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Function of the Cytoplasmic Domain of a Retroviral Transmembrane Protein: p15E-p2E Cleavage Activates the Membrane Fusion Capability of the Murine Leukemia Virus Env Protein

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In the murine leukemia viruses (MuLVs), the Env complex is initially cleaved by a cellular protease into gp70^{SU} and pre15E^{TM}. After the virus particle is released from the cell, the C-terminal 16 residues are removed from the cytoplasmic domain of pre15E by the viral protease, yielding the mature p15E^{TM} and p2E. We have investigated the function of this cleavage by generating a Moloney MuLV mutant, termed p2E$^-$, in which the Env coding region terminates at the cleavage site. This mutant synthesizes only the truncated, mature form of TM rather than its extended precursor. When cells expressing this truncated Env protein are cocultivated with NIH 3T3 cells, they induce rapid cell-cell fusion. Thus, the truncated form, which is normally found in virions but not in virus-producing cells, is capable of causing membrane fusion. We conclude that the 16-residue p2E tail inhibits this activity of Env until the virus has left the cell. p2E$^-$ virions were found to be infectious, though with a lower specific infectivity than that of the wild type, showing that p2E does not play an essential role in the process of infection. Fusion was also observed with a chimeric p2E$^-$ virus in which gp70^{SU} and nearly all of p15E^{TM} are derived from amphotropic, rather than Moloney, MuLV. In a second mutant, an amino acid at the cleavage site was changed. The pre15E protein in this mutant is not cleaved. While the mutant Env complex is incorporated into virions, these particles have a very low specific infectivity. This result suggests that the cleavage event is essential for infectivity, in agreement with the idea that removal of p2E activates the membrane fusion capability of the Env complex.

In all retroviruses, the Env proteins are synthesized in the form of a polyprotein precursor. This precursor is then cleaved by a cellular protease into the large, external glycoprotein (SU), derived from the N terminus of the precursor, and the smaller, transmembrane component (TM) (8).

TM is an integral membrane protein consisting of three distinct domains: extracellular, membrane spanning, and cytoplasmic. Studies with a variety of retroviruses have shown that the N-terminal, extracellular domain is involved in the association with SU, in the oligomerization of Env, and in the membrane fusion event by which Env delivers the contents of the infecting virus particle into the cytoplasm of the host cell (10, 12, 13, 45) (reviewed in references 9 and 29). The membrane-spanning domain functions in anchoring the Env complex in the membrane (37).

The functions, if any, of the cytoplasmic domain of TM are unknown. Indeed, in the avian type C retroviruses, mutants lacking this domain are replication competent (37). This observation suggests that the domain plays no essential role in the viral life cycle, although the replication of these mutants may be slightly less efficient than that of the wild type. On the other hand, introduction of termination codons within this coding region by oligonucleotide-directed mutagenesis has been reported to cause a loss of infectivity and a reduction in the level of Env protein present in virus particles in both human immunodeficiency virus (11, 48) and murine leukemia virus (MuLV) (19), although in one of these studies a coding change was also introduced (19).

In contrast to these mutagenesis studies, there are a number of cases in which the cytoplasmic domain is naturally truncated. Thus, propagation of equine infectious anemia virus (39), simian immunodeficiency viruses (SIVs) (5, 6, 14, 23, 27, 28, 33), or human immunodeficiency virus type 1 (41), under some conditions, selects for mutants with a termination codon in the coding region for the cytoplasmic domain. Further, TM is cleaved at a site in the cytoplasmic domain in MuLVs (20, 22, 25), Mason-Pfizer monkey virus (43), and equine infectious anemia virus (39). This cleavage event is catalyzed by the virus-encoded protease (PR) and evidently occurs only after the fully assembled virus particle has been released from the cell (7, 22, 26, 31, 39, 40, 43). It is clear that natural selection favors an extended cytoplasmic domain in some situations and a much shorter one in others and, further, that in some systems it favors synthesis of an extended form and subsequent removal of the extension by proteolysis. The reasons for the existence of these different forms are, however, completely unknown. It seems likely that an understanding of these phenomena would provide some insight into the function(s) of the cytoplasmic domain itself.

The present report describes the properties of two mutants altered at the natural cleavage site of the Moloney MuLV (Mo-MuLV) TM protein. This cleavage removes a 16-residue peptide, termed p2E, from the C terminus of the TM precursor (pre15E), yielding the mature TM protein, p15E^{TM}. The results of our studies strongly suggest that the cleavage is

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required before the Env complex can perform one of its essential functions, i.e., fusion of membranes.

MATERIALS AND METHODS

Cells, viruses, and plasmids. The cell line NIH 3T3, Kirsten sarcoma virus (KiSV)-transformed NRK rat cells, and CHO hamster cells have all been described previously (16). 293 cells (17) containing T antigen were obtained from N. Rice (NCI-Frederick Cancer Research and Development Center, Frederick, Md.). Mo-MuLV was derived from an infectious molecular clone, pRR88, originally obtained from D. Steffen (Baylor College of Medicine, Houston, Tex.). It was cloned into pGCcos3neo, a plasmid derived by Gray Crouse (Emory University, Atlanta, Ga.) from pSV2neo (16, 44). Some experiments used amphotropic MuLV (isolate 4070A) and Mo(4070A), a chimeric MuLV genome in which a 3′ portion of *pol* and nearly all of *env* (from the *Sal*I site, nucleotide 3705, to the *Cla*I site, nucleotide 7674) (42) of pRR88 were replaced by the corresponding regions of amphotropic MuLV (35, 36). Some experiments also used the 96-nucleotide deletion mutant of Mo-MuLV in the PR coding region described by Katoh et al. (26), cloned in nonpermuted form in pGCcos3neo. A MuLV-based retroviral vector encoding hygromycin resistance was obtained from G. Brady (Ontario Cancer Institute, Toronto, Canada) through David Ott. This vector was constructed by replacing the v-*src* gene of pYN (3) with the hygromycin phosphotransferase gene. pCD-ENV, a plasmid containing a cDNA clone of the Mo-MuLV Env gene (47), was obtained from M. Eiden (National Institute of Mental Health, Bethesda, Md.); the Env gene in this clone is under the control of the simian virus 40 early promoter (34).

In all experiments involving stable transfectants, viral genomes were initially transfected into cells by the calcium phosphate method (18), and transfectants were selected by the addition of 800 μg of G-418 (Life Technologies, Inc., Gaithersburg, Md.) per ml in CHO cells and 500 μg/ml in rat cells. Some experiments used transient expression. In these experiments, plasmids were transfected by the calcium phosphate method into 293 cells and the cells were tested 2 days later.

Pseudotypes of KiSV and hygromycin resistance (Hyg^r) virus were produced by stable transfection of MuLV clones into NRK cells transformed by KiSV and CHO cells previously infected with Hyg^r virus, respectively. Neither KiSV nor the Hyg^r vector encodes any viral proteins.

Mutagenesis. Oligonucleotide-directed mutations were induced in subclones of pRR88 by oligonucleotide-directed mutagenesis as described elsewhere (16) by using the mutagenesis kit of Amersham (Arlington Heights, Ill.). In mutant $p2E^-$, nucleotides 7724 to 7726 (42) were changed from the valine codon GTT to the termination codon TAG. In mutant p2Ecl$^-$, nucleotides 7721 to 7723 were changed from the leucine codon CTA to the arginine codon CGA (this mutant could also be designated L649R). The entire restriction fragment (*Cla*I, nucleotide 7674, to *Xba*I, nucleotide 8113) used in the reconstruction of the intact mutant viral genome was sequenced in each case and was wild type except for the desired mutation. For some experiments, the $p2E^-$ fragment from *Cla*I (nucleotide 7674) to *Nhe*I (nucleotide 7846) was subcloned into pCD-ENV.

An Env^- mutant of Mo-MuLV was constructed by filling in the *Bst*EII site at nucleotide 5923; the resulting frameshift introduces a termination codon after residue 18 of $gp70^{SU}$, without changing any amino acids encoded 5′ of the new termination codon.

Characterization of viral proteins. Culture fluids were filtered and virus was harvested by ultracentrifugation. Viral pellets were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then electroblotted onto Immobilon PVDF (Millipore, Bedford, Mass.) and immunoblotted with a rabbit antiserum against p15E of Mo-MuLV (a kind gift of Louis Henderson, Patrick Wesdock, and Terry Copeland of our laboratory). Bands were visualized with the Amersham enhanced chemiluminescence kit.

Relative concentrations of virus particles in culture fluids were estimated by immunoblotting a series of dilutions of each virus preparation (by using anti-$p30^{CA}$) (46) and comparing the resulting patterns to find dilutions giving similar band intensities.

Immunoelectron microscopy. CHO cells which had been stably transfected with viral clones were grown in 100-mm-diameter tissue culture dishes. When the cultures were nearly confluent, they were rinsed with cold phosphate-buffered saline (PBS) and removed from the dishes by scraping. They were pelleted by centrifugation at 100 × *g* for 5 min, and the pellets were gently resuspended in goat anti-MuLV gp70 antiserum (46) which had been diluted 1:10 in PBS containing 1 mg of bovine serum albumin per ml (PBS-BSA). All subsequent steps were performed at 0 to 4°C. The cells were incubated for 1 h in the antiserum and then washed three times in PBS-BSA. They were then fixed in 0.1% glutaraldehyde in PBS for 1 h, and glutaraldehyde was removed by incubating the cells in 50 mM Tris (pH 7.4)–50 mM NH_4Cl–0.1% BSA for 1 h. The cells were centrifuged and resuspended in the same buffer overnight. The following day, they were rinsed in PBS-BSA and suspended in a 1:25 dilution in PBS-BSA of biotinylated rabbit anti-goat antiserum (Amersham). After incubation for 1 h, the cells were rinsed three times in PBS-BSA and resuspended in a 1:20 dilution of gold-conjugated streptavidin (G10; Amersham). After incubation for 1 h followed by three washes in PBS-BSA, the cell pellet was fixed in 1.25% glutaraldehyde in cacodylate buffer (pH 7.4). It was then treated with 1% osmium tetroxide in the same buffer, stained en bloc with 1% uranyl acetate, and dehydrated in a series of graded ethanols, followed by absolute ethanol and, finally, propylene oxide. The pellet was infiltrated overnight with an LX-112 epoxy resin (Ladd Research, Burlington, Vt.) diluted with an equal volume of propylene oxide and embedded in pure LX-112 epoxy resin. After the pellet had been cured for 48 h at 60°C, thin sections were cut. The sections were mounted on copper grids and double stained with uranyl acetate and lead citrate. The sections were stabilized by carbon evaporation and photographed with a Hitachi H-7000 electron microscope operated at 75 kV.

Infectivity assays. Replication-competent MuLV was assayed on S^+L^- cells as described previously (2). KiSV was assayed on NIH 3T3 cells as described elsewhere (38). Virus inducing hygromycin resistance was assayed on NIH 3T3 cells as follows. Cells were seeded at 10^5 cells per 60-mm-diameter dish. The following day, they were infected with dilutions of virus. One day later, 100 μg of hygromycin B (Boehringer Mannheim, Indianapolis, Ind.) per ml was added to the plates. Colonies of Hyg^r cells were counted approximately 3 weeks later.

Cocultivations. In cocultivation experiments, cells were initially seeded in the absence of serum so that they would attach to the dishes rapidly. In experiments using stable transfectants, CHO cells containing viral clones were first seeded at 10^6 cells per 60-mm-diameter tissue culture dish in α-modified Eagle's medium (Life Technologies, Inc.). Next, 1.2×10^6 NIH 3T3 cells were added to the dishes. One hour after addition of the NIH 3T3 cells, fetal calf serum was added to a final concen-

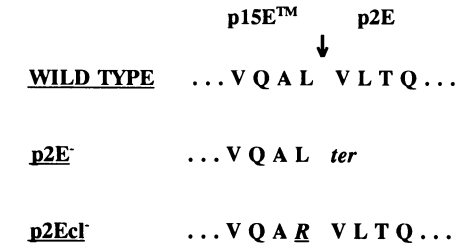

FIG. 1. Mutations introduced at the p15E-p2E cleavage site of Mo-MuLV. The top line shows the wild-type sequence around the p15E-p2E cleavage site, including amino acids 646 to 653 of the *env* primary translation product. The cleavage site is indicated by the arrow. The remaining two lines show the sequences encoded by the two mutants, p2E$^-$ and p2Ecl$^-$.

tration of 10%; times of cocultivation are times after serum addition. Cells were fixed and stained with 0.5% methylene blue–0.16% basic fuchsin in methanol (30). A similar protocol was followed in experiments using transiently transfected 293 cells instead of stably transfected CHO cells.

RESULTS

The Mo-MuLV pre15E protein is cleaved between leucine (residue 649 of the primary Env translation product) and valine (residue 650). As indicated above, the function of this cleavage event is unknown. In order to obtain some insight into this problem, we used oligonucleotide-directed mutagenesis to create two mutations at the cleavage site in Mo-MuLV (Fig. 1). In one mutant, termed p2E$^-$, we replaced the valine codon with a termination codon. This mutation should eliminate the synthesis of p2E. In the other, designated p2Ecl$^-$, we replaced the leucine codon with an arginine codon, anticipating that this drastic change in the sequence at the cleavage site would prevent cleavage.

Physical characterization of mutant particles. The two mutants were transfected into CHO cells, and stable transfectants were selected with G-418. In order to determine whether the mutants synthesized the expected proteins, we pelleted material from culture fluids and compared the electrophoretic mobilities of the TM proteins present in the respective pellets by immunoblotting with anti-TM serum (Fig. 2). As shown in Fig. 2, lane 3, the two forms, pre15E and p15E, were both present and easily resolved in the wild-type control. As expected, only p15E was present in the p2E$^-$ sample (Fig. 2, lane 1), while the p2Ecl$^-$ sample contained only a TM protein with mobility indistinguishable from that of the uncleaved form, pre15E (Fig. 2, lane 2). Thus the leucine-to-arginine change in this mutant successfully prevents cleavage of pre15E to p15E and p2E. Lanes 4 and 5 of Fig. 2 show, respectively, that cells transfected with vector alone did not release pelletable material reactive with the antiserum used here and that, as previously reported (7, 26), a PR$^-$ mutant failed to cleave pre15E into p15E and p2E. Immunoblotting of the p2E$^-$ and p2Ecl$^-$ pellets with anti-p30^{CA} and anti-gp70^{SU} sera gave normal viral patterns, except that p2E$^-$ pellets containing the same amount of p30^{CA} as the other samples had only approximately one-third as much gp70^{SU} as the others (data not shown).

The results shown in Fig. 2 demonstrate that the two mutants altered at the cleavage site synthesize the expected products and, further, that cells containing the mutants secrete TM-related proteins in pelletable form. To determine whether the mutant Env proteins are actually incorporated into virus

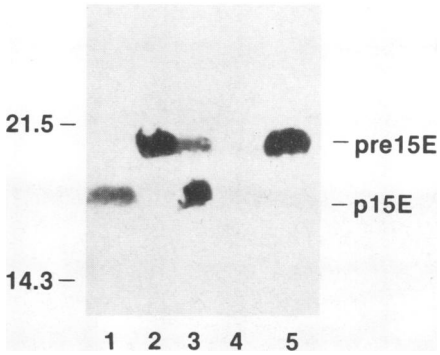

FIG. 2. Characterization of TM proteins in virus pellets. Proteins in viral pellets were fractionated on a 20% acrylamide gel and analyzed as described in Materials and Methods by using rabbit anti-p15E antiserum. Lane 1, p2E$^-$; lane 2, p2Ecl$^-$; lane 3, wild type; lane 4, culture fluid from cells transfected with pGCcos3neo vector alone; lane 5, PR$^-$. Immunoblotting with other antisera showed that lanes 1 through 3 contained equal levels of p30^{CA}, but lane 1 contained only approximately one-third as much gp70^{SU} as lanes 2 or 3 (data not shown). Numbers on the left indicate molecular weight (in thousands).

particles, we performed immunoelectron microscopy on the transfected cultures by using anti-gp70^{SU} serum. Figure 3 shows gold-labeled particles in the wild-type (panel A), p2E$^-$ (panel B), and p2Ecl$^-$ (panel C) cultures and an unlabeled particle from an Env$^-$ mutant (panel D). No labeled particles were seen in the Env$^-$ culture, while 10 to 20% of the particles were labeled in the other three cultures. While the results shown in Fig. 2 were obtained with biotinylated secondary antibody and gold-conjugated streptavidin, the use of gold-conjugated donkey anti-goat antiserum (Jackson ImmunoResearch Laboratories, West Grove, Pa.) to detect the goat anti-gp70 antibody gave virtually identical results (not shown).

Infectivity of mutant particles. The results presented above show that the two mutants, p2E$^-$ and p2Ecl$^-$, direct the synthesis of virus particles and that these particles incorporate Env proteins. We tested these virus stocks for the presence of replication-competent MuLV by using the S$^+$L$^-$ assay. As indicated in Table 1, experiment 1, no infectious MuLV was detected in the mutant stocks; on the basis of this assay, one would conclude that the ratio of infectious particles to physical particles was less than 1/500 of that in the wild-type control.

Like a plaque assay, the S$^+$L$^-$ assay only detects virus particles capable of undergoing several successive rounds of replication in a limited time interval. Therefore, viruses which are inefficient in their ability to infect cells, i.e., viruses whose ratio of infectious particles to physical particles is substantially lower than that of the wild type, will not register in this assay. The S$^+$L$^-$ assay results thus show that neither p2E$^-$ nor p2Ecl$^-$ virions infect cells as efficiently as wild-type MuLV. To determine whether either of the mutants is capable of infecting cells inefficiently, we tested them by performing assays which detect single infection events rather than multiple rounds of infection.

p2E$^-$ was tested by using it to rescue a replication-defective, MuLV-based vector encoding hygromycin resistance. The ratio of CFU of Hyg^r virus per milliliter to physical virus particles per milliliter (based on immunoblotting with anti-p30^{CA} serum) was then determined and compared with the corresponding ratio for the wild-type control. As shown in Table 1, experiment 2, this assay indicated that p2E$^-$ virions infect cells with ~1/10 of the efficiency of wild-type virions.

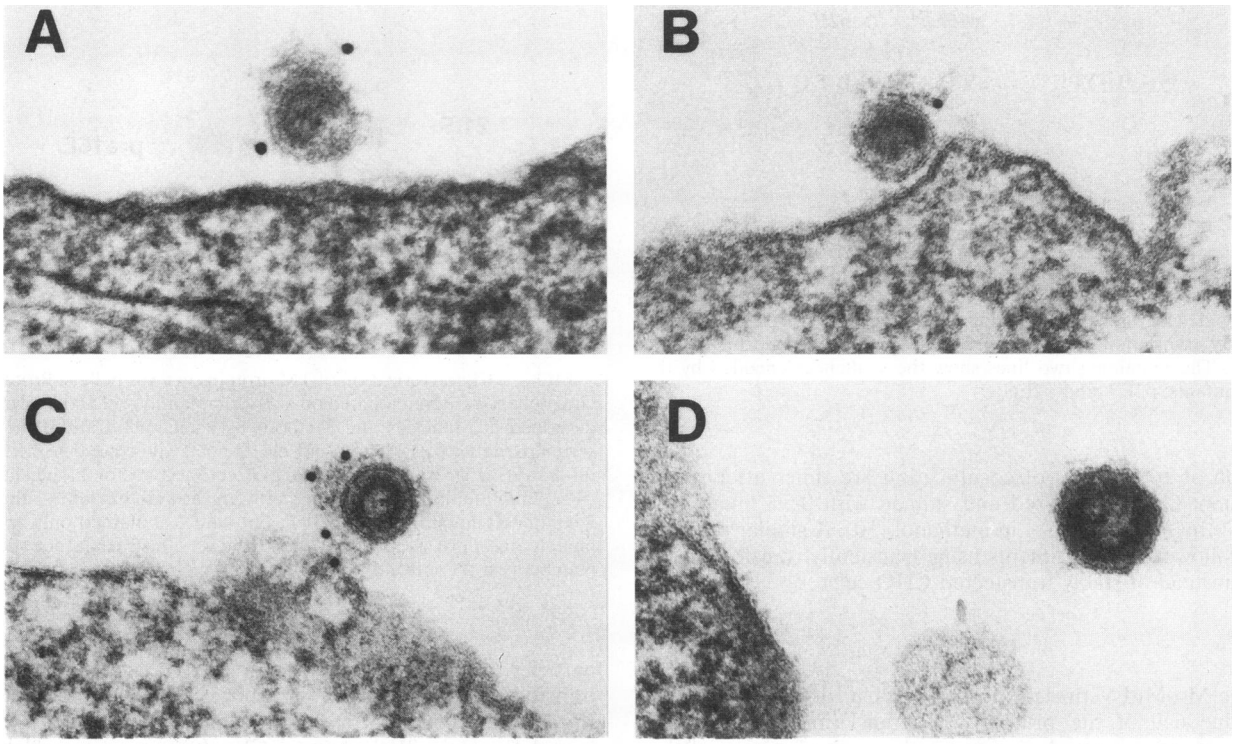

FIG. 3. Detection of Env proteins in virus particles by immunoelectron microscopy. Panels: A, wild type; B, p2E; C, p2Ecl$^-$; D, Env$^-$. Magnification, ×90,000.

p2Ecl$^-$ was tested in a similar assay, except that transformation by KiSV, rather than induction of hygromycin resistance by a retroviral vector, was scored. Table 1, experiment 3, shows that this virus infects cells only ~1/100 as efficiently as wild-type particles.

Syncytium formation by p2E$^-$ MuLV. In the course of performing infectious-center assays with the mutants, we noted the presence of syncytia in some cultures (data not shown). This observation suggested the possibility that the mutants cause cell fusion. We examined this possibility by cocultivating cultures containing the mutants (as well as control cultures) with NIH 3T3 cells, which are permissive for Mo-MuLV. As shown in Fig. 4A, CHO cells producing p2E$^-$ MuLV caused extensive fusion upon cocultivation with NIH 3T3 cells. In contrast, syncytia were not induced by cocultivating NIH 3T3 cells with CHO cells producing wild-type Mo-MuLV (Fig. 4B), control CHO cells (Fig. 4C), or cells producing p2Ecl$^-$ virus (data not shown). Significant numbers of syncytia were also never seen in control cultures containing only single cell lines (data not shown).

TABLE 1. Infectivity of viral mutants

Expt no. and virus	Virus titer (infectious units/ml)	Amt of p30/ml[a]	Infectious virus/p30 ratio[b]	Relative specific infectivity[c]
1				
p2E$^-$	<1 × 10^{0}[d]	0.12	<8	<0.002
p2Ecl$^-$	<1 × 10^{0}[d]	2.5	<0.4	<0.0001
Wild type	5 × 10^{3}[d]	1.0	5 × 10^3	1.0
2				
Hyg^r_r (p2E$^-$)	1.3 × 10^{1}[e]	0.05	3 × 10^2	~0.1
Hyg^r (Mo-MuLV)	3 × 10^{3}[e]	1.0	3 × 10^3	1.0
3				
KiSV (p2Ecl$^-$)	6 × 10^{2}[f]	0.1	6 × 10^3	~0.01
KiSV (Mo-MuLV)	8 × 10^{5}[f]	1.0	8 × 10^5	1.0

[a] Relative to wild-type level, as determined by immunoblotting.
[b] Ratio of infectivity per milliliter to p30 per milliliter.
[c] Infectivity/p30 ratio divided by infectivity/p30 ratio of wild type.
[d] Titer in the S$^+$L$^-$ focus assay.
[e] Infectious Hyg^r virus was measured in CFU.
[f] Infectious KiSV was measured in focus-forming units.

The cells depicted in Fig. 4A through C were fixed 5 h after cocultivation. To determine the rapidity with which the fusion occurs, we fixed cells after only 1 h of cocultivation. As shown in Fig. 4D, p2E$^-$ MuLV began to induce syncytium formation within 1 h or less after contact between virus-producing cells and NIH 3T3 cells.

Mo-MuLV is a member of the ecotropic class of MuLVs. It seemed possible that the fusion induced by p2E$^-$ Mo-MuLV (Fig. 4A and D) is a specific property of this class of MuLVs, particularly since it has been suggested (30) that ecotropic MuLVs penetrate cells by a mechanism different from that used by other MuLVs. Accordingly, we tested the effect of the p2E$^-$ mutation in a chimeric MuLV, Mo(4070A), in which nearly all of the Env gene, including all of gp70^{SU} and all but the C-terminal 16 residues of mature p15E^{TM}, is derived from amphotropic MuLV rather than Mo-MuLV (35, 36). Figure 4E (compared with the control, Fig. 4F) shows that p2E$^-$ Mo(4070A) MuLV causes syncytium formation in cocultivation experiments, although the syncytia tend to be somewhat smaller than those induced by p2E$^-$ Mo-MuLV.

The fusion experiments described above were performed by mixing virus-producing cells with NIH 3T3 cells. It was of interest to know whether the fusion observed here was depen-

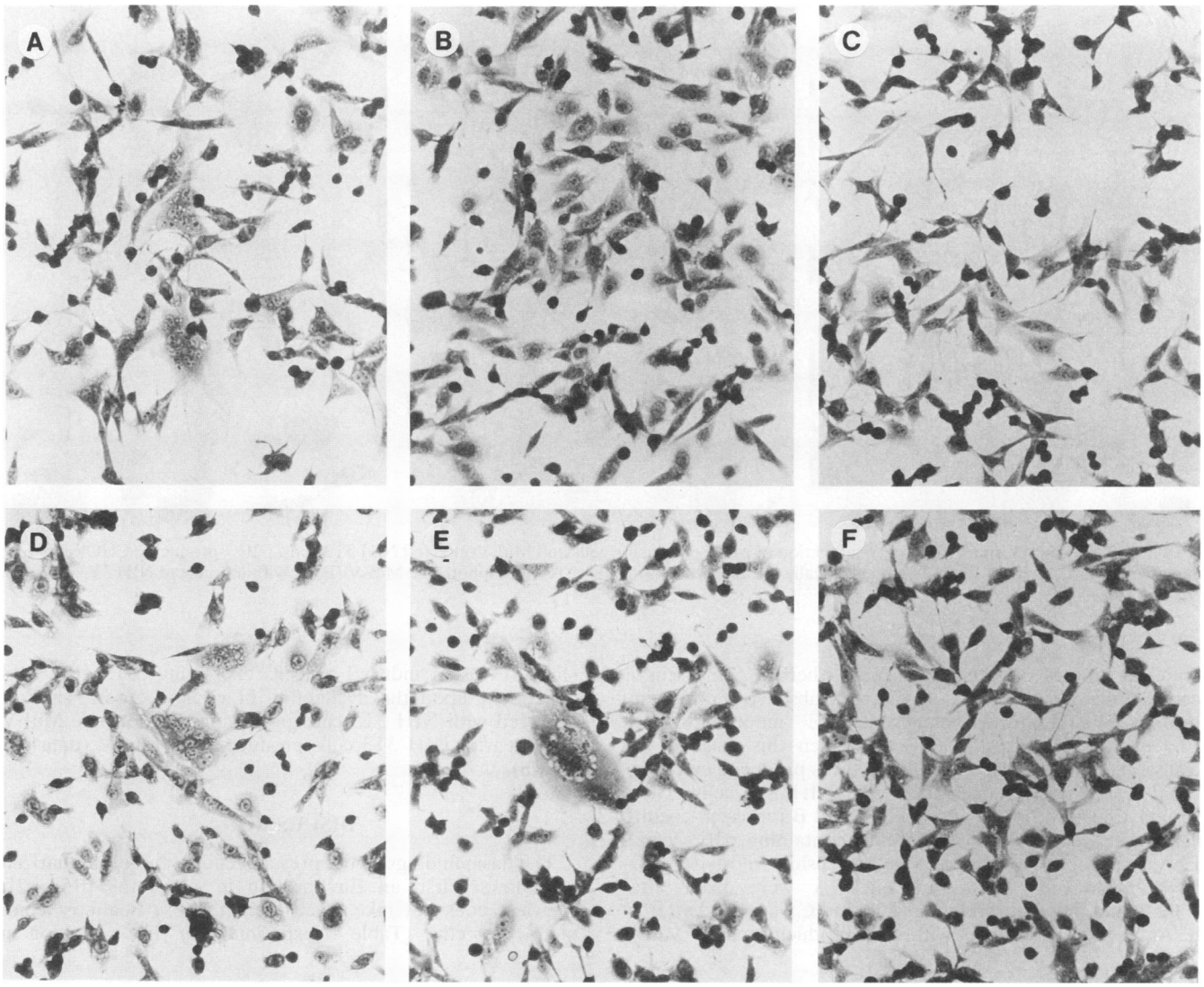

FIG. 4. Syncytium formation upon cocultivation of p2E$^-$ producing cells and NIH 3T3 cells. Cocultivations between NIH 3T3 and CHO cells stably transfected with p2E$^-$ Mo-MuLV (A and D), wild-type Mo-MuLV (B), pGCcos3neo vector alone (C), p2E$^-$ Mo(4070A) MuLV (E), and Mo(4070A) MuLV (F) are shown. Cells in panels A, B, C, E, and F were fixed after 5 h of cocultivation; cells in panel D were fixed after 1 h of cocultivation. Magnification, ×380.

dent on the availability of receptors for ecotropic (and amphotropic [Fig. 4E]) MuLV in the NIH 3T3 cells. We therefore cocultivated p2E$^-$-producing cells with NIH 3T3 cells which were productively infected with Mo-MuLV. The Mo-MuLV Env in these cells blocks their ecotropic MuLV receptors, rendering the cells specifically resistant to superinfection by ecotropic MuLV (38). Virtually no syncytia were formed in these cultures; a typical field is shown in Fig. 5A. In contrast, NIH 3T3 cells productively infected with amphotropic MuLV, which specifically blocks the amphotropic receptor (21), retained their sensitivity to syncytium formation by p2E$^-$ Mo-MuLV (Fig. 5B and C). Thus, the presence of an ecotropic MuLV is specifically required to induce resistance of NIH 3T3 cells to fusion by the mutant ecotropic virus; we conclude that fusion by p2E$^-$ MuLV requires contact between the virus-producing cell and a cell with available receptors for the virus.

We also tested the possibility that exposure to cell-free p2E$^-$ virus, rather than contact with p2E$^-$-producing cells, can induce fusion in NIH 3T3 cells. Culture fluids from the transfected hamster cells were used to infect NIH 3T3 cells. It was found that infection with p2E$^-$ MuLV caused the formation of large syncytia (Fig. 6). However, this phenomenon occurred much more slowly than the fusion observed upon cocultivation: no syncytia were seen in the infected cultures until ~48 h after infection, and the numbers and size of syncytia increased gradually over the next several days. Figure 6 shows syncytia in a culture infected 5 days previously by p2E$^-$ MoMuLV. Infection with free p2E$^-$ Mo(4070A) MuLV also induced fusion (not shown), although as in the cocultivation experiments, the syncytia tended to be smaller than those induced by p2E$^-$ Mo-MuLV. The kinetics of fusion induction by free virus suggest that in this case, syncytia cannot form until a productive infection is established in the assay cells; that is, fusion may, as in the cocultivation experiments, actually result from contact between p2E$^-$-virus-producing cells and susceptible neighboring cells.

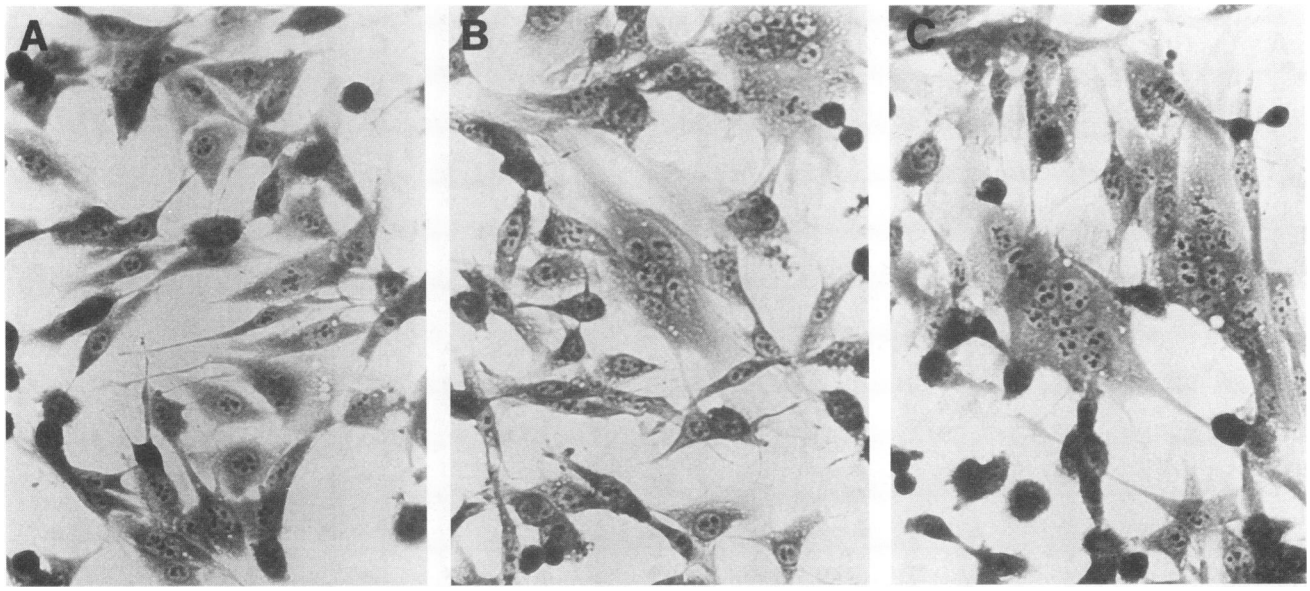

FIG. 5. Syncytium formation upon cocultivation of p2E$^-$-producing cells and MuLV-infected NIH 3T3 cells. p2E$^-$-producing CHO cells were cocultivated for 5 h with NIH 3T3 cells chronically infected with Mo-MuLV (A) or amphotropic MuLV (B) or with uninfected NIH 3T3 cells (C). Magnification, ×710.

Finally, it was of interest to know whether p2E$^-$ Env is capable of inducing cell-cell fusion in the absence of other viral proteins. We therefore subcloned the p2E$^-$ mutation into the Env expression vector pCD-ENV. When this plasmid was transfected into human 293 cells, the cells produced extensive, rapid fusion upon cocultivation with NIH 3T3 cells. These results are shown in Fig. 7: panels A and B represent cocultivations between NIH 3T3 and cells containing p2E$^-$ pCD-ENV, while panel C shows a control using wild-type pCD-ENV. The cells shown in Fig. 7A were fixed after 3 1/2 h, and those shown in panels B and C were fixed after 24 h. As in the experiments with cells producing p2E$^-$ MuLV (Fig. 5), fusion induced by cells expressing p2E$^-$ Env was dependent upon the availability of viral receptors, since it occurred with NIH 3T3 cells producing amphotropic MuLV but not with NIH 3T3 cells producing Mo-MuLV (data not shown).

DISCUSSION

The basic findings of the present report can be summarized as follows. First, an Env protein in which the p15E-p2E cleavage does not take place (i.e., p2Ecl$^-$) is almost completely defective (Table 1, experiment 3). The reduction in

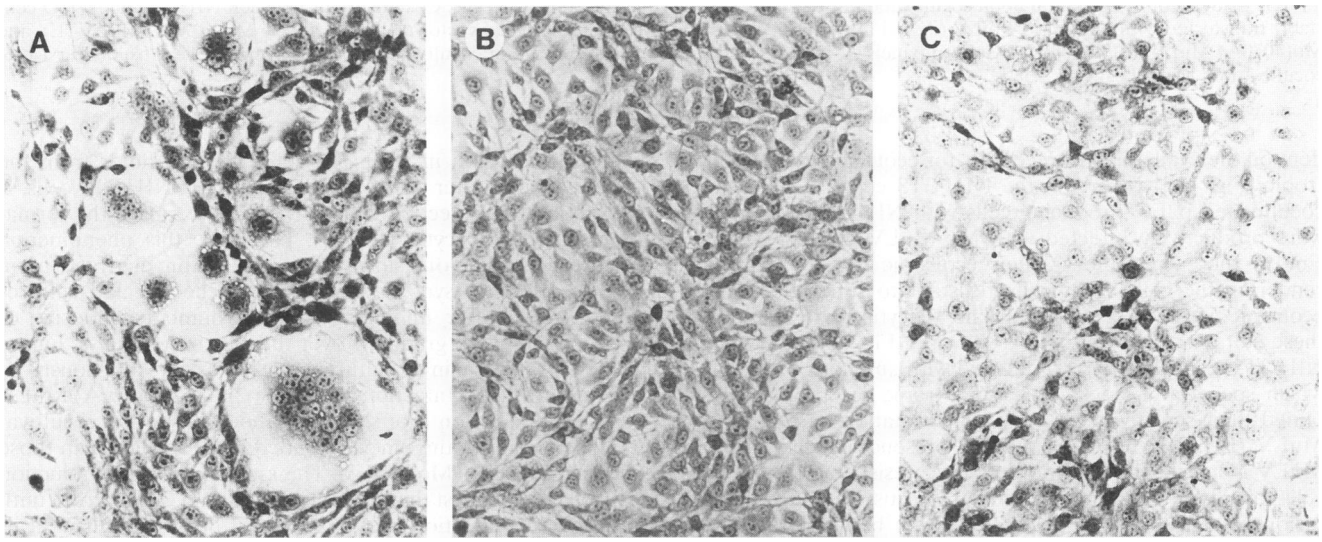

FIG. 6. Syncytium formation after infection of NIH 3T3 cells with p2E$^-$ MuLV. NIH 3T3 cells were infected with undiluted culture fluids from CHO cells stably transfected with p2E$^-$ Mo-MuLV (A), wild-type Mo-MuLV (B), or pGCcos3neo (C). Cells were fixed 5 days after infection. Magnification, ×360.

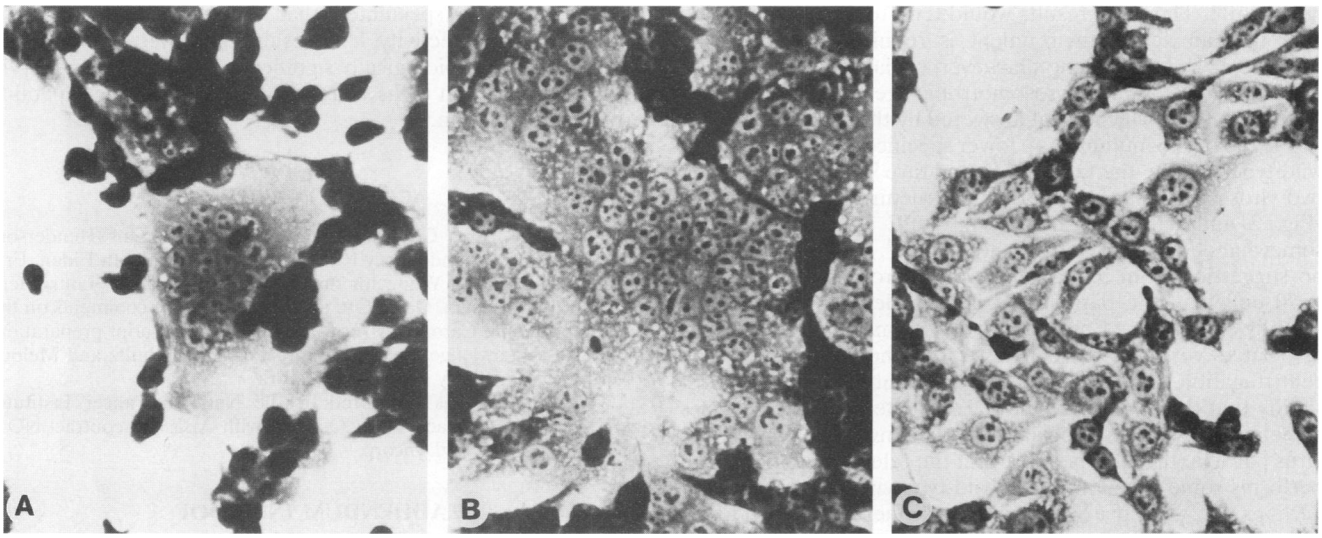

FIG. 7. Syncytium formation upon cocultivation of cells transfected with a p2E$^-$ Env expression vector and NIH 3T3 cells. 293 cells were transfected with p2E$^-$ pCD-ENV (A and B) or with pCD-ENV (C). The cells were cocultivated with NIH 3T3 cells 48 h after addition of DNA and were fixed after 3 1/2 h (A) or 24 h (B and C) of cocultivation. Magnification, ×740.

infectivity shows that the mutant protein fails to perform an essential function, despite the fact that it is successfully transported to the cell surface and incorporated into virions (Fig. 3C). In turn, its transport to the cell surface implies that it is glycosylated and oligomerized normally, since these maturation events are presumably prerequisites for export (9). The defectiveness of p2Ecl$^-$ suggests that cleavage is necessary for infectivity, though we cannot exclude the possibility that some other change at the cleavage site would block cleavage but nevertheless be compatible with Env function.

In contrast, an Env protein in which p2E is never synthesized (i.e., p2E$^-$) is functional, albeit with somewhat lower efficiency than that of the wild-type protein (Table 1, experiment 2). The primary structure of this mutant protein is the same as that of the mature, processed wild-type protein (i.e., p15E^{TM}), but the mutant never exists in the extended precursor form (pre15E^{TM}) found in the wild type.

Perhaps the most striking observation here is that cells producing the latter mutant, p2E$^-$, cause rapid cell-cell fusion when cocultivated with cells possessing available MuLV receptors (Fig. 4 through 6). Indeed, the fusion is induced by the p2E$^-$ Env protein alone, independently of other viral proteins (Fig. 7). Thus, while this mutant Env protein is capable of performing all functions required for delivery of the viral core into the cytoplasm during infection (Table 1, experiment 2), the loss of p2E has given it a new property.

One of the critical functions of Env protein molecules is to induce fusion between the viral membrane and a cellular membrane. One possible explanation for the lethality of the p2Ecl$^-$ mutant is that removal of p2E is required before an MuLV Env protein can cause membrane fusion. Since p2E is normally not removed from p15E before the virus is released from the cell, cells producing wild-type MuLV presumably never display the cleaved form of TM on their surface membranes. In view of these considerations, we suggest that the cell fusion observed in cocultivations with p2E$^-$ is a manifestation of the normal membrane fusion function of wild-type Env. According to this hypothesis, cell fusion is normally not observed because cells producing wild-type MuLV contain only the immature, inactive form of TM, containing p2E as an extension of its cytoplasmic domain.

The hypothesis presented above in turn suggests that p2E is normally synthesized in order to inhibit the fusion activity of the wild-type Env molecule. It would appear that it is advantageous for the virus to delay the activation of the fusion function of Env until the virus has left the cell. Perhaps contact between an active Env and a receptor molecule triggers an irreversible conformational change in Env, so that the virus would lose infectivity if such contact occurred prematurely in the virus-producing cell.

An alternative possibility is that the benefit for the virus lies in the health of the host cell: an active membrane-fusing protein might be toxic if it were present at internal or external cellular membranes. In this connection, we have consistently observed that the level of production of p2E$^-$ virions is somewhat lower than that of wild-type virus in stable CHO transfectants, despite the fact that these cells lack Mo-MuLV receptors. Thus, while the cells do not grow noticeably more slowly than control cells, there may be selection in our cultures against high-level expression of the mutant viral genome. It should also be noted that in the cytocidal virus human immunodeficiency virus, which evidently has not been selected to preserve the health of the host cell, expression of wild-type Env alone is sufficient to induce cell-cell fusion (reviewed in reference 32). In contrast, this is not the case with wild-type MuLV (24). We have shown (Fig. 7) that the failure of MuLV Env to induce fusion under these conditions is due to the presence of p2E.

It has been suggested (1, 30) that fusion by the Mo-MuLV Env protein requires exposure to low pH. This conclusion was inferred from the fact that agents which raise the pH of intracellular vacuoles block infection by ecotropic MuLVs such as Mo-MuLV and by their vesicular stomatitis virus pseudotypes. However, we now report that p2E$^-$ Mo-MuLV Env, which is presumably extremely similar to the Env in a mature wild-type virion, can apparently induce fusion upon contact between the surfaces of virus-producing cells and susceptible cells; this contact occurs in cultures at or near

neutral pH. Thus, our results would tend to argue against the idea that an acidic environment is required for fusion by Mo-MuLV Env. We cannot, however, explain the sensitivity of Mo-MuLV infection to lysosomotropic agents (1, 30).

One question which is not answered by the discussion above is why the p2E$^-$ mutant has a lower specific infectivity than the wild type, despite the fact that the mature TM proteins in the two viruses have the same primary structure. It was observed (Fig. 2 and data not shown) that p2E$^-$ viral pellets are somewhat deficient in gp70^{SU} and p15E^{TM}, although there was no suggestion from immunoelectron microscopy studies of a gp70 deficit in the particles. It is possible that the particles actually contain less Env protein, or Env may be more loosely associated with the particles so that more is lost during centrifugation. In either case, the observation raises the possibility that the absence of the p2E residues in these particles somehow affects the association of the Env complex with the virus particle. It is also possible that the released peptide, p2E, performs some function in the wild-type virion. Alternatively, perhaps the presence of the p2E residues in the wild-type precursor affects the postcleavage conformation of the mature wild-type TM. This suggestion is somewhat similar to that recently put forth in a study of the HA2 protein of influenza virus: Carr and Kim (4) propose that the native state of HA2 in influenza virions is not the thermodynamically most stable state for this molecule. Yet another possibility is that the pre15E molecules which are often found in wild-type MuLV preparations (25) (Fig. 2, lane 3) contribute to infectivity in some way.

It is interesting that the hypothesis discussed above implies that the presence or absence of p2E in the cytoplasmic domain of TM affects the ability of the protein to induce membrane fusion, despite the fact that fusion presumably occurs on the extracellular side of the membrane. Perhaps the presence of p2E interferes with a conformational change or multimerization in the Env complex required for the membrane fusion event; the mechanism may have some analogy with signal transduction phenomena involving transmembrane receptor molecules.

In summary, our results suggest that one function of the cytoplasmic domain of the MuLV TM protein is to somehow modulate or restrict the fusogenic activity of the Env protein complex; it also appears that this function is not absolutely essential for the process of infection. Recent results obtained with SIV are in complete agreement with these conclusions. As noted above, propagation of SIV in certain cell types selects for TM mutants with truncated cytoplasmic domains. To analyze the effects of this truncation, Zingler and Littman (49) and Ritter et al. (39a) introduced termination codons into a molecular clone encoding an extended cytoplasmic domain. In each of these studies, the new termination codon was placed at the natural truncation site. Both groups reported that truncation of the cytoplasmic domain dramatically increased the fusogenicity of the resulting Env molecule. In addition, Zingler and Littman (49) reported that the truncation resulted in enhanced infectivity for human cells. The analogy between our results with MuLV and these findings with SIV suggests that these general conclusions concerning the role of the cytoplasmic domain of TM can, to some extent, be extended to other retroviruses as well.

Finally, it has been known for many years that PR catalyzes the maturation cleavage of the Gag polyprotein in retrovirus particles; it seems likely that this major structural change in the virus is required for infectivity. We have recently described a second PR-dependent event in the maturation of MuLV particles, i.e., a change in conformation of the genomic RNA dimer (15). We speculated that this change may also be important for infectivity. The results presented here indicate that yet another biologically significant, PR-dependent step in MuLV maturation is the activation of the fusogenic capability of the Env protein.

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ADDENDUM IN PROOF

Recent observations of Mason-Pfizer monkey virus are, in a number of respects, parallel to those reported here of MuLV (B. A. Brody, S. S. Rhee, and E. Hunter, submitted for publication).

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