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Cloning of the cellular receptor for amphotropic murine retroviruses reveals homology to that for gibbon ape leukemia virus

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ABSTRACT **The host and tissue specificity of retrovirus infection is largely determined by specific cellular receptors that mediate virus entry. Genes encoding these receptors are widely distributed in the genome, and the receptors identified to date show no sequence similarity. We have identified the cellular receptor for amphotropic murine retroviruses, Ram-1, by screening a rat cDNA expression library introduced into amphotropic virus-resistant hamster cells. The 656-amino acid receptor is homologous to the gibbon ape leukemia virus receptor at both hydrophobic termini but is highly divergent in the central hydrophilic region. Both receptors appear to be integral membrane proteins having multiple membrane-spanning regions. Identification of this family of receptors will help define the evolutionary relationship between retroviruses and their cellular receptors.**

Retrovirus infection is initiated by binding of retrovirus envelope proteins to specific cellular receptors. Virus interference and chromosome mapping studies indicate the presence of many receptors that are used by different retroviruses, including at least eight receptors on human cells (1). The four retroviral receptors that have been identified to date show no sequence similarities that might suggest important features of proteins that can serve as retrovirus receptors, and the genes for these receptors are distributed on different chromosomes (2–8). Thus it is important to isolate additional receptors to see whether common features become apparent.

Identification of the receptor for amphotropic murine retroviruses is also important because of the wide use of amphotropic retroviral vectors for gene transfer, especially in human gene therapy applications (9). Certain somatic cell types are difficult to infect with amphotropic vectors (e.g., hematopoietic stem cells), and although part of this difficulty may be due to the apparent inability of these vectors to infect nondividing cells, modulation of receptor expression may also play a role.

Expression cloning of the amphotropic retrovirus receptor has been difficult due to the ability of the virus to infect many cell types from a wide range of species. Chinese hamster ovary (CHO) cells are one of the few cell types that are resistant to infection, but variants that express functional amphotropic retrovirus receptors can be easily isolated from CHO cells (see *Results*). We have found that hamster cells express amphotropic retrovirus receptors but that these receptors are blocked by a factor that is secreted by hamster cells (10, 11). Alterations in the cells that diminish production of this inhibitor, such as glycosylation mutations, render hamster cells fully infectable by amphotropic retroviruses (10, 11). Fortunately, this inhibitory factor blocks only the hamster amphotropic receptor and not amphotropic receptors from other species such as humans or mice (10). Indeed, somatic cell hybrids between hamster cells and mouse or human cells have been used to identify the chromosomal location of the mouse and human amphotropic virus receptors (12, 13), showing that the inhibitory factor from hamster cells does not affect other amphotropic receptors even when they are expressed in hamster cells.

Based on these observations, we have been able to isolate a functional cDNA clone of the receptor for amphotropic retroviruses (Ram-1) of rats by expression cloning in CHO cells.[‖] The terminal one-thirds of the Ram-1 and Glvr-1 [the receptor for gibbon ape leukemia virus (GALV)] proteins show high similarity and overall the proteins are structurally similar, showing that these receptors are in the same family. Both Ram-1 and Glvr-1 proteins show amino acid similarity with a phosphate transporter of *Neurospora crassa* (Pho-4$^+$), suggesting that these receptors may function normally as transport proteins. The ecotropic retrovirus receptor has also been shown to function as a basic amino acid transporter (14, 15), thus implicating membrane bound transport proteins as important targets for retrovirus binding and entry.

MATERIALS AND METHODS

Cell Culture. Mammalian cells were grown in α-modified minimal essential medium (GIBCO) supplemented with 10% (vol/vol) fetal bovine serum. Cells used included CHO-K1 (ATCC CCL 61), CHO-cell-derived glycosylation mutant CHO-Lec8 (ATCC CRL 1737) (16, 17), 208F rat embryo fibroblasts (18), and PC12 rat adrenal pheochromocytoma cells (ATCC CRL 1721). CHO-cell-conditioned medium was prepared by incubation of culture medium with confluent monolayers of CHO cells for 24 hr. The medium was filtered (0.45-μm pore size) and frozen at −70°C.

Retrovirus Vectors. Retrovirus vector stocks were prepared and vector titer was determined as described (19). The plasmid form of the retroviral vector DAP (20) that encodes alkaline phosphatase and neomycin phosphotransferase (neo) was kindly provided by S. C. Fields-Berry (Harvard Medical School, Boston). We constructed another vector that encodes alkaline phosphatase and neo by removing the alkaline phosphatase cDNA from DAP with *Sal* I and inserting this fragment into the *Xho* I site of pLXSN (19) to make pLAPSN. Both vectors were transfected into PE501 ecotropic retrovirus packaging cells and transiently produced virus was used to infect PA317 amphotropic packaging cells as described (19). The titer of virus produced by PA317 cells containing LAPSN was higher than that of PA317 cells containing DAP, presumably due to the extended retroviral packaging signal present in pLAPSN that is not present in DAP (19). One of 10 PA317 clones screened that produced the highest titer virus [8 × 10^6 G418-resistant colony-forming

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Abbreviations: GALV, gibbon ape leukemia virus; CHO, Chinese hamster ovary; CFU, colony-forming units; neo, neomycin phosphotransferase.

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‖The sequence reported in this paper has been deposited in the GenBank data base (accession no. L19931).

units (CFU)/ml] was used for these studies. Ecotropic, polytropic, and GALV pseudotype LAPSN virus was made by transfer of LAPSN virus from PA317 cells to PE501 ecotropic packaging cells (19), PM571 polytropic packaging cells (10), or PG13 GALV-based packaging cells (21) respectively.

The SPD retroviral vector expresses a methotrexate-resistant dihydrofolate reductase (*dhfr**) gene and is essentially identical to the SDHT vector (22) but has a different polylinker sequence around *dhfr**. PA317 cells producing SPD (PA317/SPD cells) were made as described for LAPSN, and the cell clone that was used produced SPD at a titer of 8×10^5 CFU/ml.

Sequence Analysis. The Ram-1 cDNA was cloned in both orientations into Bluescript II (Stratagene) and ordered deletions (each ≈200 bp) were generated from both ends of the cDNA using the exonuclease III procedure of Clark and Henikoff (23). Sequencing was performed on double-stranded DNA plasmids by the dideoxynucleotide sequencing method using M13 reverse dye primers and Sequenase DNA polymerase to cycle the reaction (PRISM kit, Applied Biosystems) and an Applied Biosystems model 373A DNA sequencer. The Ram-1 cDNA was completely sequenced in both directions.

Library Screen. Expression library cells were seeded on day 1 in 50 10-cm dishes at 2×10^5 cells per dish. On day 2 the medium in each dish was replaced with 2.5 ml of CHO-cell-conditioned medium, 5 ml of the *dhfr** virus from PA317/SPD cells, and 2.5 ml of fresh medium, plus Polybrene (4 μg/ml). On day 3 the medium was replaced with Dulbecco's modified Eagle's medium supplemented with nonessential amino acids (GIBCO), 10% (vol/vol) fetal bovine serum, and 2×10^{-7} M methotrexate. Methotrexate-resistant colonies were isolated on day 9 by ring cloning.

RESULTS

Expression Library Screen for the Amphotropic Retrovirus Receptor. Our strategy for cloning the amphotropic retrovirus receptor gene involved introducing a cDNA expression library into CHO cells that were resistant to amphotropic retrovirus infection and selecting for positive clones after infection with an amphotropic retroviral vector carrying a selectable marker. In an initial screen, we isolated clones after the first round of selection that were still susceptible to infection in a secondary selection; however, such clones arose at about the same rate in cells transfected with the cDNA library or in untransfected cells (J. V. Garcia and A.D.M., unpublished results). These results indicated that CHO cells can undergo epigenetic or mutational events resulting in the expression of functional amphotropic retrovirus receptors.

As described in the Introduction, we later found that CHO variants, in particular those with altered glycosylation, can express functional amphotropic retrovirus receptors. Since glycosylation mutants are readily isolated (at rates of 10^{-7} to 10^{-5} or higher) from CHO cells (24), this could explain the spontaneous occurrence of clones that were susceptible to amphotropic vector infection in our initial screen. We also found that inhibition of amphotropic vector infection of CHO cells is mediated by a factor secreted by CHO cells that has no effect on infection of 208F (rat), HeLa (human), or NIH 3T3 (mouse) cells (ref. 10 and data not shown).

In view of these findings, we added CHO-cell-conditioned medium during virus infection in our next screen to inhibit infection of cells expressing functional hamster amphotropic receptors. We screened a CHO cell expression library made by cotransfection of CHO cells with a neo selectable marker and a rat PC12 cell cDNA library (>1.5-kb insert size) cloned in the CDM8 eukaryotic expression plasmid (25). The expression library had a complexity of ≈150,000. Since the cells already expressed neo, we used an amphotropic retrovirus vector that expressed a methotrexate-resistant *dhfr** gene for infection. In a screen of 10^7 cells, we isolated 39 methotrexate-resistant clones, 17 of which were infectable on rechallenge with an amphotropic retrovirus vector that expressed alkaline phosphatase. Of the 17 clones, 11 showed similar or slightly increased infection rates in the presence of CHO-cell-conditioned medium (e.g., Table 1, clones 1, 26, and 46), as expected of CHO cells expressing the rat amphotropic retrovirus receptor, and 6 were much less infectable in the presence of conditioned medium (e.g., Table 1, clones 31 and 34), as expected of CHO cells expressing functional hamster receptors. These phenotypes parallel those of CHO H706-CL17 cells that express the human amphotropic virus receptor (13) and glycosylation mutant CHO-Lec8 cells (16, 17, 26) that express functional hamster receptors (11) (Table 1). In addition, CHO cells are poorly infectable by ecotropic retroviral vectors, and this phenotype was unchanged in CHO–rat clones 1, 26, and 46 (Table 1). CHO–rat clones 31 and 34 showed an increased susceptibility to ecotropic retrovirus infection, as did CHO Lec8 cells (Table 1), indicating that a nonspecific event was responsible for their increased infectability. Thus CHO–rat clones 1, 26, and 46 were specifically susceptible to infection by amphotropic vectors and not to retroviruses that use different receptors for entry, probably due to the expression of the rat amphotropic retrovirus receptor.

Table 1. Infection phenotypes of cell lines

Target cells	Amphotropic vector titer, CFU/ml		Ecotropic vector titer, CFU/ml	Phenotype
	− CM	+ CM		
CHO	1	<1	30	Not infectable
Rat 208F	5×10^5	6×10^5	3×10^5	Rat Ram$^+$
CHO/Rat c1	3×10^2	6×10^2	10	Rat Ram$^+$
CHO/Rat c26	3×10^4	3×10^4	10	Rat Ram$^+$
CHO/Rat c46	2×10^5	4×10^5	20	Rat Ram$^+$
CHO/Rat c31	1×10^3	<1	300	Glycosylation$^-$
CHO/Rat c34	3×10^2	<1	60	Glycosylation$^-$
CHO H706-CL17	1×10^5	3×10^5	1	Human Ram$^+$
CHO Lec8	6×10^4	3×10^2	2×10^5	Glycosylation$^-$

Cells were seeded at 2×10^5 cells per 6-cm dish and infected the next day with various dilutions of LAPSN vector produced by PA317 amphotropic or PE501 ecotropic packaging cells plus Polybrene (4 μg/ml) in the presence (+) or absence (−) of 2 ml of CHO-cell-conditioned medium (CM) plus fresh medium to bring the total to 4 ml. The cells were fed with fresh medium the day after infection, and 2 days after infection, the cells were stained for alkaline phosphatase-positive colonies as described (20). Results are average values from duplicate dishes in a representative experiment.

Table 2. CHO cells transfected with the Ram-1 expression plasmid can be efficiently transduced with amphotropic vectors

Vector pseudotype	Conditioned medium	Vector titer, CFU/ml			
		CHO/Ram-1 c3	CHO/Ram-1 c7	CHO	208F
Amphotropic	−	3×10^4	3×10^5	1	2×10^5
Amphotropic	+	5×10^4	5×10^5	<1	2×10^5
Ecotropic	−	5	3	30	1×10^5
Polytropic	−	<1	<1	<1	5×10^4

Production of alkaline phosphatase-positive colonies after infection with the LAPSN vector was determined as described in Table 1. Polytropic-pseudotype virus was made by using PM571 polytropic packaging cells (10). We generated stable cell lines that expressed pRam-1 by cotransfecting (27) CHO cells with pRam-1 and a neo gene at a 20:1 ratio and selecting G418-resistant clones. Two of seven clones analyzed are shown. Results are average values from duplicate dishes in a representative experiment.

Rescue of the Amphotropic Retrovirus Receptor cDNA. Integrated plasmid sequences in the 11 presumptive Ram-1-positive clones were examined by Southern blot analysis using a probe to expression vector sequences (data not shown). Only three distinct integration patterns were observed, two of which showed ≈50 integrants each and one showed only ≈5 integrants. Assuming that the plasmid sequences integrated in tandem arrays, we cut genomic DNA with restriction enzymes that cleave DNA infrequently and cleave the expression plasmid at only one site (*Not* I or *Sfi* I), religated the DNA at low concentration to favor plasmid circularization, and cloned the expression plasmid sequences in bacteria. Rescued plasmids were tested for their ability to enhance amphotropic virus infection of CHO cells after transient transfection. Ultimately, we obtained a positive clone (pRam-1) from size-selected DNA from presumptive Ram-1-positive CHO clone 46 that contained only five expression plasmid copies. The 2.4-kb insert in pRam-1 hybridized to apparently identical size inserts in DNA from clones displaying the rat amphotropic receptor phenotype and having each of the three integration patterns, but not to DNA from clones that displayed the hamster receptor phenotype (data not shown), suggesting that all of the clones with the rat receptor phenotype received copies of the same Ram-1 expression plasmid clone.

We generated stable cell lines that expressed the cloned Ram-1 cDNA by cotransfection. The titer of amphotropic LAPSN virus on one of the seven clones analyzed was as high as on the highly infectable rat cell line 208F and was not decreased by the addition of CHO-cell-conditioned medium (Table 2). Transfer of pRam-1 did not confer susceptibility to vectors with ecotropic or polytropic pseudotypes. Thus expression of the cloned cDNA promotes efficient and specific infection by amphotropic retrovirus.

Sequence Analysis of the Ram-1 cDNA Reveals Homology to Glvr-1 and Pho-4$^+$ Sequences. Sequencing of the 2.3-kb Ram-1 cDNA revealed a single long open reading frame with upstream stop codons in all three reading frames. A search for related protein sequences using the BLAST network service at the National Center for Biotechnology Information revealed two sequences, those of the mouse homolog of the human GALV receptor (*Glvr-1*) (28) and the *N. crassa* phosphate permease gene (*pho-4*$^+$) (29). The murine and the human (6) Glvr-1 proteins are closely related (90% identity) and show the highest similarity to Ram-1 (57–59% identity) (Fig. 1). Hydropathy plots of Ram-1, Glvr-1, and Pho-4$^+$ proteins show striking similarities, with the hydrophobic N and C terminal one-thirds showing high similarity and the central hydrophilic domains showing little similarity at the amino acid level but some common features in the hydrop-

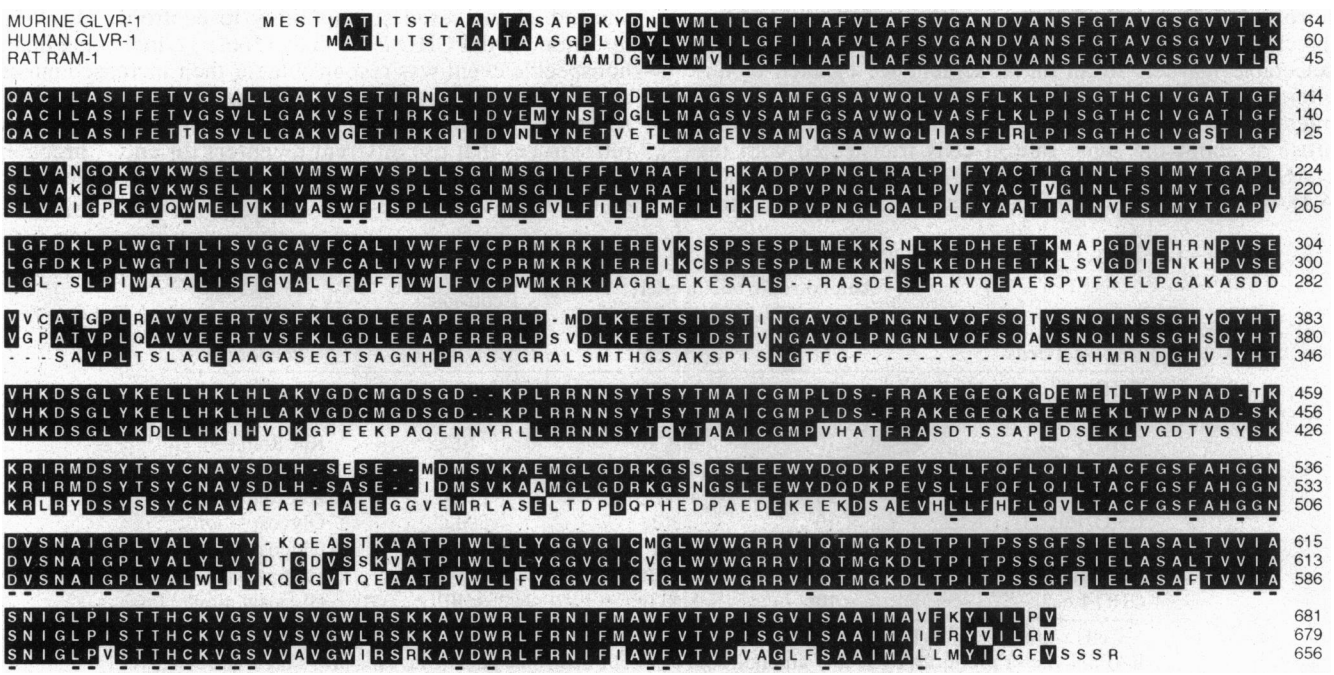

FIG. 1. Amino acid alignment of rat Ram-1 and murine and human Glvr-1. Gaps introduced to provide better sequence alignment are indicated by dashes. Identical amino acids in the two homologous regions within Ram-1 (amino acids 2–165 and 481–645) are underlined. White type on a dark background indicates regions of sequence identity.

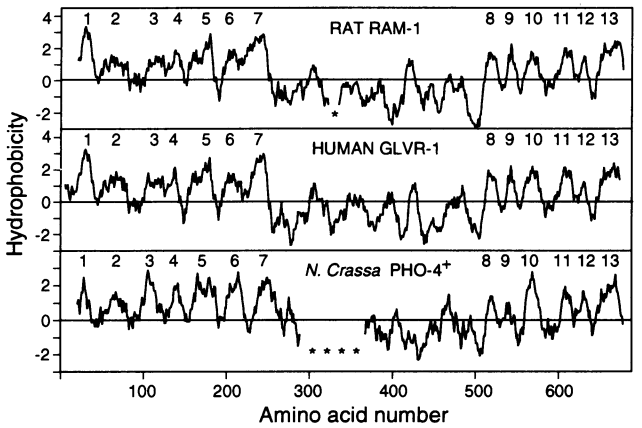

FIG. 2. Hydropathy plot of rat Ram-1, human Glvr-1, and *N. crassa* Pho-4$^+$. Average hydropathy values were calculated by the method of Kyte and Doolittle (30) using an 11-amino acid window. High values indicate hydrophobic regions and low values indicate hydrophilic regions. Ram-1 and Pho-4$^+$ were offset by 15 residues at their N termini to achieve alignment with Glvr-1. Asterisks indicate gaps introduced in the dissimilar hydrophilic regions of Ram-1 and Pho-4$^+$ to provide better alignment of the C termini of the three proteins. A 12-residue gap was included after amino acid 308 of Ram-1 and a 78-residue gap was included after amino acid 274 of Pho-4$^+$.

athy plots (Fig. 2). Potential transmembrane domains have been numbered for the three proteins. There are also regions within Ram-1 that are similar at the protein and DNA levels that extend from amino acids 2–165 and 481–645, and identical amino acids in these regions are underlined in Fig. 1.

Despite Homology to Glvr-1, Ram-1 Does Not Serve as a Receptor for GALV. Given the homology between Ram-1 and Glvr-1, we tested whether Ram-1 could serve as a receptor for GALV. NIH 3T3 cells are resistant to infection by GALV-pseudotype vectors but can be made susceptible by transfer of the *Glvr-1* gene (6). Introduction of pRam-1 into NIH 3T3 cells by a transient transfection technique did not render the cells susceptible to GALV-pseudotype vector infection, while introduction of pRam-1 into CHO cells by the same technique did render the CHO cells susceptible to amphotropic vector infection (Table 3). Cotransfection of a β-galactosidase gene showed that transfected DNA was being expressed with similar efficiency in NIH 3T3 and CHO cells. Thus Ram-1 does not act as a receptor for GALV-pseudotype virus.

Expression of Ram-1 by Other Species. Northern blot analysis using the pRam-1 cDNA insert as a probe revealed a 4-kb mRNA in RNA from human, dog, mouse, hamster, rat, and quail cells, but not from chicken, frog, or yeast cells (Fig. 3). Although the rat and hamster RNAs appear to have slightly larger size than those from the other species, the rat and

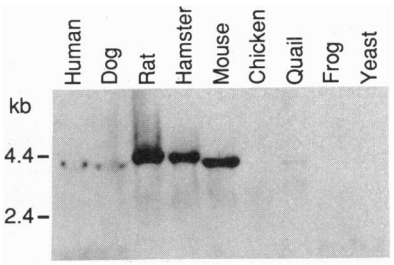

FIG. 3. Northern blot analysis of Ram-1 cDNA hybridization to RNAs from various species. Sources of RNAs were as follows: human, HeLa cells; dog, D17 cells; rat, PC12 cells; hamster, CHO cells; mouse, NIH 3T3 TK$^-$ cells; chicken, HB-1 virus-transformed chicken embryo fibroblasts; quail, QT35 cells; frog, stage 41 embryos; yeast, *Saccharomyces cerevisiae*. Total RNA (10 μg) was loaded in each lane. Hybridization was carried out at low stringency in 2× standard saline citrate (SSC) (0.3 M sodium chloride/0.03 M sodium citrate, pH 7.0) at 56°C. The positions of synthetic RNA size markers (0.24–9.5 kb, GIBCO/BRL) are indicated at left.

hamster rRNAs also migrated more slowly, indicating that differences in size are probably an electrophoresis artifact. The strongest hybridization was observed with RNA from rodent species. Although RNA from chicken cells showed no hybridization to the Ram-1 probe, these cells are still highly infectable with amphotropic retrovirus vectors and presumably express a protein related to Ram-1. These results suggest a broad distribution of Ram-1 expression in different species, as expected based on the wide range of cells that are infectable by amphotropic retroviruses.

Localization of Ram-1 to the Pericentromeric Region of Human Chromosome 8. Garcia *et al.* (13) used human–hamster hybrid cell lines to map sequences that confer susceptibility to amphotropic vector infection to the pericentromeric region of human chromosome 8. Southern blot analysis of *Hin*dIII-cut DNA from the most informative hybrids revealed a 4.2-kb band that hybridized to a Ram-1 probe only in the infectable hybrids (Fig. 4). Hybrids analyzed contain human chromosome 8 only (706-CL17), fragments of chromosome 8 only (R30-5B, 229-3A, and R30-2A), or several other human chromosomes (826-26A) (13). Analysis of total human (HeLa) cell DNA also revealed a single 4.2-kb band that hybridized with a rat Ram-1 probe. These results map the human sequences that hybridize to Ram-1 to the same region of chromosome 8 that confers sensitivity to amphotropic vector infection. In contrast, *Glvr-1* maps to human chromosome 2 (7).

DISCUSSION

Isolation of Ram-1 identifies a family of related retrovirus receptors that includes Glvr-1. Although the normal function of these proteins has not been defined, their homology with

Table 3. Ram-1 does not confer susceptibility to GALV-pseudotype vector infection

Transfected gene(s)	Transfected cells	β-gal$^+$ colonies, no. per dish	Vector pseudotype	AP$^+$ colonies, no. per dish
β-gal+Ram-1	CHO	3×10^4	Amphotropic	2×10^4
β-gal	CHO	2×10^4	Amphotropic	<1
β-gal+Ram-1	NIH 3T3	5×10^4	GALV	<1
β-gal	NIH 3T3	2×10^4	GALV	<1

Cells were plated at 5×10^5 cells per 6-cm dish on day 0. On day 1 the cells were transfected (27) with 4 μg of a β-galactosidase (β-gal) expression plasmid plus 4 μg of the Ram-1 expression plasmid or 4 μg of a β-galactosidase expression plasmid plus 4 μg of an expression plasmid without an insert. The cells were washed to remove debris and fed with fresh medium on day 2. On day 3, half of the dishes were stained for β-galactosidase activity and duplicate dishes were trypsinized and reseeded at a 1:10 dilution. On day 4, the reseeded cells were infected with 200 μl of amphotropic LAPSN vector produced by using PA317 cells or with 1 ml of GALV-pseudotype LAPSN vector produced by using PG13 cells. The cells were fed on day 5, stained for alkaline phosphatase-positive (AP$^+$) colonies on day 6, and colonies were counted using a microscope. Results are average values from duplicate dishes in a representative experiment.

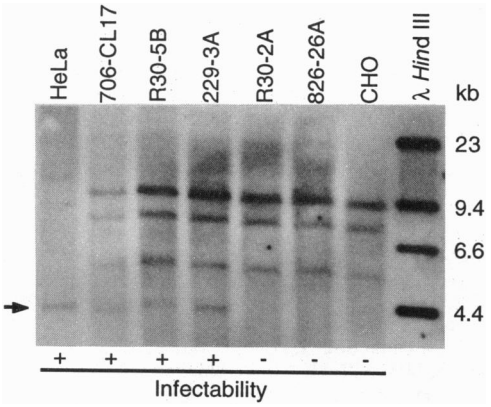

FIG. 4. pRam-1 insert hybridization to human–hamster hybrid cell lines. Cell DNA was cleaved with *Hin*dIII. The blot was washed at low stringency (2× SSC at 40°C) to allow detection of human sequences with the rat Ram-1 probe. Infectability was determined as described (13). Size markers are shown at right, and the arrow indicates the position of the putative human Ram-1 band.

Pho-4$^+$ (29) suggests that they may be involved in ion transport as is the ecotropic retrovirus receptor (4), which is a cationic amino acid transporter (14, 15). In addition to GALV, Glvr-1 serves as a receptor for feline leukemia virus subgroup B (31). Amino acid similarity of the surface proteins of feline leukemia virus subgroup B, GALV, and the amphotropic retroviruses (32) suggests that these viruses and receptors have arisen from common ancestors. The existence of other murine retroviruses that are related to these viruses but that use different cellular receptors (32, 33) raises the possibility that additional members of Ram-1/Glvr-1 family may exist.

We do not know the identity of the factor secreted by CHO cells that inhibits amphotropic retrovirus infection of these cells. Such factors are secreted by a variety of hamster cells and are present in hamster serum (11). The inhibitory factor may be encoded by endogenous retroviral *env* sequences, but it is curious that the factor only inhibits the hamster receptor and not those from other species (rat, human, and mouse). An amphotropic Env protein would be expected to block receptors on cells from all of these species since they are all permissive for amphotropic virus infection.

In an independent study, the human amphotropic receptor cDNA has recently been cloned based on sequence similarity to human Glvr-1 (34). The rat and human amphotropic retrovirus receptors show 92% amino acid similarity distributed evenly over the entire length of the proteins, showing their close relationship and providing further support that these cDNAs indeed encode amphotropic retrovirus receptors. The availability of DNA probes from this receptor family should allow better definition of factors that influence cell infectability, particularly in relation to the wide use of these vectors for human gene therapy (9).

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