Infectious Entry of Murine Retroviruses into Mouse Cells: Evidence of a Postadsorption Step Inhibited by Acidic pH

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The entry into cells by many enveloped RNA viruses is accomplished by endocytosis and subsequent penetration of the endosomal membrane by an acidic pH-dependent fusion event. In the current study, we examined early events in the infectious entry of mouse retroviruses, using as a framework the observation that infection of a mouse tail skin cell line by the ecotropic virus Friend murine leukemia virus was inhibited at mildly acidic pH (pH 6). This inhibition operated on a postadsorption step, since binding of virus was unaffected at this pH. The rate of penetration of preadsorbed virus, which displayed first-order kinetics, was markedly affected by changes in the pH of the medium. The half-time for disappearance of infectious cell surface virus at 37°C was approximately 10 min at pH 7.6. At pH 6.0, however, >98% of the adsorbed infectivity remained at the cell surface after 45 min. This cell surface virus, though not infecting the cell at pH 6.0, retained its capacity to enter and infect the cell when the pH of the medium was raised. Acidic pH had little effect on the rate of fluid uptake by the cells, as measured by internalization of [³H]sucrose, indicating that global inhibition of endocytosis had not occurred. In contrast, cell fusion induced by Friend murine leukemia virus was optimal at pH 7.6 but markedly inhibited at a pH of <6.4. This inhibitory effect of acidic pH on membrane fusion is unique among the enveloped viruses which have been studied and would preclude entry of Friend murine leukemia virus from within acidified endocytic vesicles. Entry of other members of the ecotropic, mink cell focus-forming, and xenotropic host range groups displayed similar pH sensitivity. However, one xenotropic virus was relatively resistant to the effect of acidic pH, suggesting that differences might exist in the requirements for entry of different retroviruses.

The initial step in the infectious cycle of all viruses is adsorption to cell membrane receptors. Although specific, saturable receptors for retroviral gp70 have been defined (2, 12), their chemical nature remains an enigma. In addition, the role of these receptors in virus adsorption is unclear, since virus appears to bind equally to both permissive and nonpermissive cells (27). It has been suggested that the high degree of host cell specificity expressed by retroviruses is conferred by postadsorption steps in the entry process (27). The postadsorption events which precede the uncoating of retroviruses are not well understood. By inference from studies on the entry of other enveloped viruses (for review, see reference 37), it is likely that penetration of retroviral cores into the cytosol is accomplished by fusion of the viral envelope with the cell membrane, mediated by an as yet unidentified fusion protein in the viral envelope.

The entry of myxo-, rabdo-, and togaviruses involves at least two steps: endocytosis of virions and fusion of viral envelope and the membrane of endocytic vesicles. The fusion proteins of these viruses are activated in the mildly acidic environment of the endosome, fusion being markedly inhibited at the physiological pH of the extracellular milieu (38). In contrast, the fusion protein of paramyxoviruses is relatively insensitive to pH (9), and these viruses appear to enter the cytosol by direct fusion with the plasma membrane (13, 24).

In the current study, we examined the entry of Friend murine leukemia virus (F-MuLV) into a tail skin cell line derived from *Mus dunni*. This model offers several advantages for studying early entry events. At pH 7.6 the entry of preadsorbed virus was very rapid, with a half-time of approx-

imately 10 min. In addition, since infection of this cell line with F-MuLV results in cell fusion (18), we were able to quantify both adsorption and postadsorption (fusion) steps in the entry process. Finally, these cells are unique in their permissiveness to viruses of all host range groups (18), thus allowing a comparison of the entry of different retroviruses in the same cell line.

The results of these studies indicate that, unlike other enveloped RNA viruses, the entry of ecotropic viruses, as well as two mink cell focus-forming (MCF) viruses and a xenotropic virus, was markedly inhibited at acidic pH. For F-MuLV, this pH effect appeared to operate on the postadsorption fusion step, since cell fusion induced by this virus was also inhibited at a pH of <6.4.

MATERIALS AND METHODS

Cells and viruses. The cells used throughout this study were a tail skin cell line derived from *M. dunni* (18) and were originally supplied by S. Chattopadhyay (National Institutes of Health, Bethesda, Md.). These cells were maintained in RPMI 1640 medium plus 10% fetal calf serum. SC-1 cells (31) were maintained in Eagle minimal essential medium plus 5% fetal calf serum. F-MuLV strain I⁻⁵ (22) was kindly provided by S. Gisselbrecht, Laboratoire d'Immunologie et Virologie des Tumeurs, Hôpital Cochin, Paris. The molecularly cloned ecotropic virus Akv623 (19), derived from AKR mice, was supplied by D. Lowy (National Institutes of Health), and PBR-NE-8, from feral mice (15), was supplied by P. Jolicoeur, Institut de Recherches Cliniques de Montreal, Quebec. The xenotropic viruses BALB-IU-1 and AKR-6 and the MCF virus AKR 247 (10, 11) were originally provided by M. Cloyd (Duke University Medical Center, Durham, N.C.). The MCF virus 98D (7) was supplied by B. Chesebro (Rocky

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TABLE 1. Effect of pH on infection^a

Temp and pH	No. of foci per dish
37°C	
7.4.....	41 ± 2.5
6.0.....	1 ± 1.7
2°C	
7.4.....	0
6.0.....	0

^a Cells were incubated with virus for 30 min at 37°C or 2°C at pH 7.4 or 6.0. Dishes were then washed at 2°C, treated with trypsin, and reseeded in growth medium. Infectivity was scored on day 3 and is expressed as number of foci per dish ± 1 standard deviation.

Mountain Laboratories, Hamilton, Mont.). Stocks of ecotropic viruses were prepared from acutely infected SC-1 cells; stocks of xenotropic and MCF viruses were prepared from acutely infected mink or SC-1 cells as described previously (28). Viruses were quantified by a focal immunofluorescence assay (34) with gp70-specific monoclonal antibodies on live cell monolayers. The antibodies used were 24-8 (29) for Akv623, 48 (8) for F-MuLV, 667 (F. J. McAtee and J. L. Portis, submitted for publication) for PBR-NE-8, 18-6 (29) and 603 (28) for the xenotropic viruses AKR-6 and BALB-IU-1, respectively, and 514 (6) for the MCF viruses. In some experiments, F-MuLV was quantified by its cytopathic effect on *M. dunni* cells (18). Dishes were fixed with methanol and stained with Giemsa. Foci of infection were identified by collections of polykaryons surrounding a cleared area of the monolayer. The results of this assay were comparable in sensitivity to those of the focal immunofluorescence assay but allowed examination of the entire dish. Thus, the cytopathic effect assay yielded more consistent results at low levels of infectivity.

Media and solutions. Growth medium was RPMI 1640 medium plus bicarbonate and 10% fetal calf serum. Except where stipulated, washes were carried out with normal saline containing 1% fetal calf serum, 5 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), and 5 mM MES (2[*N*-morpholino]ethanesulfonic acid) adjusted to the pH of the adsorption medium. Adsorption medium was RPMI 1640 medium without bicarbonate plus 2% fetal calf serum buffered with 10 mM HEPES and 10 mM MES and containing 8 µg of polybrene per ml. Penetration medium consisted of adsorption medium without polybrene. The pH was adjusted with 5 N NaOH. All 37°C incubations with either adsorption or penetration medium were carried out in an incubator without CO₂ supplementation, under which conditions the pH remained stable throughout the experiments.

Kinetic studies. The dilution of virus stocks were chosen empirically to result in 200 to 300 foci per dish after a 30-min adsorption period. The general approach was that described by Andersen and Nexø (1). *M. dunni* cells (4 × 10⁴ per 60-mm dish), seeded the day before, were cooled on ice (2°C) for 10 min, washed twice with 4 ml of iced wash solution to remove the medium, and exposed to virus diluted in pH-adjusted adsorption medium at 2°C. After a 30-min incubation, dishes were washed four times with 4 ml of iced wash solution and then either fed with growth medium or treated with trypsin (0.5 mg of trypsin per ml, 0.05 mM EDTA, 1 mg of glucose per ml, and 0.58 mg of NaHCO₃ per ml in normal saline) for 10 min at 37°C to inactivate extracellular virus (1). Cells were reseeded in growth medium, and foci of infection

were counted on day 3. Total infectivity was determined in dishes not treated with trypsin but instead fed with growth medium (i.e., penetration was allowed to progress indefinitely). All determinations were carried out in duplicate or triplicate.

Cell fusion. *M. dunni* cells (4 × 10⁵ per well) were seeded in 35-mm wells of six-well culture dishes. The following day, monolayers were washed twice and 2 ml of pH-adjusted adsorption medium per well was added along with 0.2 ml of F-MuLV at various dilutions (2 × 10⁷ focus-forming units per ml, undiluted). Plates were incubated at 37°C for various periods, after which monolayers were fixed with methanol and stained with Giemsa. Cell fusion was quantified by counting the number of polykaryons in at least 10 fields at a magnification of ×150. The sensitivity of cell fusion to UV irradiation was tested by exposure of virus in 40-mm watch glasses to two germicidal G15T8 Sylvania lamps at a target distance of 40 cm (3). This delivered a dose of 40 ergs per mm²/s. A constant volume of virus was removed at intervals for titration of infectivity and quantitation of fusion as described above.

Fluid-phase endocytosis. *M. dunni* cells (4 × 10⁵) were seeded in 60-mm dishes. The following day, dishes were washed twice and 0.4 ml of pH-adjusted penetration medium containing 0.1 mM sucrose and 8 × 10⁻⁷ mM [³H]sucrose (10 Ci/mM; New England Nuclear Corp., Boston, Mass.) (21) was added. Dishes were incubated at 37°C, and at intervals monolayers were washed six times at room temperature with 4 ml of Dulbecco phosphate-buffered saline containing 10 mM sucrose, lysed in situ with 1% sodium dodecyl sulfate, and counted. In an initial experiment, after the monolayers were washed with Dulbecco phosphate-buffered saline-sucrose, cells were removed from the dishes with trypsin, centrifuged through a cushion of fetal calf serum, and washed twice before being lysed and counted. The results of this experiment were comparable to those obtained when the cells were lysed in situ, indicating that the radioactivity was cell associated, a negligible amount being bound to the dishes.

RESULTS

Infection by F-MuLV is inhibited at acid pH. The entry of many enveloped viruses requires that the virions be in an acidic environment. The proton pump of endocytic vesicles (36) provides such an environment, and it is by fusion with the vesicular membrane that the viral nucleocapsids gain entry to the cytosol. Our initial approach to studying the entry of retroviruses into *M. dunni* cells was to examine the effect of ambient pH and temperature on infectivity. *M. dunni* cells were exposed at 37 or 2°C to F-MuLV at either pH 7.4 or 6.0 for 30 min, after which free virus was washed away and cells were treated with trypsin to inactivate virus remaining at the cell surface. The results (Table 1) show that incubation at pH 6.0 resulted in an infectivity that was approximately 40-fold lower than that obtained with incubation at pH 7.4. At 2°C, no infectivity was detected at either pH 6.0 or 7.4, indicating that trypsin treatment was effective in inactivating extracellular virus. The nature of this pH effect on the infectivity of F-MuLV is the subject of the following series of experiments.

Virus binding to the cells is not inhibited at pH 6. The first step in the replication cycle is the binding of virus to cell membrane receptors. It is known that acidic pH inhibits the interaction of certain ligands with their respective membrane receptors (5), and it is this relationship that is responsible for the dissociation of receptor and ligand within the acidic

FIG. 1. Effect of pH on kinetics of virus entry. (A) Virus was adsorbed at 2°C for 30 min at pH 6.0 (▲), 6.4 (△), 6.8 (□), 7.4 (○), and 7.6 (●). After unadsorbed virus was washed away, the temperature was abruptly shifted (time 0) to 37°C with penetration medium which had been prewarmed and adjusted to the same pH as the adsorption medium. Dishes were treated at intervals with trypsin, and infectivity was assayed on day 3. Data are expressed as the log percentage of total infectivity [log (100% - percentage of trypsin-resistant infectivity)]. (B) Virus was adsorbed for 30 min at 2°C at pH 6. After the dishes were washed, the temperature was shifted to 37°C with prewarmed penetration medium which had been adjusted to pH 6, and incubation at 37°C continued for 15 min. At time 0, a premeasured volume of 2 N NaOH was added to one set of dishes to abruptly shift the pH to 7.6 (●). Another set of dishes was left at pH 6 (○). Dishes were treated at intervals with trypsin, and infectivity was assayed on day 3. For comparison, results of a temperature shift experiment carried out at pH 7.6 (□) are shown.

environment of the endosome. It therefore seemed likely that inhibition of infectivity at pH 6 might simply be the result of decreased binding of virus to the cell surface. To study this possibility, we examined the adsorption of infectious virus at various pHs. Virus was adsorbed to cells at 2°C for 30 min in pH-adjusted adsorption media. After being washed, cell surface virus was not removed with trypsin but instead was allowed to penetrate during the subsequent incubation in growth medium at 37°C. No difference in infectivity was seen as a function of pH (Table 2), indicating that adsorption of infectious virus was not inhibited by acidic pH. In addition, virus was not inactivated at 2°C under these mildly acidic conditions.

A second experiment (Table 3) was designed to test the effect of pH on the penetration of preadsorbed virus. After an adsorption period at 2°C, cells were incubated at either pH 6.0 or 7.6 at 37°C to allow penetration. The penetration period was terminated by treatment with trypsin. Acidic pH during the penetration period markedly inhibited infectivity. It was again apparent that the adsorbed virus was not simply inactivated at acidic pH, since infectivity was not abolished when the cells were not trypsinized but instead were incubated in growth medium.

Rate of penetration is sensitive to ambient pH. It is clear

that acidic pH had little effect on virus adsorption but had a marked inhibitory effect on penetration. To quantify this effect, we examined the kinetics of penetration as a function of pH. After an adsorption period at 2°C, the temperature was abruptly shifted to 37°C and penetration was allowed to progress at different pHs for various intervals. The penetration period was terminated with trypsin. The data (Fig. 1) are expressed as the percentage of total infectious virus (see above) remaining at the cell surface (trypsin sensitive) as a function of time. At pH 7.6, less than 20% of the virus remained at the cell surface 45 min after the temperature shift (Fig. 1A). The rate of disappearance of adsorbed virus decreased with decreasing pH. At pH 6.0, 98% of the total infectious virus was still sensitive to trypsin at 45 min. Note that the curves contained an initial shoulder of 5 to 10 min which could be eliminated by preincubation of cells at 37°C before allowing penetration to proceed (Fig. 1B). In this experiment, virus was preadsorbed at 2°C and the cells were then treated for 15 min with prewarmed penetration medium at pH 6.0, during which time no penetration occurred (Fig. 1A). When the pH was abruptly shifted to pH 7.6 by the addition of 2 N NaOH, virus penetration progressed in a

TABLE 2. Effect of pH on virus adsorption^a

pH	No. of foci per dish
6.0.....	205
6.4.....	205
6.8.....	262
7.2.....	229
7.6.....	223
8.0.....	235

^a Virus was adsorbed at 2°C for 30 min at the indicated pH. Dishes were then washed at 2°C and fed with growth medium without prior trypsin treatment. Infectivity was assayed on day 3.

TABLE 3. Effect of pH on virus entry

Penetration pH ^a	Trypsin treatment ^b	No. of foci per dish ^c
7.6	-	242
7.6	+	98
6.0	-	227
6.0	+	1

^a Virus was adsorbed at 2°C for 30 min at pH 7.6, and free virus was washed away. The penetration step was carried out for 30 min at 37°C with prewarmed penetration medium adjusted to pH 6 or 7.6.

^b After the penetration period, dishes were either treated with trypsin (+) or fed with growth medium (-). Trypsin-treated cells were reseeded in growth medium.

^c Infectivity was assayed on day 3.

FIG. 2. Effect of acidic pH on fluid-phase endocytosis. *M. dunni* cells were incubated at 37°C with 0.1 mM sucrose plus a trace amount of [³H]sucrose in penetration medium adjusted to pH 6 (●) and 7.6 (○). Dishes were harvested at various times, and the monolayers were washed and lysed with 1% sodium dodecyl sulfate.

straight-line relationship with no initial delay. Thus, the disappearance of cell surface infectivity displayed first-order kinetics, with a half-time at pH 7.6 of approximately 10 min. In addition, these data suggest that a temperature-sensitive step may precede the pH-sensitive event.

The marked effect of acidic pH on internalization of virus could simply be a reflection of a global inhibition of endocytosis by the cells. This possibility was examined by quantifying the rate of internalization of a fluid phase marker, [³H]-sucrose. Sucrose is not degraded, and its internalization is not receptor mediated (35). Only a small difference between the rate of sucrose uptake at pH 6 and that at pH 7.6 was noted (Fig. 2); the respective rates of fluid uptake during this period were 0.016 and 0.022 μ l/10⁶ cells per h. Thus, fluid-phase endocytosis by *M. dunni* cells, when compared with infectivity (Fig. 1), appeared relatively resistant to mildly acidic pH.

F-MuLV-induced membrane fusion is inhibited by acidic pH. Infectious penetration by RNA-enveloped viruses depends in part on the fusion of viral envelope and cell membrane. With one notable exception (paramyxoviruses), this fusion event has a discrete, slightly acidic pH optimum, fusion and virus entry being markedly inhibited at a pH of >6. Since penetration of F-MuLV into *M. dunni* cells was inhibited at an acidic pH, it seemed likely that the postattachment pH-sensitive step might be the fusion event. It has been previously reported that *M. dunni* cells undergo polykaryocyte formation within several days after infection with F-MuLV (18). To examine the effect of pH on fusion induced by F-MuLV, we developed a rapid assay for cell

fusion. The time course of cell fusion depended on the multiplicity of infection (Fig. 3A). At a multiplicity of infection of <1 focus-forming unit per cell, cell fusion reached a peak at 28 to 30 h after infection, thus correlating with the expected time of appearance of progeny virus. In contrast, cell fusion was relatively rapid at a high multiplicity of infection, being seen as early as 2 h and reaching maximum levels at 5 to 6 h. This form of cell fusion was analogous to fusion from without (4) induced by other RNA viruses, since it was insensitive to the effects of UV irradiation (Fig. 3B) and thus was probably due to direct interaction of the viral envelope and cell membrane. We determined the pH sensitivity of fusion from without by incubating *M. dunni* cell monolayers with a high multiplicity of infection of F-MuLV in adsorption medium adjusted to different pHs (Fig. 4). Cell fusion was optimal at pH 7.6 and markedly inhibited at a pH of ≤ 6.4 , thus correlating with the pH sensitivity of infectivity (Fig. 1).

Effect of acidic pH on penetration of other mouse retroviruses. *M. dunni* cells are unique among the mouse cell lines used in retrovirology in that they are susceptible to infection by a wide variety of viruses of each host range group (18). It was therefore possible to compare the effect of acidic pH on the penetration of preadsorbed ecotropic, dualtropic, and xenotropic viruses in the same cell line (Table 4). Like that of F-MuLV, the entry of two ecotropic viruses (Akv 623 and PBR-NE-8) was markedly inhibited at pH 6.0, as was that of two MCF viruses (247 and 98D) and one xenotropic virus (AKR 6). Penetration by the xenotropic virus BALB-IU-1, however, was relatively resistant to acidic pH as compared with other viruses tested.

DISCUSSION

Infection of a tail skin cell line from *M. dunni* by several different mouse retroviruses was found to be highly sensitive to mildly acidic ambient pH. This finding does not appear to be a peculiarity of *M. dunni* cells, since a similar pH effect has been observed for infection of NIH 3T3 cells by F-MuLV (data not shown). In the current study, we have shown that this inhibitory effect operated on a postadsorption step, since binding of infectious virus at 2°C was not inhibited by acidic pH. Internalization of infectious virus at 37°C, however, was approximately 100-fold lower at pH 6 than at pH 7.6 (Table 3). We further observed that cell fusion induced by F-MuLV was markedly inhibited at a pH of <6.4, suggesting that the inhibitory effect of acidic pH on internalization of this virus was due to inhibition of the fusion of the viral envelope with the cell membrane. In other virus systems, this fusion event appears to be the terminal step in the entry process and is generally highly pH dependent.

Acidic pH had little inhibitory effect on the rate of fluid-phase endocytosis by *M. dunni* cells (Fig. 2). It is predicted, however, that the inhibitory effect of acidic pH on membrane fusion (Fig. 4) might limit the efficiency of infectious penetration at the level of acidified endocytic vesicles (pH 4.5 to 5.5). In this regard it is of interest that the toxicity of two plant toxins, abrin and ricin, has also been found to be optimal at slightly alkaline pH and inhibited at pH 6.0 (32). Toxin internalized by endocytosis at pH 6.0, however, was still toxic when the pH of the medium was increased. These toxins thus appear to gain entry to the cytosol within a nonacidified vesicular compartment, the pH of which is influenced by that of the extracellular milieu. In contrast, infectivity of F-MuLV was not expressed at pH 6.0 when cell surface virus was inactivated by trypsin before the pH

FIG. 3. (A) Kinetics of cell fusion. *M. dunni* cells were exposed to F-MuLV in growth medium at high (7 [○]) and low (0.7 [●]) multiplicities of infection. Cells were incubated at 37°C and fixed with methanol at various times after infection. The number of polykaryons (containing three or more nuclei per cell) per field were counted after staining with Giemsa. Note that the number of polykaryons decreased after reaching a maximum. These cells became detached from the plastic surface and were found floating in the medium. (B) UV sensitivity of cell fusion induced by F-MuLV at high multiplicity of infection. Undiluted virus (2×10^7 focus-forming units [ffu] per ml) was exposed to UV light for various intervals. Samples were assayed for infectivity by titration on *M. dunni* cells. Cell fusion was quantified by incubation of samples on *M. dunni* cell monolayers for 5 h at 37°C. Polykaryons were counted as in panel A. Although infectivity was inactivated by more than 100-fold after 4 min of UV exposure, fusion activity was relatively resistant to this level of irradiation.

was raised (Table 3 and Fig. 1). These observations suggest that, over short intervals, endocytosis is not an important mechanism for the entry of F-MuLV into *M. dunni* cells. One might speculate that the efficiency of infection may depend on a balance between the rate of fusion and the rate of endocytosis, the latter inexorably leading to degradation.

Direct penetration of the plasma membrane by murine

retroviruses has been observed in electron micrographs (23), although there was no morphological evidence for fusion of viral envelopes with cell membranes. However, cell fusion can be induced at neutral pH by several groups of retroviruses, including lentiviruses (20), some members of the type D primate viruses (25), and the ecotropic group of murine type C viruses (39). Furthermore, nonpermissive cells lacking virus receptors can be productively infected if an external fusigen, such as UV-inactivated Sendai virus, is added (14). These observations suggest that, like the paramyxoviruses, retroviruses might gain entry to the cytosol by direct fusion with the plasma membrane. There is evidence that infection by one murine ecotropic virus, however, is inhibited by drugs which raise endosomal pH (1). In addition, fusion of mink lung cells in vitro by mouse mammary tumor virus, a type B retrovirus, has been found to occur only at acidic pH (30). It therefore seems apparent that the mechanisms mediating entry might vary among the different groups of retroviruses. Whether viral or host cell factors are responsible for these differences is not known. In this regard it is of interest that the inhibitory effect of acidic pH on the pene-

FIG. 4. pH sensitivity of cell fusion induced by F-MuLV at high multiplicity of infection. Monolayers of *M. dunni* cells were exposed to F-MuLV at a multiplicity of infection of 10 focus-forming units per cell in adsorption medium adjusted to a pH between 6.0 and 9.0. Incubation was carried out at 37°C, and monolayers were fixed with methanol after only 2.5 h to avoid changes in the pH. Polykaryons were counted after staining with Giemsa.

TABLE 4. Effect of acidic pH on entry of other mouse retroviruses^a

Virus	% Relative infectivity at pH 6.0
Ecotropic	
F-MuLV.....	2
Akv623.....	1
PBR-NE-8.....	0.5
Xenotropic	
AKR-6.....	2
BALB IU-1.....	34
MCF	
98D.....	0.5
AKR247.....	1

^a Virus was adsorbed for 30 min at 2°C in adsorption medium adjusted to pH 7.6, after which dishes were washed. Prewarmed penetration medium, adjusted to pH 6 or 7.6, was added, and dishes were incubated at 37°C for 45 min. All dishes were treated with trypsin, and cells were reseeded in growth medium. Infectivity was assayed on day 3. Data are expressed as percentage of infectivity detected at pH 7.6 [(number of foci per dish at pH 6.0/number of foci per dish at pH 7.6) × 100].

tration of *M. dunni* cells was observed with ecotropic and MCF viruses as well as with one xenotropic virus (AKR-6) (Table 4). However, entry of another xenotropic virus, BALB-IU-1, was relatively resistant to acidic pH. These two xenotropic viruses have been shown to cross-interfere in *M. dunni* cells (7), suggesting that they interact with related receptors. We interpret the differences in acid sensitivity to reflect differences in the structure of the respective envelope proteins in a region distinct from that responsible for receptor binding.

In enveloped viruses which gain entry to the cytosol by endocytosis, acidic pH induces a conformational change in the fusion protein, exposing a hydrophobic region, usually near the N-terminus, which can then interact with the endosomal membrane (for review, see reference 37). In the case of F-MuLV, it is possible that such a hydrophobic region may be exposed at neutral and slightly alkaline pH but buried at acidic pH. The best candidate for a retroviral fusion protein is p15(E) because of its structural similarity to fusion proteins of other RNA viruses (17) and the fact that, unlike gp70, it is firmly inserted in the lipid bilayer of the viral envelope (33). The association of gp70 and p15(E) by covalent and noncovalent bonds is well documented (26), and we have found, using monoclonal antibodies, that this association produces significant conformational changes in both polypeptides (6, 8, 28, 29). We are currently examining the effect of pH on this association.

The time course for infectious penetration of *M. dunni* cells by F-MuLV was short, with a half-time of approximately 10 min at pH 7.6, a rate which compares favorably with that for penetration of BHK-21 cells by Semliki Forest virus (21). The rate of penetration reported for another mouse ecotropic retrovirus, C57MC, was considerably slower, with a half-time of approximately 3 h (1). Differences in the cell type and virus or in the pH of the medium might explain this apparent discrepancy. In this regard, we have observed that rates of penetration differ among mouse retroviruses (data not shown) and in some instances are comparable to that reported for C57MC.

Two additional points of interest were the first-order nature of the penetration process and the initial lag observed

in the temperature shift experiments. First-order kinetics generally indicate that the response being studied involves only one rate-limiting reaction, which occurs randomly. This would suggest that cell-bound F-MuLV behaved as a homogeneous population, each virion having an equal chance to penetrate in a given interval. The initial lag in the entry of adsorbed virus when the temperature was abruptly shifted from 2 to 37°C at pH 7.6 (Fig. 1A) suggests that temperature-sensitive constraints preceded the initiation of entry. When temperature was eliminated as a variable by shifting the pH abruptly from pH 6 to 7.6 (Fig. 1B), such a lag in the initiation of virus entry was not seen, indicating that acidic pH did not inhibit this temperature-sensitive reaction. If virus entry requires multipoint binding to several gp70 receptors and if these receptors are sparsely distributed in the plasma membrane, virus adsorption at 2°C may not allow sufficient multivalent binding. After restoration of membrane fluidity by a shift to 37°C, a measurable period could elapse before sufficient virus-receptor interaction occurred to promote virus entry. Such a delay has not been observed in the entry of Semliki Forest virus (21) or macromolecular ligands such as transferrin (16), perhaps because the concentration of membrane receptors is not rate limiting in the former case and multivalent binding is not required in the latter.

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