

Substitution of a Single Amino Acid Residue Is Sufficient To Allow the Human Amphotropic Murine Leukemia Virus Receptor To Also Function as a Gibbon Ape Leukemia Virus Receptor

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We have previously reported the unique properties of a receptor for amphotropic murine leukemia viruses (A-MuLVs) expressed on Chinese hamster E36 cells (C. A. Wilson, K. B. Farrell, and M. V. Eiden, *J. Virol.* 68:7697–7703, 1994). This receptor, HaPiT2 (formerly designated EAR), in contrast to the human form of the A-MuLV receptor (PiT2), functions as a receptor not only for A-MuLVs but also for gibbon ape leukemia virus (GALV). Comparison of the deduced amino acid sequences of the HaPiT2 and PiT2 proteins suggested that differences in the amino acid composition of the extracellular region(s) of the hamster and human proteins account for their functional differences. We substituted extracellular regions of HaPiT2 for those of PiT2 to map the region of the HaPiT2 protein required for GALV receptor function. Only those PiT2-HaPiT2 chimeric receptors containing the fourth and fifth extracellular regions of HaPiT2 functioned as GALV receptors. We have now determined that the substitution of a single amino acid residue, glutamic acid, for the lysine residue at position 522 in the fourth extracellular region of the PiT2 protein is sufficient to render PiT2 functional as a GALV receptor.

Although they share many structural and biological features, the mammalian type C retroviruses are genetically diverse viruses isolated from a broad range of animals. These retroviruses have been isolated from mice and other rodents, cats, cows, primates, and humans, as well as from many other types of animals (18). The murine leukemia viruses (MuLVs) have been further divided on the basis of host range and interference properties. Five different MuLV receptor classes, the ecotropic (E-MuLVs), amphotropic (A-MuLVs), xenotropic, dualtropic (mink cell focus-forming viruses), and the 10A1 MuLV, have been identified. E-MuLVs, A-MuLVs, xenotropic MuLVs, and mink cell focus-forming viruses use discrete receptors for viral entry (14). 10A1 MuLV is unusual in its ability to use two different viral receptors. Although capable of using the same receptor as A-MuLVs, 10A1 MuLV can also infect cells by means of a second auxiliary receptor. Therefore, cells infected with A-MuLV remain susceptible to 10A1 MuLVs (14).

The receptor for the E-MuLVs was the first type C retroviral receptor identified. This receptor is a widely expressed multimembrane-spanning protein that functions as an amino acid transporter (1). The human cDNA encoding the receptor for the gibbon ape leukemia viruses (GALVs) has also been identified (11). This receptor, PiT1 (formerly designated *glvr1*), also functions as a receptor for the subgroup B feline leukemia viruses (FeLV B) (17) and is one of the two receptors used by 10A1 MuLV (10, 21). More recently, the rat (RaPiT2 [formerly designated *Ram-1*] [9]) and human (PiT2 [formerly designated *glvr2*] [22]) forms of the A-MuLV receptor were cloned. These receptors are markedly similar to PiT1 in both their

structure and normal cellular function (5, 12). Despite the similarities of their receptors, GALVs and A-MuLVs do not usually exhibit any overlap in their receptor utilization (15, 20). Chinese hamster E36 cells are an exception because they express a PiT2 homolog that functions as a receptor for both A-MuLV and GALV (3). We have cloned, sequenced, and functionally characterized this receptor, HaPiT2 (formerly designated EAR), and determined that it closely resembles PiT2, with 97% amino acid identity (21). Characterization of the HaPiT2 protein allows the comparative assessment of A-MuLV and GALV receptor requirements. We introduced alterations in the PiT2 coding region consistent with those present in HaPiT2 to determine the basis for the ability of HaPiT2 to function as a GALV receptor. We have found that the substitution of a glutamic acid for the lysine residue at position 522 within the PiT2 protein is sufficient to convert this receptor to one capable of facilitating both A-MuLV and GALV entry.

MATERIALS AND METHODS

Cells and viruses. *Mus dummi* tail fibroblasts (MDTF) were obtained from Olivier Danos (Institute Pasteur, Paris, France). The canine fibroblast cell line D17 and CHO K1 cells were obtained from the American Type Culture Collection (CCL 183 and CCL 61, respectively). All cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum and 40 mM glutamine, with the exception of CHO K1 cells, which were maintained in alpha minimal essential medium.

The PA317/G1BgSvN and PG13/G1BgSvN retroviral vector producer cell lines have been previously described (21). The G1BgSvN vector genome contains the bacterial genes for neomycin phosphotransferase and β -galactosidase (6). The molecular clone of FeLV B, pEEZZ (2), was generously provided by Julie Overbaugh (University of Washington, Seattle). The ProFectin method (Promega, Madison, Wis.) of calcium phosphate-mediated gene transfer was used to introduce the pEEZZ and G1BgSvN plasmids into D17 cells. Transfected D17 cells were passaged in medium containing 400 μ g of G418 per ml for 2 weeks, at which time a reverse transcriptase assay of the culture supernatant confirmed

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productive infection by FeLV B. The pseudotyped FeLV B vectors produced from selected D17 cells are designated FeLV B/G1BgSvN.

Construction of chimeric receptor and AAAA*A cDNA plasmids. Nine chimeric receptor cDNAs were constructed by exchanging regions of PiT1 and PiT2, PiT1, and HaPiT2, or PiT2 and HaPiT2. All plasmid constructions were cloned into the pSp72 plasmid (Promega) and then subcloned into the pLNSX retroviral vector (8) for expression analysis. To facilitate cloning between putative extracellular domains of some receptors, it was necessary to introduce restriction enzyme sites at corresponding regions in one or both of the cDNAs being exchanged. Briefly, synthetic oligonucleotides containing one or more nucleotide changes to introduce a specific restriction site without changing the encoded amino acid residue were designed. PCRs were performed (50°C, 20 cycles), and the resulting products were directly cloned into the TA pCRII vector (Invitrogen, San Diego, Calif.). Plasmids containing PCR-generated DNA fragments were sequenced to rule out unscheduled PCR changes.

The nomenclature for chimeric receptor cDNAs is based on the use of a single letter to refer to the origin of each of the five extracellular regions, where G, A, and E are PiT1-, PiT2-, and HaPiT2-derived sequences encoding the extracellular regions, respectively. Nucleotide (nt) 1 is the first nucleotide of the receptor open reading frame.

The PiT1-HaPiT2 chimeras, EEEGG and GGGEE, were made by exchanging the fourth and fifth putative extracellular domains at the *Sph*I site (nt 1280 in PiT1 and nt 1181 in HaPiT2) present in both cDNAs. In chimera EEGEE, the putative third extracellular domain of HaPiT2 was replaced with that of PiT1 between the *Age*I and *Sph*I sites (nt 593 to 1280 for PiT1 and nt 549 to 1181 for HaPiT2). The *Age*I sites were introduced by PCR with synthetic oligonucleotides. The resulting chimeric cDNAs contained no changes in encoded amino acid residues.

Chimeric receptor cDNAs involving exchanges between PiT2 and HaPiT2, EEEAA, AAAEE, AAEEE, and EEAAE, were made by the following strategy. The chimeric cDNAs AAEEE and EEEAA were constructed by reciprocal exchanges of the fourth and fifth extracellular domains at the *Bgl*II site (nt 1065 for both). The *Bgl*II site was introduced into HaPiT2 cDNA at nt 1062 by PCR-based mutagenesis. AAEEE cDNA was constructed by using the *Age*I sites introduced by PCR-based mutagenesis at nt 549 in both the PiT2 and HaPiT2 molecules. The chimeric cDNA EEAAE contains the region encoding the third domain of PiT2 (nt 549 to 1184) joined to HaPiT2 cDNA at the introduced *Age*I site at nt 549 and the *Sph*I site at nt 1184. The PiT1-PiT2 chimera designated GGGAA was constructed by replacing the 3' end of PiT1 (nt 1551 to 2039) cDNA with the fourth and fifth putative extracellular domains of PiT2 (nt 1468 to 2097) at the *Pst*I site present in both genomes. GAAEE was constructed by PCR mutagenesis to introduce an *Nhe*I site at nt 151 of PiT2 cDNA to allow ligation to the analogous *Nhe*I site at nt 265 of PiT1 cDNA. The fourth and fifth

extracellular regions of HaPiT2 were adjoined at the *Bgl*II site to form GAAEE cDNA.

AAAA*A cDNA was made by using a PCR-based mutagenesis strategy similar to that previously described (21). A glutamic acid codon was introduced in place of the lysine codon at nt 1566 of PiT2.

Assays for A-MuLV infection. CHO K1 cells were seeded at 3×10^4 cells per well on 12-well dishes. The ProFectin calcium phosphate-mediated transfection protocol (Promega) was used. Chimeric or control receptor plasmid DNA (9 µg) was used to form precipitates, and precipitate was then applied to CHO K1 cells in each of three wells. Each plasmid was tested in two to four independent transfection-infection experiments. On the next day, transfected cells were washed two times and fresh medium was applied. At 48 h posttransfection, 2 ml of supernatant from PA317/G1BgSvN retroviral vector-producing cells were filtered (pore size, 0.45 µm), adjusted to 3 µg of Polybrene per ml, and added to each well of cells. At 72 hour postexposure to PA317/G1BgSvN vectors, cells were stained with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) as described previously (19).

Assays for FeLV B and GALV infection. MDTF cells expressing PiT1 and HaPiT2 cDNAs have been previously described (21). PiT2 cDNA, each of the nine chimeric receptors, and AAAA*A cDNA were subcloned into the retroviral vector pLNSX (8). Each of these retroviral vector plasmids was introduced into PA317 packaging cells by calcium phosphate-mediated transfection; the only exception was the GGGAA plasmid, which was transfected directly into MDTF cells. Transfected cells were selected in 400 µg of G418 (active) per ml for 10 to 14 days, at which time G418-resistant cells were pooled. Supernatants from each of the receptor-containing packaging cells were used to infect MDTF cells, as previously described (21). Transduced MDTF cells were selected in 400 µg of G418 per ml. At the end of G418 selection, G418-resistant cells were pooled and seeded onto 12-well dishes to assess receptor function.

MDTF cells expressing PiT1, PiT2, and HaPiT2 cDNAs were exposed to 2 ml of filtered supernatant from D17 cells producing FeLV B/G1BgSvN pseudovirions and assayed for infection, as described below.

MDTF cells expressing different chimeric receptor cDNAs were exposed to GALV-enveloped retroviral vectors produced from PG13 cells (7). The efficiency of infection was assessed at 72 h postexposure to viral supernatants by enumerating blue-stained cell foci, as previously described (19). A large preparation of PG13/G1BgSvN supernatant was pooled and frozen. This stock was used in all PG13/G1BgSvN infections. Three individual experiments in which the control and various receptor-expressing MDTF cells were plated in triplicate and exposed to 10-fold serial dilutions (1, 0.1, and 0.01 ml) of the stock PG13/G1BgSvN supernatant were carried out. The reported titer for PG13/G1BgSvN on each of the target cells is the average number of blue foci per ml obtained in the three experiments with at least two different virus concentrations.

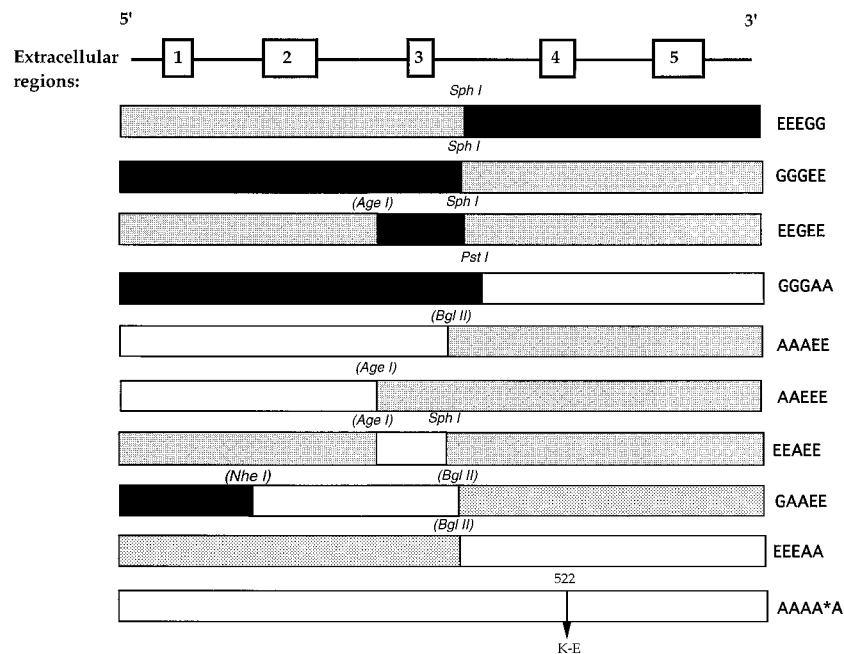


FIG. 1. Schematic representation of the chimeric receptor cDNAs used to examine virus receptor function. The relative positions of the five extracellular regions of the PiT1, PiT2, and HaPiT2 receptors are depicted by numbered boxes. The regions of chimeric receptors derived from PiT1, PiT2, and HaPiT2 are represented by black, white, and gray bars, respectively. Capital letters in chimeric receptor designations denote the origin of each of the five extracellular regions. The restriction enzyme sites that served to join receptor cDNA regions are designated at their appropriate locations in receptor cDNAs. The *Age*I, *Bgl*II, and *Nhe*I sites are shown in parentheses because they represent sites introduced as silent mutations in either PiT1, PiT2, or HaPiT2 cDNA.

TABLE 1. A-MuLV infection of CHO K1 cells transiently expressing chimeric viral receptors

Chimeric viral receptor	% Infection ^a
PiT2	100
HaPiT2	96.4 ± 2.9
PiT1	<0.01 ^b
EEEEA	188 ± 19.6
AAAE	129 ± 11.5
AAEEE	114 ± 5.5
EEAEE	53.3 ± 19.6 ^c
EEGEE	58.8 ± 15.45 ^c
GAAEE	183.5 ± 9.5
GGGEE	3.45 ± 3.05
EEEEG	13.8 ± 5.5
GGGAA	55.2 ± 25.4 ^c
No DNA	0

^a As described in Materials and Methods, data are the means ± the standard errors of the means for two independent transfection-infection experiments. Each transfection precipitate was placed on triplicate wells of a 12-well dish. Triplicate wells were infected with 2.0 ml of the PA317/G1BgSvN retroviral vector. The average number of β-galactosidase-positive foci in each of the three wells transfected with PiT2 DNA was assigned a value of 100% for each independent experiment. All other values were accordingly normalized to these results.

^b One β-galactosidase-positive cell in three wells of a 12-well dish.

^c Data are the means ± the standard errors of the means for four independent transfection experiments.

RESULTS

HaPiT2 does not function as a receptor for FeLV B. PiT1 functions as a receptor for both GALV and FeLV B. Murine MDTF cells are resistant to both of these retroviruses. We have previously demonstrated that HaPiT2 is a receptor for GALV (21). To determine if the HaPiT2 protein functions as a receptor for FeLV B, MDTF cells expressing HaPiT2 cDNA were exposed to FeLV B/G1BgSvN pseudovirions. Although MDTF cells expressing PiT1 were efficiently infected by FeLV B/G1BgSvN pseudovirions, MDTF cells expressing HaPiT2 remained resistant to FeLV B (data not shown). Thus, PiT2 does not function as an FeLV B receptor.

Characterization of the HaPiT2 chimeric receptors as A-MuLV receptors. To determine which extracellular region(s) of the HaPiT2 protein is required for A-MuLV or GALV receptor function, we used a combination of PCR technology

and standard subcloning techniques to generate a series of chimeric cDNAs, as described in Materials and Methods. Figure 1 contains a schematic representation of the chimeric receptor cDNAs we used to map the region(s) of HaPiT2 that correlated with A-MuLV or GALV receptor function. Chimeric receptor cDNA designations are series of letters corresponding to the origin of each of the five extracellular domains. Extracellular coding regions derived from PiT1 are designated G, A is used for regions derived from PiT2, and E is used for regions derived from HaPiT2.

To compare the relative efficiencies of the HaPiT2 and PiT2 proteins to function as A-MuLV receptors, receptor cDNAs were transiently expressed in CHO K1 cells, which are resistant to A-MuLV infection. CHO K1 cells expressing either PiT2, HaPiT2, EEEAA, AAEE, AAEEE, EEAEE, EEGEE, GA AEE, GGGEE, EEEGG, or GGGAA cDNA were rendered susceptible to amphotropic enveloped retroviral vectors (PA317/G1BgSvN) (Table 1). Control CHO K1 cells and CHO K1 cells expressing PiT1 cDNA were resistant to infection by A-MuLV-enveloped retroviral vectors.

Characterization of the HaPiT2 chimeric receptors as GALV receptors. To test the chimeric receptors for GALV receptor activity, each receptor cDNA was stably expressed in MDTF cells. MDTF cells are resistant to GALV infection. MDTF cells expressing human PiT1 cDNA are susceptible to GALV-enveloped PG13-derived vectors (16). The results of experiments assessing the GALV receptor function of each HaPiT2-PiT2 and HaPiT2-PiT1 chimeric receptor are shown in Fig. 2. Control MDTF cells expressing either PiT1 or HaPiT2 cDNA are efficiently infected by PG13 vectors, while MDTF cells expressing PiT2 cDNA remain resistant to infection by PG13 vectors. MDTF cells expressing PiT1- and HaPiT2-derived chimeric cDNA, EEEGG, GGGEE, or EE GEE, are susceptible to infection to PG13 vectors. MDTF cells expressing PiT2- and HaPiT2-derived chimeric cDNA, AAA EE, GAAEE, AAEEE, or EEAEE, are also susceptible to PG13 infection. In contrast, GGGAA and EEEAA cDNAs fail to express a functional GALV receptor. Only those chimeric receptors containing the fourth and fifth extracellular regions of either PiT1 or HaPiT2 retain GALV receptor function.

On the basis of these results, we concluded that the fourth and/or fifth extracellular domains of HaPiT2 play a critical role in GALV receptor function. HaPiT2 contains several amino acid residues in the fourth and fifth extracellular regions that

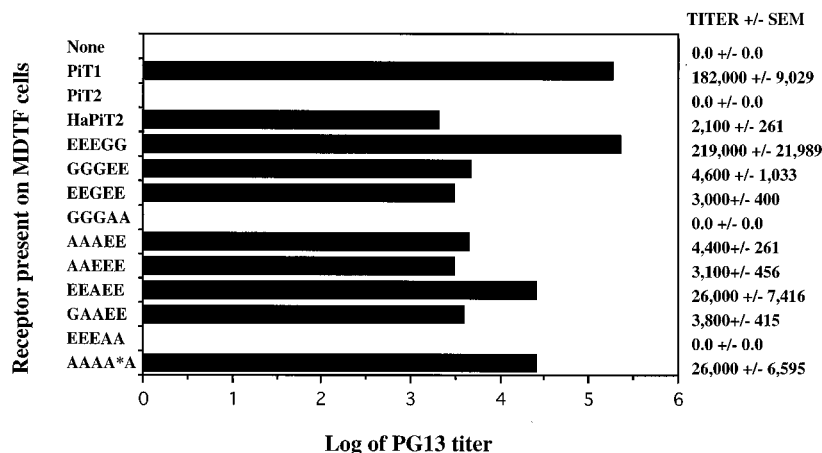


FIG. 2. MDTF cells expressing receptor cDNAs were exposed to GALV PG13/G1BgSvN retroviral vectors, as described in Materials and Methods. SEM, standard error of the mean.

TABLE 2. Alignment of amino acid residues in the carboxy termini of the fourth extracellular regions of GALV receptor homologs

Receptor	Sequence ^a
PiT1	D T G D V S S K V
HaPiT2	E Q - G - M Q E A
RaPiT1	E - R D - T T - E
PiT2	K Q - G - T Q E A

^a Residues 550 to 558 for PiT1 and homolog and residues 522 to 530 for PiT2 and homolog. —, conserved residue.

differ from those in each of the previously characterized A-MuLV receptors, the RaPiT2 homolog (9) and PiT2 (22) (Table 2). The compositions of the fifth extracellular domains of these receptors are identical except for a single conservative amino acid substitution at position 618 of the HaPiT2 protein. HaPiT2 contains a histidine residue at this position, whereas both PiT2 and RaPiT2 contain an arginine residue at position 618. The fourth extracellular domain of HaPiT2 contains glutamic acid and threonine residues at positions 522 and 527, respectively, whereas both RaPiT2 and PiT2 contain a lysine residue at position 522 and a methionine residue at position 527.

To determine if substituting a negatively charged amino acid residue for the positively charged residue at position 522 of PiT2 was sufficient to render the PiT2 receptor functional as a GALV receptor, we replaced the lysine codon present at position 522 of PiT2 with a glutamic acid codon. This cDNA, designated AAAA*A (Fig. 1), was tested for A-MuLV receptor function in CHO K1 cells. CHO K1 cells expressing AAAA*A cDNA were infected by A-MuLV at an efficiency

equivalent to that of CHO K1 cells expressing wild-type PiT2 cDNA (data not shown). Therefore, the K-to-E mutation at position 522 did not compromise the A-MuLV receptor function of the AAAA*A receptor. AAAA*A cDNA was expressed in MDTF cells to assess GALV receptor function. As shown in Fig. 3, this mutant form of PiT2 functions as a GALV receptor.

DISCUSSION

Cells coexpressing distinct A-MuLV and GALV receptors are common. Cells expressing receptors capable of facilitating the entry of both A-MuLV and GALV are atypical. The HaPiT2 protein expressed on hamster E36 cells is one example of a receptor that functions as both a GALV receptor and an A-MuLV receptor (21). There are other examples of receptors which facilitate the entry of distantly related viruses. For example, PiT1 has been shown to function as a receptor for both GALV and the seemingly unrelated C-type feline retrovirus FeLV B. However, not all functional GALV receptors facilitate FeLV B infection. The rat homolog of PiT1 (RaPiT1) allows GALV infection, but not FeLV B infection (16). Although HaPiT2 appears to function as a receptor for a broad group of C-type retroviruses, allowing GALV, A-MuLV, and 10A1 MuLV infections (21), we have now determined that HaPiT2 does not function as a receptor for FeLV B (Table 3). It has been previously reported that the nine amino acids contained in the carboxy terminus of the fourth extracellular region of the PiT1 receptor (referred to as the A region) are critical for GALV and FeLV B receptor function (4, 16). The results obtained with site-directed mutagenesis studies of the RaPiT1 receptor (16), together with our results demonstrating

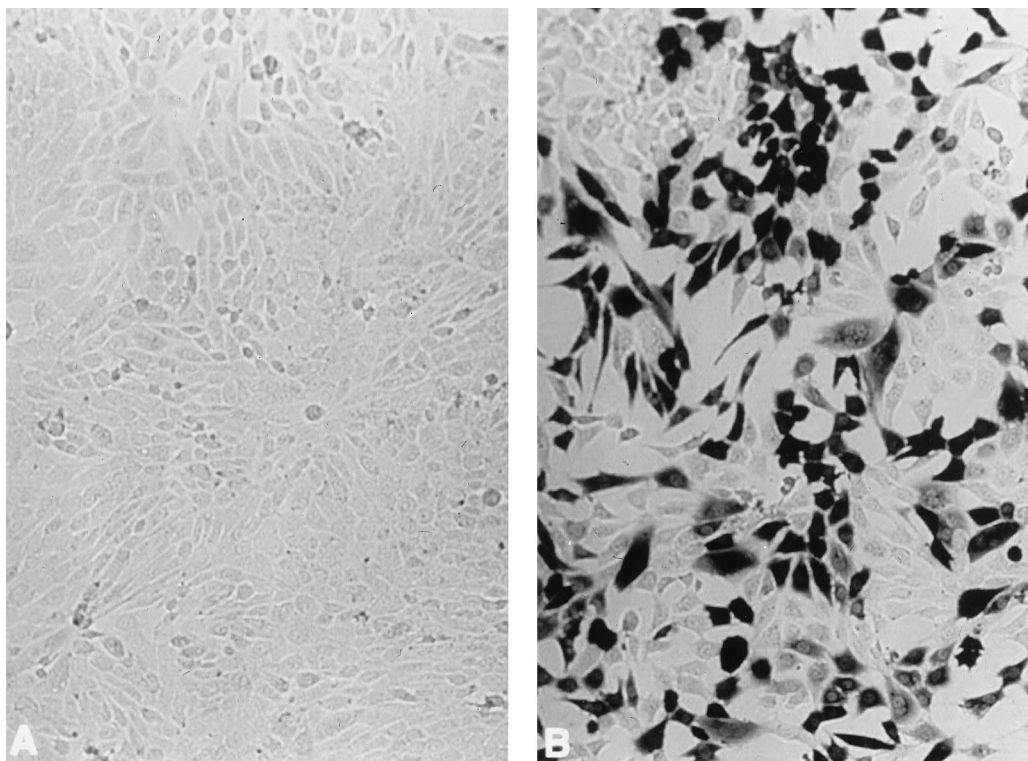


FIG. 3. Control MDTF cells (A) and MDTF cells expressing AAAA*A cDNA (B) were exposed to 2.0 ml of supernatant containing PG13/G1BgSvN retroviral vectors. Cells histochemically stained at 48 h after PG13/G1BgSvN exposure are shown. Dark-stained foci represent β -galactosidase-expressing cells infected by GALV vectors. Magnification, $\times 100$.

TABLE 3. Retroviruses that use PiT1, PiT2, or their homologs as receptors

Receptor	Use by ^a :				Reference(s)
	GALV	A-MuLV	FeLV B	10A1 MuLV	
PiT1	Yes	No	Yes	Yes	10, 11, 17, 21
RaPiT1	Yes	No	No	Yes	4, 16
PiT2	No	Yes	No	Yes	9, 13, 22
HaPiT2	Yes	Yes	No	Yes	21

^a Yes, virus can use this receptor to infect cells; no, virus can't use this receptor to infect cells.

the inability of HaPiT2 to function as a FeLV B receptor, provide evidence that FeLV B and GALV have different functional requirements within this nine-amino-acid region.

The results we obtained with HaPiT2-PiT1 chimeric receptors to map the extracellular regions of HaPiT2 that controlled A-MuLV receptor function were similar to previously published results obtained with PiT2-PiT1 chimeric receptors (10, 13). We found that the substitution of either the first three domains of HaPiT2 or the last two extracellular regions of HaPiT2 for the corresponding region of PiT1 is sufficient to confer A-MuLV receptor function to PiT1 receptor chimeras. One distinguishing finding of the present study was the observation that HaPiT2-PiT1 chimeric A-MuLV receptors were 5- to 10-fold less efficient than the corresponding PiT2-PiT1 chimeric A-MuLV receptors were. On the other hand, the substitution of regions of HaPiT2 for corresponding regions of PiT2 did not result in a decrease in A-MuLV receptor function. This suggests that the combinatorial conformations obtained between HaPiT2 and PiT2 are more optimal for A-MuLV receptor function than are those obtained with HaPiT2 and PiT1.

Alignment of the carboxy termini of the fourth extracellular regions of PiT1 and PiT2 reveals that seven of the nine amino acid residues contained in the A region of these proteins are not conserved (Table 2). HaPiT2 and PiT1 also differ by seven residues within the A region (Table 2). Of the seven residues that differ between PiT1 and PiT2, only two represent conservative amino acid substitutions (the S→T at position 555 and the V→A at position 558). PiT1 contains negatively charged glutamic acid residues at positions 550 and 554 and a positively charged lysine residue at position 557. In contrast, PiT2 contains a positively charged lysine, an uncharged glycine, and a negatively charged glutamic acid residue at the positions corresponding to 550, 554, and 557, respectively (Table 2). HaPiT2 contains the same residues as does PiT2 at positions 554 and 557 but differs from PiT1 and PiT2 in that it contains a glutamic acid residue at the position corresponding to 550 (Table 2). We have now determined that AAAA*A (a modified form of PiT2 in which a glutamic acid residue replaces the lysine residue at position 522) functions as a GALV receptor. Of the seven amino acids in the fourth extracellular region that distinguish between PiT1 and PiT2, it is solely the lysine residue at position 522/550 that is incompatible with GALV receptor function.

Absolute conservation of the nine amino acid residues within the A region is not required for GALV receptor function. Considerable flexibility in both the positions and identities of charged residues in this region exists. Although a negatively charged residue (either aspartic acid or glutamic acid) at position 550 is critical for GALV receptor function, the charged residues at positions 553 and 557 do not appear to play such an essential role. Our results with both HaPiT2 and the AAAA*A mutant demonstrate that receptors in which a neutral residue is substituted for the aspartic acid residue at 553

and an acidic residue is substituted for the basic lysine residue at position 557 function as GALV receptors (Table 3). Both polar uncharged and hydrophobic nonpolar residues are tolerated at position 555 (16, 21) (Table 2). It is important to note that the seven residues that differ within the fourth extracellular regions of PiT1 and HaPiT2 may play a role in the relative efficiency of GALV receptor function. Such a role is supported by the finding that chimeric receptors containing the last two extracellular regions of HaPiT2 in combination with the first three extracellular regions of either PiT1 or PiT2 functioned at least 10-fold less efficiently than did either wild-type PiT1 or the EEEGG chimeric receptor.

In contrast to the highly polymorphic nature of the carboxy terminus of the fourth loop, the amino terminus of this proposed extracellular region is invariant in all GALV and A-MuLV receptors characterized to date. The invariant nature of this region extends to nonfunctional GALV receptor homologs. At present, it is not clear if the highly polymorphic C-terminal region of the fourth extracellular domain of PiT1 represents a virus binding site. On the other hand, it is obvious that the presence of a lysine residue at position 522 of the PiT2 protein is incompatible with GALV receptor function. It is possible that the presence of a positively charged lysine residue at this position blocks virus access to an adjacent region critical for GALV entry rather than directly disrupting the site required for viral entry. Further experiments are under way to determine more precisely the roles of specific regions within the fourth extracellular domain of these receptors for GALV binding and entry.

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