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Analysis of the Functional and Host Range-Determining Regions of the Murine Ecotropic and Amphotropic Retrovirus Envelope Proteins

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A series of Moloney murine leukemia virus (Mo-MuLV) envelope gene constructs were analyzed for biological activity. Three classes of recombinant envelopes were examined: insertions, deletions, and chimeras. Insertion (4 to 5 amino acids) and deletion (31 to 62 amino acids) mutants spanned most of the SU (gp70)-coding region and were all biologically inactive. Radioimmunoprecipitation demonstrated that the mutant envelope proteins were incorrectly processed. The Pr80^{env} envelope precursor proteins failed to obtain the proper posttranslational modifications and were not cleaved into SU (gp70) and TM (p15E), suggesting that disruption of Pr80^{env} structure prevents intracellular transport and processing. To analyze the functional domains of the SU portion of the Env protein, we assembled several chimeric constructs. In these constructs, portions of the ecotropic Mo-MuLV envelope gene were replaced with corresponding sequences from the 4070A amphotropic MuLV envelope. Using a retroviral vector pseudotyping assay, 5 of 12 chimeric envelope proteins were shown to be biologically active. Host range was determined by retroviral vector transduction of the appropriate cell, by viral interference studies, and by the productive infection of Chinese hamster ovary cells expressing the murine ecotropic receptor. These results permit assignment of the amino acids responsible for host range determination. Ecotropic host range is determined by the first 88 amino acids of the Mo-MuLV SU, while the amphotropic host range-determining region spans the first 157 amino acids of the 4070A SU.

The Moloney murine leukemia virus (Mo-MuLV) is an ecotropic virus with a host range confined to mice and rats. The virus was isolated from a murine sarcoma (23). Mo-MuLV encodes an envelope precursor, Pr80^{env}, that is initially glycosylated in the endoplasmic reticulum, further processed in the Golgi apparatus, and proteolytically cleaved to generate the mature SU (gp70) and TM (p15E) protein heterodimer (34, 42, 43). Host range determinants reside in the SU moiety, while TM contains a typical membrane-spanning region (7, 8, 20, 33). TM is postulated to form noncovalent and covalent bonds with SU (32). Envelope proteins from other ecotropic murine retroviruses express a larger envelope precursor, Pr90^{env}, that appears to process and transport in a manner similar to Mo-MuLV Pr80^{env} (10, 15, 25). Biochemical studies with human, primate, and avian retroviral envelope proteins have shown that oligomerization of monomeric precursor precedes transport and proteolytic processing (9, 35). Envelope proteins appear to retain their oligomeric structure in virions as well (9, 31).

Naturally occurring murine retroviruses are classified into five subgroups based on host range and interference patterns (ecotropic, xenotropic, amphotropic, polytropic, and 10A1 [29]). Different host ranges can arise by recombination between ecotropic and endogenous nonecotropic sequences (4, 5, 16, 39, 40). Sequence comparisons between murine retroviral envelopes reveal large regions of sequence conservation, with the most divergent regions found in the amino-terminal half of SU. Protein sequences from the carboxyl-terminal region of SU through the end of TM have a high degree of homology among all host range types as well as nonmurine retroviruses (28, 39). Sequence analysis has shown that the ecotropic SU protein diverges significantly from the other murine host range classes primarily in the N-terminal one-third of SU (28, 39).

Chimeric envelope constructs have been described that are biologically active based on standard assays for replication-competent virus (2, 4, 24, 26, 27, 30). In an early report, a polytropic virus (mink cell focus-forming virus) was derived from Mo-MuLV and shown to contain mink cell focus-forming virus sequences from the leader through most of SU, thus localizing host range determinants to the amino-terminal three-fourths of the protein (4). The assignment of host range determinants has been refined for the xenotropic, polytropic, 10A1, and amphotropic viruses to include just the N-terminal one-third of SU (2, 30). Protein sequence comparisons between different MuLV host range types suggest two small regions within this area that are likely to play a major role in receptor binding.

Results presented in this study identify regions throughout the SU protein that are necessary for transport and processing of biologically active Mo-MuLV Pr80^{env}. Disruption of Mo-MuLV envelope protein function results from linker insertion, from deletions, and can occur in chimeric constructs. Several functional chimeras with an ecotropic host range or an amphotropic host range permit localization of the envelope region responsible for host range determination to 88 and 157 amino acids at the amino terminus of SU for the ecotropic and amphotropic viruses, respectively.

MATERIALS AND METHODS

Envelope constructs. Infectious cloned Mo-MuLV DNA, construct pMLV-K (Fig. 1), was obtained from S. Ruscetti

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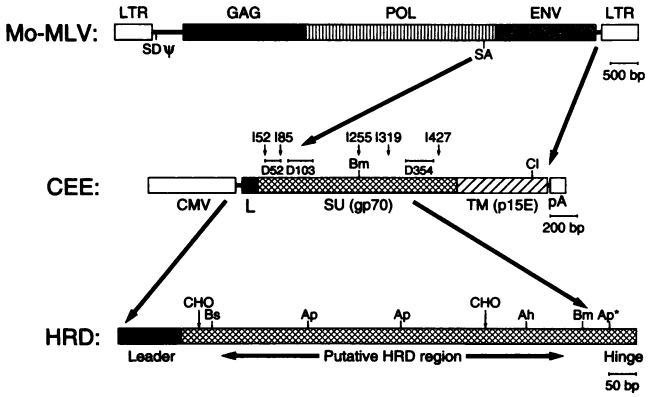

FIG. 1. Schematic representation of Mo-MuLV and expression constructs. Shown on the top of the figure is a schematic representation of the integrated form of the Mo-MuLV. In the middle of the figure is a diagram of the CEE expression plasmid indicating the location of the insertion and deletion mutants. On the bottom of the figure are shown landmark restriction enzyme sites Bm (*Bam*HI), Cl (*Cla*I), Bs (*Bst*EII), Ap (*Apa*I), and Ah (*Aha*II) and putative glycosylation sites (CHO). Other abbreviations: L, leader sequence; pA, polyadenylation signal; HRD, host range-determining region; LTR, long terminal repeat.

and has been described previously (22). Amphotropic envelope gene, up to the *Cla*I site in TM (pAM-MLV), was obtained from D. Miller and was constructed by insertion of a 3.9-kbp *Sal*I-*Cla*I fragment from the 4070A amphotropic MuLV into corresponding sites in Mo-MuLV. The amphotropic sequences include part of the polymerase gene and all of the envelope through the carboxyl-terminal region of TM. All recombinant envelopes were subcloned into pCXS, a cytomegalovirus-promoted, simian virus 40-terminated expression vector. The Mo-MuLV envelope sequence from pENV (21) was removed by *Eco*RI digestion and inserted into pCXS, yielding pCEE (Fig. 1). The Mo-MuLV sequences in pCEE span coordinate 5408 (38) in the polymerase gene to 7847 in U3 of the 3′ long terminal repeat and are flanked by *Eco*RI linkers. The parallel amphotropic envelope expression plasmid (pCAE) was constructed by removing the amphotropic envelope from pAM-MLV by *Xma*III-plus-*Nhe*I digestion followed by attachment of *Xba*I linkers and ligation into the pCXS expression plasmid. Insertion and deletion mutants were constructed with available restriction enzyme sites as follows: *Bst*EII (I52, D52), *Nco*I (I85), *Apa*I (D103), *Bam*HI (I255), *Acc*I (I319), *Kpn*I (D354), and *Nsi*I (I427). Figure 1 shows the approximate locations of these insertions/deletions, and Table 1 reports the specific changes in amino acid sequence.

To facilitate production of chimeric ecotropic and amphotropic envelope constructions, we used site-directed mutagenesis to create homologous restriction enzyme sites according to the manufacturer's instructions (Oligonucleotide Directed In-vitro Mutagenesis System, version 2; Amersham). Nucleotide and amino acid designations derive from the sequence of 4070A reported by Ott et al. (29) and from the Mo-MuLV sequence communicated by Shinnick et al. (38). First, an additional *Apa*I site (Ap*) was created in the ecotropic sequence by changing nucleotide 6987 from A to C (without changing any amino acid). The same techniques were used to create a *Bst*EII site (Bs*) in the amphotropic sequence, by changing nucleotide 158 from T to G (changing the amino acid from Asn to Lys). This change does not effect biological activity (see Results). The symbols Bs*, Ap*, Ap^1, Ap^2, Ap^3, and Ap^4 denote specific restriction enzyme sites (see Fig. 4 for locations).

The chimeric envelopes were constructed as follows.

(i) AE1. An insert from pCAE, from an *Xba*I in the polylinker between the promotor and the envelope gene to the *Cla*I site in the transmembrane region, was inserted into pCEE, previously cut at *Xba*I and *Cla*I.

(ii) AE2. An insert from pCAE, from the polylinker *Xba*I to the $Apa I^4$ site, was inserted into pCEE previously cut at *Xba*I and *Apa*I*.

(iii) AE3. As for AE2, but using the $Apa I^3$ of the amphotropic envelope gene.

(iv) AE4. An insert from pCAE, from the polylinker *Xba*I to the *Xho*I site of the amphotropic envelope gene (Xh), was inserted into pCEE previously cut at *Xba*I and at the *Aha*II (Ah) site in the ecotropic envelope gene. The insertion was made with *Xho*I-*Aha*II adaptor oligonucleotides, resulting in no change in the amino acid sequences.

(v) AE5. As for AE4, but the *Bam*HI site of the ecotropic envelope gene (Bm) and an *Xho*I-*Bam*HI adaptor oligonucleotide were used.

(vi) AE6. As for AE1, but with the further substitution of 5′ ecotropic sequences up to the *Bst*EII site.

(vii) AE7. As for AE2, but including the 5′ ecotropic sequences up to the *Bst*EII-*Bst*EII* junction.

(viii) AE8. Using polymerase chain reaction techniques, a DNA fragment from the amphotropic envelope gene from nucleotide 344 to $Apa I^4$ was made. The oligonucleotides used create an *Apa*I site near the amphotropic *Mae*II site by changing nucleotide 346 from C to G and nucleotide 349 from A to C. The DNA fragment was inserted into pCEE, previously cut partially with *Apa*I, at the $Apa I^1$ and *Apa*I* positions.

TABLE 1. Amino acid sequences of Mo-MuLV envelope mutants

Construct	Wild-type sequence[a]	Mutant sequence[a]
I52	Val-Thr-Asn	Val-Thr-GLY-ILE-PRO-VAL-THR-Asn
D52[b]	Val-Thr-Asn. . .His-His-Gly	Val-Thr-GLY-ILE-PRO-His-Gly
I85	His-His-Gly	His-His-ALA-LEU-GLU-HIS-Gly
D103	Pro-Pro-Gly. . .Cys-Pro-Gly	Pro-Pro-Gly-ILE-Pro-Gly
I255	Gly-Ile-Arg	Gly-Ile-GLN-ILE-TRP-ILE-Arg
I319	Leu-Val-Asp	Leu-Val-ALA-THR-ARG-ARG-Asp
D354	Val-Leu-Gly. . .Gly-Thr-Met	Val-Leu-ALA-GLU-PHE-ARG-Met
I427	Thr-Pro-Cys-Ile-Ser	Thr-Pro-ARG-ARG-VAL-VAL-Ser

[a] Residues introduced by linkers and envelope junctions are indicated by capital letters, and deleted residues are underlined.
[b] Deletion mutants show sequences at both deletion junctions. D52 deletes residues 52 to 83; D103 deletes residues 103 to 152; and D354 deletes residues 354 to 416.

(ix) **AE9.** Using polymerase chain reaction techniques, two DNA fragments were synthesized, a DNA fragment from the amphotropic envelope gene, from nucleotide 412 to *Apa*I[4], and a DNA fragment from the ecotropic envelope gene, from *Bst*EII to nucleotide 6139. The two fragments were ligated together, digested with *Bst*EII plus *Apa*I, and inserted into pCEE previously cut with *Bst*EII and *Apa*I.

(x) **AE10.** As for AE8, but using *Apa*I[2] and the polymerase chain reaction-generated DNA fragment from nucleotide 478 to *Apa*I[4] of the amphotropic envelope gene, creating an *Apa*I site near the *Afl*II site (Af) by changing nucleotide 478 from T to G, nucleotide 481 from G to C, and nucleotide 482 from A to C.

(xi) **AE11.** As for AE8, but using *Apa*I[1], *Apa*I[2], and polymerase chain reaction-generated DNA fragments from nucleotides 344 to 518 of the amphotropic envelope gene, creating a second *Apa*I site near *Afl*II, by changing nucleotide 514 from T to G, nucleotide 517 from G to C, and nucleotide 518 from A to C.

(xii) **AE12.** An insert from pCEE, from an *Apa*I site in the polylinker in front of the cytomegalovirus promotor, to *Apa*I* was inserted into pCAE previously cut with *Apa*I polylinker site and *Apa*I[4].

All the chimeric constructs were sequenced prior to use (Sequenase 2.0 Kit; United States Biochemical Co.).

Cell lines and virus assays. NIH 3T3 cells, mink lung fibroblasts, and COS-1 cells were maintained in Dulbecco's modified essential medium (DMEM; Biofluids, Rockville, Md.) supplemented with 10% fetal calf serum (FCS, Hyclone, Logan, Utah). XC cells were obtained from the American Type Culture Collection and maintained as recommended. The CHO and CHO-2 cells were a gift from M. Kaden (Genetic Therapy, Inc., Gaithersburg, Md.) and were cultured in alpha MEM plus 10% FCS. The CHO-2 cell line contains a plasmid that expresses the murine ecotropic receptor protein (pJET [1]). For the construction of envelope test cell lines, NIH 3T3 cells were transfected with the plasmid pGag-polGpt (containing the Mo-MuLV *gag* and *pol* regions) and were then selected in HXM medium as described previously (21). Twelve clones were screened for reverse transcriptase and the highest reverse transcriptase producer, clone 8, was chosen and named GP8. GPL cells and GPNZ cells were made by transducing GP8 cells with either the retroviral vector LNL6 (to yield GPL) or LBgSN (to yield GPNZ). The LNL6 retroviral vector carries the neomycin resistance (*neo*) gene from Tn*5* and has been described previously (3). LBgSN is a retroviral vector that expresses the *Escherichia coli* β-galactosidase (β-gal) (*lacZ*) gene from the retroviral long terminal repeat and a *neo* gene via an internal simian virus 40 early region promoter (this vector was obtained from Genetic Therapy and is a derivative of the LXSN vector). Both GPL and GPNZ were selected for stable integration of the vector sequences by growth in G418-containing medium for 14 days.

Transfection, titer determination, interference assays, and LacZ staining. DNA (30 μg) was transfected into GPL or GPNZ cells (5×10^5 cells in a 100-mm dish) by the calcium phosphate precipitate method (6). Sixteen-hour posttransfection cell culture medium was changed and, after 48 h, collected for titer determinations. The culture supernatant was either used immediately or stored at −70°C. For titration or β-gal staining, NIH 3T3, mink, and COS-1 cells were plated (2.5×10^4 cells in a 30-mm well) in 2 ml of DMEM containing 10% FCS (D10) medium, and 18 to 24 h later, the medium was replaced with 0.5 ml of serial-diluted fresh or thawed viral supernatant containing Polybrene (8 μg/ml) for 2 h at 37°C, after which 2 ml of D10 was added. Eighteen to 24 h posttransduction, the cells were selected for G418 resistance by growth in D10 containing G418 (500 μg/ml) for 12 to 14 days. Colonies were scored by methylene blue staining. For in situ β-gal staining, 18 to 24 h posttransduction, the culture medium was replaced with fresh D10, and the cells were grown for 48 h. Cells were stained for in situ β-gal activity as previously described (37). For viral interference studies, approximately 2×10^4 3T3 (or mink) cells were incubated with 1 ml of high-titer ($>1 \times 10^6$ G418^r CFU/ml) ecotropic or amphotropic retroviral vector supernatant for 60 min at 4°C. Supernatant (1 ml) from GPNZ cells (previously transfected with various chimeric envelope constructs) was then added and incubated for a further 30 min at 4°C. Following the 4°C incubations, the test supernatants were removed and fresh DIO medium was added. The cells were incubated for 36 h at 37°C and then stained for β-gal activity. XC assays were performed by cocultivation of 3T3 cells expressing envelope with XC cells as described previously (18) and by direct transfection of XC cells with envelope DNA followed by growth of the cells to confluence.

Cell labeling and immunoprecipitation. NIH 3T3 cells were analyzed for viral envelope gene expression by labeling with [^{35}S]methionine (Amersham). Cells were grown to near confluence in 60-mm tissue culture dishes, then starved in 3 ml of methionine-free medium (45 min), and then labeled for 30 min at 37°C in 100 μCi/ml. The cells were lysed at 4°C as described previously (6). The lysates were precleared with normal goat immunoglobulin G IgG (Sigma) and 50 μl of protein G-Sepharose 4B beads (Zymed). Envelope protein was immunoprecipitated at 4°C with 4 μl of goat anti-Rauscher virus SU antiserum (lot no 79S000771; Microbiological Associates). Overnight labeling with [^{3}H]fucose and [^{3}H]mannose was done with cells that were transfected 36 h previously. The cells were labeled for 3 h with 500 μCi of labeled sugar in 1.5 ml of glucose-free DMEM with 2% dialyzed FCS, followed by an additional 9-h incubation in low (0.5-g/liter)-glucose DMEM with 2% FCS. Glucose levels were adjusted by addition of DMEM (containing glucose) with 2% FCS to give a final volume of 4.0 ml. Samples were electrophoresed in 7.5% acrylamide gels as described previously (19). Gels were stained with Coomassie blue, dried, and fluorographed with Enlightening (Dupont, NEN Research Products) with Kodak X-AR film at −70°C.

Immunostaining and fluorescence-activated cell sorter analysis. Plasma membrane localization of chimeric envelope molecules was determined by immunofluorescence staining and flow cytometric analysis as previously described (14). After transient expression of transfected GPL cells with chimeric envelope constructs, supernatants were collected, and the cells were trypsinized, washed with phosphate-buffered saline (PBS), and brought to 10^7 cells per ml in PBS containing 10% goat serum. The collected supernatant was incubated with 3T3 or mink cells as follows. After trypsinization and washing with PBS, 10^6 cells were incubated with 3 ml of viral supernatant in 15-ml polypropylene screw-top tubes on a rotator for 2 h at 4°C. Following the incubation, cells were collected by centrifugation and washed three times with 1 ml of PBS containing 10% goat serum. All cells (10^6) were immunostained by incubation in 250 μl of undiluted rat anti-SU monoclonal antibody (83A25) for 1 h at 4°C on a rotator. Cells were collected, washed as above, and resuspended in 100 μl of goat anti-rat immunoglobulin G labeled with phycoerythrin (TAGO) diluted 1:75 in PBS containing 10% goat serum. After 0.5 h of incubation as

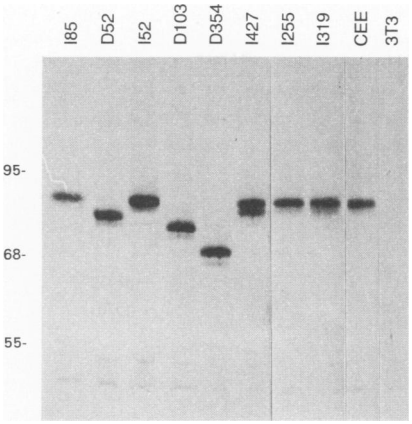

FIG. 2. Expression of insertion and deletion mutants. Immunoprecipitation of [^{35}S]methionine-labeled Mo-MuLV envelope insertion and deletion mutants was performed as described in Materials and Methods. Cells were transfected with 40 μg of plasmid DNA and immunoprecipitated 48 h later. CEE expresses wild-type Mo-MuLV envelope protein. The sizes of protein molecular mass standards (in kilodaltons) are indicated on the left.

above, cells were washed and resuspended in 0.3 ml of 4% paraformaldehyde and analyzed by flow cytometry (FAST System, Inc., Gaithersburg, Md.). Untransfected or uninfected GPL, 3T3, or mink cells were used as negative controls, and background fluorescence was subtracted from experimental samples.

RESULTS

Construction and expression of insertion and deletion constructs. As a first step in the analysis of the structure of the Mo-MuLV envelope protein, we constructed a series of insertion and deletion mutants. The sites chosen for mutagenesis were existing restriction enzyme sites spread throughout the length of the envelope protein. The approximate location of each mutation is displayed in Fig. 1, while the changes in amino acid sequence are shown in Table 1. The DNA coding for these envelope mutations was constructed into an expression plasmid and tested by transfection into the NIH 3T3 cell line by calcium phosphate coprecipitation. Forty-eight hours after removal of the Ca-DNA complex from the tissue culture plates, the cells were examined for envelope expression by radioimmunoprecipitation. The polyclonal antiserum used for these precipitation assays has broad cross-reactivity that permits detection of gross perturbations in envelope structure.

A 30-min pulse-labeling was used to detect the unprocessed envelope protein Pr80^{env} (43). The $t_{1/2}$ for Mo-MuLV Pr80^{env} processing in murine fibroblasts is 80 to 90 min and is similar to that seen for other MuLV (33, 42). Examination of the results from this analysis (Fig. 2) demonstrated that all the insertion and deletion mutants are capable of producing Pr80^{env}-like precursor envelope proteins. All mutant constructs expressed protein that migrated as expected except for the I427 envelope (Fig. 2). I427 consistently expressed a precursor protein that ran as a doublet. The bottom band of the doublet resolved to the top position based on pulse-chase experiments (data not shown). D52 and D103 constructs migrate as predicted from the loss of amino acids in the envelope gene. The D354 construct deletes three potential N-linked glycosylation sites and migrates with an apparent molecular mass of 72 kDa compared with 85 kDa for the wild-type protein. The calculated molecular mass reduction for D354 is 7.1 kDa based on loss of amino acids alone. The observed reduction of 13 kDa is consistent with the loss of three high-mannose oligosaccharides in addition to the amino acids deleted in D354. This result agrees with previous studies showing that all ecotropic MuLV envelope N-linked glycosylation sites are occupied by sugars (36).

As a further test of the mutant envelopes, NIH 3T3 cells were transfected with mutant envelope constructs and then cocultivated with the XC indicator cell line. XC cells form syncytia in the presence of ecotropic MuLV and are used to quantitate infectious virus by plaque assay (18). Using wild-type MuLV envelope, syncytia were observed within 6 h after the plating of XC cells onto previously transfected 3T3 cells. The number and size of syncytia increased over a 24-h period and resulted in numerous cells containing up to 40 nuclei (data not shown). However, none of the mutant envelope constructs generated multinucleated cells at any time as the cultures grew to confluence and were indistinguishable from control 3T3-XC cell cocultures (data not shown). Identical results were obtained by direct transfection of XC cells with pCEE and the mutant constructs. These results do demonstrate (for the first time) that wild-type ecotropic Mo-MuLV envelope expression alone can generate syncytia with XC cells.

Intracellular transport and processing of mutant envelope proteins. To attempt to identify the presumed block to surface expression of the mutant envelope proteins, we used radiolabeled to monitor intracellular processing. Immunoprecipitation following radiolabeling with [^{3}H]mannose and [^{3}H]fucose was used to determine whether the mutant envelope proteins were transported to the Golgi apparatus and further processed by the addition of fucose and proteolytic cleavage to SU and TM. Fucose is added to complex N-linked oligosaccharides in the Golgi apparatus, and thus the presence of labeled fucose indicates that transport from the endoplasmic reticulum has occurred. As seen in Fig. 3, the wild-type Mo-MuLV protein is processed to generate an SU that contains fucose in this overnight labeling. The envelope antiserum used in this study was raised against purified SU and does not precipitate TM. Immunoprecipitated samples were denatured under reducing conditions prior to gel electrophoresis, causing dissociation of TM. The heterogenous label seen above Pr80^{env} represents a small amount of envelope precursor with mature N- and O-linked glycosylation that is proteolytically cleaved to SU and TM (34). Compared with wild-type Mo-MuLV envelope, little fucose was incorporated into the mutant proteins.

Biological activity of ecotropic and amphotropic chimeric envelopes. A series of chimeric envelopes (Fig. 4) were constructed by inserting different regions of the 4070A amphotropic envelope into ecotropic envelope expression plasmid pCEE. Each chimera was then tested in a pseudotyping assay. For this assay, two Gag-Pol-expressing retroviral vector-containing cell lines were constructed: GPL cells, which contain a *neo* retroviral vector, and the GPNZ cells, which contain a *neo/lacZ* retroviral vector. The GPNZ cells permit rapid qualitative analysis of biological activity (by staining for β-gal), while the GPL line is used to obtain quantitative results (by scoring for resistance to the neomycin analog, G418). With the exception of the AE1 chimera (which contains amphotropic sequences from the leader sequence through the transmembrane region), all chimeras were constructed by switching the heterologous domain of SU that is located between the leader sequence and the

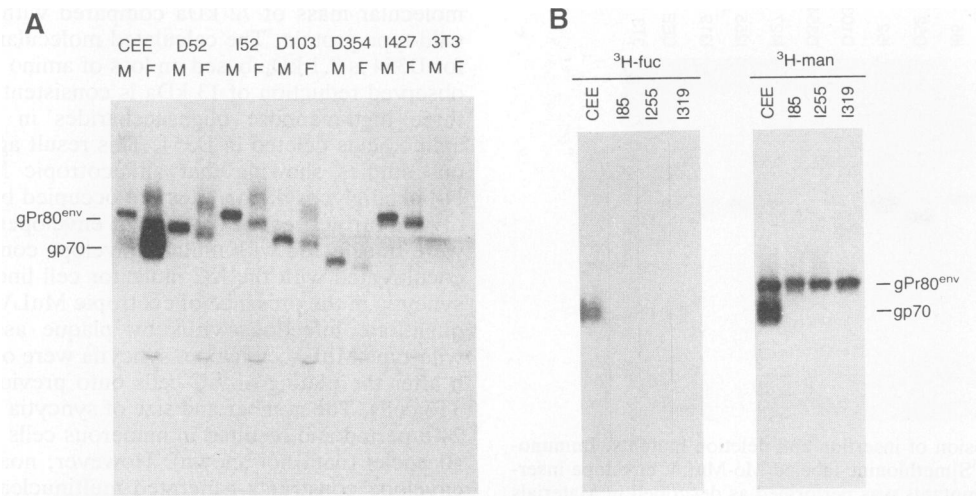

FIG. 3. Processing of insertion and deletion mutants. Immunoprecipitation of [^{3}H]sugar-labeled Mo-MuLV envelope insertion and deletion mutants. 3T3 cells were transfected with 40 μg of plasmid DNA and, 30 h after transfection, radiolabeled with [^{3}H]mannose (M) or [^{3}H]fucose (F). Following labeling for 16 h, cell lysates were analyzed by immunoprecipitation and SDS-PAGE with fluorography. (A) Deletion mutants D52, D103, and D354 and insertion mutants I52 and I427. (B) Insertion mutants I52, I255, and I319.

proline-rich hingelike region (approximately amino acids 35 through 250).

The various envelope constructs were transfected into the GPL or GPNZ cell lines by calcium phosphate coprecipitation, and after an overnight incubation, culture medium was removed and replaced with fresh medium. Following culture for 48 h to permit transient expression, cell culture medium was removed, filtered, and assayed. To determine the tropism of the chimeric envelope-containing retroviral vector particles, culture medium was used to transduce (infect) the murine NIH 3T3 cell line (permissive for ecotropic and amphotropic viruses), or two nonmurine cell lines, mink lung fibroblasts and rhesus monkey COS-1 cells (both permissive for amphotropic viruses only).

The results from multiple independent transfections are shown in Table 2. The titer generated by the control constructs, CEE and CAE, was generally in the range of 10^4 to 10^5 G418^r CFU/ml when measured on 3T3 cells (for each individual transfection, the data are normalized by setting the control vector titer to 100%; titers on mink cells and COS-1 cells were 2-fold and 10-fold lower, respectively, than those on 3T3 cells). Five of the envelope chimeras yielded biologically active retroviral particles: AE1, AE2, AE4, AE9, and AE12. AE1, AE2, and AE4 had amphotropic host ranges as determined by titer on mink cells and β-gal staining on COS-1 cells. AE9 and AE12 displayed an ecotropic host range by being capable of titer and β-gal staining only on NIH 3T3 cells. The titers of the chimeras, with the exception of AE9, were nearly equivalent to those of the controls (between 65 and 115%). The AE9 construct consistently yielded titers of a few percent of the control, and this was independent of the specific plasmid DNA preparation used for transfection (data not shown).

To further analyze the tropism of the two biologically active chimeric ecotropic envelope constructs (AE9 and AE12), we determined the titers of supernatants from GPNZ cells on Chinese hamster ovary cells containing the ecotropic receptor (CHO-2 cells). The ecotropic receptor protein is a cationic amino acid transporter whose transfer to nonpermissive cells is sufficient to permit infection by ecotropic MuLV (1, 17). The control CEE construct produced retro-

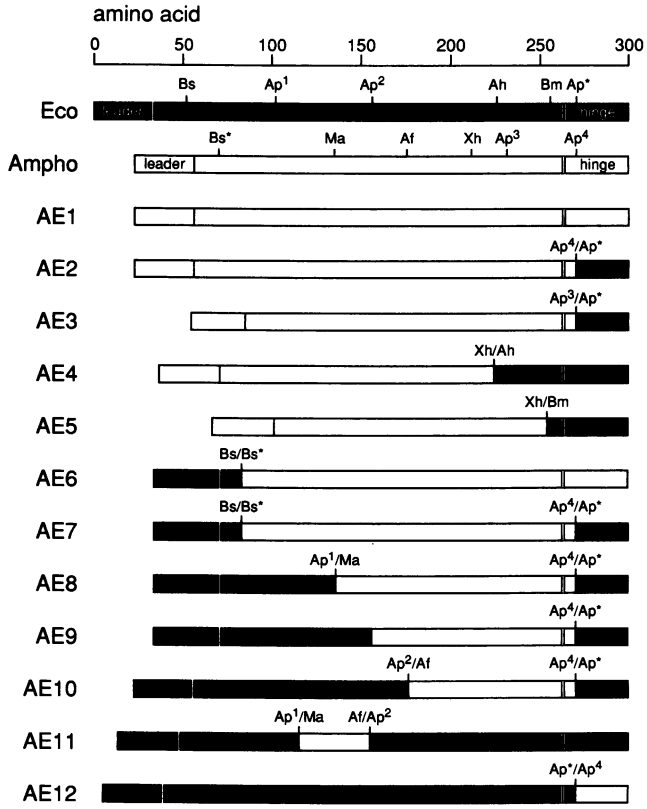

FIG. 4. Chimeric envelope constructs derived from the Mo-MuLV and 4070A envelope genes. Shown are diagrams of the chimeric envelope constructs in relation to the two control ecotropic (CEE) or amphotropic (CAE) expression plasmids. The sequences are aligned at the proline-rich hingelike region to emphasize the difference in the size of the amino-terminal region of SU. Amphotropic-ecotropic junctions in AE2 and AE6 constructs are at a conserved *Cla*I site located near the carboxyl terminus of TM (not shown in this figure; see Fig. 1). Restriction sites generated by in vitro mutagenesis are indicated by asterisks. Restriction site abbreviations: Af, *Afl*II; Ah, *Aha*II; Ap, *Apa*I; Bm, *Bam*HI; Bs, *Bst*EII; Ma, *Mae*II; Xh, *Xho*I Bs*, Ap*, Ap^1, Ap^2, Ap^3, and Ap^4 denote specific restriction enzyme sites.

TABLE 2. Biological activity and host range of chimeric envelope proteins

Envelope expression construct	β-Gal-positive GPNZ[a]		Relative G418[r] CFU/ml[b]				Host range[c]
			GPL		GPNZ		
	3T3	COS-1	3T3	Mink	3T3	Mink	
CEE	+	−	100	ND	100	ND	E
CAE	+	+	100	100	100	100	A
AE1	+	+	95 ± 20	85 ± 25	115 ± 15	110 ± 20	A
AE2	+	+	65 ± 10	67 ± 7	93 ± 33	80 ± 20	A
AE3	−	−	ND	ND	ND	ND	
AE4	+	+	70 ± 12	65 ± 10	107 ± 33	100 ± 30	A
AE5	−	−	ND	ND	ND	ND	
AE6	−	−	ND	ND	ND	ND	
AE7	−	−	ND	ND	ND	ND	
AE8	−	−	ND	ND	ND	ND	
AE9	+	−	2.5 ± 1	ND	2.5 ± 1	ND	E
AE10	−	−	ND	ND	ND	ND	
AE11	−	−	ND	ND	ND	ND	
AE12	+	−	94 ± 23	ND	102 ± 24	ND	E

[a] Supernatants from GPNZ cells were used to transduce the indicated cell type and the cells were stained for β-gal activity as described in Materials and Methods.

[b] Supernatants from GPL cells were used to transduce the indicated cell type. Target cells were selected for G418[r] as described in Materials and Methods. In each experiment, the G418[r] CFU per milliliter values for CEE and CAE were set to 100% to normalize results obtained in different transfections (titers were in the range of 10^4 to 10^5 on 3T3 cells and 10^3 to 10^4 on mink cells). Each construct was tested three to five times, and the results are the mean ± SD for the normalized CFUs (ND, not detected, where the limit of detection in this assay is 1 G418[r] CFU/ml).

[c] E, ecotropic; A, amphotropic.

viral vector supernatants capable of transducing NIH 3T3 and CHO-2 cells but not the mink or CHO control cell lines (Table 3). The amphotropic envelope constructs (CAE and AE4) yielded vector preparations capable of transduction of 3T3 and mink cells but not the CHO lines. When ecotropic constructs AE9 and AE12 were tested on CHO-2 cells, they produced levels of transduction (relative to 3T3 cells) similar to that observed for the control CEE construct (44 and 29% for AE12 and AE9, respectively, versus 50% seen with CEE).

As a final test of biological activity, a viral interference assay was conducted. In this experiment, 3T3 or mink cells were preincubated with high-titer ecotropic or amphotropic retroviral vector preparations (see Materials and Methods). These cells were then exposed to supernatant from GPNZ cells previously transfected with CEE, CAE, AE4, AE9, or AE12. The cells were stained for β-gal activity 36 h later, and results obtained are shown in Table 4. Based on the behavior of the control constructs (CEE and CAE), preincubation of cells with a virus of the identical tropism significantly interferes with the ability of the test virus to infect the cells (approximately 90% reduction in the number of β-gal-positive cells). For the chimeric construct AE4, preincubation

TABLE 3. Analysis of chimeric envelope infectivity of CHO-2 cells

Construct	Relative titer on indicated cell line[a]			
	3T3	Mink	CHO	CHO-2
CEE	100	ND	ND	50
CAE	100	80	ND	ND
AE4	100	86	ND	ND
AE9	100	ND	ND	29
AE12	100	ND	ND	44

[a] For each envelope construct the number of G418[r] CFU per milliliter obtained with GPNZ supernatant to transduce NIH 3T3 cells was set to 100. The number of colonies on the other cell lines was then normalized to this value (ND, not detected, where the limit of detection in this assay is 1 G418[r] CFU/ml).

TABLE 4. Viral interference assay

Construct	Test cell	Interfering virus	LacZ staining[a]
CEE	3T3	None	++++
CEE	3T3	Ecotropic	+
CEE	3T3	Amphotropic	+++
AE9	3T3	None	++++
AE9	3T3	Ecotropic	−
AE9	3T3	Amphotropic	++
AE12	3T3	None	++++
AE12	3T3	Ecotropic	+
AE12	3T3	Amphotropic	+++
CAE	3T3	None	++++
CAE	3T3	Ecotropic	+++
CAE	3T3	Amphotropic	+
AE4	3T3	None	++++
AE4	3T3	Ecotropic	+++
AE4	3T3	Amphotropic	+
CAE	Mink	None	++++
CAE	Mink	Ecotropic	+++
CAE	Mink	Amphotropic	+
AE4	Mink	None	++++
AE4	Mink	Ecotropic	+++
AE4	Mink	Amphotropic	+

[a] For each individual envelope construct, the qualitative amount of blue-staining cells observed when no interfering virus was used was set to ++++. Relative to no interfering virus, +++ equals approximately 75% of the number of blue cells observed with no interfering virus, ++ equals approximately 50% of the number of blue cells observed with no interfering virus, + equals approximately 10% of the number of blue cells observed with no interfering virus, and − equals less than 1% of the number of blue cells observed with no interfering virus.

TABLE 5. Fluorescence flow cytometry analysis of chimeric envelope expression and function[a]

Envelope construct	Antigen on producer cells	Antigen on target cells	
		3T3	Mink
None	−	−	−
CAE	+	+	+
CEE	+	+	−
AE2	+	+	+
AE6	+	−	−
AE7	−	−	−
AE8	−	−	−
AE12	+	+	−

[a] GPL producer cells were transfected with the indicated constructs. Immunostaining of the producer or target cells was performed as described in Materials and Methods. The − indicates a less than 1% shift in fluorescence, while + indicates a greater than 10% shift in fluorescence.

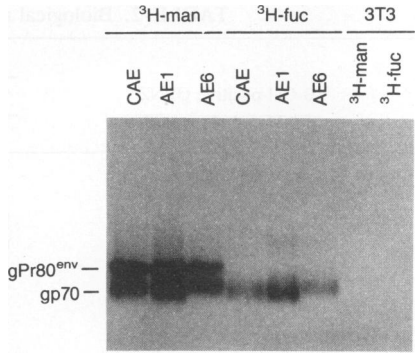

FIG. 5. Immunoprecipitation of [^{3}H]sugar-labeled GPL cells transfected with either CAE, AE1 or AE6. Cells were transfected and labeled with [^{3}H]mannose (^{3}H-man) or [^{3}H]fucose (^{3}H-fuc), and the proteins were immunoprecipitated as described in Materials and Methods. Untransduced 3T3 cells were used as a negative control. Immunoprecipitation of [^{3}H]sugar-labeled CAE (amphotropic envelope) and chimeric envelope constructs AE1 and AE6 was analyzed by SDS-PAGE with fluorography. Cells were transfected with 40 μg of plasmid DNA and labeled for 16 h beginning 30 h after transfection.

with the ecotropic virus had little effect, while preincubation with amphotropic virus greatly reduced the number of transduced cells. The opposite results were obtained with AE9 and AE12 (preincubation with ecotropic virus significantly reduced titer, while amphotropic virus had little effect).

Expression and activity of chimera envelope proteins: immunostaining and gel electrophoretic analysis. As seen in Table 2, seven of the chimeric constructs failed to produce biologically active retroviral particles. To investigate the lack of activity for three of these constructs, flow cytometry binding analysis was preformed on the transfected producer cells GPL (14). This technique is a rapid assay for testing whether the chimeric envelope proteins were being expressed on the cell surface membrane (1% wild-type binding activity can be visualized by this assay). Using essentially the same technique, transfected cell culture medium (containing presumptive retroviral vector particles) was collected and exposed to cells expressing the appropriate envelope receptor protein. A second fluorescence-labeled antienvelope antibody was then used to tag virus-bound cells. The results of this assay are shown in Table 5.

As expected, transfection of GPL cells with biologically active constructs CAE, CEE, AE2, and AE12 resulted in readily detectable binding of labeled antienvelope antibody to the cell surface. No antigen was detected on the surfaces of untransfected GPL, 3T3, or mink cells. When culture medium from the transfected GPL cells was exposed to target cells, virus binding was observed with all biologically active constructs. Testing of biologically inactive constructs AE7 and AE8 demonstrated no antigen was present on the GPL producer cell's surface (or the target cells). When the AE6 construct was tested, viral antigen was detected on the surface of the GPL producer cells, but no antigen was detected on the surface of the target cells following incubation with putative virus-containing cell culture medium.

The lack of the retroviral vector envelope-mediated binding to the target cells, even though it appears on the surface of the producer cells (as with AE6), could be associated with defective processing of the chimera's protein. ^{3}H-labeled sugars were again used to study the role of gPr80^{env} processing from the endoplasmic reticulum (where mannose is added) to the Golgi apparatus (the site of fucose addition) and to the outer cell membrane, where cleavage of Pr80 to SU and TM occurs. The results of this analysis are shown in Fig. 5. As expected, both the control construct CAE and the biologically active chimera AE1 gave rise to properly processed envelope proteins (overnight labeling with [^{3}H]mannose and [^{3}H]fucose yields radiolabeled and appropriately migrating SU). When AE6 was subjected to the identical analysis, it also processed to SU and incorporated [^{3}H]mannose and [^{3}H]fucose. The alteration in protein structure which render AE6 biologically inactive may thus be more subtle than observed with the insertion and deletion mutants (see discussion).

DISCUSSION

Of the five classes of murine retroviruses identified to date, four (amphotropic, polytropic, xenotropic, and 10A1) show a remarkable degree of conservation upon sequence alignment, and the name polytropic-related MuLV (PRM) family has been suggested for this group (29). The fifth class, the ecotropic MuLVs, display far less homology to the PRM family and contain an insertion of sequences not found in the PRMs. Previous reports from ours and other groups have shown that chimeras between ecotropic viruses or the similar PRM class of viruses can be assembled and are biologically active (2, 24, 27, 30). In this report, we sought to analyze the structural domains involved in host range determination by the ecotropic Mo-MuLV and the PRM family member, the 4070A amphotropic virus. This was accomplished by the construction of three classes of envelope mutations: insertions, deletions, and chimeric constructs.

Deletion mutants of the ecotropic MuLV envelope protein have been previously reported and shown to produce biologically inactive virus particles (12, 13). Some of these mutants are capable of binding the ecotropic receptor as judged by interference studies. We created a series of mutations using both insertion and deletion techniques. The deletion mutations D52 and D103 remove sequences from the putative host range-determining region of SU and were not expected to be biologically active, as was observed. Deletion mutant D354 is in the carboxyl-terminal one-third of the SU protein, and its biological properties were not immediately predictable. D354 is also biologically inactive.

Five insertion mutations were created by linker insertion: two (I52 and I85) in the putative host-range-determining

region, one (I255) near the start of the proline-rich region, and two (I319 and I427) in the conserved carboxyl-terminal one-third of SU. As with the deletion mutations, none of the insertions were biologically active. The negative results observed in XC syncytium assays suggest that either cell surface expression of the mutant envelopes did not occur in the XC cell line or it did occur but was nonetheless incapable of mediating cell fusion, perhaps because of rapid degradation or shedding from the cell surface. In pulse-labeling experiments, the $Pr80^{env}$ form of the I427 mutant consistently ran as a doublet in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis (Fig. 2). The amino acid sequence of I427 shown in Table 1 contains two charged residues (arginine) which are inserted near the center of a long hydrophobic stretch of residues at the carboxyl-terminal region of SU. Charged amino acids located in the hydrophobic domain may retard one of the initial steps in synthesis or glycosylation of $Pr80^{env}$, possibly causing a pause in translocation of the protein into the endoplasmic reticulum.

Because of the failure of any of the insertion and deletion mutants to produced biologically active virus, we analyzed the processing of the precursor $Pr80^{env}$ to the mature SU (gp70) protein (Fig. 3). The deficiency of 11 mutants to be processed suggests that the $Pr80^{env}$ protein contains domains spread throughout SU that are necessary for biological activity. The lack of significant fucose incorporation into the mutant proteins indicates a failure to efficiently transport to the Golgi apparatus. Based on these observations, none of the mutants appeared to be efficiently processed and transported through the appropriate cell organelles. An alternative explanation for the observed results is that processing or transport occurs, but the mutant protein is unstable and rapidly degraded and therefore not observed in large quantities. The *ts*3 Mo-MuLV mutant is defective for virion assembly at nonpermissive temperatures and expresses an unstable envelope precursor that does not generate detectable levels of SU (41). Other conditional Mo-MuLV envelope mutants have been described that are defective for processing and accumulate $Pr80^{env}$ intracellularly (44). For the spleen focus-forming virus, whose envelope transports inefficiently, envelope protein degradation occurs over a period of hours (11). Conversion of $Pr80^{env}$ to SU in 3T3 cells transfected with pCEE confirms previous observations suggesting that a cellular protease cleaves $Pr80^{env}$ (43). The sensitivity of $Pr80^{env}$ to structural perturbation is also consistent with a disruption in the process of oligomerization similar to that seen in Rous sarcoma virus which precedes transport from the endoplasmic reticulum (9).

The construction of 12 chimeric envelope constructs yielded 5 that were biologically active. While chimeras between different ecotropic viruses or members of the PRM family have been previously reported (2, 24, 27, 30), this is the first description of hybrids between these two most-divergent classes of viruses. In all constructs but AE1 and AE6, the switches are within the putative host-range-determining region. In AE1 and AE6, the switch included a part of TM, leaving the transmembrane domain of the ecotropic envelope protein (Fig. 4). Constructs AE1, AE2, and AE4 (switches that included the amphotropic host-range-determining region) pseudotyped retroviral vector particles capable of transducing both the murine NIH 3T3 and the nonmurine COS-1 and mink cell lines (Table 2). In addition, when interference studies were performed, construct AE4 behaved similar to the control amphotropic construct CAE (Table 4). From these data, we conclude that sequences conferring amphotropic host range are within the first 157 amino acids of the mature SU protein. This agrees with the finding of Battini et al. (2) that the first 120 amino acids are sufficient for receptor choice in the PRM family. Chimeric envelopes AE9 and AE12 are capable of pseudotyping retroviral vector particles that will only productively transduce NIH 3T3 cells or CHO cells containing the ecotropic receptor (Tables 2 and 3). In viral interference studies, both AE9 and AE12 were significantly blocked from transduction by preincubation with ecotropic virus but not amphotropic virus (Table 4). From this observation, we conclude that the minimal region necessary for ecotropic host range resides within the first 88 amino acids of the mature Mo-MuLV SU protein. The lack of full activity of the AE9 recombinant suggests that additional amino acids may be required for complete wild-type activity.

The chimeric envelopes which are biologically inactive may provide additional information into the functional domains of the SU protein. Examination of Table 2 and Fig. 4 shows that constructs containing *Apa*I[3]-*Apa*I* (AE3) and *Xho*I-*Bam*HI (AE5) junctions between ecotropic and amphotropic sequences do not generate infectious virus. For the AE3 and AE5 chimeras, the envelope has a deletion relative to the AE2 chimera, which is infectious. The junction in the AE2 construct (*Apa*I[4]-*Apa*I*) is located in a 12-amino-acid span that is identical in both ecotropic and amphotropic SU molecules. Highly diverse amino acid sequences flank the conserved region containing the AE2 (*Apa*I[4]-*Apa*I*) junction and their deletion in the AE3 and AE5 constructs may account for the lack of biological activity. The junction region in the infectious AE4 construct is in a region of limited amino acid homology, showing that SU can be modified in some regions without disrupting envelope function.

The lack of biological activity of the AE8, AE10, and AE11 constructs is not related to any gross deletion of sequence information and is more likely due to the juxtaposition of incompatible protein domains. In the AE6 construct, which is similar to the biologically active construct AE1, there is a short region of ecotropic sequences at the amino terminus, yet this construct did not generate detectable $G418^r$ titers or β-gal-staining cells on any cell type tested. Other than the change in amino acid sequences, there is no obvious change that may account for the lack of biological activity (the junction retains the N-linked glycosylation site in the Mo-MuLV SU at residue 12). Whatever the nature of the defect caused by the *Bst*EII-*Bst*EII* junction, it is likely a part of the reason why the AE7 construct is also inactive (both have the *Bst*EII-*Bst*EII* junction).

Data presented in Table 5 and Fig. 5 demonstrate that for the AE6 construct there is no overt defect in SU protein processing and that the protein is present at the cell surface. This result is in contrast to our previous findings with the insertion and deletion mutants (Fig. 3). Virions produced from cell lines expressing AE6 were unable to bind any cell type tested, suggesting that the junction alters the structure of SU so that the biological function (i.e., receptor binding) of the AE6 envelope is impaired. SU molecules with a *Bst*EII-*Bst*EII* junction (like AE6) may affect envelope function by subtle alterations of the protein structure. While it is possible that the AE6 SU protein is not incorporated into virions, we consider this unlikely on the basis of the observation that even SU proteins with gross structural changes are still found in virions (12).

The results obtained in this study localized the receptor-

binding region of two MuLV envelope proteins to short amino-terminal regions of SU. It is possible that additional small-scale chimeric constructs may further narrow down the most critical regions involved in host range determination. This may prove to be difficult given our finding that small-scale insertion mutants and minor changes at the junctions of some of the chimeric molecules render the SU protein biologically inactive. Taken together, our data and the observations of others may permit the elucidation of sufficient detail of the structure-to-function properties of the SU protein to be useful in the construction of novel SU proteins capable of targeting viruses to specific cell types.

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