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pH-Independent Murine Leukemia Virus Ecotropic Envelope-Mediated Cell Fusion: Implications for the Role of the R Peptide and p12E TM in Viral Entry

JACK A. RAGHEB* AND W. FRENCH ANDERSON†

Molecular Hematology Branch, National Heart Lung and Blood Institute, Bethesda, Maryland 20892

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Murine leukemia virus ecotropic and amphotropic envelope expression vectors were genetically engineered to generate truncations of the p15E TM cytoplasmic tail. The ecotropic construct CEET has the entire cytoplasmic tail of TM deleted, while the CEETR construct has only the R peptide portion of the tail deleted, thereby producing a TM subunit (p12E) that is identical to the one found in mature virions. The analogous amphotropic constructs were called CAET and CAETR. These envelopes, as opposed to their p15E TM counterparts, mediate cell-to-cell fusion at neutral pH in both transformed and nontransformed cell lines. Though the TM cytoplasmic domain is not required, its presence appears to augment such cell-to-cell fusion. This envelope-dependent fusion requires the presence of the viral receptor on the cell surface. Ecotropic virions bearing the p12E TM contain wild-type levels of the envelope complex and have near-normal titers. In contrast, virions which lack the cytoplasmic domain of TM (e.g., CEET) have 10- to 100-fold-lower titers but contain normal amounts of envelope. Both of the corresponding amphotropic virions contain normal amounts of envelope but have 10- to 100-fold-lower titers. Using immunofluorescent detection of envelope to monitor the fate of receptor-bound virions, we found that ecotropic murine leukemia virus envelope disappears from the cell surface while amphotropic envelope persists on the cell surface after virus binding. This pattern of immunofluorescence is consistent with the proposed routes of cell entry for these viruses, i.e., by endocytosis and direct fusion, respectively. In this assay, ecotropic virions bearing the genetically engineered p12E TM also appear to be internalized despite the ability of their envelope to mediate fusion at neutral pH in the same target cells. Our results show that direct fusion at neutral pH is a natural consequence of the surface expression of the mature ecotropic envelope and its receptor. We propose that the processing of the R peptide from the envelope TM (p15E) to yield p12E, at the time of virus budding or within virions, renders the envelope competent to fuse.

The murine leukemia viruses (MuLV) are classified into five subgroups (ecotropic, amphotropic, polytropic, xenotropic, and 10A1) on the basis of their host range (23, 34, 35). Viral tropism is determined in large part by the specific interaction between the viral receptor on the host cell and the retroviral envelope protein (6, 27). The envelope complex is an oligomer of heterodimers composed of two polypeptides, SU and TM (26). These proteins are encoded by the *env* gene and processed by a host cell protease from a common precursor. In the ecotropic Moloney MuLV, SU is a 70-kDa glycoprotein (gp70) and TM is a 15-kDa polypeptide (p15E) (26, 39, 40). p15E is processed further by a viral protease in virions or at the time of budding to produce a 12-kDa protein (p12E) and a 16-amino-acid oligopeptide (R peptide) (5, 7).

Among the MuLV subgroups, the envelope protein sequence is highly conserved from the carboxyl-terminal region of SU through the end of TM (23). We and others have recently mapped the determinants of host range to the N-terminal one-third of the SU protein (3, 19, 24). TM is an integral transmembrane protein which anchors SU to the virion surface through covalent and noncovalent interactions and is believed to be a vital component of the viral entry process (9, 14, 28). The function of the R peptide, however, is unknown. As for other enveloped retroviruses, upon binding of SU to the viral receptor, TM mediates fusion of the virion membrane to the host membrane, which may occur at the cell surface or within an endocytic compartment (8, 16, 17). Mutations within the N-terminal region of TM block this fusion (9).

It has been proposed that the MuLV subgroups utilize different routes of entry into host cells (16, 21). Infection of murine cells by ecotropic MuLV is thought to occur through an endocytic pathway following binding of the virus to its cell surface receptor (2, 16, 17, 29). It has been proposed that the acidic pH of the endosome induces a conformational change in the envelope which is required to mediate the fusion of virion and host membranes (16, 29). In contrast, amphotropic MuLV is thought to enter cells by direct fusion of the virion membrane to the cell surface membrane (i.e., at neutral pH) following receptor binding (16, 17, 35).

Neither amphotropic nor ecotropic MuLV produce syncytia in the cells that they infect. However, for ecotropic MuLV, there are two transformed cell lines, XC and 3T3/DTras, which are exceptions to this generalization (9, 13, 31, 38). As the envelope-dependent formation of syncytium occurs at neutral pH, presumably a pH-induced conformational change in the ecotropic envelope is not required for fusion to occur in these cell lines.

We and others have recently described the use of envelope expression vectors to study envelope function in the absence of

* Corresponding author. Mailing address: Molecular Hematology Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Building 10, Room 7D-18, Bethesda, MD 20892. Phone: (301) 496-5844. Fax: (301) 496-9985.

† Present address: Norris Cancer Center, University of Southern California School of Medicine, Los Angeles, CA 90033.

TABLE 1. Carboxyl amino acid sequence of envelope TM[a]

Virus	Sequence
	633 R peptide →
Ecotropic wild type	N R L V Q F V K D R I S V V Q A L:V L T Q Q Y H Q L K P I E Y E P
	633
CEET	N R
	633
CEET2	N R d
	633
CEETR	N R L V Q F V K D R I S V V Q A L
	622 R peptide →
Amphotropic wild type	N R L V Q F V K D R I S V V Q A L:V L T Q Q Y H Q L K P I E Y E P
	622
CAET	N R d
	622
CAETR	N R L V Q F V K D R I S V V Q A L

[a] Amino acid substitutions are shown in lowercase. :, R peptide cleavage site.

other viral proteins (9, 19, 22). Using syncytium formation in XC cells as an indicator of envelope-mediated fusion, we demonstrated that the membrane-spanning, but not the cytoplasmic, domain of p15E is required for fusion (30). We now report that in the ecotropic envelope TM cytoplasmic tail, the R peptide acts to inhibit cell-to-cell fusion, and in its absence, nontransformed murine cells readily undergo receptor-specific envelope-mediated syncytium formation.

MATERIALS AND METHODS

Envelope constructs. Nucleotide and amino acid positions for the ecotropic envelope correspond to the numbering of Schinnick et al. (32); numbering for the amphotropic envelope corresponds to that of Ott et al. (23). In both cases, amino acid 1 is the first amino acid of the envelope signal peptide. All envelope constructs were expressed from a cytomegalovirus promoter-driven expression vector which contains a simian virus 40 poly(A) termination signal. pCEE, which contains the entire Moloney MuLV ecotropic envelope sequence (positions 5408 to 7847), has been previously described (19). pCEET was constructed by linearizing pCEE at the unique *Cla*I site, Klenow fill-in of ends, and ligation of a 14-bp *Xba*I STOP linker (New England Biolabs catalog no. 1062) (30). pCEET2 was constructed by linearizing pCEE at the unique *Cla*I site, Klenow fill-in of ends, and recircularization. pCEETR was constructed by linearizing pCEE at the unique *Cla*I site and introducing an oligonucleotide that encodes the cytoplasmic domain (amino acids 634 to 649) followed by a stop codon and a unique *Bgl*II site. pCAE, which contains the 4070A amphotropic envelope in the same backbone as pCEE, has been previously described (19). pCAET was generated by linearizing pCAE at the unique *Cla*I site, Klenow fill-in of ends, and recircularization. pCAETR was constructed by linearizing pCAE at the unique *Cla*I site and introducing an oligonucleotide that encodes the cytoplasmic domain (amino acids 623 to 638) followed by a stop codon and a unique *Bgl*II site. All plasmid constructs were confirmed by DNA sequencing (Sequenase 2.0; U.S. Biochemical, Cleveland, Ohio). Table 1 shows the predicted amino acid sequences of the envelope constructs.

Cell lines, DNA transfection, and viral titers. All cell lines except XC and Chinese hamster ovary (CHO) lines were maintained in Dulbecco's modified essential medium (Biofluids, Rockville, Md.) XC cells were grown in Eagle's minimal essential medium (Biofluids); CHO cell lines were grown in Alfa modified minimum essential medium (Biofluids). All media were supplemented (10%) with heat-inactivated fetal calf serum (HyClone, Logan, Utah). The GPL and CHO-2 lines have been described elsewhere (10, 19). XC cells were a gift from Janet Hartley. DNA (40 μg, total) was transfected into GPL or NIH 3T3 cells (5×10^5 cells in a 100-mm-diameter dish) by the calcium phosphate precipitation method, using reagents from 5′-3′ Inc. (Boulder, Colo.). At 16 to 20 h posttransfection, the cells were washed and fresh medium was added. Viral supernatant was harvested 48 h later by collecting and filtering the cell medium. Viral supernatant was used immediately or stored at −70°C prior to assay. G418^r CFU-per-milliliter viral titer was determined as previously described (19). Virus obtained from transient expression of an envelope vector in GPL cells will have a titer of $\sim 10^4$ CFU/ml, while that of virus from a recombinant retroviral producer line established by the micro ping-pong method will have a titer of 10^6 CFU/ml.

Flow cytometry. The virus binding assay was performed as described previously (10) except that 3.5 ml of viral supernatant was used for 10^6 NIH 3T3 cells and all incubations were performed at 4°C for 3 h for ecotropic virus and 37°C for 45 min for amphotropic virus unless indicated otherwise. Flow cytometry was performed by Fast Systems Inc. (Gaithersburg, Md.) on an EPIC 752 flow cytometer (Coulter, Hialeah, Fla.). For orange fluorescence signals, the cytometer is used at a high voltage setting of 1150. Settings were calibrated daily to channel number 185, using standardized fluorescent beads from Coulter. Transient expression of wild-type ecotropic envelope in the *gag-pol* (GPL) line produces envelope bearing viral particles that have a relative fluorescence intensity in the virus binding assay that is approximately 25 to 50% of that seen with viral particles from a stable *gag-pol-env*-expressing line such as GPE+86. Virus binding is expressed as the median relative fluorescence intensity normalized to the signal obtained from a positive control.

In temperature shift experiments, virus binding was carried out at 4°C for 2 h, after which the cells were washed to remove unbound virus and resuspended in complete medium ($t = 0$). Aliquots of the washed, cell-bound virus were then incubated at 37 and at 4°C. At 15-min intervals, 10 volumes of ice-cold phosphate-buffered saline (PBS) was added to the samples to return the cells to 0 to 4°C. Samples were maintained on ice until the end of the experiment and then immunostained as usual.

Detection of cell surface envelope on 10^6 transfected cells was performed as in the virus binding assay after the virus adsorption step. The transfected cells were dissociated with

enzyme-free dissociation solution (Specialty Media, Inc., Lavallette, N.J.) and washed in PBS with 10% normal goat serum prior to immunostaining.

Cocultivation fusion assay. About 1×10^5 to 2×10^5 3T3 cells in a 60-mm-diameter dish were transfected with 30 μg of envelope expression vector DNA as described above. Twenty-four hours after fresh medium had been added, $\sim 10^6$ XC cells were added to the plate and the culture was incubated for an additional 24 h. Alternatively, if the cells were too dense after transfection, the cells were trypsinized (trypsin-EDTA, 0.05%/0.02%; Biofluids) briefly, $\sim 10^6$ XC cells were added, and the coculture was replated on a 100-mm-diameter dish. Plates were stained with 1% methylene blue in methanol prior to photography.

RESULTS

Ecotropic envelope-mediated membrane fusion. In previous studies defining the functional domains of the TM polypeptide, we determined that an envelope construct (CEET) in which the entire p15E cytoplasmic domain had been deleted produced syncytia in the transformed XC cell line to an equal extent as wild-type envelope (30). Ecotropic envelope-mediated cell-to-cell fusion has been reported to occur only in XC cells and another transformed cell line, 3T3/DTras (38). We now show that expression of the CEET envelope in nontransformed cell lines also results in syncytium formation.

The nontransformed cell line GPL, which is derived from NIH 3T3 cells, constitutively expresses the MuLV *gag* and *pol* products and contains a retroviral vector genome (LNL6) that carries a neomycin resistance gene (19). Transient expression of the CEET envelope, but not wild-type ecotropic envelope, produces extensive syncytia in GPL cells (Fig. 1).

To help characterize the determinants of syncytium formation in the GPL line, the CEET envelope was transiently expressed in two other 3T3-based cell lines that constitutively produce the MuLV *gag* and *pol* products: PA317 and GPE+86 (15, 18). The PA317 and GPE+86 lines are distinguished from the GPL line in that they constitutively express a MuLV envelope in addition to the *gag* and *pol* products. The PA317 line expresses an amphotropic envelope, while the GPE+86 line expresses an ecotropic envelope. As a consequence of the constitutive expression of the amphotropic envelope in the PA317 line, the cell surface expression of the amphotropic viral receptor is severely down regulated, but the ecotropic receptor is unaffected. The converse is true of the GPE+86 line, in which the ecotropic envelope is constitutively produced.

There is abundant syncytium formation when the ecotropic CEET envelope is expressed in PA317 cells but not when it is expressed in GPE+86 cells (Fig. 1). These results indicate that the *gag* and *pol* products, together with the CEET envelope, are not sufficient for cell-to-cell fusion, as expression of these proteins did not result in syncytia in GPE+86 cells. It may also be inferred that the presence of the ecotropic receptor on the cell surface is required for syncytium formation. None of the lines formed syncytia when expressing the wild-type ecotropic envelope (Fig. 1). These observations indicate that the ability to mediate cell fusion is a property of the truncated envelope, but that there are additional cellular requirements for syncytium formation.

Having demonstrated that Gag and Pol proteins are not sufficient for cell-to-cell fusion, we next determined whether they are necessary for GPL cell syncytia. Transient expression of the CEET envelope in NIH 3T3 cells (which produce no Gag or Pol protein) results in the formation of large multinucleated syncytia but not as extensively as in GPL or PA317 cells (Fig. 2). This result indicates that while the *gag* and *pol* products are not required, they do appear to augment membrane fusion in cells expressing the CEET envelope.

Requirement for the viral receptor in membrane fusion. The inability of the CEET envelope to form syncytia in the GPE+86 cell line (see above) suggests that fusion is dependent on the presence of the ecotropic receptor on the cell surface. To address directly whether the cell-to-cell fusion that we observed requires the ecotropic receptor, we transiently expressed the CEET envelope in several cell lines that are positive or are negative for the ecotropic MuLV viral receptor. Only those cells which express the ecotropic receptor on their surface form syncytia when transfected with the CEET envelope expression plasmid (Table 2).

CHO-2 is a CHO cell line into which the gene for the ecotropic receptor has been stably transfected. The ability of CEET to form syncytia in CHO-2 but not CHO cells demonstrates directly the necessity for the ecotropic receptor in cell-to-cell fusion (Table 2; Fig. 3). None of the lines formed syncytia when transfected with a plasmid that expresses the wild-type envelope.

To exclude the possibility that the absence of syncytia in the transfected receptor-negative cell lines was due to poor expression of envelope, immunofluorescence flow cytometry was performed. Envelope protein was found to be present on the cell surface of all transfected lines to comparable extents except for mink cells (data not shown).

To determine whether the CEET envelope expressed on the surface of cells that do not bear the ecotropic receptor is capable of mediating fusion with cells that do carry the receptor, HeLa cells transiently expressing the CEET envelope were cocultivated with rodent XC or GPL cells. The CEET envelope mediated fusion between HeLa and GPL cells just as well as between HeLa and XC cells (Fig. 4). This result also demonstrates that the nontransformed GPL cell line can be as sensitive an indicator of CEET envelope-mediated cell-to-cell fusion as the transformed XC line.

***in*8125-2 envelope-mediated membrane fusion.** The CEET envelope is very similar to the envelope encoded by the proviral mutant *in*8125-2 described by Granowitz et al. (4). However, in contrast to CEET, the *in*8125-2 envelope was found not to be incorporated into virions; in addition, syncytium formation in NIH 3T3 cells transiently or stably transfected with the proviral DNA was not reported. To examine the *in*8125-2 mutant in our virus-free system, we recreated the identical mutation in our envelope expression vector to produce CEET2 (Table 1). The CEET2 envelope was found to be equivalent to CEET in its ability to form syncytia among GPL cells (Fig. 5).

Role of the R peptide in membrane fusion. The truncation in the CEET envelope occurs at the membrane-cytosol junction (Table 1). CEET thus lacks both the cytoplasmic domain of TM and the R peptide which is ordinarily cleaved from the carboxyl end of p15E to produce p12E at the time of viral budding or within virions.

To determine the contribution of the R peptide to the phenotype observed with CEET, we generated an envelope, CEETR, that terminates precisely at the end of the cytoplasmic domain of the mature TM protein p12E (Table 1). When CEETR is expressed in GPL cells, it mediates syncytium formation even more effectively than CEET does (Fig. 5).

Amphotropic envelope-mediated membrane fusion. The 4070A and Moloney retroviruses belong to the amphotropic and ecotropic MuLV subgroups, respectively. Both infect murine cells, though they utilize different receptors and enter through different pathways. In general, neither forms syncytia

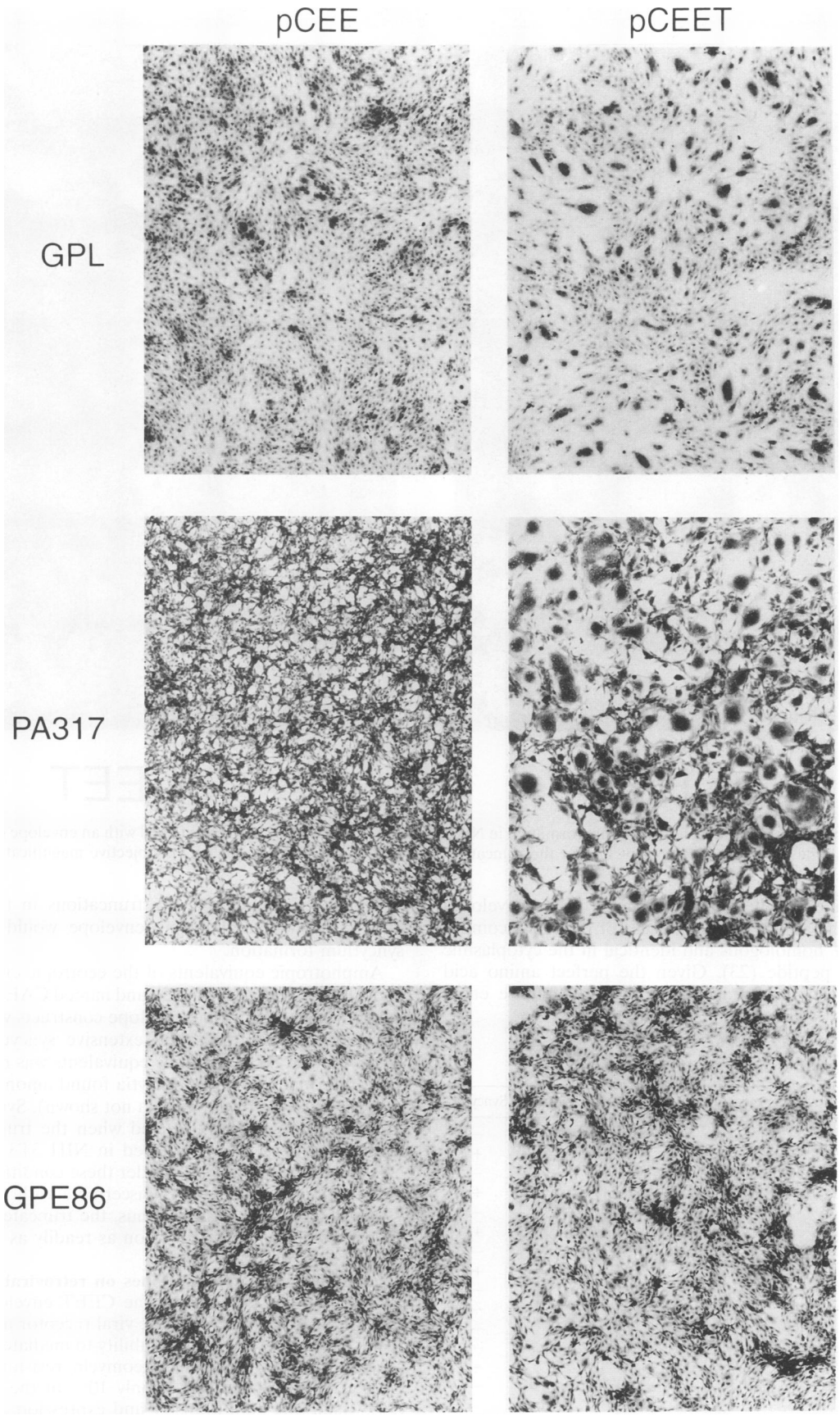

FIG. 1. Ecotropic envelope-mediated syncytium formation in *Gag-pol* cell lines. The indicated cell line was transfected with an envelope expression vector encoding the wild-type ecotropic envelope (pCEE) or the truncated CEET ecotropic envelope (pCEET). Objective magnification, $\times 50$.

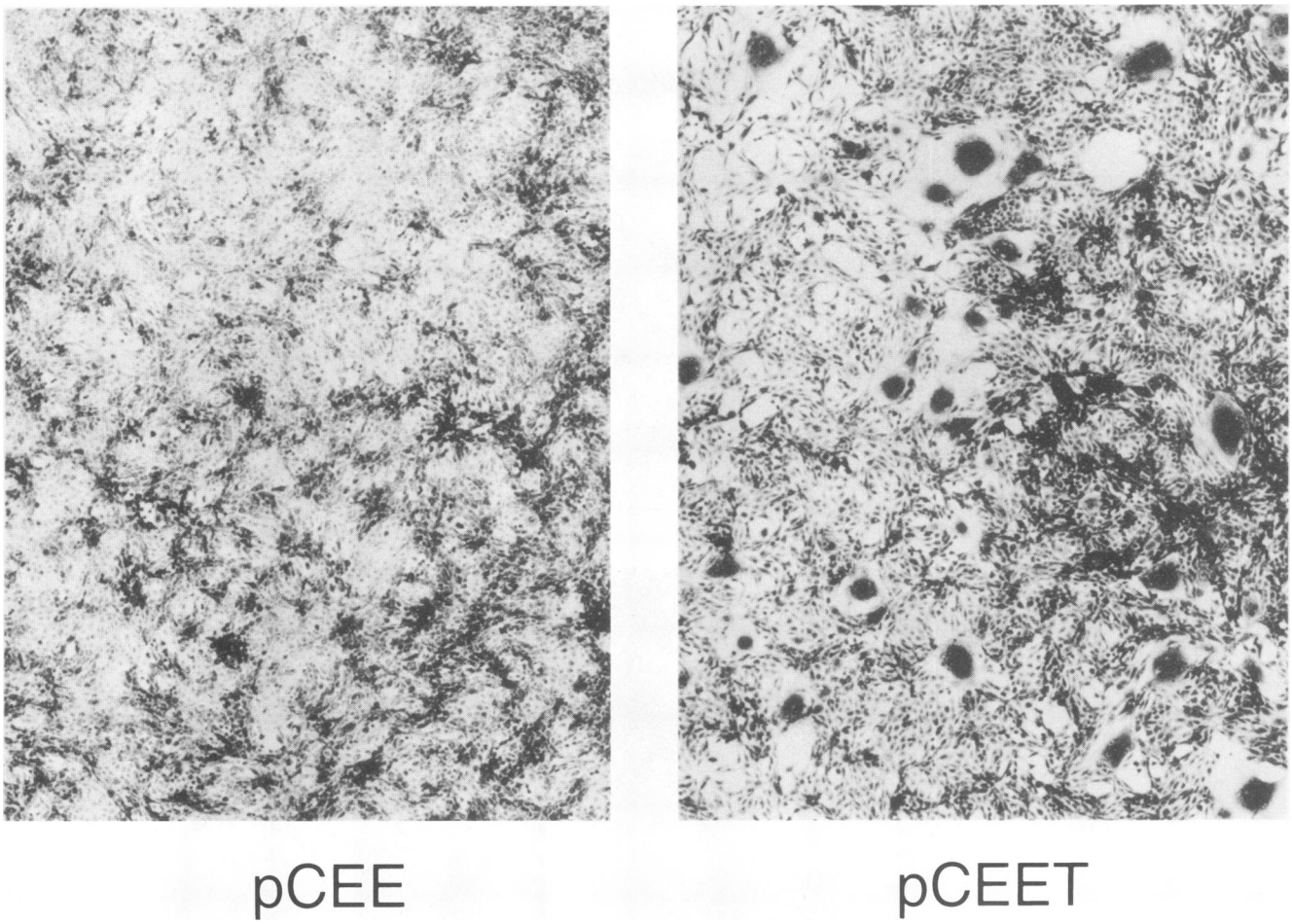

FIG. 2. Ecotropic envelope-mediated syncytium formation in NIH 3T3 cells. NIH 3T3 cells were transfected with an envelope expression vector encoding the wild-type ecotropic envelope (pCEE) or the truncated CEET ecotropic envelope (pCEET). Objective magnification, ×50.

in murine cell lines. While the SU subunits of their envelopes are divergent, the amino acid sequences of their TM components are ~80% homologous and identical in the cytoplasmic domain and R peptide (23). Given the perfect amino acid homology between the intracellular domains of these envelopes, we examined whether truncations in the cytoplasmic domain of the amphotropic envelope would also result in syncytium formation.

TABLE 2. Summary of syncytium formation

Cell line	Ecotropic receptor	Envelope	Syncytia[a]
GPL	+	wt[b]	–
		CEET	+
PA317	+	wt	–
		CEET	+
NIH 3T3	+	wt	–
		CEET	+
CHO-2	+	wt	–
		CEET	+
GPE+86	–	wt	–
		CEET	–
HeLa	–	wt	–
		CEET	–
Mink	–	wt	–
		CEET	–
CHO	–	wt	–
		CEET	–

[a] +, >10 syncytia per ×100 field with >4 nuclei.
[b] wt, wild type.

Amphotropic equivalents of the ecotropic envelopes CEET and CEETR were constructed and named CAET and CAETR. When these amphotropic envelope constructs were transiently expressed in GPL cells, the extensive syncytium formation observed with their ecotropic equivalents was not seen. There were rare multinucleated syncytia found among cells expressing the CAETR envelope (data not shown). Syncytium formation was more readily detected when the truncated amphotropic envelopes were expressed in NIH 3T3 cells and then cocultivated with XC cells. Under these conditions, a low level of syncytium formation was discernible with the CAET and CAETR envelopes (Fig. 6). Thus, the truncated amphotropic envelopes do not mediate fusion as readily as their ecotropic counterparts do.

Effects of truncated envelopes on retroviral gene transfer. We previously reported that the CEET envelope is incorporated into virions and binds the viral receptor nearly as well as the wild-type envelope, yet its ability to mediate the transfer of a retroviral vector-encoded neomycin resistance gene from GPL cells to a target cell is only 10% of the wild-type level (30). Using the gene transfer and expression assay described previously (19), we examined the ability of constructs lacking the R peptide to mediate postfusion binding events. As before, the titer of virions with the CEET envelope is ~10% of that

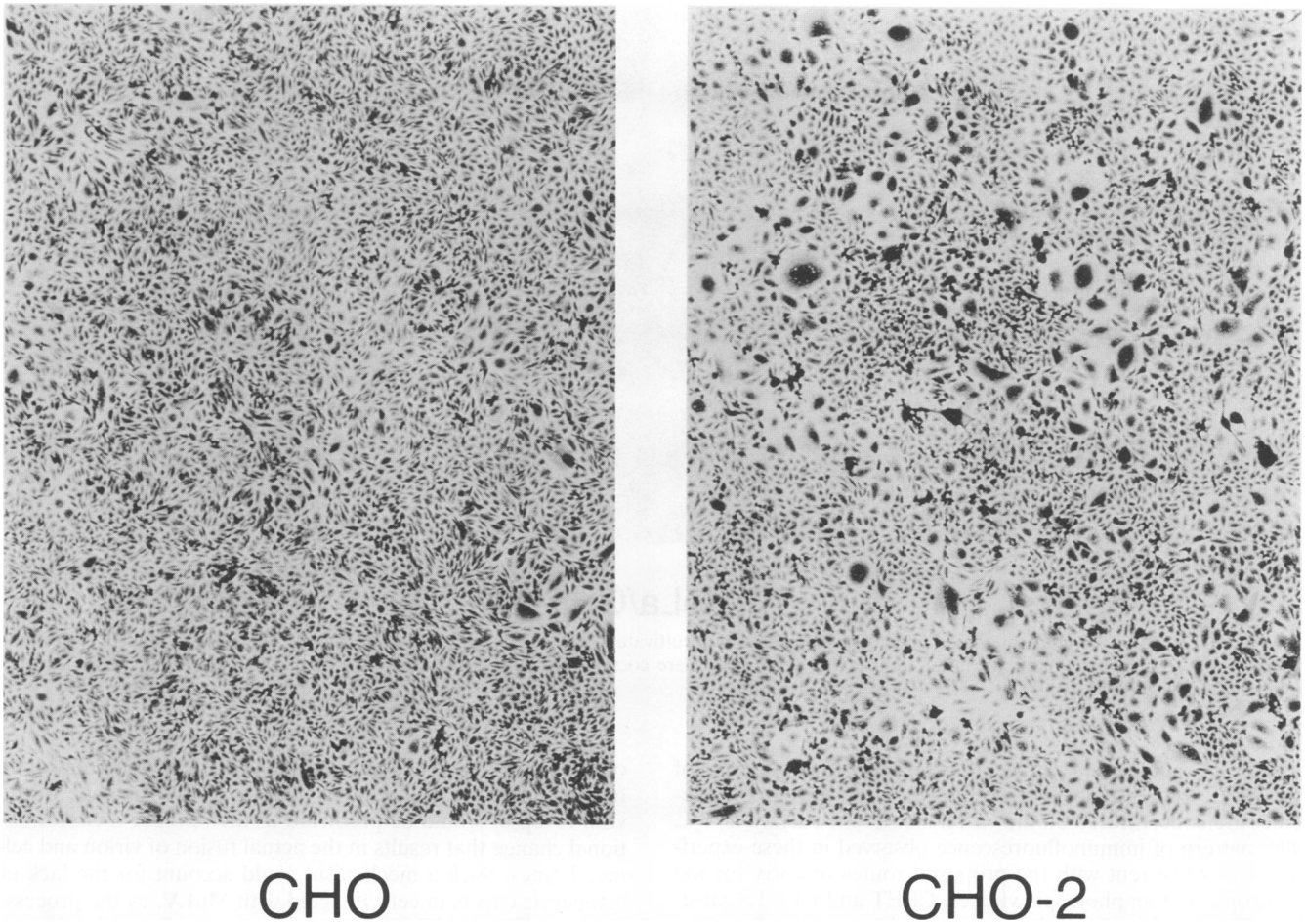

FIG. 3. Ecotropic envelope-mediated syncytium formation in CHO cell lines. CHO cells and CHO-2 cells (a stable CHO cell line which expresses the MuLV ecotropic receptor) were transfected with an envelope expression vector encoding the truncated CEET ecotropic envelope (pCEET). Objective magnification, ×50.

seen with virions expressing the wild-type envelope, while that of virions with the CEETR envelope is comparable to the wild-type titer (Table 3). Amphotropic CAETR virions, in contrast to ecotropic CEETR virions, had a titer that was only ~10% of the control value (Table 3).

We also determined the titers of virions carrying the amphotropic or ecotropic envelopes with the *in*8125-2 mutation. Virions with the CAET or CEET2 envelope gave a titer that was ~1% of that obtained with their respective wild-type envelopes.

To determine whether the differences in titer could be accounted for on the basis of differential incorporation of the envelopes into virions, the same viral supernatants were tested in the virus binding assay (10, 19). All envelopes of a given subgroup were incorporated into virions and bound target cells to comparable extents. The results for syncytium formation (Fig. 1 to 6), titer (Table 3), and virus binding are summarized in Table 4. This relationship between alterations of the cytoplasmic tail, envelope incorporation, and viral titer suggest that an association between the cytoplasmic domain and core may play a role in postfusion events.

Route of viral entry. The route of MuLV entry had been previously established in studies that monitored the fate of radioactively labeled virions and/or examined the pH dependence of infection. It is believed that ecotropic MuLV enter cells by endocytosis, while amphotropic MuLV enter by direct fusion (2, 16, 17, 38). We sought to visualize the route of viral entry into cells by tracking the path of envelope incorporated in the virion membrane. To do so, we slightly modified our standard virus binding assay (10, 19). Virus adsorption to cells at 4°C allows virus binding to receptor but not internalization of the complex and/or membrane fusion (2). By performing virus binding at 4°C, removing unbound virus, and then shifting the cell-bound virus to 37°C to permit fusion or endocytosis, we could monitor the route of viral entry by examining the fate of envelope incorporated in the virion membrane. Internalization of the receptor-virus complex by endocytosis would be expected to result in a loss of envelope from the cell surface. In contrast, entry by direct fusion results in incorporation of the viral envelope into the cell surface membrane and would not be expected to lead to a loss of envelope from the cell surface.

When the virus binding temperature shift assay is performed with MuLV ecotropic and amphotropic virions, the predicted results are observed, as shown in Fig. 7. When maintained at 4°C, neither virus shows a change in the amount of surface-bound envelope over the time course of the experiment. However, at 37°C, the ecotropic envelope disappears from the cell surface while the apparent level of amphotropic envelope increases slightly. The small increase in fluorescence at 37°C is presumably due to redistribution of amphotropic envelope

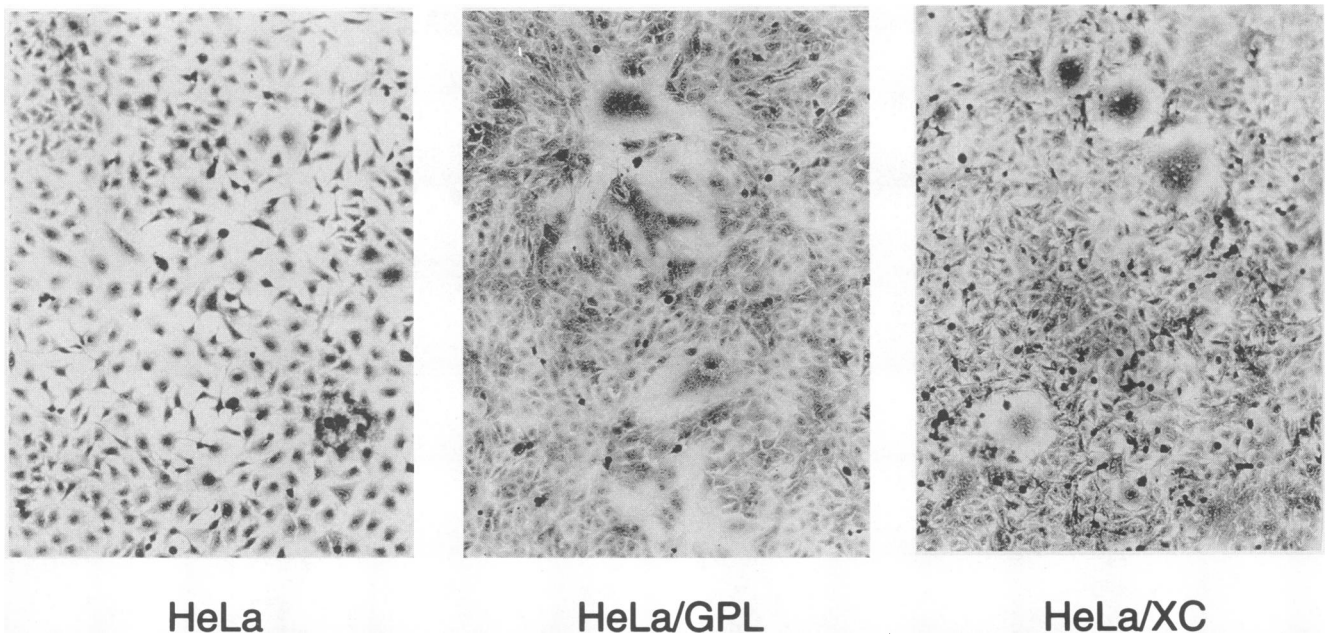

FIG. 4. Ecotropic envelope-mediated syncytium formation in cocultivated HeLa Cells. HeLa cells transiently expressing the CEET envelope were allowed to grow to confluence after transfection (HeLa) or were cocultivated with GPL cells (HeLa/GPL) or with XC cells (HeLa/XC). Objective magnification, ×100.

molecules over the cell surface, thus relieving the quenching of the fluorescence signal that occurs when the envelope molecules are concentrated on the virion membrane surface at 4°C. The pattern of immunofluorescence observed in these experiments is consistent with the proposed routes of entry for the ecotropic and amphotropic viruses. CEET and CEETR envelope-bearing virions also demonstrate a decrease in cell surface fluorescence at 37°C, thus suggesting that despite the ability of these envelopes to mediate direct membrane fusion at neutral pH, the route of viral entry into NIH 3T3 cells may still be through endocytosis (Fig. 7).

DISCUSSION

We have demonstrated that a genetically engineered truncation of the envelope cytoplasmic domain that is identical to the natural form of the processed envelope (p12E) found in mature virions mediates extensive membrane fusion among nontransformed cells at neutral pH.

It has been proposed for pH-dependent enveloped viruses and retroviruses, such as the ecotropic MuLV, that exposure to a low-pH environment is necessary for envelope-mediated membrane fusion to take place (36). The endosome is the most likely site for this pH-dependent fusion event to occur (16, 21, 29). For several enveloped viruses, such as influenza virus and vesicular stomatitis virus, reducing the pH of the extracellular medium permits receptor-bound virus to enter the cell by directly fusing to the cell membrane (16, 21, 37). This finding suggests that low pH induces a conformational change in the envelope which renders the envelope competent to fuse. The pH-dependent ecotropic MuLV cannot be induced to fuse directly to the cell membrane by reducing the extracellular pH (22). Consequently, for Moloney virions, alternative proposals that invoke the action of a pH-dependent cellular protease have been suggested (1, 17).

We propose that it is the processing of the R peptide from p15E to yield p12E, not a pH-dependent event at the surface of or within target cells, that renders the ecotropic envelope fusion competent. Subsequent binding of the envelope to the viral receptor would be predicted to then induce a conformational change that results in the actual fusion of virion and cell membranes. Such a mechanism could account for the lack of cytopathic effects in cells infected with MuLV, as the processing of p15E to p12E occurs by a viral protease at the time of virus budding or within virions. The definitive proof of the role of p12E in viral entry awaits the creation of a mutant MuLV envelope that is blocked in the processing of p12E from p15E.

The temporal and spatial characteristics of the processing of p15E to p12E presumably would exclude it as the pH-dependent step in ecotropic viral entry. The mechanism whereby the cleavage of the R peptide from p15E to yield p12E influences fusion is not clear, since the fusion domain has been mapped to the amino-terminal end of TM, which resides on the opposite side of the cell membrane (9). It can be postulated that despite the spatial separation, a conformational change is induced at the amino terminus upon clipping of the R peptide. In the case of human immunodeficiency virus, it is well known that alteration of the envelope cytoplasmic domain can affect its ability to mediate fusion (33).

While the cytoplasmic domain of TM is not required for incorporation of envelope into virions or for fusion, its presence appears to augment fusion. This is illustrated by the relative ability of the CEET and CEETR envelopes to form syncytia. In contrast, minor alterations of the cytoplasmic domain have a dramatic effect on viral titer. The addition of a single negatively charged amino acid at the carboxyl end of the CEET2 TM relative to the CEET TM decreases the titer 10-fold without comparably affecting incorporation of envelope into virions or membrane fusion (Table 4). For human immunodeficiency virus, truncation of the cytoplasmic tail results in diminished viral titer as a consequence of defective incorporation of envelope into virions. The relationship between alterations of the MuLV envelope cytoplasmic domain, envelope incorporation, and viral titer suggests that interac-

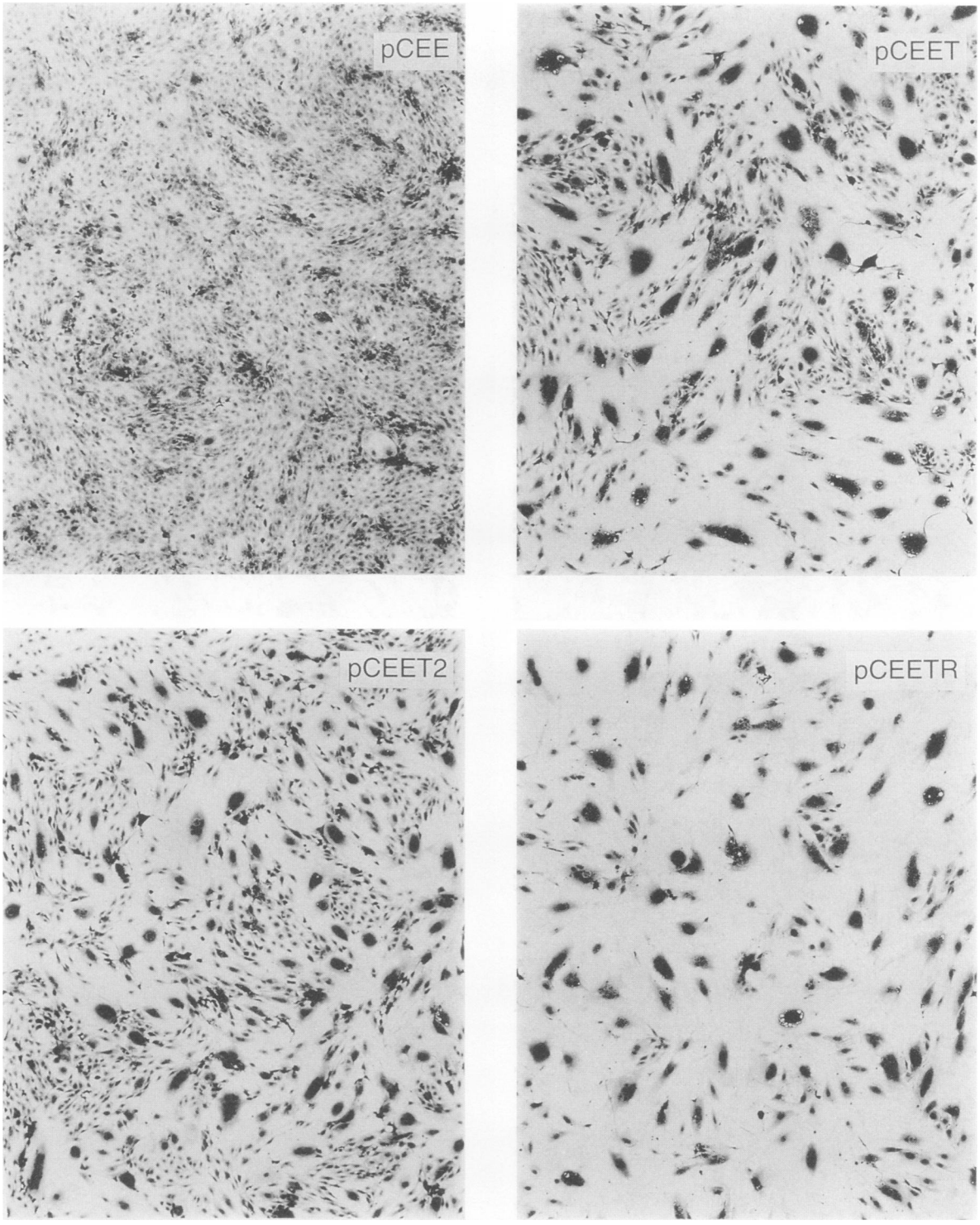

FIG. 5. Ecotropic envelope-mediated syncytium formation in GPL cells. GPL cells were transfected with an envelope expression vector encoding the wild-type ecotropic envelope (pCEE) or the indicated truncated ecotropic envelope (pCEET, pCEET2, or pCEETR). Objective magnification, ×50.

tions between MuLV core and envelope affect some step after viral entry.

The TM subunits of the amphotropic and ecotropic MuLVs are ~80% homologous overall and identical in their cytoplasmic domains (23). Nonetheless, truncations of the amphotropic TM identical to those made in the ecotropic TM had quite different effects on membrane fusion and viral titer. Whereas removal of the R peptide leads to extensive syncytium formation in cells expressing the CEETR envelope, a much lower level of syncytium formation is observed with the

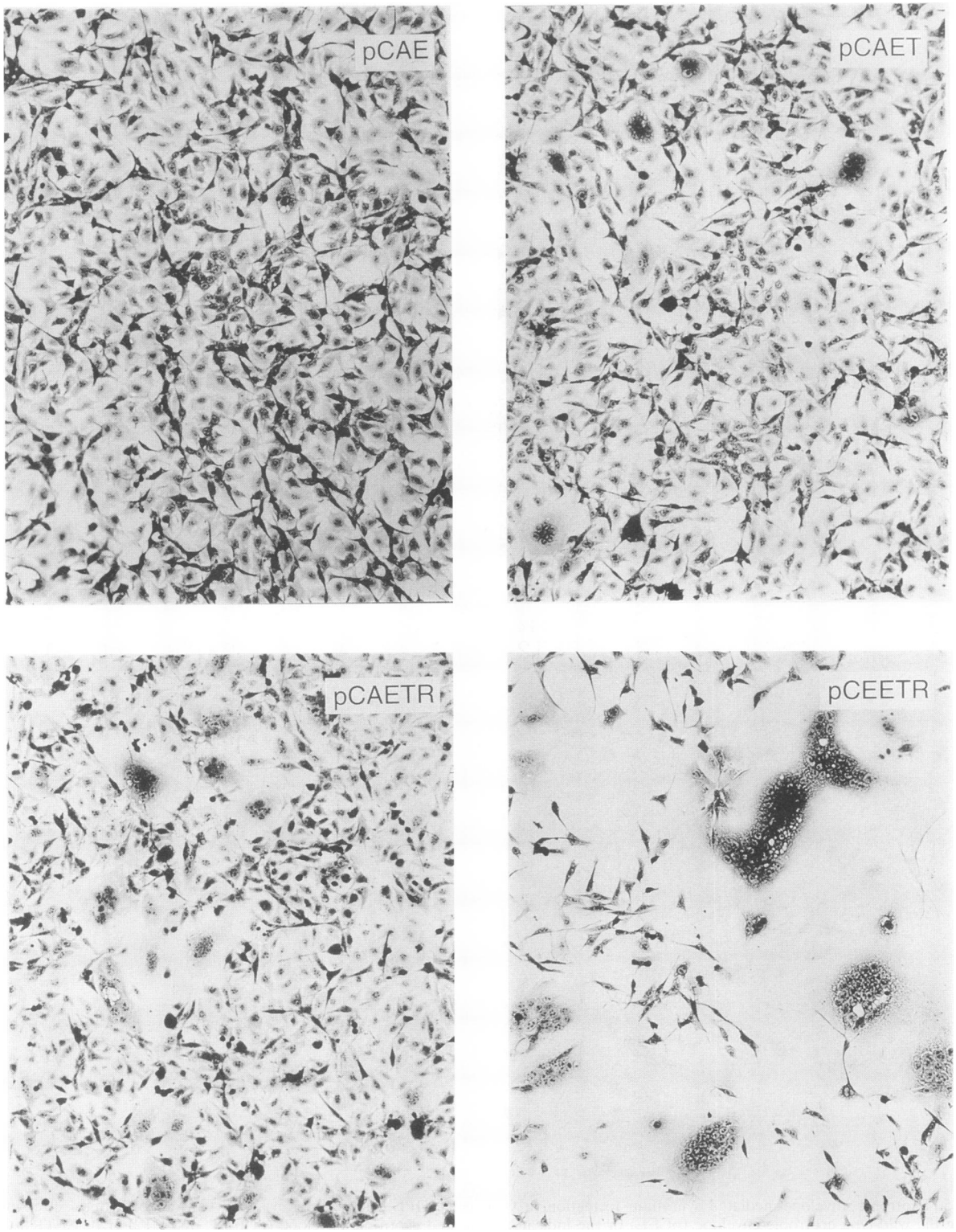

FIG. 6. Amphotropic envelope-mediated syncytium formation in cocultivated NIH 3T3 cells. NIH 3T3 cells were transfected with an envelope expression vector encoding the wild-type amphotropic envelope (pCAE), the indicated truncated amphotropic envelope (pCAET or pCAETR), or a truncated ecotropic envelope (pCEETR). Objective magnification, ×50.

CAETR envelope. Similarly, virions with the CEETR envelope had nearly normal titers, while those with CAETR had titers 10-fold lower (Table 3). While cell death secondary to syncytium formation may account for some of the decrease in viral titer, it is unlikely to contribute to more than a twofold reduction, as the envelope (CEETR) which produces the most extensive syncytia yields a viral titer that is nearly normal. Differences between the ecotropic and amphotropic SU sub-

TABLE 3. Titer of viral supernatant from GPL cells transiently expressing envelope construct

Envelope construct[a]	G418^r CFU/ml	% of wt titer
CEE	5.4×10^4	100
CEET	4.3×10^3	8
CEET2	4.6×10^2	1
CEETR	2.4×10^4	44
CAE	3.4×10^4	100
CAET	1.7×10^2	1
CAETR	4.4×10^3	13

[a] CEE and CAE are the wild-type (wt) ecotropic and amphotropic envelopes.

TABLE 4. Summary of relative envelope-mediated function

Envelope construct[a]	Binding[b]	Syncytia[c]	% of wt titer[d]
CEE	1.0	−	100
CEET	1.2	+++	8
CEET2	1.4	+++	1
CEETR	1.6	++++	44
CAE	1.0	−	100
CAET	1.4	+	1
CAETR	1.5	++	13

[a] CEE and CAE are the wild-type ecotropic and amphotropic envelopes.
[b] Median relative fluorescence intensities of CEE and CAE are 85 and 14, respectively.
[c] Relative syncytium formation in GPL cells for ecotropic envelopes and in 3T3/XC cocultivation for amphotropic envelopes, from Fig. 1 to 6.
[d] See Table 3.

unit, the extracellular TM domain, or the viral receptor might account for these contrasting phenotypes.

We monitored the fate of viral receptor-bound envelope by immunofluorescence to examine the route of cell entry for the ecotropic and amphotropic MuLVs. The disappearance of the ecotropic envelope and the persistence of the amphotropic envelope on the cell surface following virus binding is consistent with the routes of entry previously proposed for these viruses, that is, endocytosis of the ecotropic virus and direct fusion for the amphotropic virus (2, 16, 17, 38). Several lines of evidence suggest that the disappearance of the ecotropic envelope reflects the internalization of MuLV. The temperature dependence of virus binding argues against the elution of virus from the cell surface at 37°C as being responsible for the loss of surface immunofluorescence (10). The disappearance of radioactively labeled virus from the cell surface corresponds to the accumulation of reverse transcriptase activity (2). Immunofluorescent staining of permeabilized cells indicates that the total amount of envelope does not decrease at 37°C, only the amount of envelope present on the cell surface (31a). Finally, data recently obtained for a virus that is directly labeled with a fluorochrome shows that the virus does not elute off the cell surface at 37°C (31a).

Virions bearing the CEET or CEETR envelope also appear to enter cells by internalization, rather than by direct fusion, despite the ability of these envelopes to mediate cell-to-cell membrane fusion. Several alternative explanations might account for this observation. A discordance between pH-dependent infection (i.e., presumptive viral internalization) and cell-to-cell fusion at neutral pH has been observed with the

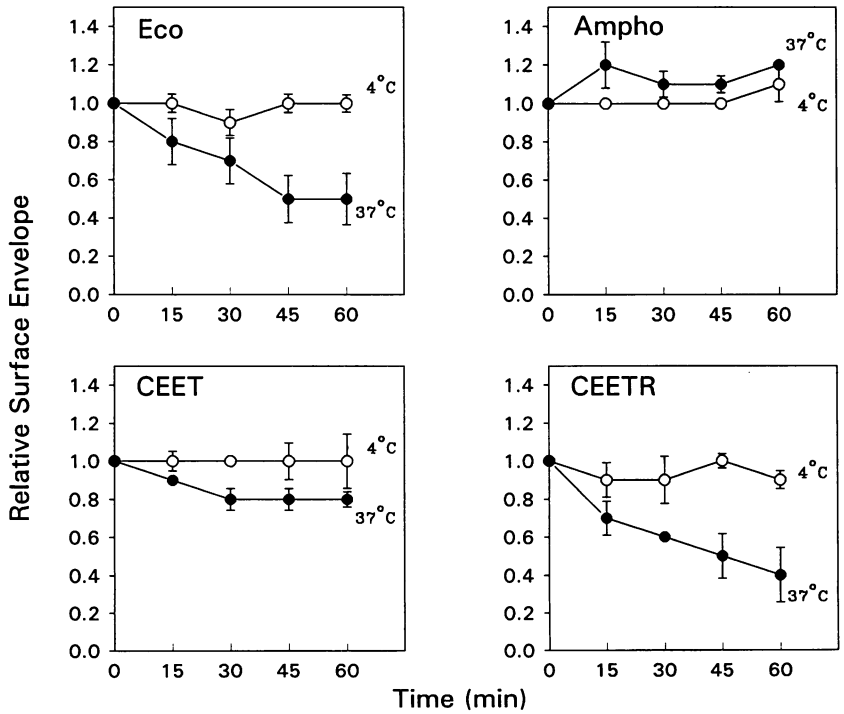

FIG. 7. Viral route of cell entry. Virus binding to NIH 3T3 cells with the indicated envelope-bearing virions was carried out at 4°C for 2 h. At $t = 0$, washed, virus-adsorbed cells were shifted to 37°C (closed circles) or maintained at 4°C (open circles) and incubated for the indicated times (x axis). Relative surface envelope (y axis) is defined as the ratio of the relative fluorescence intensity of a sample at the indicated time and temperature to the relative fluorescence intensity of that same sample at $t = 0$ and 4°C. The error bars indicate standard deviation. Eco, wild-type ecotropic envelope; Ampho, wild-type amphotropic envelope.

ecotropic virus on 3T3/DTras cells (38). This discrepancy suggests either that the ability to form syncytia does not accurately reflect membrane fusion during viral entry or that the pH-dependent step in ecotropic MuLV entry occurs after membrane fusion. Having demonstrated that syncytium formation in nontransformed cell lines can be mediated by an envelope that is identical to one naturally found in mature virions and is a receptor-specific phenomenon, we suggest that the latter is the case.

The ability of envelope to mediate direct membrane fusion does not preclude the possibility that the virus utilizes an endocytic route of entry (for reasons unrelated to fusion) or that fusion occurs within the endosome even though there is no pH requirement for it to do so. We also cannot exclude the possibility, since our conclusions are based on the fate of the envelope protein, that virions bearing the CEET or CEETR envelope fuse directly to the cell membrane with extrusion of the core into the cytoplasm, but that the envelope-receptor complex continues to be internalized.

It is intriguing that CEET virions appear to not be internalized as well as wild-type ecotropic or CEETR virions are (Fig. 7). This observation, along with the reduced titer of CEET virions, may suggest that the envelope cytoplasmic domain plays a role in the internalization of ecotropic virus.

XC cells are the only line for which ecotropic MuLV entry is reported not to be pH dependent (17). It will be of interest to examine the retroviral route of entry in these cells by immunofluorescent measurement of envelope as well as the route of entry of virions carrying our amphotropic-ecotropic chimeric envelopes (19). As these chimeras have switched their host range (i.e., receptor) but retain their original TM subunit, elucidating their route of entry should be quite informative (22). In addition, these chimeras should be helpful in distinguishing between the relative roles of SU, the extracellular TM domain, and the viral receptor in syncytium formation.

Until now, the ecotropic envelope has been shown to mediate syncytium formation only in transformed cell lines (17, 38). We have shown that an engineered ecotropic envelope, identical to the mature form naturally found in virions, mediates cell-to-cell fusion in several nontransformed rodent cell lines. Results with CHO cells that have been stably transfected with the ecotropic receptor gene demonstrate directly that the receptor is required for syncytium formation. In addition, this envelope will mediate fusion between a human cell line and a murine cell line bearing the ecotropic receptor. It has been noted that ecotropic envelope expressed in XC cells has a glycosylation pattern different from that produced in NIH 3T3 cells (9). Our experiments reported here and elsewhere, which utilize expression of envelope in NIH 3T3 cells with subsequent cocultivation with XC cells, indicate that these differences in glycosylation do not appear to be relevant to syncytium formation (30).

We did note that nontransformed cells which constitutively express the retroviral *gag* and *pol* products form syncytia much more readily and showed that one of these lines was as sensitive an indicator for CEET envelope-mediated syncytium formation as XC cells. Such lines are continuously budding bare particles from their cell surface as a result of the expression of the *gag* and *pol* products. Two probable consequences of budding which may contribute to syncytium formation are a destabilization of the cell membrane and an increased rate of membrane turnover, possibly accompanied by an increase in membrane fluidity. Transformed cells have long been recognized to have altered membrane properties (20). In addition, agents which alter the lipid composition and thus the fluidity of nontransformed cell membranes have been reported to alter fusogenicity (11, 12, 25). It may be these features which are common to the *gag-pol* cell lines and to transformed cells. Transformed cell lines are likely to have additional features that increase their propensity to fuse, as these lines, but not the *gag-pol* lines, form syncytia in response to the p15E TM form of the ecotropic envelope and truncated amphotropic envelopes. Nonetheless, our results demonstrate that direct fusion at neutral pH is the natural consequence of the surface expression of a fusion-competent ecotropic envelope and its receptor and not a peculiarity of transformed cell lines.

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