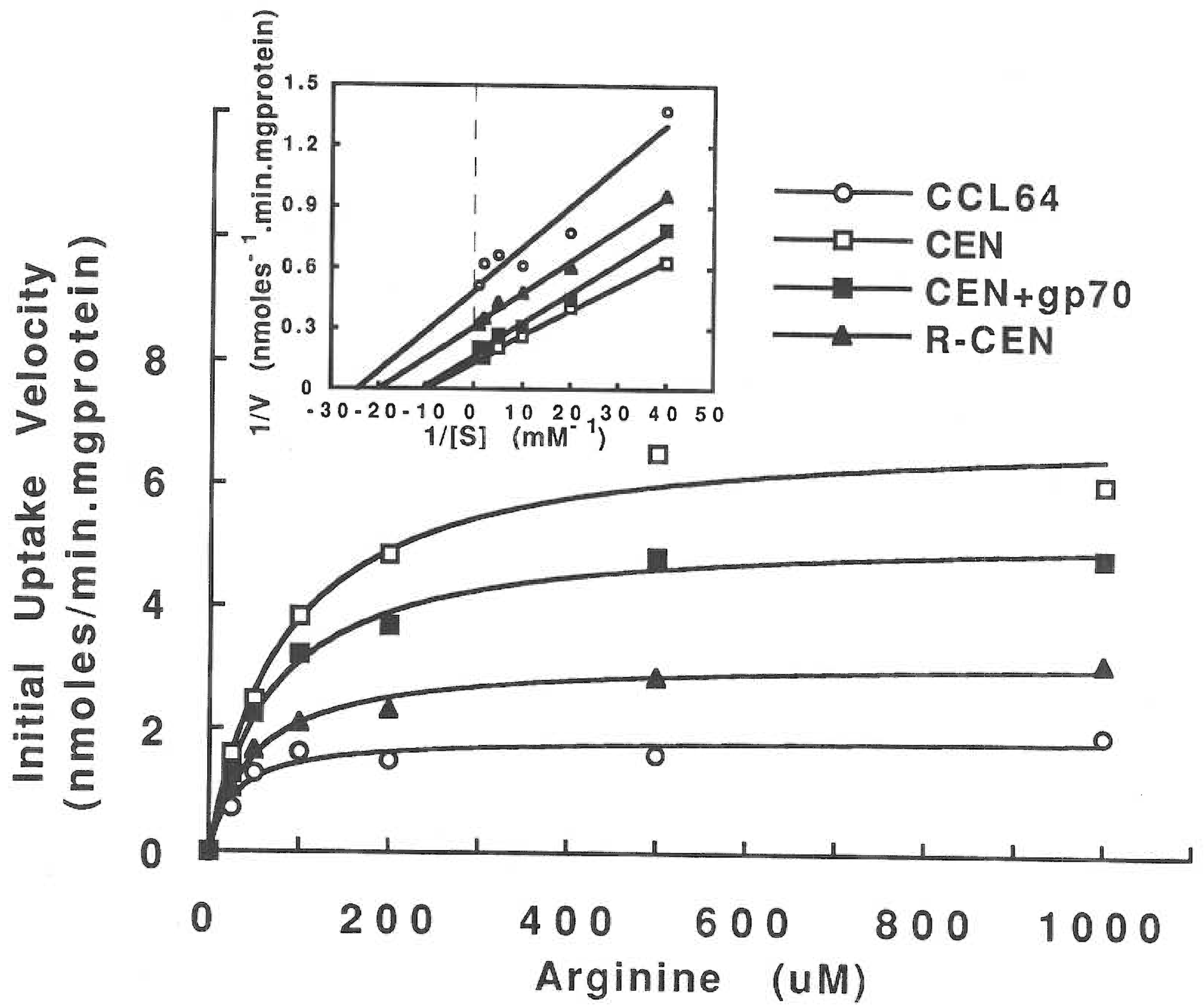

Na+
A
Balb-Lys
Balb-Homoser
Balb-Pro
Percentage of Arginine Uptake Velocity
Inhibitor (mM)
0
20
40
60
80
100
120
2
4
6
8
10


Choline+
B
Balb+Lys
Balb+Homoser
Balb+Pro
Percentage of Arginine Uptake Velocity
Inhibitor (mM)
0
20
40
60
80
100
120
2
4
6
8
10

Although significant and reproducible, these effects of virus and of gp70 were difficult to quantitatively evaluate. In order to perform Michaelis-Menten analysis, it was necessary to subtract the nonsaturable component of uptake. For the results in Fig. 1A, such subtraction suggests that infection with ecotropic MuLV reduces y^+ activity by approximately 50%, and that adsorption of extracellular gp70 reduces y^+ import activity by approximately 25%. It is also difficult with these cells to be certain that all of the high affinity L-[^{3}H]arginine uptake is due to system y^+; some cells contain another high affinity Na^+-independent transporter (31). Consequently, these studies of Balb/c 3T3 fibroblasts do not establish whether infection causes complete loss of y^+ with residual activity due to another transporter or whether it causes only partial depletion of y^+.

y^+ transporter activities in mink CCL64 fibroblasts--To circumvent these problems inherent in studies using mouse cells, we analyzed y^+ transport using CCL64 mink lung fibroblasts and a derivative cell line (CEN) that expresses a large quantity of recombinant mouse ecoR (25). Fig. 3 shows an initial comparison of L-[^{3}H]arginine uptake into CCL64 cells, CEN cells, CEN cells chronically and efficiently (greater than 99%) infected with the Rauscher strain of ecotropic MuLV (R-CEN), and CEN cells that had adsorbed extracellular gp70 at 37°C. These data are plotted as 1/v versus 1/[S] in the Fig. 3 insert. Clearly, CEN cells that express ecoR contain substantially more apparent y^+ transporter activity than the CCL64 cells that contain only the endogenous mink transporter. Although the chronically infected R-CEN cells have much less y^+ than the uninfected CEN cells, they nevertheless contain more than the background amount that occurs in the control CCL64 cells. This implies that infection causes incomplete loss of mouse y^+. Moreover, as with mouse fibroblasts (Fig. 1), adsorption of gp70 at 37°C onto CEN cells

Fig. 3. Effect of gp70 adsorption and viral infection on L-[^{3}H]arginine uptake into mink cells. CCL64 (o), CEN (□), CEN after being incubated with gp70 (4ug/ml) at 37°C for 2 h (■), and CEN chronically infected with Rauscher MuLV (R-CEN) (▲) were exposed to different concentrations (25, 50, 100, 200, 500, and 1000 uM) of radioactive arginine (specific activity 50 uCi/umole) at 37°C for 0.5 min. After two washings with ice-cold PBS, the radioactive arginine accumulated in the cells was extracted with 5% TCA and measured as described in the Materials and Methods. The lines were drawn by computer using least squares fitting to the Michaelis-Menten equation. The insert is a double reciprocal plot of the same data. The computer-derived Km values are 28$\pm$9 uM, 84$\pm$13 uM, 70$\pm$7 uM, and 51$\pm$6 uM for CCL64, CEN, CEN+gp70, and R-CEN cells, respectively. The corresponding Vmax values for these same cells were 1.9$\pm$0.1, 7.0$\pm$0.3, 5.3$\pm$0.1, and 3.2$\pm$0.1 nmoles/min-mgprotein, respectively.



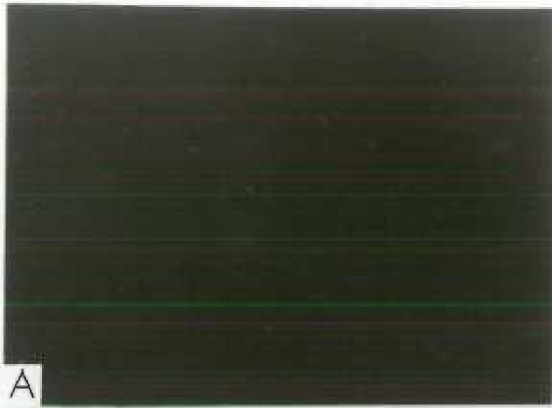
Initial Uptake Velocity
(nmoles/min.mgprotein)
0
2
4
6
8
0
200
400
600
800
1000
Arginine (uM)
CCL64
CEN
CEN+gp70
R-CEN
1/V (nmoles⁻¹.min.mgprotein)
0
0.3
0.6
0.9
1.2
1.5
-30 -20 -10 0 10 20 30 40 50
1/[S] (mM⁻¹)

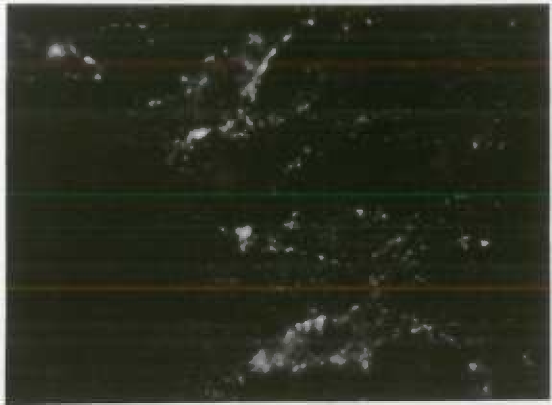
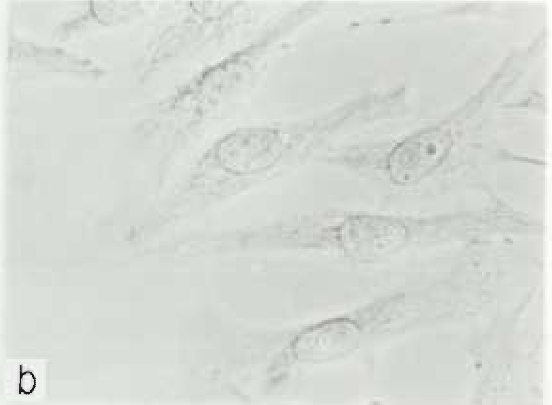
causes partial (c.a., 25%) noncompetitive inhibition of L-[^{3}H]arginine uptake. Support for these conclusions is described below.

This mink cell system is advantageous for analyzing ecotropic MuLV effects on y^+ transporter activities. First, because CEN cells express recombinant mouse ecoR, there is no doubt that the difference between CEN and CCL64 cells is due to mouse y^+. Second, the L-[^{3}H]arginine uptake curve for CCL64 mink cells has a simple shape consistent with the Michaelis-Menten equation and it lacks the secondary slope that occurs in Balb/c 3T3 cells. Indeed, the endogenous transport activity in mink CCL64 cells has properties consistent with system y^+, including its K_m for arginine, Na^+-independency, inhibition by other basic amino acids, and Na^+-dependent inhibition by a high concentration of homoserine (results not shown). Third, as illustrated further below, the endogenous transport activity of the mink CCL64 cells provides a baseline for quantitative evaluation of the effects of infection or of gp70 adsorption.

To determine whether the partial loss of y^+ caused by infection with ecotropic MuLV is specific to mouse y^+, we expanded the latter investigation (i.e., Fig. 3) to include analysis of mink CCL64 cells that were infected with ecotropic Rauscher MuLV. Because mink CCL64 cells lack ecotropic receptors, the infection was accomplished using virus that had been pseudotyped with an amphotropic host-range envelope (see Experimental Procedures). Fig. 4 shows an immunofluorescence analysis that demonstrates expression of gp70 on the surfaces of mink CCL64 and CEN cells that were infected with Rauscher ecotropic MuLV. These mink cell lines were efficiently infected (greater than 99% positive for gp70) and they released virions that had the expected ecotropic host-range (data not shown).

Fig. 4. Expression of gp70 on the surfaces of cells infected with ecotropic host-range Rauscher murine leukemia virus (R-MuLV). The immunofluorescence microscopy procedure is described in Materials and Methods. Each microscopic fields is shown in both fluorescence (left panels) and phase-contrast (right panels). (A, a) Mink CCL64 cells; (B, b) Mink CCL64 cells that had been infected with R-MuLV pseudotyped with an amphotropic envelope; (C, c) CEN cells (these are CCL64 cells that express ecoR); and (D, d) CEN cells chronically infected with R-MuLV.


A

a


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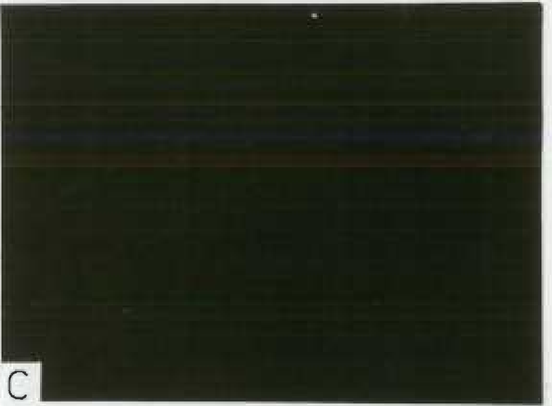
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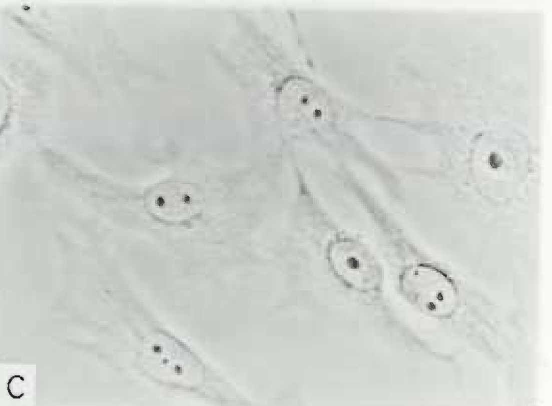
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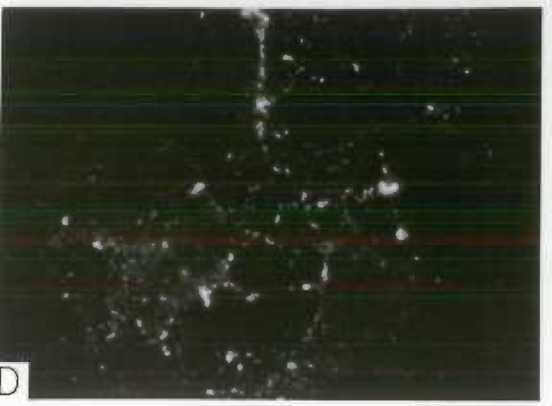
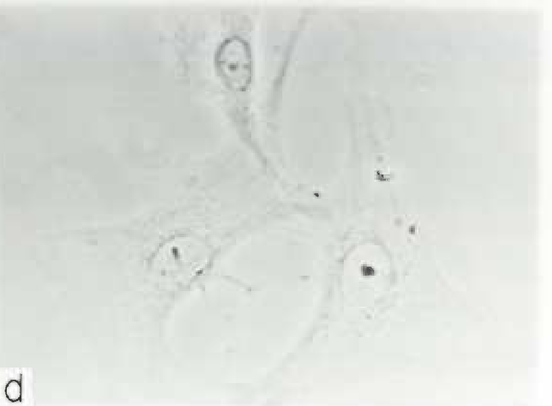

D

d

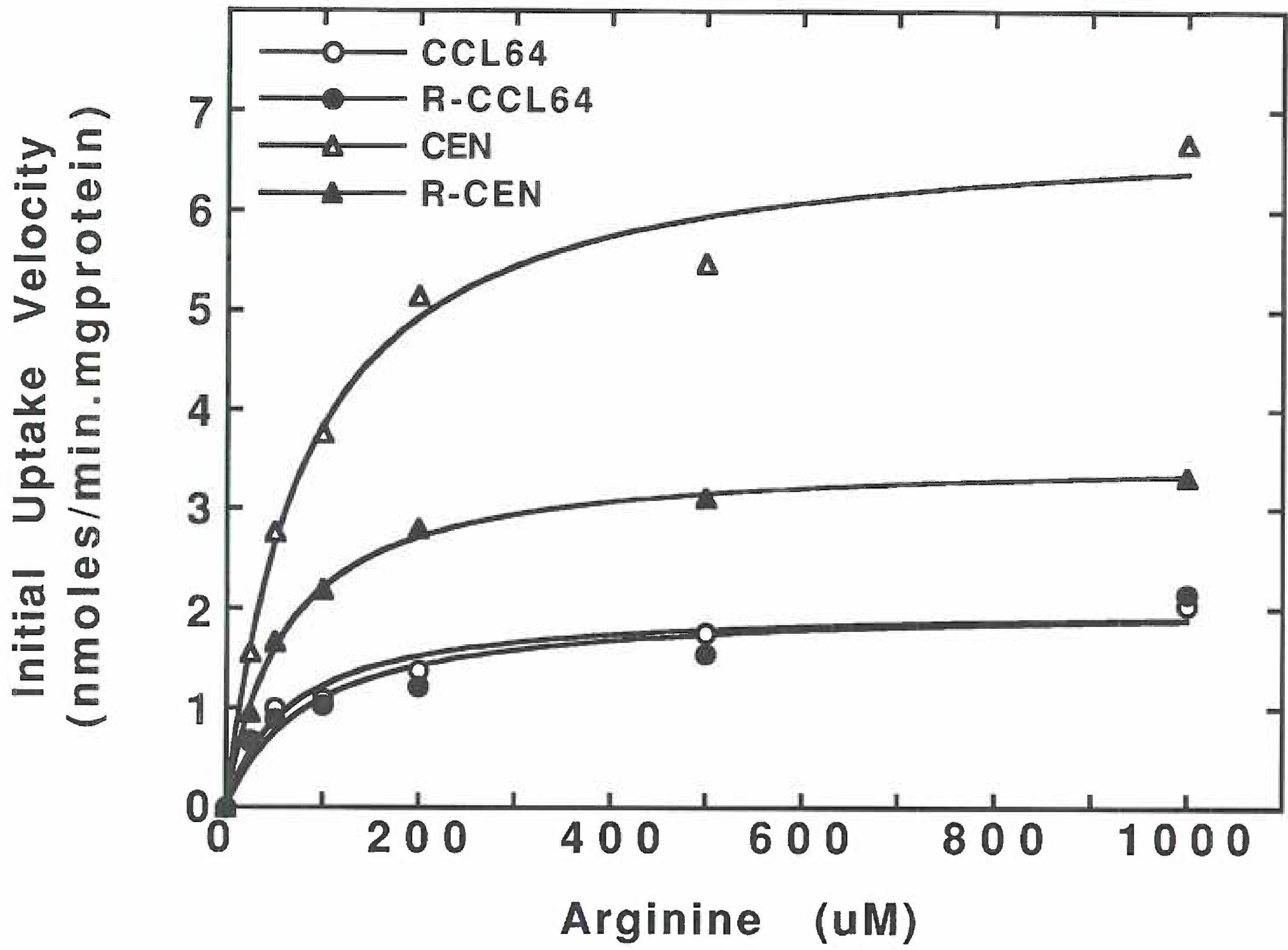
Fig. 5 shows a comparison of L-[^{3}H]arginine uptake into the uninfected and infected CCL64 cells and into the uninfected and infected CEN derivative cells that contain recombinant ecoR. Consistent with the previous data, infection of CEN cells caused substantial but incomplete loss of mouse y^+ uptake activity. However, infected and uninfected mink CCL64 cells had indistinguishable amino acid uptake curves, suggesting that ecotropic MuLV has no effect on mink y^+. This strongly suggests that ecotropic MuLV infection causes partial down-modulation of mouse y^+ amino acid uptake activity, but has no effect on the endogenous mink y^+ transporter.

Fig. 6 presents evidence that the inhibitory effect of extracellular ecotropic gp70 on L-[^{3}H]arginine uptake is also specific to mouse y^+. Thus, addition of a saturating amount of ecotropic gp70 at 37°C reduced mouse y^+ import activity in CEN cells. Although this inhibitory effect of gp70 on CEN cells was small, it was reproducible and was determined to be statistically highly significant (see below). On the contrary, gp70 had no effect on the endogenous mink transporter in the control CCL64 cells. Because ecotropic gp70 binds to mouse y^+ but not to mink y^+ (20,21,25), the inhibitory effect of gp70 must be caused by its binding to the transporter.

Table I summarizes results of many studies in which we measured effects of infection and of gp70 adsorption on L-[^{3}H]arginine uptake into CCL64 mink fibroblasts and their CEN derivative that contains mouse ecoR/y^+. These results suggest that infection with Rauscher MuLV or adsorption of ecotropic gp70 at 37°C significantly inhibit V_{max} for uptake by mouse y^+ with no significant effects on K_m's.

Analysis of L-[^{3}H]arginine export from these mink cell lines is shown in Fig. 7. For export, cells were preloaded to equilibrium by incubation for 1.5 h with 1 mM L-[^{3}H]arginine. Because system y^+ is electrogenic (2), it can

Fig. 5. Comparison of L-[^{3}H]arginine uptake into mink CCL64 fibroblasts, CEN fibroblasts (mink CCL64 that express recombinant mouse ecoR), R-CEN (CEN infected with Rauscher ecotropic MuLV),and R-CCL64(CCL64 cells that were infected with Rauscher ecotropic MuLV as described in Materials and Methods). Conditions for uptake studies are described in legend to Fig.3 and in Materials and Methods. The lines were drawn by computer using least squares fitting to the Michaelis-Menten equation. The computer Km estimates were 80$\pm$11 uM, 10$\pm$0.4 uM, 65$\pm$15 uM and 84$\pm$31 uM for CEN, R-CEN, CCL64 and R-CCL64 cells, respectively. The corresponding Vmax estimates for these same cells were 6.9$\pm$0.3, 3.5$\pm$0.1, 1.9$\pm$0.1, and 2.0$\pm$0.2 nmoles/min-mgprotein, respectively.


CCL64
R-CCL64
CEN
R-CEN
Initial Uptake Velocity
(nmoles/min.mgprotein)
0
1
2
3
4
5
6
7
0
200
400
600
800
1000
Arginine (uM)

Fig, 6. Effect of ecotropic gp70 on L-[^{3}H]arginine uptake into mink CCL64 fibroblasts and into CEN fibroblasts (CCL64 that express recombinant mouse ecoR), in the absence or presence of a saturating concentration of ecotropic gp70. Conditions for uptake studies were described in legend of Fig. 3 and in Materials and Methods. Cells were incubated with purified gp70 (4ug/ml) at 37°C for 2 h prior to uptake studies. The lines were drawn by computer to the Michaelis-Menten equation. The computer Km estimates were 57$\pm$13 uM, 87$\pm$21 uM, 27$\pm$7 uM, and 22$\pm$8 uM for CEN, CEN+gp70, CCL64, and CCL64 +gp70, respectively. The corresponding Vmax estimates for these same cells were 6.2$\pm$0.4, 5.7$\pm$0.4, 2.0$\pm$0.1, and 2.0$\pm$0.1 nmoles/min-mgprotein, respectively. Statistical analysis of many replicate experiments of gp70 effects are summarized in Table I.

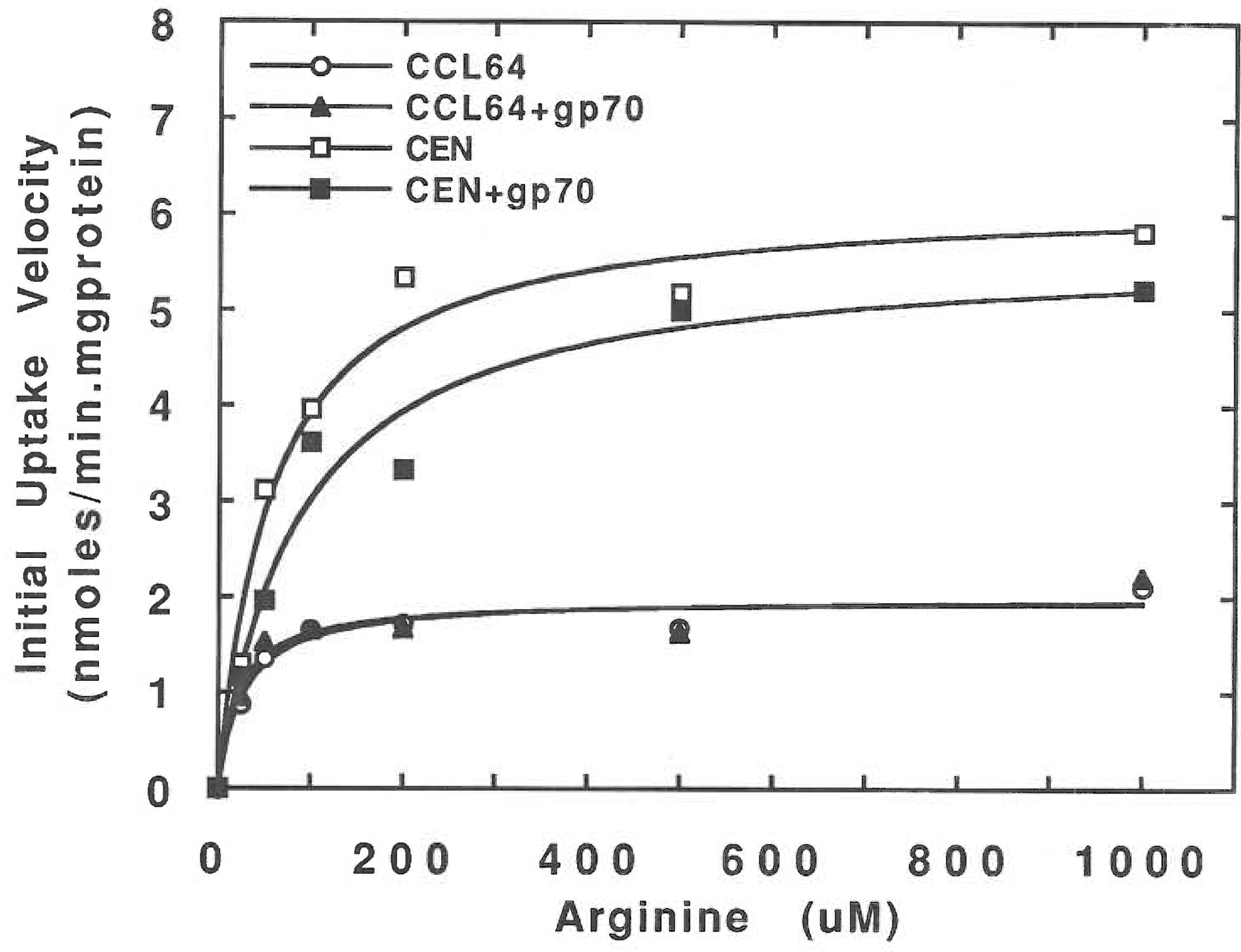

CCL64
CCL64+gp70
CEN
CEN+gp70
Initial Uptake Velocity
(nmoles/min.mgprotein)
Arginine (uM)
0
1
2
3
4
5
6
7
8
0
200
400
600
800
1000

TABLE I

Effects of gp70 adsorption and viral infection on the Vmax and Km of arginine uptake in mink cells

Cells Used	Vmax (nmol/min.mgprotein)[1]	Km (uM)[1]	Number[2]
CCL64	2.0 ± 0.1	60.4 ± 11.9	7
CEN	7.5 ± 0.8	97.0 ± 11.1	7
CEN+gp70	5.8 ± 0.4	91.1 ± 11.6	4
R-CEN	3.8 ± 0.3	80.0 ± 15.2	4

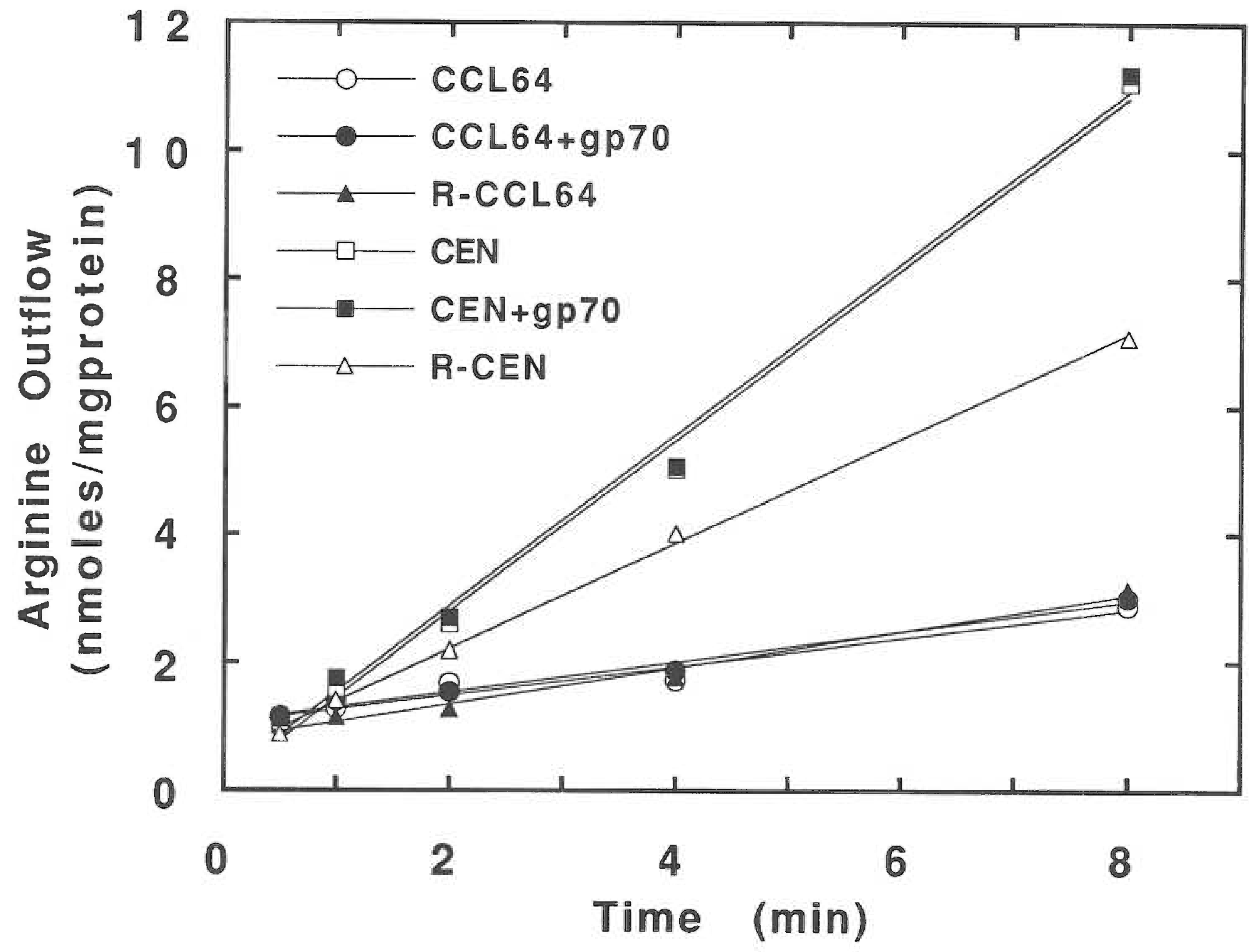
1. Values are presented as means ± standard errors of means. The Vmax differences are significant by the paired-comparisons t-test (32) to at least 95% confidence for all of the cells indicated. The effect of gp70 on Vmax of CEN cells is significant to 98% confidence. Reproducibly, the endogenous mink y^+ transporter of control CCL64 cells has a significantly lower Km for arginine import than the mouse ecoR/y^+ transporter. The small apparent Km differences between CEN, CEN+gp70, and R-CEN, while not significant to 95% confidence limits, are consistent with the lower Km for mink y^+ and with the fact that these CEN cells contain an endogenous mink y^+ in addition to their different amounts of mouse y^+. These mean Km estimates differ somewhat from the values of the individual experiments reported in the legends of Figs. 3, 5, and 6.

2. Number of independent experiments.

mediate a substantial (10-fold, assuming a -60 mV resting potential) accumulation of substrate; such values are consistent with previous measurements in fibroblasts (4-7). This concentration is substantially above the K_m for export of this amino acid by system y^+ (c.a., 0.8 mM) (5). These considerations predict that the rate of L-[^{3}H]arginine release after removing extracellular arginine should be constant and equal to V_{max} for export for several minutes until the intracellular concentration declines toward the K_m. As expected, the initial rates of export were constant (see Fig. 7). Moreover, the rate of export from CEN cells was substantially higher than from CCL64 cells, consistent with a larger amount of y^+ transporter in the CEN cells. The initial rate of export from ecotropic MuLV-infected R-CEN cells was considerably slower than from CEN cells but faster than from CCL64 cells. Thus, both import and export measurements suggest that there is less mouse y^+ transporter activity in the surface membranes of infected CEN cells. This reduction is specific to mouse y^+ because infected and uninfected mink CCL64 cells have the same quantity of L-[^{3}H]arginine transport activity. As with mouse Balb/c 3T3 cells (see Fig. 1), gp70 does not inhibit L-[^{3}H]arginine export from any of these cells. Moreover, this lack of effect of gp70 on outflow is true not only for the initial rate (i.e., V_{max} conditions) but also throughout the entire time course of outflow as the amino acid concentration approaches the intracellular K_m and then continues to decline toward zero (results not shown). This implies that intracellular K_m is also unaffected by gp70 binding to mouse y^+.

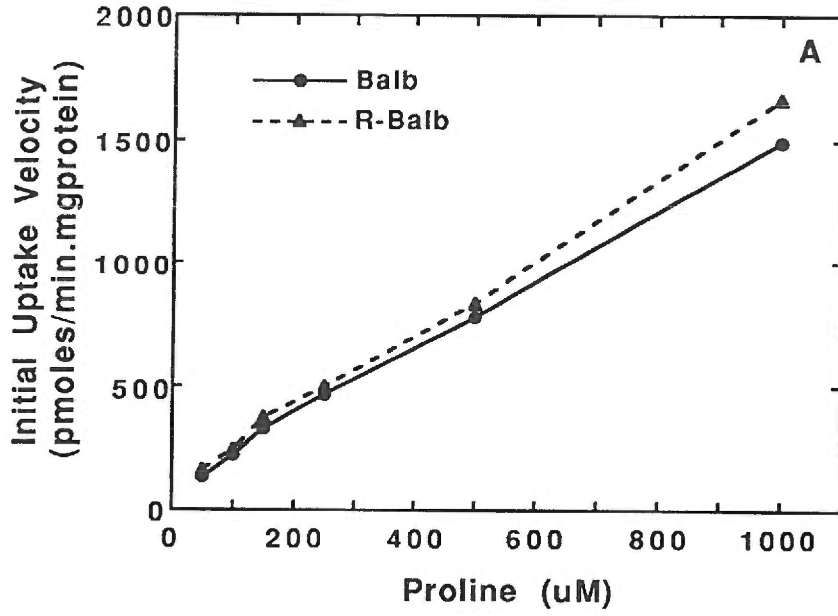
Lack of effect of ecotropic MuLV on proline transport--As shown in Fig. 8A, L-[^{3}H]proline uptake into cells was identical in uninfected and Rauscher MuLV-infected mouse Balb/c 3T3 fibroblasts. Similarly, L-[^{3}H] proline uptake was not significantly different in mink CCL64, CEN, and

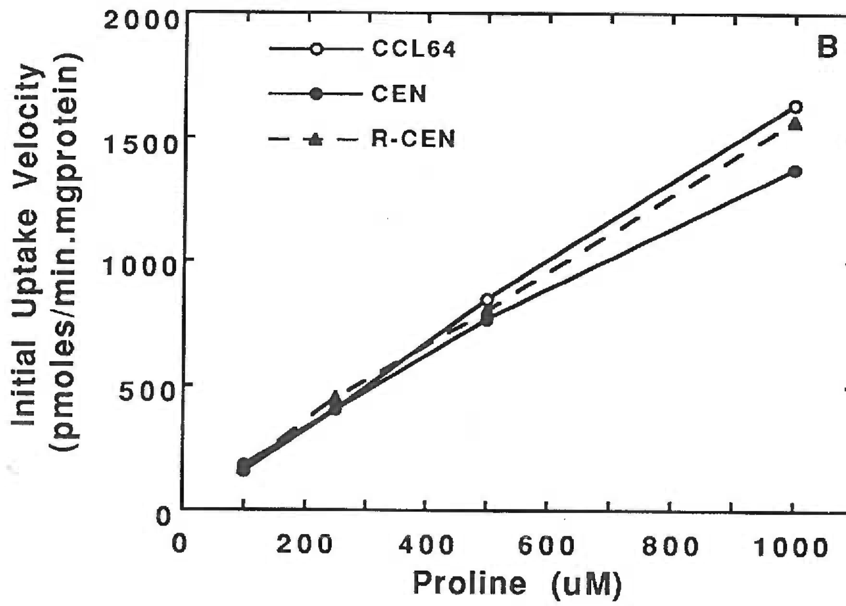
Fig. 7. Effect of gp70 adsorption and viral infection on L-[^{3}H]arginine outflow from mink cells. cells were preloaded with 200 ul of 1 mM L-[^{3}H] arginine (50 uCi/umole) at 37°C for 1.5 h. The radioactive arginine outside the cells was then quickly removed by washing three times with ice-cold EBSS, before 1 ml of EBSS at 37°C was added to the cells. Samples of the media were taken at 0.5, 1, 2, 4, and 8 min with a multi-channel pipette (Applied Scientific, San Francisco, CA) while the cells were incubating at 37°C. The CEN and CEN + gp70 cells release label rapidly; the R-CEN cells release label at an intermediate rate; and the CCL64, R-CCL64, and CCL64+gp70 cells release the label at the slowest rate.



Arginine Outflow (nmoles/mgprotein)
Time (min)
0
2
4
6
8
10
12
CCL64
CCL64+gp70
R-CCL64
CEN
CEN+gp70
R-CEN

Fig. 8. Proline uptake into mouse and mink fibroblasts. Cell monolayers were exposed to different concentrations of L-[^{3}H]arginine (50 uCi/umole) for 1 min at 37°C. After rapidly washing the cells twice with ice-cold PBS solution, the radioactivity in the cells was extracted with 5% TCA and measured in the liquid scintillation spectrophotometer as described in the materials and Methods. Panel (A) shows initial rates of proline uptake in mouse Balb/c 3T3 fibroblasts and in Balb/c 3T3 fibroblasts chronically infected with Rauscher MuLV (R-Balb). Panel (B) shows the initial rates of proline uptake in mink CCL64 fibroblasts, CEN (mink CCL64 that express recombinant ecoR) and R-CEN (CEN infected with Rauscher MuLV).


A
Balb
R-Balb
Initial Uptake Velocity
(pmoles/min.mgprotein)
Proline (uM)
0
500
1000
1500
2000
200
400
600
800
1000


B
CCL64
CEN
R-CEN
Initial Uptake Velocity
(pmoles/min.mgprotein)
Proline (uM)
0
500
1000
1500
2000
200
400
600
800
1000

Rauscher MuLV-infected CEN cells (Fig. 8B).

Highly efficient interference to superinfection by ecotropic retroviruses in both mink and mouse cells--The above data indicates that infection of cells with ecotropic Rauscher MuLV causes only partial (2-3-fold) loss of cell surface mouse y^+ transporter, implying that ecoR must remain in a substantial amount on surfaces of infected cells. To determine whether the infected cells exhibit interference to superinfection, we attempted to superinfect them with an ecotropic host-range retrovirus that encodes human growth hormone and we analyzed the cells 48 h later for growth hormone-specific immunofluorescence. From the proportion of cells that were negative for growth hormone, and assuming the binomial distribution for virus infections in the cell populations, we calculated the multiplicities of infection (i.e., the average numbers of virus infections per cell) (see Materials and Methods). As shown in Table II, mouse Balb/c 3T3 and mink CEN that have ecoR were both highly susceptible to infection by the growth hormone-encoding virus and high proportions of the cells expressed growth hormone. Cells that were preinfected with Rauscher ecotropic MuLV were completely resistant to superinfection with this growth hormone-encoding virus; no cells with growth hormone were detected among at least 1000 cells examined. However, cells infected with virus of another host-range (MCF-13) remain susceptible to superinfection by the ecotropic growth hormone-encoding virus.

TABLE II

Infection of cells by an ecotropic virus that encodes human growth hormone

Cell type[1]	Multiplicity of infection[2]
Balb	1.48 ± 0.13
R-Balb	<0.001[3]
M-Balb	1.94 ± 0.14
CEN	0.48 ± 0.02
R-CEN	<0.001[3]
M-CEN	0.41 ± 0.04

1. Balb, R-Balb and M-Balb are Balb c/3T3 mouse fibroblasts that are uninfected, infected with ecotropic Rauscher MuLV, and infected with dualtropic MCF-13 virus, respectively. CEN, R-CEN and M-CEN are CCL64 mink fibroblasts expressing ecoR that are uninfected, infected with ecotropic Rauscher MuLV, and infected with dualtropic MCF-13 virus, respectively.

2. Multiplicities of infection by the helper-free ecotropic host-range virus that encodes human growth hormone were determined by analysis of growth hormone-specific immunofluorescence (see Materials and Methods). Multiplicity of infection is the average number of infections that occurred per cell in the culture.

3. No positives were found among at least 1000 cells scanned.

DISCUSSION

Infection with ecotropic MuLV causes incomplete removal of mouse y^+ activity from cell surfaces--These studies with mammalian cells confirm evidence obtained with *Xenopus* oocytes (1,2) that mouse ecoR is a basic amino acid transporter with characteristics of system y^+. Our results suggest that this mouse y^+ activity is partially down-modulated by chronic infection with ecotropic MuLV (see Figs. 1, 3, 5, 7). This incomplete loss of transporter, detected as a reduction in V_{max} for both import and export of L-[^{3}H]arginine without any significant effect on K_m's, is specific to mouse y^+ and does not affect mink y^+ (Figs. 5 and 7 and Table I).

Down-modulation of mouse y^+ caused by infection with ecotropic MuLV is incomplete. This is seen in all of our studies as a residual mouse y^+ activity after efficient infection of both mouse and mink cells (Figs. 1, 3, 5, and 7 and Table I). In mink cells that express mouse ecoR, infection reduces y^+ activity to a residual level that is substantially higher than the baseline of endogenous transport activity that occurs in the control mink cells. Infection by ecotropic MuLV reduces mouse y^+ activity both for import and export of amino acids by approximately 50-70% in different experiments. Further understanding of this down-modulation would require direct studies of ecoR synthesis, processing, and turnover, and we are attempting to make antiserum for this purpose.

Implications for the mechanism of viral interference--The partial loss of mouse ecoR/y^+ from surfaces of cells infected with ecotropic MuLV cannot explain the essentially complete resistance of these cells to superinfection by ecotropic host-range retroviruses (e.g., see Table II). Indeed, we have presented evidence that CEN cells contain a vast excess of ecoR, which is not the limiting factor in infection (25). A 50-70% loss of ecoR would not be expected

to reduce efficiency of infection.

Consequently, a mechanism other than loss of receptor must be responsible for viral interference in these cells. One possibility is that the ecoR/y^+ on surfaces of infected cells is saturated with gp70 and therefore inaccessible to extracellular virus. According to this idea, interference is accomplished by blocking receptor sites on cell surfaces. A second possibility is that the ecoR/y^+ remaining on surfaces of infected cells is nonfunctional in mediating superinfection because of a posttranslational modification. For example, the third extracellular loop of ecoR, which is believed to contain the site for virus attachment, also contains potential sites for N-linked glycosylation (3). If these sites were incompletely glycosylated with heterogeneously structured oligosaccharides, as is common for glycoproteins (33), only a portion of mouse ecoR/y^+ might be able to interact with gp70. Presumably, only this interactive portion would be eliminated by infection and the remaining y^+ would be incapable of mediating superinfection. A third possibility is that infection may eliminate an accessory factor other than y^+ that is required for ecotropic viral entry into cells (25).

Inhibition of mouse y^+ transporter by gp70 adsorption at 37°C--In contrast to chronic infection, which may reduce receptor expression metabolically by affecting its processing and/or rate of degradation (10-13,15), adsorption of a saturating amount of gp70 onto cell surface ecoR at 37°C affects mouse y^+ transporter activity as an impermeant inhibitor. Specifically, gp70 significantly inhibits the V_{max} for import of L-[^{3}H]arginine by mouse y^+ by approximately 25% without any significant effect on K_m (see Figs. 1, 3, 6, and Table I). In contrast, gp70 has no significant effect on either V_{max} or K_m for export of arginine by mouse y^+ (see Results), and it has no effect on any parameter of mink y^+ transport. Because it is uncertain whether all mouse y^+

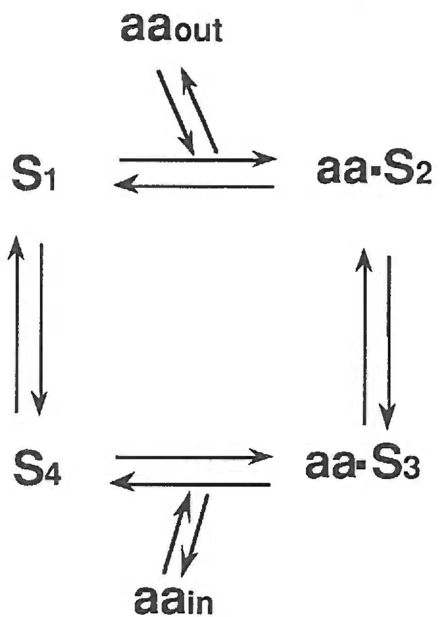
on uninfected cells is capable of binding gp70 (see previous paragraph), we do not know the extent to which gp70 binding slows the mouse y^+ import cycle. Nevertheless, because gp70 binding has no significant effect on export of arginine (Figs. 1, 7), it is clear that gp70 is not an irreversible inhibitor that locks mouse y^+ into one conformational state. Therefore, ecoR with bound gp70 remains able to transport amino acids.

Generally, facilitated transport can be well-described by a four-state model in which import operates in a clockwise direction and export in a counterclockwise direction (see Fig. 9A). In each cycle, transporter binds solute on one side, undergoes a conformational change that allows movement of solute through the membrane, releases the solute, and undergoes another conformational change that returns the empty transporter to its initial state (5,34).

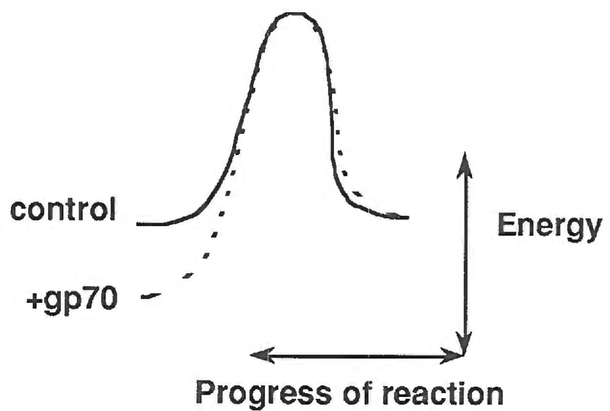
Previous workers have thoroughly analyzed kinetic properties of system y^+ (5-7). Like other transporters, the conformational changes of y^+ are several orders of magnitude slower than the rates of association and dissociation of solute. Indeed, by electrophysiological analyses we have estimated that each y^+ maximally cycles in the import direction at approximately 200 times/sec (M. Kavanaugh and R.A. North, unpublished results), a rate typical for transporters but orders of magnitude less than turnover numbers for many enzymes (34). Furthermore, evidence strongly suggests that the conformational change of the empty y^+ transporter [i.e., interconversion of states $S_4 <=> S_1$ (see Fig. 9)] is much slower than the conformational change that moves bound amino acid through the membrane (5-7). This evidence is based principally on the existence of "trans-stimulation", a process whereby an unlabeled cationic amino acid on either side of the membrane greatly stimulates the rate of transport of a radioactive cationic amino acid from the

Fig. 9. Four-state model of y+ transporter function and interpretation of gp70 inhibitory effects. (A) The four-state model is shown. In the import cycle (clockwise), state S1 binds extracellular amino acid to form the aa.S2 complex. The transporter then changes conformation to form aa.S3 from which the amino acid can dissociate into the cytoplasm to form the S4 state. The final step in the import cycle occurs by conformational change of S4 to regenerate S1. The amino acid export cycle operates in the counterclockwise direction. Substantial evidence (5-7) suggests that the S4 —> S1 transition is the rate-limiting step of the amino acid import cycle and that the opposite S1—>S4 transition rate-limits the export cycle. This evidence drives in part from the existence of trans-stimulation. (B) Effects of gp70 binding are interpreted in terms of the energetics of the S4<=>S1 transition, since these are believed to be the rate-limiting steps of the import and export cycles (see text). The basic interpretation of our data is that gp70 binding to ecoR stabilizes state S4 and has relatively little effect on state S1. In the curve, the ordinate scale represents the potential energy and the abscissa represents the progress of the reaction. Since by transition-state theory the rate of any reaction is proportional to the probability of reaching to the transition state and since this probability is related by the Boltzman distribution to the energy required (35), this model predicts that gp70 would inhibit the y+ amino acid import cycle (i.e., S4 —>S1 transition) but have no effect on the y+ export cycle (i. e., limited by the S1—>S4 transition) (as observed).

(A)



(B)



opposite side. Moreover, such trans-stimulation of L-[^{3}H]arginine uptake into CEN cells occurs both in the presence and absence of adsorbed gp70 (unpublished results), strongly suggesting that S_4 —> S_1 conformational change is rate-limiting for L-[^{3}H]arginine import both in the presence and absence of gp70. Similar studies (5) have demonstrated that export of cationic amino acids by y^+ is limited by the opposite S_1 —> S_4 conformational change (see Fig. 9A).

Based on these considerations and on our results (Figs. 1, 3, 6, 7), we propose the model for gp70 interaction with y^+ described in Fig. 9B. According to this model, gp70 adsorption onto mouse y^+ at 37°C inhibits the rate-limiting S_4 —> S_1 step of the amino acid import cycle but has no effect on the rate-limiting step of the export cycle (i.e., S_1 —> S_4 conversion). This can be readily understood from the perspective of transition state theory (35) by assuming that gp70 binds strongly to conformational state S_4 and only relatively weakly to state S_1. This would increase the energy required to convert S_4 to the S_4 <–> S_1 transition state and would therefore inhibit uptake of amino acid. However, as illustrated in Fig. 9B, the energetics of converting S_1 to the S_4 <–> S_1 transition state would be unaltered by gp70, and the rate of export by y^+ would therefore be unaffected. This result occurs because the rate of S_4 —> S_1 conversion (or vice-versa) depends on the transition-state barrier that separates these states (35), and because we propose that gp70 heightens this barrier in the S_4 —> S_1 direction but not in the S_1 —> S_4 direction.

We conclude that ecotropic MuLVs bind to a conformationally mobile site on mouse y^+. Indeed, this site with bound gp70 evidently continues to change conformation during amino acid transport at a rate of several hundred times per second. Conceivably, this could perturb stability of the viral membrane in a manner necessary for infection.

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Manuscript #4

Glycosylation of a Cell Surface Receptor Blocks Its Interaction With a Retrovirus

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Summary: The cell surface receptor (ecoR) for ecotropic host-range [infection limited to mice or rats] murine leukemia viruses (ecoMuLVs) is the widely-expressed transporter (system y^+) for cationic amino acids lysine, arginine, and ornithine (1,2). As with other retroviruses (3-6), infection by ecoMuLVs eliminates functional virus-binding receptors from cell surfaces and results in complete interference to superinfection. Surprisingly, however, infection causes only partial (50-60%) loss of mouse y^+ transporter from cell surfaces (7). The ecoR contains 14 hydrophobic potential membrane-spanning sequences, and there is evidence that extracellular loop 3 is required for virus binding; loop 3 also contains two potential sites for N-linked glycosylation (8). Site-directed mutagenesis studies demonstrate that these glycosylations are not required in a positive manner for ecoR/y^+ processing or functions. However, ecoR proteins that lack the glycosylation site (site 2) closest to the virus-binding site are quantitatively eliminated from cell surfaces by infection with ecoMuLVs. These results suggest that wild-type mouse ecoR/y^+ is heterogeneously glycosylated, that only a portion is able to bind virus, that this portion is down-modulated by infection, and that elimination of glycosylation site 2 renders the protein fully interactive with virus. Loop 3 glycosylation sites and adjacent sequences are also highly divergent in mammals. These results demonstrate a new role for glycosylation, blocking the binding site of a virus receptor.

Several lines of evidence suggest that the proposed extracellular loop 3 of ecoR (8) contains a site necessary for ecoMuLV binding. The loop 3-encoding sequences of hamster and mink y^+ were amplified by messenger RNA-PCR and are shown in Fig. 1 compared with previously reported mouse (8) and human (9, 10) sequences. Although loop 3 sequences are highly divergent, each contains two N-X-(S/T) sites for potential N-linked glycosylation. Using

FIG 1. Predicted amino acid sequences of the third extracellular loop in ecoR among various species and the sequences of ecoR mutants. Sequences of the third extracellular loop in ecoR are indicated by the bar above the sequences. The two potential sites for N-linked glycosylation are indicated by asterisks. The putative virus binding site is underlined. M1, M2 and M3 are mouse ecoR mutants that lack of either or both sites for N-linked glycosylations by substituting N with E or V as indicated.

METHODS. Total RNA were extracted from Chinese hamster ovary cells or from mink lung fibroblasts by guanidinium thiocyanate method (11). Reverse transcription were performed in 20 ul of 1xPCR reaction buffer (Perkin Elmer Cetus) containing 1 ug of total RNA, 2.5 mM of each deoxyribonucleotide triphosphate, 20 units of RNasin, 2.5 uM of random hexamers, and 100 units of Mo-MuLV reverse transcriptase (BRL). Incubate 10 min at room temperature, then 30 min at 42°C. Heat the reaction at 95°C for 5 min, then quickly chill on ice and add PCR components as following: 22.5 ul of water, 3 ul of 10xPCR buffer, 20 pmoles of each primer (primer 1: 5'GATCGGATCCTGGAACCTGATTCTCTCCTAC 3'; primer 2: 5'GATCGAATTCCATCTGGTATACCAGATTAGG 3'), 2.5 units of Taq polymerase, and 50 ul of mineral oil on the top of reaction. The PCR was run at 94°C for 30 sec, 50° C for 1 min, 72°C for 1.5 min and was run for 30 cycles. The amplified PCR fragments (nucleotide 538 to 1506) (8) were subcloned and sequenced by standard dideoxyribonucleotide termination method (12). Site-directed mutagenesis was performed using the in vitro mutagenesis system from Amersham. Two synthetic oligodeoxyribonucleotide primers (5'CTCACGGAGAAAGAATTCTCCT 3' and 5'GTAACAACGTCGACACAAACGTG 3') were used in the mutagenesis reaction. The mutations in M1, M2 and M3 were then verified by sequencing.

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                 ------------------------------------
                                *        *
human    CINVLVLGFIMVSGFVKGSVKNWQLTEEDFGNTSGRLCLNNDTKEGKPGVGGFMPFGFSGVLSGAATCFY
mink     CVNVLVLGFIMVSGFVKGSIKNWQLTEEDFQNTSSHRCLSNDTKQGTLGAGGFMPFGFSGVLSGAATCFY
hamster  CINVLVLCFIMVSGFVKGSIKNWQLTEEDFLNRSSPLCGNNDT.NVKHGEGGFMPFGFSGVLSGAATCFY
mouse    CINVLVLCFIVVSGFVKGSIKNWQLTEK...NFS...CNNNDT.NVKYGEGGFMPFGFSGVLSGAATCFY
M1       ...............................E......................................
M2       ........................................V.............................
M3       ...............................E........V.............................
```

the Nco I site sequences from nucleotide 759 to 1272 (8), we constructed a mouse-hamster y^+ chimera that contained this substituted hamster region. The y^+ chimera was processed to cell surfaces and it functioned in amino acid transport; however, this chimeric y^+ did not bind the gp70 envelope glycoprotein purified from an ecoMuLV or mediate virus infection (unpublished results). Cunningham and co-workers (personal communication) previously found that the YGE loop 3 sequence of mouse y^+ was essential for virus binding and for infection, and that a human y^+ with YGE replacing the PGV sequence (see Fig. 1) was a functional virus receptor.

To study the role of N-linked glycosylation in ecoR functions, we constructed mutants that lack these N-X-(S/T) sites (see Fig. 1). Mutant 1 (M1) lacks site 1, mutant 2 (M2) lacks site 2, and mutant 3 (M3) lacks both sites. Each y^+ mutant was then verified by sequencing and was expressed in mink CCL64 fibroblasts using a retroviral vector as described previously (13). The expressive cells were all susceptible to infection by a helper-free ecotropic pseudotype of MSV-*neo* virus (14), and this enabled us to isolate the cell clones by G418 selection (13). All of the tested cell clones bound ecotropic gp70, and we selected clones for further study that were high-expressors by this criterion (see Table I). As also indicated, the mink cell clones that express ecoR mutants 1, 2, and 3 were also highly susceptible to infection by an ecotropic host-range helper-free virus that encodes human growth hormone (16). Thus, ecoR mutant proteins 1, 2, and 3 are processed to cell surfaces where they function as viral receptors. As summarized in Table I and described further below, the wild-type and mutant ecoRs were also all active as y^+ transporters. Samples of each of these cell clones were infected with the replication-competent Friend strain of ecoMuLV. The ecoMuLV infections were complete (greater than 99% of cells infected) as determined by immunofluorescence assay for viral antigens.

Table I. Characteristics of Wild-type and Mutant Murine Ecotropic Receptors Expressed in Mink Cells

ecoR	gp70 binding[1] (cpm/ugprotein)	m.o.i[2]	mouse y^+ activity [3]		
			Vmax (nmol/min.mgprotein)	Km (uM)	N[4]
wild type	23.6 ± 0.6	0.69 ± 0.05	5.5 ± 0.9	120 ± 18	7
M1	25.7 ± 2.0	0.68 ± 0.07	5.7 ± 0.9	141 ± 32	4
M2	13.3 ± 0.3	0.56 ± 0.07	3.3 ± 0.2	154 ± 38	6
M3	19.0 ± 1.1	0.63 ± 0.05	2.7 ± 0.5	141 ± 19	4

1. Quantitative gp70 binding assay was done by sequentially incubating 2×10^5 cells with purified gp70 (15) (4ug/ml) for 2 h, with antibody against gp70 for 2 h, and with [^{125}I]-protein A (0.4 uCi/ml) for 30 min. All incubations were done at 37°C. The radioactivity bound to the cells was standardized by measuring the protein concentration of each culture. The results for each cell line were from three independent cultures and shown by means±s.e.m. The binding of gp70 to the control CCL64 cells was below detectable level (<0.02 cpm/ugprotein). The radioactivity bound was also below the detectable level when gp70 was eliminated during the sequential incubations.

2. Multiplicities of infection were measured by infecting cells with an ecotropic host-range virus that encodes human growth hormone. The infected cells were detected 48 h later by an immunofluorescence assay specific for human growth hormone. The multiplicities of infection were calculated from the fraction of infected cells as described elsewhere (13). These results demonstrated that the mink cells with these different ecoRs are similarly susceptible to infection. Control mink cells are completely resistant to infection by ecotropic retroviruses (13).

3. Mouse-specific y^+ activities were obtained by subtracting data points of arginine inflow into control mink CCL64 cells from the data points of arginine inflow into cells expressing the wild-type or mutant ecoRs, and by fitting the results to the Michaelis-Menten equation. Vmax and Km are given as means $\pm$ s.e.m. (N=4-7 as indicated in the table).

4. Number of independent experiments in the transport assay.

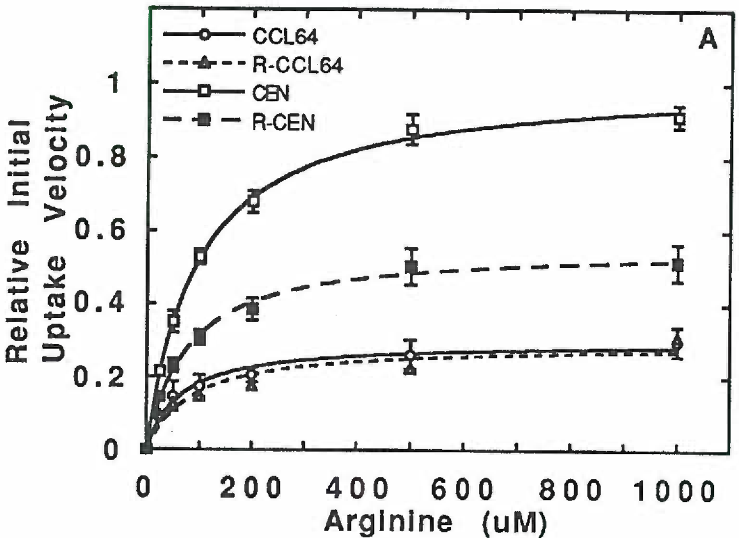
As reported previously for mink cells that express wild-type ecoR (13), these ecoMuLV-infected cells were all completely resistant to superinfection by the growth hormone-encoding virus described above.

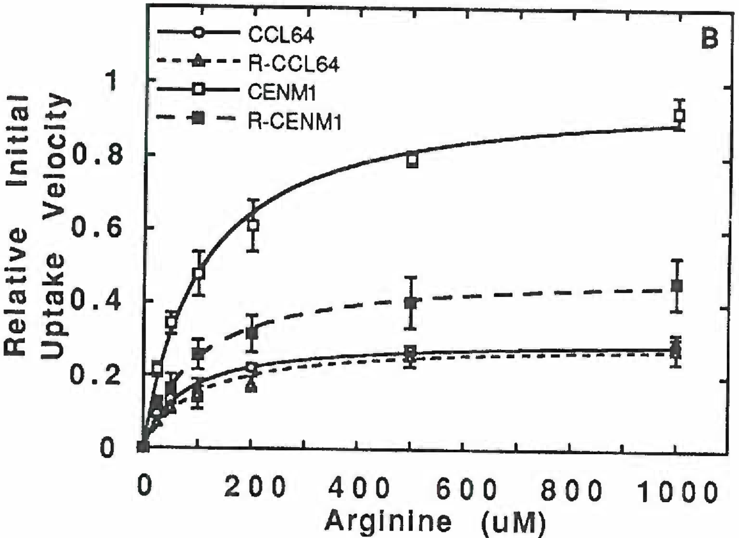
Fig. 2 shows results of our assays for L-[^{3}H]arginine uptake into these mink cell clones and into control CCL64 mink cells that were either uninfected or infected with Friend ecoMuLV. As described previously (7), this infection of control mink cells that lack ecoR was accomplished using a Friend ecoMuLV (from the retroviral packaging cell line PA12[17]) that had been pseudotyped with an amphotropic envelope; as expected, these infected mink cells released ecotropic host-range virions. The results in Fig. 2A are in agreement with our previous detailed analysis (7) of mink cells that contain wild-type ecoR. Specifically, these cells contain y^+ transporter activity above the background of endogenous mink y^+. Moreover, infection with ecoMuLV caused only partial (c.a., 60%) loss of wild-type mouse y^+, whereas the same infection caused no loss of the endogenous mink y^+. Thus, infection with ecoMuLV only down-modulates a y^+ transporter that can bind the virus. Figs. 2B, C, D show data for the mink cell clones that express ecoR mutants 1, 2, and 3, respectively. Although these computer-drawn best-fit curves to the Michaelis-Menten equation are similar to those in Fig. 2A, the down-modulations caused by infection were complete within experimental error in the cases of mutants 2 and 3. On the contrary, infection down-modulated mutant 1 y^+ transport activity by only approximately 70%.

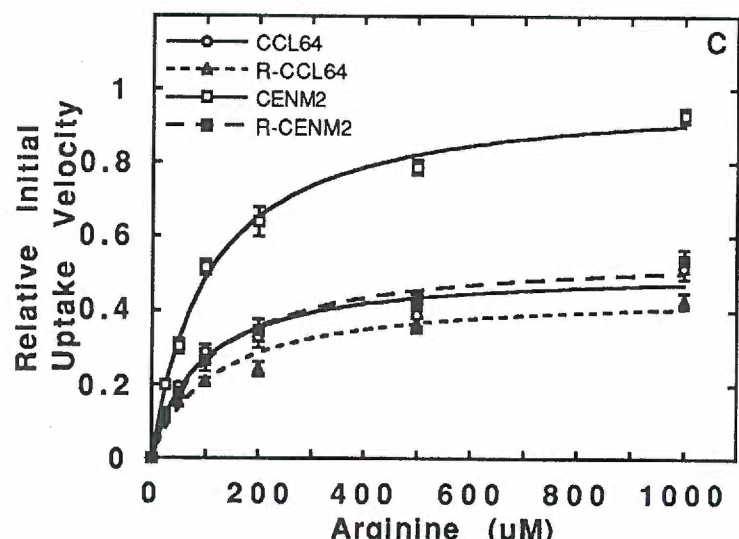
Table 2 summarizes many assays of L-[^{3}H]arginine uptake into all of these cells. By statistical analysis (paired-comparisons t-test [18]), it is clear that: (a) Infection with ecoMuLV causes only partial (c.a., 60%-70%) down-modulation of mouse wild-type or of mutant 1 y^+ uptake activities, with no effect on mink y^+. (b) Infection with ecoMuLV causes complete loss

FIG 2. Down-modulation of arginine transport in cells infected with Rauscher MuLV. CCL64 are mink lung fibroblasts. CEN, CENM1, CENM2, and CENM3 are CCL64 derivatives that express wild type ecoR, M1, M2, and M3 respectively. R-CCL64, R-CEN, R-CENM1, R-CENM2, and R-CENM3 are cells that have been chronically infected with Rauscher MuLV.

METHODS. Details of transport assay have been described elsewhere (Wang, 1992). Briefly, cells were exposed to different concentrations of L-[^{3}H] arginine (50 uCi/umole) at 37^{o}C for 30 sec. After rapidly washing twice with 2 ml ice-cold PBS, the radioactive arginine accumulated in the cells was extracted with 5% TCA, and the radioactivity was measured by a liquid scintillation counter. The rates of amino acid uptake for individual cultures were standardized to their protein concentrations and fitted by least squares to the Michaelis-Menten equation. Results of individual experiments were normalized by setting the Vmax measured in the uninfected CEN, CENM1, CENM2, or CENM3 as 1. Values were presented as means $\pm$ s.e.m. (n=4-6).


A
CCL64
R-CCL64
CEN
R-CEN
Relative Initial Uptake Velocity
0
0.2
0.4
0.6
0.8
1
0
200
400
600
800
1000
Arginine (uM)


B
CCL64
R-CCL64
CENM1
R-CENM1
Relative Initial Uptake Velocity
0
0.2
0.4
0.6
0.8
1
0
200
400
600
800
1000
Arginine (uM)


C
CCL64
R-CCL64
CENM2
R-CENM2
Relative Initial Uptake Velocity
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0.4
0.6
0.8
1
0
200
400
600
800
1000
Arginine (uM)

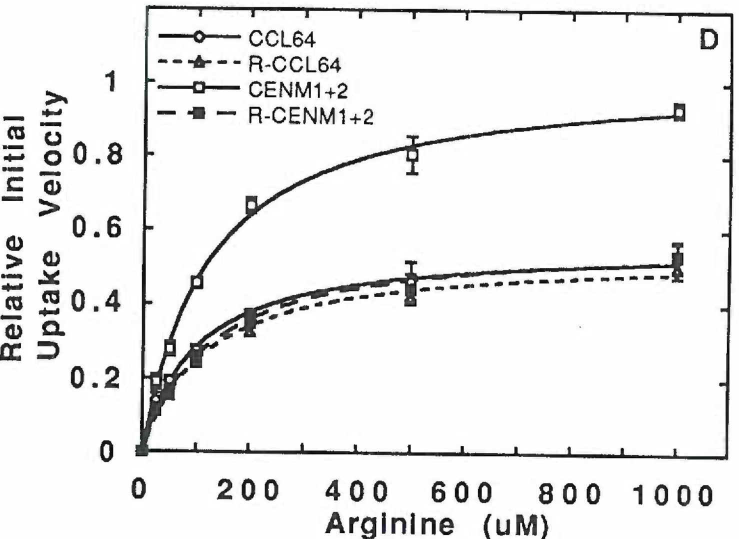
D
CCL64
R-CCL64
CENM1+2
R-CENM1+2
Relative Initial Uptake Velocity
0
0.2
0.4
0.6
0.8
1
0
200
400
600
800
1000
Arginine (uM)

Table II. Down-modulation of y^+ activity in cells infected with R-MuLV.

Cells Expressing Wild Type or Mutant ecoR	Relative Vmax			Down-modulation (%)[2]	N[3]
	uninfected	infected	control[1]		
Wild Type	1.00	0.57 ± 0.06	0.31 ± 0.05	57 ± 6	4
M1	1.00	0.49 ± 0.08	0.31 ± 0.02	73 ± 9	4
M2	1.00	0.56 ± 0.03	0.53 ± 0.03	95 ± 9	6
M3	1.00	0.57 ± 0.05	0.57 ± 0.05	104 ± 17	4

1. Control is the relative Vmax of arginine inflow into mink CCL64 cells. Paired comparison t-test (18) analysis indicated that mink cells expressing wild-type or M1 ecoRs and infected with R-MuLV have more y^+ activity than control CCL64 cells and is significant to 95% and 93% confidence, respectively. On the contrary, there is no significant difference between infected cells that express mutants M2 or M3 and control cells.

2. Percentage of down-modulation is calculated by the equation [(1-Vmax of infected cells)/(1-Vmax of control cells)]x100%.

3. Number of independent experiments.

of mutant 2 and 3 y^+ activities from cell surfaces. Thus, infection reduced these y^+ activities to the low level characteristic of the endogenous mink y^+.

These results suggest a simple model for virus binding onto mouse ecoR. Essentially, the site for virus binding may be partially blocked due to heterogeneity of N-linked glycosylation at site 2 (see Fig. 1). Such heterogeneity could result from inefficiency of glycosylation at this site and/or from the normal structural diversity of processed N-linked oligosaccharides (19). For example, many N-X-(S/T) sites in proteins are incompletely glycosylated (20, 21) and oligosaccharides at single glycosylation sites are generally extremely diverse (22). In the case of ecoR, it is conceivable that glycosylation at site 1 could sterically preclude subsequent glycosylation at site 2, and vise-versa, so that a family of different mouse y^+ proteins would result. Furthermore, after infection of the cells, the interference mechanism(s) (3-6, 23) would deplete the cell surfaces only of y^+ proteins that could interact with viral gp70. Indeed, our results show that mink y^+, which cannot bind gp70, is not down-modulated by ecoMuLV infection. In the case of wild-type mouse ecoR/y^+, the infected cells would be completely resistant to superinfection although they would retain sufficient y^+ transporter (c.a., 40% of the original amount) for viability. However, since mutants 2 and 3 lack site 2 glycosylation, all of the encoded y^+ would interact with virus and be down-modulated in infected cells. Although we propose that the ecoR down-modulation that we have detected is caused by loss of ecoR from cell surfaces, we emphasize that it may remain on cell surfaces in a form incapable of functioning either as a transporter or as a viral receptor. Receptors for other retroviruses are known to be removed from surfaces of infected cells (3-6, 23). In any case, we conclude that N-linked glycosylation of mouse y^+ controls its down-modulation caused by infection.

Glycosylation at site 2 may help to preserve enough y^+ transporter to ensure viability of mice endemically infected with ecoMuLVs.

There is a fascinating diversity in loop 3 sequences and glycosylation sites of y^+ from different species (see Fig. 1), consistent with the hypothesis that this protein sequence may have been a battleground in an evolutionary struggle between retrovirus and host. In this context, the hamster sequence is especially interesting because hamster cells become susceptible to ecoMuLVs after treatment with tunicamycin (24, 25), a drug that blocks N-linked glycosylation of proteins but has several side-effects (26). We have confirmed these results, and have also found that N-glycanase treatment of Chinese hamster ovary cells renders them susceptible to infection by ecoMuLVs (unpublished results). Because of the sequence diversity in the extracellular loop 3 (Fig.1), the glycosylation in the hamster ecoR homologue could differ from the glycosylation in murine ecoR. These results imply that N-linked glycosylation may control interaction of y^+ with ecoMuLV not only in mice but also in other species; we are currently testing this possibility.

Our results suggest a role for protein glycosylation in blocking interaction of a virus with its receptor. By a related mechanism, influenza A host-range properties appear to be controlled by species differences in structures of sialic acid receptors (27, 28, 29). Further studies will be required to understand the details of this mechanism of ecoR receptor blockade and its applicability to other viruses.

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Discussion and Conclusions

Different amounts of ecoR were expressed by a retroviral vector in cells from various species such as human, mink and hamster which do not naturally express ecoR. The abilities of these cells to bind the ecotropic viral glycoprotein gp70 and to be infected with ecotropic virus was demonstrated. Thus, cells expressing high level of ecoR bind more gp70 than cells expressing low levels of ecoR. Although these cells differentially bound gp70, they were equally susceptible to infection by an ecotropic host-range virus. Although this result might imply that virus infections were limited at a post-adsorption step, this was clearly not the case. In the conditions of our studies, virus adsorption onto functional receptors was the limiting step of infection. Consequently, we conclude that cells with different amounts of recombinant ecoR all express the same amount of functional ecoR. These results suggest that there is a limiting accessory factor that cooperates with ecoR for infection of cells by ecotropic virus. This limiting factor could be a subunit of the receptor, or an accessory protein such as a modifying enzyme that activates the receptor.

Expressing CD4 in murine cells and some human cells does not confer susceptibility to HIV infection (15, 45). It is possible that similar accessory factors are involved in the infection with HIV or with ecotropic murine retroviruses. In fact, Hela cells that express different amounts of CD4 on their surfaces are equally susceptible to HIV infection (D. Kabat, unpublished results). Elucidating the nature of the unknown limiting accessory factor(s) involved in retroviral infection needs further investigation.

Several attempts were made to obtain antiserum against ecoR. These attempts included using synthetic peptides, E.coli fusion proteins and a vaccinia virus expression system. Two synthetic peptides, one from the

amino terminus (KNLLGLGQQMLR), the other from the carboxyl terminus (SEEASLAAGQAKTPDSNLDQCK) of ecoR, were used to immunize rabbits. None resulted in a ecoR-specific antiserum. ecoR amino acid sequences from 188 to 353, 425 to 552, and 553 to 622 have been successfully expressed as malE fusions in E.coli using pMAL-c vector (New England Biolabs, Beverly, MA). The ecoR amino acid sequence from 553 to 622 was also expressed as a trypLE fusion protein using pNCV vector. Injection of rabbits with these fusion proteins resulted in antisera against the fusion proteins. However, none of these produced antisera could detect any ecoR-specific proteins by various approaches including Western blotting, immunoprecipitation and immunofluorescence. In collaboration with Dr. Gary Thomas, we constructed a vaccinia virus encoding ecoR. Infection of non-murine cells with this vaccinia virus resulted in the expression of ecoR and adsorption of gp70 onto these cells. However, inoculation of the vaccinia virus encoding ecoR into rabbits did not result in an ecoR-specific antiserum. In summary, despite numerous efforts, we have not succeeded in making ecoR-specific antibody. At least two possibilities could explain the failure. First, ecoR might not be very immunogenic in rabbits since it is a very conservative protein among mammalian species such as mouse, human, hamster and mink (see Appendix II). Secondly, the antiserum produced against ecoR might not be able to recognize the protein extracted from cell culture because the conformation of the immunogen could be different from the conformation of the protein existing in each experimental assay. Raising antibody against ecoR in non-mammalian species such as chickens (116, 117), or modifying the antigen before injection could increase the immunogenecity of the antigen and induce a stronger immune response (118). It would be important and necessary to have an ecoR- specific antibody in order to

biochemically characterize the size, heterogeneity, glycosylation, phosphorylation and processing of ecoR. In addition, ecoR-specific antibody could help to identify the putative accessory factor(s) involved in ecoR-mediated viral infection. For instance, cross-linking experiments could be used to identify molecules that are physically associated with ecoR.

By expressing ecoR in Xenopus laevis oocytes using in vitro synthesized ecoR mRNA, we have demonstrated with both electrophysiological and chemical methods that ecoR is a major mammalian transport system for basic amino acids, generally known as system y^+. ecoR is the first molecularly cloned mammalian amino acid transporter and the first instance of a virus subverting a transporter molecule as its receptor.

The predicted protein sequence of the putative receptor for the gibbon ape leukemia virus (GALV-R) also contains multiple membrane-spanning domains and resembles the sequence of transporter molecules (18). In fact, it was found that GALV-R is similar in sequence to a phosphate transporter in yeast (20). We have made GALV-R mRNA with SP6 RNA polymerase using an in vitro transcription system. Oocytes injected with these mRNA did not show any enhanced phosphate uptake with either electrophysiological or chemical measurements. Further investigation is needed to clarify whether the receptor for GALV is indeed a phosphate transporter.

Tea, a gene originally isolated from a T-cell lymphoma by MacLeod et al, was found to have extensive sequence similarity to ecoR (111). However, the existing cDNA clone of Tea appeared not to represent a full length clone, apparently lacking the 5' sequence of the gene. We have isolated a full length cDNA clone, designated as mlTea, from a mouse liver library using the existing Tea as a probe (see Fig.1 in Appendix I). The mlTea encodes a protein of 657 amino acids which includes a sequence of 69 amino acids at the amino

terminus that is not present in MacLeod's Tea clone. Interestingly, there is a sequence of 43 amino acids (355-397) in mlTea which is different (53.5% identical and 90.7% similar) from the corresponding sequence in MacLeod's Tea although the rest of the sequence is identical in both clones (Appendix I, Fig. 1, and 2). This divergent sequence in Tea happens to correspond to the exonVI of ecoR. It is likely that Tea and ecoR are evolutionary related and that the two versions of Tea might be the result of alternative splicing.

There is extensive sequence similarity between mlTea and ecoR at both DNA and amino acid levels. The amino acid sequence of mlTea is 58.1% identical and 75.5% similar to that of ecoR. Also, the topology of mlTea protein is extremely similar to that of ecoR and resembles a transporter molecule (Appendix I, Fig.3 and 4). Because of the structural similarity, Tea may also encode a transporter for amino acids. In fact, preliminary results have shown that Tea also encodes a transporter for cationic amino acids (unpublished results). These results indicate that there is a family of y+ transporters. Further studies on the mechanism of amino acid transport by this family of transporters will now be possible by constructing site-specific mutants and chimeras between Tea and ecoR.

The Tea gene has been mapped to chromosome 8 (111) as has the gene encoding the receptor for amphotropic murine leukemia virus (104). It is reasonable to think that Tea, homologous to ecoR, might encode the receptor for the amphotropic murine leukemia virus. If this is true, Tea and ecoR would provide an extremely useful model to study virus-host interactions and to elucidate the mechanisms that control host-range specificity in retroviral infections. However, our preliminary results suggest that Tea is not an amphotropic receptor.

MacLeod's Tea was isolated from mouse lymphoma while mlTea was isolated from normal mouse liver. As described earlier, there is a sequence-divergent region between MacLeod's Tea and the mlTea we isolated. The products of the two versions of Tea may function differently because of this sequence diversity. In fact, it was shown that there was no measurable y+ activity in mouse liver unless the liver cells are transformed (119, 120). It has also been known that mouse liver cells are not susceptible to ecotropic virus unless they are transformed (121). Additionally, the ecoR transcripts could not be detected in liver RNA (17). Therefore it is possible that there is another version of ecoR in mouse liver, perhaps functionally and structurally different from the ecoR clone isolated from mouse fibroblasts.

Incubation of gp70 with oocytes expressing ecoR at 25^{o}C did not have any effect on cationic amino acid transport in these oocytes. These results are not conclusive however, since gp70 binding is a time- and temperature-dependent event (43, 115, 122). Using mink fibroblasts that express a high level of ecoR, we have shown that chronic infection with ecotropic MuLV causes 50-70% down-modulation of the mouse y^+ transporter. Although the down-modulation of the transport activity of ecoR is not complete, viral interference in the infected cells appears to be total. We conclude that viral interference does not result from a complete removal of y^+ from the surfaces of infected cells. Possible mechanisms for viral interference include the blocking of receptor sites by gp70 expressed on the surfaces of infected cells or the inactivation of receptors by a posttranslational modification such as glycosylation (see blow).

The third extracellular loop of ecoR, which is believed to contain the site for virus attachment, also contains two potential sites for N-linked glycosylation. It is possible that the heterogenously structured

oligosaccharides on these sites could affect virus binding and infection. It has been shown that treatment of hamster cells with tunicamycin, an inhibitor of N-linked glycosylation (123, 124), or with N-glycanase (Appendix II, Fig.1), causes these cells to become susceptible to ecotropic viruses. In addition, CHO15B (125), a hamster mutant cell line that is defective in N-linked glycosylation due to absence of glycoprotein N-acetylglucosaminyltransferase activity is highly susceptible to ecotropic virus (Appendix II, Fig.1)

To investigate the possible roles of N-linked glycosylation in controlling viral infection, we have made ecoR mutants that lack of the first, second or both sites for N-linked glycosylation, designated as M1, M2, and M3 respectively . These mutants were expressed in mink cells by a retroviral vector. The cells expressing these mutants bind significant amounts of gp70 and can be infected with a multiplicity of infection similar to cells expressing wild type ecoR. A mouse y^+ transport activity was also measured in the mink cells expressing M1, M2 or M3. Interestingly, in contrast to cells expressing wild type ecoR or the M1 mutant, infection appeared to completely down-modulate mouse y^+ activity in cells expressing M2 or M3. These results demonstrate that the second N-glycosylation on the third extracellular loop of ecoR has an inhibitory effect on the virus-host interaction, presumably through interfering with virus binding.

These important findings imply that the host may have utilized glycosylation as a mechanism to protect itself from chronic viral infection. By this mechanism, the infected cells preserve a substantial quantity (c.a., 40%) of the indispensable physiological function of the cell surface protein that has been exploited by the virus as its receptor.

To further investigate the role of glycosylation in determining host-range specificity of retroviral infection, we have isolated the homologous sequence

of mouse ecoR (amino acids 122 to 436) from hamster and mink cells by reverse transcription-PCR (126, 127) using RNA extracted from Chinese hamster ovary cells and from mink lung fibroblasts. The hamster and mink sequences appeared to be very similar to the mouse ecoR sequence except for the sequence in the third extracellular loop of ecoR (see Appendix II, Fig. 2 and 3). The 314 amino acid sequences of hamster and mink isolated by PCR are 92.2% and 90.3% identical to that of mouse, respectively. However the amino acid sequence of the third extracellular loop has only 66.6% and 50.0% identity between hamster and mouse or mink and mouse. The previously reported sequence of human ecoR homologue is also very similar to that of mouse except for the extracellular loop 3 region (112, 113). Although the number of potential sites for N-linked glycosylation are conserved in all species from which ecoR has been isolated, the two sites are separated by eight amino acids in hamster, mink and human in contrast to five amino acids in mouse because of an insertion of nine nucleotides in the nonmurine sequences. The sequence diversity in the third extracellular loop of ecoR suggests the importance this region may have played in the evolutionary struggles between host and virus. Whether the glycosylation in nonmurine species also regulates virus binding as it does in mice awaits further investigation.

Recently, cDNA isolated from rabbit or rat was found to induce a transport system for neutral and dibasic amino acids(128, 129). The predicted amino acid sequence of the isolated cDNAs represents a membrane protein with a single membrane-spanning domain and is highly homologous to the 4F2 heavy-chain cell surface antigen and to a family of α-glucosidases. Moreover, expression of the human 4F2 surface antigen in *Xenopus laevis* oocytes stimulates the y^+-like amino acid transport system(130). The 4F2 antigen is

homologous to a family of α-glucosidases and is known to be involved in regulating cell growth in a way that has not yet been determined. Stimulation of y^+ by 4F2 antigen indicates that 4F2 might function as a regulator for the y^+ transporter, perhaps by altering glycosylation of the transporter, or by acting as a regulatory subunit of an heterooligomeric transporter. It is possible that N-linked glycosylation may not only regulate the receptor function but also modulate the transport function of ecoR/y^+.

As discussed earlier, there is an unidentified accessory factor that limits infection of cells with ecotropic virus. It is possible that 4F2 antigen, the regulator of the y^+ system, also regulates the entry of ecotropic virus which is known to use the y^+ transporter as its receptor. If the 4F2 antigen is the limiting accessory factor, we would predict that overexpression of the 4F2 antigen in cells that express high levels of ecoR would result in a higher multiplicity of infection than in cells that express low levels of ecoR. This hypothesis could be tested using the hamster or human clones that express different amounts of ecoR which have been described in Manuscript #1. The results of these studies would be important because they could demonstrate that molecules other than the receptor itself may control viral entry, perhaps at the step of viral penetration.

We have shown that viral infection down-modulates the transport activity of ecoR. It remains unknown whether the transport of amino acids affects viral infection. The answer to this question will be important because it will show whether conformational changes of the transporter that occur during the amino acid transport cycle are directly linked to the process of viral entry. We showed that gp70 caused a slight inhibition on the uptake of arginine, but had no effect on the outflow of arginine. The effects of gp70 on transport kinetics suggest that the virus binding site on ecoR is in a mobile region that

changes conformation during the amino acid transport cycle (Manuscript #3). If this is true, one would predict that the transport of amino acids could affect the kinetics of viral infection. It would be interesting to know whether the infection with ecotropic virus is stimulated or inhibited by the presence of cationic amino acids using cells that had been depleted of amino acids prior to infection. Results from these studies could provide further insights into our understanding of basic mechanisms involved in the entry of a retrovirus, particularly the process of viral penetration.

The following conclusions are made based upon the studies performed in this thesis:

(1) Characterization of the receptor for the ecotropic MuLV indicates that an accessory factor limits viral infection.

(2) The receptor for the ecotropic MuLV is the transporter for basic amino acids, generally known as system y^+.

(3) Infection with ecotropic virus partially down-modulates mouse y^+, with no effect on mink y^+.

(4) The slight inhibition by gp70 on the uptake but not on the outflow of arginine indicates that the virus binding site on ecoR is in a mobile region that changes conformation during the amino acid transport cycle.

(5) Elimination of the second site for N-linked glycosylation in the third extracellular loop of ecoR renders a receptor fully interactive with virus.

(6) The sequence diversity among various species in the third extracellular loop of ecoR indicates the importance of this region in the evolutionary struggles that may have developed between the host and the virus.

(7) Studies on Tea indicate that there is a family of y^+ transporters.

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Appendix I. A Gene Related to ecoR: Tea

Figure 1. Nucleotide and deduced amino acid sequence of Tea cDNA isolated from mouse liver (mlTea). The (▾) symbols indicate a site in which G is substituted by T at nucleotide 717, and a site in which a T is inserted at nucleotide 1093 when the sequence is compared with the partial mouse T lymphocyte Tea sequence isolated by MacLeod et al (111). The divergent region between the two versions of Tea is indicated by a dotted line above the nucleotide sequence. Four potential sites for N-linked glycosylation at amino acids 157, 227, 239 and 456 are indicated by asterisks. The putative membrane-spanning domains are underlined.

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   1  CTACGATGTTAGAGCCCAAGTCTCCTAAGGCCAAGGACAGGGGGCGAGACTTGCAGGCAG
  61  CAATCTCCTGAGGTTGTGTTACTGTTGATGGGTATGAGGAAGTTGAAGGGAGCAACACCC
 121  CACACCAGCAGGTCATTGTACAGGTGTGCAGCCAGTGAAGTACAAGACTGACACACAACA
 181  CACTCCACCCCTCCTCCCTGACTGCCTTCAGTCTGCTGAACTGTGTGTCCCTGTAGCTAA
 241  CACCAAATAGACCATTTAAATTTACAGTATTTCTTTTGAACTTAGGTTGTACATATTCCT
 301  TGGAATTAATGTGGAGATAGATAGTAATAATAATATGTGTTGATGAAATACATAGAAGTA
 361  AGAAATTAACTCACCATCAAATTCTTAAGCCTCCTGGGTGCTCTGAACCAAGTATCTATT
 421  AAAGAGAGGCCCACTGCCCTTCTGTGTTCAACGGGAGGGGAGAAGGCCTCAACCCCCTCC
 481  CCTCCCCCCTCTCCTACAGGTTGCTCTCTTCCACCATGATTCCCTGCAGAGCAGTGCTGA
                                                M  I  P  C  R  A  V  L      8

 541  CTTTCGCGCGATGTCTGATCCGGAGAAAAATTGTCACACTGGACAGCCTTGAAGATTCCA
      T  F  A  R  C  L  I  R  R  K  I  V  T  L  D  S  L  E  D  S     28

 601  AACTCTGCCGCTGCTTAACCACCGTGGACCTCATCGCTTTGGGGGTTGGAAGCACTCTGG
      K  L  C  R  C  L  T  T  V  D  L  I  A  L  G  V  G  S  T  L     48

 661  GCGCTGGGGTCTACGTCCTGGCTGGGGAAGTCGCCAAAGCCGATTCTGGCCCGAGTATCG
      G  A  G  V  Y  V  L  A  G  E  V  A  K  A  D  S  G  P  S  I     68
      ▼
 721  TGGTGTCTTTCCTCATCGCTGCCCTGGCCTCGGTTATGGCCGGCCTTTGCTATGCTGAAT
      V  V  S  F  L  I  A  A  L  A  S  V  M  A  G  L  C  Y  A  E     88

 781  TTGGGGCCCGAGTACCCAAGACTGGATCTGCGTATCTATACACTTACGTCACGGTCGGAG
      F  G  A  R  V  P  K  T  G  S  A  Y  L  Y  T  Y  V  T  V  G    108

 841  AGCTGTGGGCCTTCATCACTGGCTGGAATCTCATCCTGTCATATGTCATAGGTACGTCCA
      E  L  W  A  F  I  T  G  W  N  L  I  L  S  Y  V  I  G  T  S    128

 901  GTGTCGCAAGAGCATGGAGTGGCACCTTTGACGAACTTCTTAATAAACAGATTGGCCAGT
      S  V  A  R  A  W  S  G  T  F  D  E  L  L  N  K  Q  I  G  Q    148

 961  TTTTCAAAACGTACTTCAAAATGAATTACACTGGTCTGGCAGAGTATCCAGACTTCTTTG
      F  F  K  T  Y  F  K  M  N  Y  T  G  L  A  E  Y  P  D  F  F    168

1021  CCGTGTGCCTTGTATTACTCCTGGCAGGTCTTTTATCTTTTGGAGTAAAAGAGTCTGCTT
      A  V  C  L  V  L  L  L  A  G  L  L  S  F  G  V  K  E  S  A    188
                     ▼
1081  GGGTGAATAAATTTTTTACAGCTATTAATATCCTGGTCCTTCTCTTTGTCATGGTGGCTG
      W  V  N  K  F  F  T  A  I  N  I  L  V  L  L  F  V  M  V  A    208

1141  GGTTTGTGAAAGGAAATGTGGCTAACTGGAAGATCAGTGAAGAGTTTCTCAAAAATATAT
      G  F  V  K  G  N  V  A  N  W  K  I  S  E  E  F  L  K  N  I    228
                                                             *
1201  CAGCAAGTGCTAGAGAACCACCTTCTGAGAACGGAACAAGCATCTACGGGGCTGGCGGCT
      S  A  S  A  R  E  P  P  S  E  N  G  T  S  I  Y  G  A  G  G    248
                                      *
1261  TTATGCCCTATGGCTTTACAGGGACGTTGGCTGGTGCTGCAACGTGCTTTTATGCCTTTG
      F  M  P  Y  G  F  T  G  T  L  A  G  A  A  T  C  F  Y  A  F    268

1321  TGGGCTTTGACTGCATTGCAACAACCGGTGAAGAGGTTCGGAATCCACAAAAGGCGATCC
      V  G  F  D  C  I  A  T  T  G  E  E  V  R  N  P  Q  K  A  I    288

1381  CCATCGGAATAGTGACGTCCTTACTTGTCTGCTTTATGGCTTACTTTGGGGTTTCTGCAG
      P  I  G  I  V  T  S  L  L  V  C  F  M  A  Y  F  G  V  S  A    308

1441  CTTTAACGCTTATGATGCCTTACTACCTCCTGGATGAGAAAAGTCCACTCCCAGTCGCGT
      A  L  T  L  M  M  P  Y  Y  L  L  D  E  K  S  P  L  P  V  A    328

1501  TTGAGTATGTCAGATGGGGCCCCGCCAAATACGTTGTCGCAGCAGGCTCCCTCTGCGCCT
      F  E  Y  V  R  W  G  P  A  K  Y  V  V  A  A  G  S  L  C  A    348
                        ...............................................
1561  TATCAACAAGTCTTCTGGGTTCTATGTTCCCCTTACCCCGAATTCTGTTTGCCATGGCCC
      L  S  T  S  L  L  G  S  M  F  P  L  P  R  I  L  F  A  M  A    368
      ............................................................
1621  GGGATGGCTTACTGTTTAGATTTCTTGCAAGAGTGAGTAAGAGGCAGTCACCCGTTGCTG
      R  D  G  L  L  F  R  F  L  A  R  V  S  K  R  Q  S  P  V  A    388
      ...........................
1681  CCACGATGACTGCAGGGGTCATTTCTGCTGTGATGGCCTTTCTTTTTGACCTGAAGGCCC
      A  T  M  T  A  G  V  I  S  A  V  M  A  F  L  F  D  L  K  A    408

1741  TCGTGGACATGATGTCTATTGGCACCCTCATGGCCTACTCTCTGGTGGCAGCCTGTGTGC
      L  V  D  M  M  S  I  G  T  L  M  A  Y  S  L  V  A  A  C  V    428

1801  TTATTCTCAGGTACCAACCTGGCTTGTGTTACGAGCAGCCCAAATACACCCCTGAGAAAG
      L  I  L  R  Y  Q  P  G  L  C  Y  E  Q  P  K  Y  T  P  E  K    448

1861  AAACTCTGGAATCATGTACCAATGCGACTTTGAAGAGCGAGTCCCAGGTCACCATGCTGC
      E  T  L  E  S  C  T  N  A  T  L  K  S  E  S  Q  V  T  M  L    468
                            *
1921  AAGGACAGGGTTTCAGCCTACGAACCCTCTTCAGCCCCTCTGCCCTGCCCACACGACAGT
      Q  G  Q  G  F  S  L  R  T  L  F  S  P  S  A  L  P  T  R  Q    488

1981  CGGCTTCCCTTGTGAGCTTTCTGGTGGGATTCCTGGCTTTCCTCATCCTGGGCTTGAGTA
      S  A  S  L  V  S  F  L  V  G  F  L  A  F  L  I  L  G  L  S    508

2041  TTCTAACCACGTATGGCGTCCAGGCCATTGCCAGACTGGAAGCCTGGAGCCTGGCTCTTC
      I  L  T  T  Y  G  V  Q  A  I  A  R  L  E  A  W  S  L  A  L   528

2101  TCGCCCTGTTCCTTGTCCTCTGCGCTGCCGTCATTCTGACCATTTGGAGGCAGCCACAGA
      L  A  L  F  L  V  L  C  A  A  V  I  L  T  I  W  R  Q  P  Q    548

2161  ATCAGCAAAAAGTAGCCTTCATGGTCCCGTTCTTACCGTTTCTGCCGGCCTTCAGCATCC
      N  Q  Q  K  V  A  F  M  V  P  F  L  P  F  L  P  A  F  S  I    568

2221  TGGTCAACATTTACTTGATGGTCCAGTTAAGTGCGGACACTTGGATCAGATTCAGCATCT
      L  V  N  I  Y  L  M  V  Q  L  S  A  D  T  W  I  R  F  S  I    588

2281  GGATGGCGCTTGGCTTTCTGATCTATTTCGCCTATGGCATTAGACACAGCTTGGAGGGTA
      W  M  A  L  G  F  L  I  Y  F  A  Y  G  I  R  H  S  L  E  G    608

2341  ACCCCAGGGACGAAGAAGACGATGAGGATGCCTTTTCAGAAAACATCAATGTAGCAACAG
      N  P  R  D  E  E  D  D  E  D  A  F  S  E  N  I  N  V  A  T    628

2401  AAGAAAAGTCCGTCATGCAAGCAAATGACCATCACCAAAGAAACCTCAGCTTACCTTTCA
      E  E  K  S  V  M  Q  A  N  D  H  H  Q  R  N  L  S  L  P  F    648

2461  TACTTCATGAAAAGACAAGTGAATGTTGATGCTGGCCCTCGGTCTTACCACGCATACCTT
      I  L  H  E  K  T  S  E  C  *

2521  AACAATGAGTACACTGTGGCCGGATGCCACCATCGTGCTGGGCTGTCGTGGGTCTGCTGT
2581  GGACATGGCTTGCCTAACTTGTACTTCCTCCTCCAGACAGCTTCTCTTCAGATGGTGGAT
2641  TCTGTGTCTGAGGAGACTGCCTGAGAGCACTCCTCAGCTATATGTATCCCCAAAACAGTA
2701  TGTCCGTGTGCGTACATGTATGTCTGCGATGTGAGTGTTCAATGTTGTCCGTTATTAGTC
2761  TGTGACATAATTCCAGCATGGTAATTGGTGGCATATACTGCACACACTAGTAAACAGTAT
2821  ATTGCTGAATAGAGATGTATTCTGTATATGTCCTAGGTGGCTGGGGAAATAGTGGTGGTT
2881  TCTTTATTAGGTATATGACCATCAGTTTGGACATACTGAAATGCCATCCCCTGTCAGGAT
2941  GTTTAACAGTGGTCATGGGTGGGGAAGGGATAAGGAATGGGCATTGTCTATAAATTGTAA
3001  TGCATATATCCTTCTCCTACTTGCTAAGACAGCTTTCTTAAACGGCCAGGGAGAGTGTTT
3061  CTTTCCTCTGTATGACAAGATGAAGAGGTAGTCTGTGGCTGGAGATGGCCAATCCTGGTT
3121  TTGAGAGCCTTGGTCCTTAAATATAACACTTCTACCTGCTGTAAATGATATACCCATCAA
3181  CTCCTTGAAAGTCTCAGGAAAGGTACCGTGGCCTACTTGGGACCCTTTAAGCCAGAGAGT
3241  GAACGTAGTCCCAGTGGCTTCTAAAGATCAGATGACTTACAAAGATGCTTGGCAGAGACT
3301  GAAGGCAGAATAGGCCTCCGAATCACACATTTGGTAGACTGGTTCAGGCTGTTTCCCATT
3361  ACTTCAGACCTAATCACAGCTCAGCGTCTGCTCCTTCATCACAGAACCCAGTCTTTTGTT
3421  TCTCATCTTTCCTTTGATCTGGATGAAAACGAGTGTGGTAGCAAATTCTTTTTTTACTGT
3481  GGACAAAAAAGACACAGATGGAGGGGTATAGGAAAATGCCCTTAGTCCAGTCTCACAGGG
3541  CGCTTGAGCCCAGTTGAAGGATTTTAAACCTAATTTTCCTCTGATATTTCTTTTAGAAAT
3601  GTTTAGAGATTCTATCTATCTCCTACCCTATCTCATCATTTAGCACTTATAAATTTATTT
3661  TCTCCCTGTCTGTCTGTCCCC
```

Figure 2. Comparison of the amino acid sequences in the divergent region of mlTea and MacLeod's Tea. Comparison of the predicted amino acid sequence was done by using the GCG computer program (Genetics Computer Group, Madison, Wisconsin). The mlTea sequence is shown on the top. Identical residues are indicated by vertical lines between the sequences. The residues that have comparison values greater than or equal to 0.50 or 0.10 are indicated by two dots or one dot, respectively. The comparison value equals 1.00 when two residues are identical.

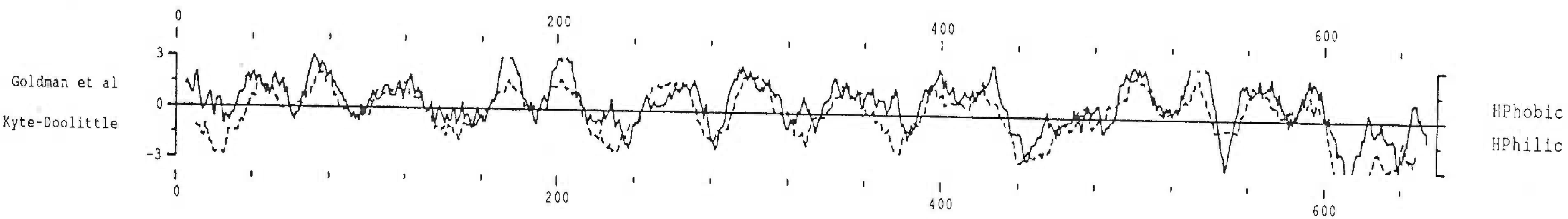
```
301 MAYFGVSAALTLMMPYYLLDEKSPLPVAFEYVRWGPAKYVVAAGSLCALS 350
    ||||||||||||||||||||||||||||||||||||||||||||||||||
301 MAYFGVSAALTLMMPYYLLDEKSPLPVAFEYVRWGPAKYVVAAGSLCALS 350

351 TSLLGSMFPLPRILFAMARDGLLFRFLARV.SKRQSPVAATMTAGVISAV 399
    ||||||:||:||:::||| |||||: ||.: || ..|| ||:..|.:.||
351 TSLLGSIFPMPRVIYAMAEDGLLFKCLAQINSKTKTPVIATLSSGAVAAV 400

400 MAFLFDLKALVDMMSIGTLMAYSLVAACVLILRYQPGLCYEQPKYTPEKE 449
    ||||||||||||||||||||||||||||||||||||||||||||||||||
401 MAFLFDLKALVDMMSIGTLMAYSLVAACVLILRYQPGLCYEQPKYTPEKE 450
```

Figure 3. Alignment of the predicted mlTea protein sequence with ecoR protein sequence. The predicted amino acid sequences of mlTea (top) and ecoR (bottom) are shown. The alignment was made using the GCG computer program.

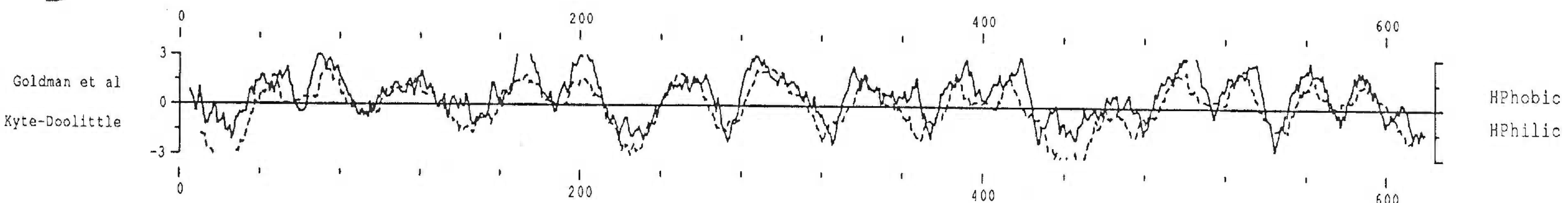
```
                .         .         .         .         .
  1 MIPCRAVLTFARCLIRRKIVTLDSLEDSKLCRCLTTVDLIALGVGSTLGA 50
     :.|:.:|.::. ::|||:|.  | |:|:|:|||.| ||:||||||||||
  1 .MGCKNLLGLGQQMLRRKVVD.CSREESRLSRCLNTYDLVALGVGSTLGA 48
                .         .         .         .         .
 51 GVYVLAGEVAKADSGPSIVVSFLIAALASVMAGLCYAEFGARVPKTGSAY 100
    |||||||.||:.:.||.||:||||||||||:|||||:|||||||||||||
 49 GVYVLAGAVARENAGPAIVISFLIAALASVLAGLCYGEFGARVPKTGSAY 98
                .         .         .         .         .
101 LYTYVTVGELWAFITGWNLILSYVIGTSSVARAWSGTFDELLNKQIGQFF 150
    ||.||||||||||||||||||||:||||||||||||:|||||:.|.||:|
 99 LYSYVTVGELWAFITGWNLILSYIIGTSSVARAWSATFDELIGKPIGEFS 148
                .         .         .         .         .
151 KTYFKMNYTG.LAEYPDFFAVCLVLLLAGLLSFGVKESAWVNKFFTAINI 199
    : .: :| .| ||: ||:|||.::::|.|||.:|||||| |||:||.||:
149 RQHMALNAPGVLAQTPDIFAVIIIIILTGLLTLGVKESAMVNKIFTCINV 198
                .         .         .         .         .
200 LVLLFVMVAGFVKGNVANWKISEEFLKNISASAREPPSENGTSI.YGAGG 248
    ||| |::|.|||||.: ||.:.|    ||:|.       .:|:|.: ||.||
199 LVLCFIVVSGFVKGSIKNWQLTE...KNFSC......NNNDTNVKYGEGG 239
                .         .         .         .         .
249 FMPYGFTGTLAGAATCFYAFVGFDCIATTGEEVRNPQKAIPIGIVTSLLV 298
    |||:||.|.|.|||||||||||||||||||||||:|||||||:|||.|||:
240 FMPFGFSGVLSGAATCFYAFVGFDCIATTGEEVKNPQKAIPVGIVASLLI 289
                .         .         .         .         .
299 CFMAYFGVSAALTLMMPYYLLDEKSPLPVAFEYVRWGPAKYVVAAGSLCA 348
    ||:||||||||||||||||: || .||||.||..  |:.|||.|| |||||
290 CFIAYFGVSAALTLMMPYFCLDIDSPLPGAFKHQGWEEAKYAVAIGSLCA 339
                .         .         .         .         .
349 LSTSLLGSMFPLPRILFAMARDGLLFRFLARVSKR.QSPVAATMTAGVIS 397
    |||||||||||:||:::||| |||||:|||::..| ..|| ||:|.|.|.
340 LSTSLLGSMFPMPRVIYAMAEDGLLFKFLAKINNRTKTPVIATVTSGAIA 389
                .         .         .         .         .
398 AVMAFLFDLKALVDMMSIGTLMAYSLVAACVLILRYQPG...LCYEQPKY 444
    |||||||:||.|||:||||||:|||||||||||:|||||:    |.|: ::
390 AVMAFLFELKDLVDLMSIGTLLAYSLVAACVLVLRYQPEQPNLVYQMART 439
                .         .         .         .         .
445 TPEKETLESCTNATLKSESQVTMLQ.GQGFSLRTLFSPSAL.PTRQSASL 492
    |.| : ::  .: .  ||||..:|. :: |||:.::||..: |.: |: :
440 TEELDRVDQ.NELVSASESQTGFLPVAEKFSLKSILSPKNVEPSKFSGLI 488
                .         .         .         .         .
493 VSFLVGFLAFLILGLSILTTYGVQAIARLEAWSLALLALFLVLCAAVILT 542
    |.: .|:|| ||:.::|:...| :|:|  . |.: ::.  ::||  |. .
489 VNISAGLLAALIITVCIVAVLGREALAEGTLWAVFVMTGSVLLCMLVTGI 538
                .         .         .         .         .
543 IWRQPQNQQKVAFMVPFLPFLPAFSILVNIYLMVQLSADTWIRFSIWMAL 592
    |||||:.. |:.|.|||:|.||.:||:|||||||:||..:||:||.:|| :
539 IWRQPESKTKLSFKVPFVPVLPVLSIFVNIYLMMQLDQGTWVRFAVWMLI 588
                .         .         .         .         .
593 GFLIYFAYGIRHSLE.....GNPRDEEDDEDAFSENINVATEEKSVMQAN 637
    || |||:|||:|| |      |.::..:.: |. .
589 GFTIYFGYGIWHSEEASLAAGQAKTPDSNLDQCK*
                .
638 DHHQRNLSLPFILHEKTSEC*
```

Figure 4. Hydrophobicity plots of mlTea predicted protein (top) and ecoR predicted protein (bottom).

A

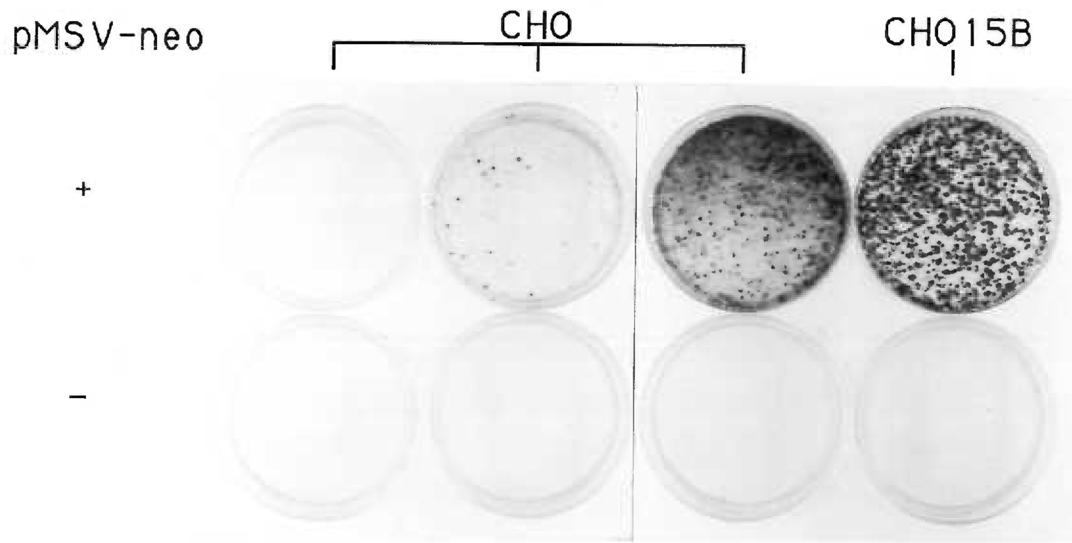


B



Appendix II. Role of N-linked Glycosylation in Controlling Infection of Hamster Cells by Ecotropic Mouse Retroviruses

Figure 1. G418 resistant colonies from hamster cells infected with ecotropic host-range pMSV-neo virus. As indicated in the figure, CHO cells were treated either with N-glycanase (Genzyme, Cambridge, MA) (1 unit/ml) in serum-free *a*-MEM for 6 h at 37°C or with tunicamycin (0.15 ug/ml) in *a*-MEM plus 10% FBS for 18 h at 37°C. Cells were then exposed to the pMSV-neo virus of ecotropic host-range, and were selected by G418. CHO15B, a CHO mutant that is defective in N-linked glycosylation due to absence of glycoprotein N-acetylglucosaminyltransferase activity (125) , were also exposed to pMSV-neo virus and were selected by G418. G418 concentrations used for selection were 500, and 300 ug/ml for CHO, and CHO15B cells, respectively. G418-resistant colonies appeared on the dishes after selection for 8 to 10 days and were stained with methylene blue. CHO cells become susceptible to infection after treatment with tunicamycin or N-glycanase, suggesting that N-linked oligosaccharide(s) may block a potential viral receptor. The 15B mutant cells are highly susceptible, also supporting this idea.



N-glycanase	−	+	−	−
tunicamycin	−	−	+	−

Figure 2. Nucleotide and deduced amino acid sequences of hamster and mink ecoR homologues. (A) Hamster sequences. (B) Mink sequences. Nucleotide sequences were obtained by RNA-PCR and by subsequent sequencing of the PCR products as described in details in Manuscript #4. Amino acid sequences were deduced from the nucleotide sequences by using GCG computer program. The sequences amplified and sequenced correspond to nucleotides 562 to 1485 (amino acids 122 to 436) of the full-length ecoR.

A.

```
ATCATTGGTACTTCAAGTGTGGCAAGAGCCTGGAGTGCGACTTTTGACGAGCTGATAGGC
 I  I  G  T  S  S  V  A  R  A  W  S  A  T  F  D  E  L  I  G

AAACCCATTGGAGAGTTCTCTCGGAAACACATGGCCCTGAATGCCCCTGGGGTGCTGGCC
 K  P  I  G  E  F  S  R  K  H  M  A  L  N  A  P  G  V  L  A

CAAAACCCTGACATATTAGCTGTGATTATAATTCTCATCTTAACAGGATTGTTAACGCTT
 Q  N  P  D  I  L  A  V  I  I  I  L  I  L  T  G  L  L  T  L

GGTGTGAAAGAATCAGCCATGGTCAACAAAATCTTTACCTGTATTAATGTTCTGGTCTTA
 G  V  K  E  S  A  M  V  N  K  I  F  T  C  I  N  V  L  V  L

TGCTTCATCATGGTGTCGGGATTCGTGAAAGGGTCCATTAAAAACTGGCAGCTCACGGAG
 C  F  I  M  V  S  G  F  V  K  G  S  I  K  N  W  Q  L  T  E

GAGGACTTCTTGAATAGATCCAGCCCTCTCTGTGGCAACAATGACACAAATGTAAAGCAT
 E  D  F  L  N  R  S  S  P  L  C  G  N  N  D  T  N  V  K  H

GGTGAGGGAGGGTTCATGCCCTTCGGATTCTCTGGCGTCCTGTCGGGGGCAGCGACCTGC
 G  E  G  G  F  M  P  F  G  F  S  G  V  L  S  G  A  A  T  C

TTTTACGCCTTTGTGGGCTTTGACTGCATTGCCACCACAGGTGAAGAAGTCAAGAACCCC
 F  Y  A  F  V  G  F  D  C  I  A  T  T  G  E  E  V  K  N  P

CAGAAGGCCATCCCCGTGGGCATCGTGGCGTCCCTCCTCATTTGCTTCGTAGCTTACTTC
 Q  K  A  I  P  V  G  I  V  A  S  L  L  I  C  F  V  A  Y  F

GGGGTGTCTGCAGCCCTCACGCTCATGATGCCTTACTCCTGCCTGGACACTGACAGCCCA
 G  V  S  A  A  L  T  L  M  M  P  Y  S  C  L  D  T  D  S  P

CTGCCTGGTGCCTTCAAGTACAGTGGCTGGGAAGGAGCTAAGTATGCAGTGGCTGTCGGC
 L  P  G  A  F  K  Y  S  G  W  E  G  A  K  Y  A  V  A  V  G

TCCCTCTGCGCACTCTCTGCCAGTCTCCTAGGCTCCATGTTTCCCATGCCCCGAGTAATC
 S  L  C  A  L  S  A  S  L  L  G  S  M  F  P  M  P  R  V  I

TACGCCATGGCTGAAGACGGACTACTGTTTAAATATTTGGCCAGAGTCAACAAGAGGACC
 Y  A  M  A  E  D  G  L  L  F  K  Y  L  A  R  V  N  K  R  T

AAAACACCAGTAATTGCTACACTGACCTCAGGCGCCATTGCTGCTGTGATGGCCTTCCTC
 K  T  P  V  I  A  T  L  T  S  G  A  I  A  A  V  M  A  F  L

TTTGAATTGAAGGACCTGGTAGACCTACTGTCCATTGGCACGCTCCTGGCTTACTCTTTG
 F  E  L  K  D  L  V  D  L  L  S  I  G  T  L  L  A  Y  S  L

GTGGCTGCCTGTGTATTAGTCTTAAGGTACCAGCCCGAGCAA
 V  A  A  C  V  L  V  L  R  Y  Q  P  E  Q
```

B.

```
ATCATCGGTACTTCAAGTGTAGCGAGAGCCTGGAGTGCGACGTTTGACGAGCTGATTGGC
 I  I  G  T  S  S  V  A  R  A  W  S  A  T  F  D  E  L  I  G

AATCCCATCAGGGAGTTCTCACGGACGCACATGGCCCTGAATGCCCCTGGAGTGCTGGCC
 N  P  I  R  E  F  S  R  T  H  M  A  L  N  A  P  G  V  L  A

GAAAACCCAGACATATTTGCTGTGATTATCATTCTCATCTTAACAGGACTTTTAACTCTC
 E  N  P  D  I  F  A  V  I  I  I  L  I  L  T  G  L  L  T  L

GGTGTGAAAGAGTCGGCCATGGTCAACAAAATATTCACTTGTGTCAATGTTCTGGTCTTG
 G  V  K  E  S  A  M  V  N  K  I  F  T  C  V  N  V  L  V  L

GGCTTCATAATGGTGTCAGGATTTGTGAAAGGATCGATTAAAAACTGGCAGCTCACAGAG
 G  F  I  M  V  S  G  F  V  K  G  S  I  K  N  W  Q  L  T  E

GAGGATTTCCAGAACACATCCAGTCATCGCTGCTTGAGCAATGACACAAAACAAGGGACG
 E  D  F  Q  N  T  S  S  H  R  C  L  S  N  D  T  K  Q  G  T

CTTGGCGCTGGTGGATTCATGCCCTTTGGATTCTCTGGTGTCCTGTCTGGGGCAGCGACG
 L  G  A  G  G  F  M  P  F  G  F  S  G  V  L  S  G  A  A  T

TGCTTCTATGCCTTCGTGGGCTTTGACTGCATCGCCACAACAGGTCAAGAGGTCAAGAAC
 C  F  Y  A  F  V  G  F  D  C  I  A  T  T  G  Q  E  V  K  N

CCTCAGAAGGCGATCCCCGTGGGGATTGTCGCGTCCCTCCTAATTTGCTTCATCGCTTAC
 P  Q  K  A  I  P  V  G  I  V  A  S  L  L  I  C  F  I  A  Y

TTCGGGGTGTCGGCTGCCCTCACACTCATGATGCCATACTTCTGCCTGGACAAGGATAGT
 F  G  V  S  A  A  L  T  L  M  M  P  Y  F  C  L  D  K  D  S

CCCTTGCCCGACGCCTTCAAGTATGTGGGCTGGGAAGGTGCCAAGTATGCGGTGGCCGTC
 P  L  P  D  A  F  K  Y  V  G  W  E  G  A  K  Y  A  V  A  V

GGCTCACTCTGCGCTCTTTCCACCAGTCTTTTGGGTTCCATGTTTCCTATGCCTCGAGTC
 G  S  L  C  A  L  S  T  S  L  L  G  S  M  F  P  M  P  R  V

ATCTATGCCATGGCTGAAGATGGACTGCTATTTAAATTTTTGGCCAAAATCAACGATAGG
 I  Y  A  M  A  E  D  G  L  L  F  K  F  L  A  K  I  N  D  R

ACCAAAACGCCAATAATTGCCACGTTGACCTCAGGTGCCATTGCTGCTGTGATGGCCTTT
 T  K  T  P  I  I  A  T  L  T  S  G  A  I  A  A  V  M  A  F

CTCTTTGACCTGAAGGACTTGGTGGACCTCATGTCGATTGGCACTCTCCTGGCTTACTCT
 L  F  D  L  K  D  L  V  D  L  M  S  I  G  T  L  L  A  Y  S

TTGGTGGCTGCCTGTGTGTTGGTCTTACGGTATCAGCCAGAGCAA
 L  V  A  A  C  V  L  V  L  R  Y  Q  P  E  Q
```

Figure 3. Alignment of deduced amino acid sequences of ecoR and its human, mink and hamster homologues. Symbols: (▼): amino acid insertions in non-murine species. (∇): amino acid insertions in mink and human. (*): potential sites for N-linked glycosylations. (Δ): The amino acid residues that are different in all four species listed. The amino acid residues that are identical among three or more than three species are boxed. The extracellular loop 3 regions that contain the YGE virus binding site of mouse ecoR occur between positions 214 and 246 as indicated by the arrows. This is the most divergent region of the sequences from these species.

```
         101                                                  150
human    SYVTVGELWA FITGWNLILS YIIGTSSVAR AWSATFDELI GRPIGEFSRT
mink     .......... ..........IIGTSSVAR AWSATFDELI GNPIREFSRT
hamster  .......... ..........IIGTSSVAR AWSATFDELI GKPIGEFSRK
mouse    SYVTVGELWA FITGWNLILS YIIGTSSVAR AWSATFDELI GKPIGEFSRQ

         151                                                  200
human    HMILNAPGVL AENPDIFAVI IILILTGLLT LGVKESAMVN KIFTCINVLV
mink     HMALNAPGVL AENPDIFAVI IILILTGLLT LGVKESAMVN KIFTCVNVLV
hamster  HMALNAPGVL AQNPDILAVI IILILTGLLT LGVKESAMVN KIFTCINVLV
mouse    HMALNAPGVL AQTPDIFAVI IIIILTGLLT LGVKESAMVN KIFTCINVLV

         201                                                  250
human    LGFIMVSGFV KGSVKNWQLT EEDFGNTSGR LCLNNDTKEG KPGVGGFMPF
mink     LGFIMVSGFV KGSIKNWQLT EEDFQNTSSH RCLSNDTKQG TLGAGGFMPF
hamster  LCFIMVSGFV KGSIKNWQLT EEDFLNRSSP LCGNNDT.NV KHGEGGFMPF
mouse    LCFIVVSGFV KGSIKNWQLT EK...NFS.. .CNNNDT.NV KYGEGGFMPF
                                      *            *

         251                                                  300
human    GFSGVLSGAA TCFYAFVGFD CIATTGEEVK NPQKAIPVGI VASLLICFIA
mink     GFSGVLSGAA TCFYAFVGFD CIATTGQEVK NPQKAIPVGI VASLLICFIA
hamster  GFSGVLSGAA TCFYAFVGFD CIATTGEEVK NPQKAIPVGI VASLLICFVA
mouse    GFSGVLSGAA TCFYAFVGFD CIATTGEEVK NPQKAIPVGI VASLLICFIA

         301                                                  350
human    YFGVSAALTL MMPYFCLDNN SPLPDAFKHV GWEGAKYAVA VGSLCALSAS
mink     YFGVSAALTL MMPYFCLDKD SPLPDAFKYV GWEGAKYAVA VGSLCALSTS
hamster  YFGVSAALTL MMPYSCLDTD SPLPGAFKYS GWEGAKYAVA VGSLCALSAS
mouse    YFGVSAALTL MMPYFCLDID SPLPGAFKHQ GWEEAKYAVA IGSLCALSTS

         351                                                  400
human    LLGSMFPMPR VIYAMAEDGL LFKFLANVND RTKTPIIATL ASGAVAAVMA
mink     LLGSMFPMPR VIYAMAEDGL LFKFLAKIND RTKTPIIATL TSGAIAAVMA
hamster  LLGSMFPMPR VIYAMAEDGL LFKYLARVNK RTKTPVIATL TSGAIAAVMA
mouse    LLGSMFPMPR VIYAMAEDGL LFKFLAKINN RTKTPVIATV TSGAIAAVMA

         401                                                  450
human    FLFDLKDLVD LMSIGTLLAY SLVAACVLVL RYQPEQPNLV YQMASTSDEL
mink     FLFDLKDLVD LMSIGTLLAY SLVAACVLVL RYQPEQ...............
hamster  FLFELKDLVD LLSIGTLLAY SLVAACVLVL RYQPEQ...............
mouse    FLFELKDLVD LMSIGTLLAY SLVAACVLVL RYQPEQPNLV YQMARTTEEL
```