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Characterization of Chimeras between the Ecotropic Moloney Murine Leukemia Virus and the Amphotropic 4070A Envelope Proteins

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A series of 22 chimeric envelope (*env*) genes were generated between the ecotropic Moloney murine leukemia virus and the amphotropic 4070A isolate. The chimeric envelopes were expressed within the complete, replication-competent provirus and tested for virus viability by transient expression assays. Eleven of the 22 viruses were viable. Five of these chimeric viruses showed an ecotropic host range, and six exhibited an amphotropic host range and viral interference. The host range determinants map to the first half of the surface (SU) protein. The N-terminal 72 amino acids of 4070A (42 of processed SU) are not required for amphotropic receptor usage. Ecotropic and amphotropic viruses differ in their ability to form large, multinucleated syncytia when cocultured with the rat XC cell line. Ecotropic murine leukemia virus forms large syncytia with XC cells, whereas no syncytia are reported for amphotropic virus. All chimeras which contained the N-terminal half of the ecotropic SU protein, encoding the receptor binding domain, formed the large multinucleated syncytia with XC cells.

Entry of a retrovirus involves the binding of the viral envelope (*env*) gene products with the host cell receptor followed by a fusion event between the viral and cell membranes. Murine leukemia viruses (MuLVs) have been classified by the competitive viral interference patterns defined by their receptor specificities (50, 52, 59). Viral envelope proteins can bind to their cognate host cell receptor and block superinfection of virus which utilizes the same receptor. The five groups defined are ecotropic, amphotropic, xenotropic, polytropic, and 10A1 virus. The unique receptors recognized by the ecotropic, amphotropic, and 10A1 retroviruses have been molecularly identified. The ecotropic receptor encodes the cationic amino acid transporter (1, 24, 66). The amphotropic and 10A1 receptors are members of the sodium-dependent phosphate symporters (21, 35, 36, 65).

The viral *env* gene encodes two proteins, the surface (SU) and transmembrane (TM) proteins, which are initially expressed as a precursor protein. Processing of the precursor protein is extensive during transport to the cell surface. This includes cleavage of the signal peptide, the addition of seven N-linked glycans (12, 22) as well as O-linked glycans (47), and proteolytic cleavage of the SU and TM proteins by a cellular protease (25). Proteolytic processing of the SU/TM precursor releases the hydrophobic membrane fusion domain located at the N terminus of the TM protein (13, 19). The C-terminal 16 amino acids (R peptide) of the TM protein is proteolytically cleaved during or after budding by the viral protease (62, 64). The virus-associated SU/TM complex is a multimer, consisting of between four to six SU/TM subunits by cross-linking studies (46).

A considerable effort has been made to define the functional domains of the SU/TM proteins (5, 6, 15, 16, 37, 42) and to alter the host range of the virus (9, 20, 38, 55, 63). The ability to change the repertoire of cells which can be infected by retroviral vectors has direct applications for gene therapy. Sequence alignments of MuLV have indicated that the N-terminal two-thirds of the SU protein shows the greatest diversity (6, 32, 40, 41, 61). Two hypervariable regions have been noted within the N-terminal half of the SU protein, termed VRA and VRB (variable regions A and B, respectively) (6). These are followed by a proline-rich region of approximately 30 to 50 amino acids. The N terminus of the proline-rich region is punctuated by an ordered proline or hinge sequence conserved between ecotropic, amphotropic, xenotropic, and polytropic MuLVs with the consensus sequence GPRI/VPIGPNP (15). The carboxy terminus of SU protein is highly conserved. The association with the amino-terminal extracellular domain of TM protein has been mapped to this region (15, 45, 48). The cystine maps of isolated Friend MuLV and mink cell focus-forming virus SU proteins indicate general conservation of the disulfide bonds (28, 29). Three distinct cysteine-looped structures are predicted within the N terminus of SU. The first two cysteine loops comprise the VRA region, and the third loop encodes VRB. Among different host range isolates, variation is seen in the size and composition of the amino acids within the cysteine loops.

Analyses of receptor binding domains have indicated various roles of the VRA, VRB, and proline region, depending on the viral class (5, 6, 16, 37, 42). The VRA region is a key determinant in host range (6, 37). Additional sequences, however, are required for efficient binding and entry (5, 6, 37, 42). Analyses of amphotropic, polytropic, and xenotropic chimeras have indicated that the VRB is important for stabilizing the receptor-specific structure (6). For polytropic, xenotropic, and 10A1 virus, receptor choice is influenced by the proline region, either through direct binding or through the induction of an appropriate allosteric shape (5, 42).

In addition to receptor recognition, each class of MuLVs maintains unique characteristics for viral entry. The ampho-

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tropic and ecotropic MuLVs differ from each other at several postbinding steps in entry. Entry of amphotropic virus is pH independent (33, 39), indicative of a direct fusion of the viral and cell membranes. In contrast, the mechanism of entry of ecotropic MuLV is dependent on the host cell line. In most cells, including NIH 3T3 cells, entry is pH dependent (33), utilizing a receptor-mediated endocytosis pathway. Entry of ecotropic MuLV into rat XC cells is pH independent. Although the pH requirements for entry into XC cells are similar for ecotropic and amphotropic MuLVs, they differ in their ability to form syncytia. In the presence of ecotropic virus, XC cells fuse and form large multinucleated syncytia. These syncytia are not detected with amphotropic virus and XC cells.

In this study, a series of 22 chimeric ecotropic and amphotropic envelope proteins were constructed to dissect the functional domains of the *env* gene product. Eight positions of crossover between the two genes were generated within regions of general sequence conservation between the two proteins. Viruses bearing the chimeric envelopes were examined for viral spread, host range, viral interference, and syncytium formation. The results support the conclusion that the sequence that determines receptor choice resides at the amino terminus of the SU moiety of the envelope protein (5, 6, 16, 37, 42). The ability to form large multinucleated syncytia with XC cells segregated with the receptor binding domain of the ecotropic virus.

MATERIALS AND METHODS

Cell lines. NIH 3T3, D17, and XC cells were grown in Dulbecco's modified Eagle's medium (GIBCO-BRL) supplemented with 10% newborn calf serum (HyClone). All cell lines were kept at 37°C in a 5% CO_2 atmosphere.

Plasmids. Plasmid pNCA-C expresses the Moloney MuLV (M-MuLV) provirus and has been previously described (10, 12). The 4070A amphotropic envelope was expressed in a M-MuLV provirus by replacement of the *Sfi*I-*Cla*I fragment from pEnvAm (30) into pNCA-C, generating pNCA-Am. In all experiments labeled 4070A, pNCA-Am was used.

Mutagenesis oligonucleotides. For the mutagenesis oligonucleotides listed below, numbers in parentheses correspond to the 4070A envelope sequence (accession number M33469 [41]) or to the RNA sequence of M-MuLV (57). The restriction sites generated are underlined. The restriction enzyme is indicated in parentheses. Mutations are indicated by lowercase letters. The following oligonucleotides were used: (i) amphotropic 4070A, position 1 (268 to 247), 5′ CCA GATCACATAGATCtAAATA 3′ (*Bgl*II); position 2 (462 to 447), 5′ CCAG TAAGCtTGTCCG 3′ (*Hin*dIII); position 4 (762 to 745), 5′ GGGGACgCGtG GTCCCAC 3′ (*Mlu*I); position 5 (966 to 949), 5′GGCTCCgTcGACTAGAGC 3′ (*Sal*I/*Acc*I); (ii) ecotropic M-MuLV, position 1 (6016 to 6001), 5′ CATACATA gATCTGGG 3′ (*Bgl*II); position 2 (6331 to 6315), 5′ CCAGTAAGCTtTAC CGG 3′ (*Hin*dIII); position 3 (6454 to 6424), 5′ CCCGGCGTCTGTgAAttc AATAACTAAGGGG 3′ (*Eco*RI); position 4 (6576 to 6560), 5′ GGGACGCGt GGTCCTAG 3′ (*Mlu*I); position 5 (6739 to 6724), 5′ GGCTCCGTCgACTAAG 3′ (*Sal*I/*Acc*I); position 6 (6886 to 6907), 5′ CCAACACAAGcTtACCCTGTCC 3′ (*Hin*dIII); and position 7 (7004 to 6954), 5′ GATAATAGGAtCCTCGAC 3′ (*Bam*HI).

Mutagenesis. Mutagenesis of ecotropic M-MuLV positions 1 to 5 and 7 was performed on plasmid p150-18 (53) by the gapped heteroduplex method (18). The gap was introduced between the unique *Sph*I and *Cla*I sites; the DNA was linearized with *Pvu*I. The ecotropic position 6 mutation was generated by the method of Kunkel et al. (26, 27) with plasmid pHS (12), generating p2074.

For mutagenesis of positions 1, 2, 4, and 5 of the 4070A amphotropic MuLV, the 2.5-kb *Sph*I-*Cla*I fragment of 4070A was substituted for the *Sph*I-*Cla*I fragment of pNCA-C. The fragment from *Sph*I to *Sac*I was then subcloned into plasmid pTZ19U, creating pTZ19/Am. Mutagenesis was performed with pTZ19/Am gapped heteroduplexes (18). Briefly, a gap was generated in a circular species by hybridization of two overlapping DNAs of unequal lengths. This gap was generated between *Sph*I and *Bam*HI sites within the *pol* and *env* genes. The full-length linear plasmid was generated by digestion of pTZ19/Am at the *Xmn*I site within the *amp* gene. Sequence analysis of position 4 indicated that the region downstream of the oligonucleotide had additional mutations. This fragment was regenerated by PCR with VENT DNA polymerase (New England Biolabs) and primer 4075 (position 4 [PCR] [747 to 766] 5′ GGGACC aCGCgTCCCCATAG 3′) and primer 2848 (5′ GTAGAATTCTCCTAGTAG AAGGGCCAG 3′) containing a seven-nucleotide tail encoding an *Eco*RI restriction site and nucleotides 1429 to 1447 of 4070A MuLV *env*. The PCR product was digested with *Mlu*I and *Nsi*I, and the approximately 700-bp fragment was substituted for the amphotropic sequences, which were changed during oligonucleotide-directed mutagenesis.

Construction of chimeric ecotropic/amphotropic *env* genes. Constructs were named according to the host range from the N terminus to the C terminus of the protein and the position of the junctions. For example, construct AE1 contains amphotropic sequence (A) through position 1 followed by ecotropic sequence (E) through the *Cla*I site. The numbering of the amino acids is based on that of the precursor envelope protein; the initiator methionine is position 1.

The AE series was initially constructed in pEnvAm (30). The mutagenesis scheme created a restriction enzyme site which was used to generate the chimeras. Wild-type 4070A amphotropic virus encoded the restriction sites at positions 3, 6, 7, and 8 and did not require mutagenesis. Restriction digestion of pEnvAm indicated a unique *Bgl*II site within the *pol* gene. In general, constructs were generated with a three-fragment ligation consisting of the 6.6-kb fragment from pEnvAm digested with *Bgl*II-*Cla*I, amphotropic virus *env* genes from *Bgl*II to the junction restriction site, and ecotropic virus from the introduced restriction site to *Cla*I. For position 6, pEnvAm was partially digested with *Hin*dIII and then completely digested with *Cla*I, and the *Hin*dIII-*Cla*I fragment of ecotropic *env* was inserted. For position 7, pEnvAm was directly digested with *Bam*HI and *Cla*I and the *Bam*HI-*Cla*I fragment of ecotropic M-MuLV was inserted. For position 8, a four-fragment ligation consisting of a pEnvAm 1.9-kb *Hin*dIII-*Bgl*II fragment, a pEnvAm 1.1-kb *Bgl*II-*Eco*RI fragment, a pEnvAm 0.84-kb *Eco*RI-*Nco*I fragment, and a pEnv (31) 5.0-kb *Nco*I-*Hin*dIII fragment was used. The chimeric AE *env* genes were later moved from pEnvAm into the full-length MuLV provirus through the exchange of the 2.3-kb *Sfi*I-*Cla*I fragment into pNCA-C.

The chimeric ecotropic/amphotropic *env* genes (EA series) were directly constructed into the full-length MuLV provirus. For EA1-5, the 2.5-kb *Sph*I-*Cla*I fragment of pNCA-C was replaced by two fragments: the *Sph*I-junction restriction site from the ecotropic mutagenesis and the junction restriction site-*Cla*I fragment from the amphotropic mutagenesis. For EA1, partial *Bgl*II digestion was required in order to isolate the *Sph*I-*Bgl*II fragment. pEA6 was generated by introduction of the 2,000-bp *Hin*dIII-*Hin*dIII fragment (from nucleotide positions 4891 to 6892) from p2074 (described above) into pNCEA5, which was digested with *Hin*dIII. For EA8, pNCA-C was digested with *Sfi*I and *Cla*I. The ecotropic 1.85-kb *Sfi*I-*Nco*I fragment was isolated after partial digestion with *Nco*I and complete digestion with *Sfi*I. The amphotropic 450-bp *Nco*I-*Cla*I fragment was isolated and used in the ligation. The 8,307-bp *Hin*dIII-*Cla*I fragment of pEA8 was used as the vector for construction of pEA7. The inserts consisted of a 2-kb *Hin*dIII-*Bam*HI fragment generated by partial *Bam*HI digestion of p150-18 containing the novel *Bam*HI site and complete digestion with *Hin*dIII and the 682-bp *Bam*HI-*Cla*I fragment of 4070A.

The N-terminal amphotropic *env* fragment through position 1 was moved into pEA4, pEA5, and pEA6, generating pAEA1/4, pAEA1/5, and pAEA1/6, respectively. pAE1 was digested with *Hin*dIII and *Bam*HI, generating an approximately 1.6-kb fragment, and with *Hin*dIII-*Cla*I (8,307-bp fragment). pEA4, pEA5, and pEA6 were digested to completion with *Cla*I and partially digested with *Bam*HI. Fragments in which the *Bam*HI cleavage occurred within the ecotropic *env* sequence (at 6534) were isolated and ligated with the two pAE1 fragments described above.

Similarly, the N-terminal ecotropic *env* fragment was moved into pAE4, pAE5, and pAE6, generating pEAE1/4, pEAE1/5, and pEAE1/6, respectively. The 978-bp *Sfi*I-*Eco*RI fragment was isolated from pEA1 and inserted into pAE4, -5, and -6 digested with *Sfi*I and *Eco*RI.

The sequence at the junction of each of the constructs was confirmed by dideoxynucleotide DNA sequencing (56). Sequencing of the AE3 construct was performed with primers spanning the entire *env* gene and the AmpliCycle sequencing kit (Perkin-Elmer) according to the manufacturer's instructions.

Transfections. Plasmid DNA was prepared by CsCl gradients. For each provirus, 2.5 μg of DNA was transfected into 10^5 or 10^6 NIH 3T3 cells, as indicated. A total of 2.5 μg of DNA was transfected into 10^5 D17 cells per 60-mm-diameter dish. The DEAE-dextran method was used to transfect the DNA (34).

Reverse transcriptase assay. Supernatants (10 μl) were collected periodically after transfections and/or infections, starting from day 1 for a period up to 40 days. Virus spread was tested by the release of reverse transcriptase activity into the media (14).

Interference assays. Cells were split to 4.0×10^5 cells per 60-mm plate. After 24 h, the cells were challenged with ecotropic or amphotropic virus. Virus used for the challenge was isolated from the helper cells ΨCRE (ecotropic) or ΨCRIP (amphotropic) containing the MFG-NB vector. This vector expresses a modified form of the *Escherichia coli lacZ* gene containing a nuclear localization sequence (gift of J. M. Heard) (16). The titer of the amphotropic virus varied between 2.7×10^5 and 9.9×10^5 CFU/ml; the titer of the ecotropic MuLV varied between 2.0×10^4 and 4.7×10^6 CFU/ml, as indicated. Viral infection was performed in the presence of 8 μg of Polybrene per ml. Forty-eight hours after infection, the cells were fixed in phosphate-buffered saline containing 2% paraformaldehyde and stained with 1 mg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) per ml in 20 mM $K_3Fe(CN)_6$–20 mM $K_4Fe(CN)_6$–2 mM $MgCl_2$. Cells were stained for a period of 12 to 24 h, and the positive-staining cells were counted.

Hirt DNA isolation. NIH 3T3 cells (50% confluence in a 100-mm-diameter dish) were infected with 3 ml of filtered (0.45-μm-pore size; Acrodisc) medium from viral producer cells in the presence of 8.0 μg of Polybrene per ml at 37°C for 2 h. Cells were harvested 48 h later, and the extrachromosomal DNA was

isolated as described by Hirt (17). DNA samples were treated with DEPC (pyrocarbonic acid diethyl ester) and were further purified by phenol-chloroform extraction followed by ethanol precipitation.

PCR restriction analysis. DNA from a Hirt extract from one 100-mm-diameter dish (total volume, 200 μl) was isolated and titrated for optimal PCR amplification. For EA4-7, the HIRT DNA was amplified, the product was isolated, and the DNA was reamplified with the same primers. The oligonucleotide primers for PCR analysis of Hirt DNA include the following: 1086, 5′ CAGTA AACAATATTCTCACCTCTG (6360 to 6383; M-MuLV [57]); 2213, 5′ CCGC ATAGAAGCTACATTC (complementary to 7457 to 7475 of M-MuLV [57]); 2846, 5′ CCAAGGGGCTACTCGAGGGGG (582 to 603, 4070A [41]); 2848, 5′ GTGAATTCTCCTAGTAGAAGGGCCAG (complementary to 1429 to 1447 of 4070A *env* [41]); 2922, 5′ CCCCTACAAGTCCAAG (4070A *env* 893 to 908 [41]); 3270, 5′ GGACCACTGATATCCTGT (complementary to 7698 to 7715 of M-MuLV [57]); 3807, 5′ GATATACATATGCCGTTAACAGGGA (complementary to 4926 to 4940 of M-MuLV [57]); 4256, 5′ CCAGTACTGCAAGC CCACAT (complementary to 7025 to 7044 of M-MuLV [57] and 1246 to 1266 of 4070A [41]); and 6320, 5′ CCTTAAGGCCCCCCTTTTTCTGGAGACTAA ATA, (complementary to 7815 to 7791 of M-MuLV [57]).

Pairs of primers which hybridize with both ecotropic and amphotropic sequences were used for the amplification of the Hirt DNAs. For AE6, -7, and -8 and 4070A, a region with a size of about 800 bp was amplified. For AE4, the approximately 2,900-bp product was produced with a mixture of *Taq* and *Pfu* (Stratagene) polymerase at a ratio of 20:1 (U/U) (4). All other PCR mixtures utilized *Taq* polymerase. In constructs AE5, EAE1/6, and wild-type M-MuLV, a region with a size of about 1,115 bp was amplified. For the EA series, ecotropic and amphotropic primers, spanning a region with an average size of 880 bp, were used for constructs EA4, -5, -6, and -7; a region with a size of about 1,355 bp was used for the wild-type M-MuLV. The amplified DNAs were separated by gel electrophoresis, and products with the correct size were isolated by glass powder isolation. The DNAs were digested with a restriction enzyme to check for the presence of the mutated site.

XC syncytium formation. XC syncytium assays in which the producer cells were UV irradiated, overlayed with rat XC cells for 48 h, fixed with 37% formaldehyde, and stained with hematoxylin (Sigma) were performed essentially as described previously (15). The number of syncytia and the number of nuclei per syncytium were counted by visual inspection under a microscope.

Cell labeling and immunoprecipitation. Viral proteins were labeled with Trans-label ([^{35}S]Met and [^{35}S]Cys [ICN]) as previously described (15). Intracellular proteins were metabolically labeled for 40 min as previously described (12). Immunoprecipitation of viral and intracellular proteins was performed as previously described (12). Goat anti-MuLV Env (792-842) and goat anti-MuLV CA (75S-287) were from the NCI-BCB Repository (distributed by Microbiological Associates, Inc). Immunoprecipitation of TM protein was performed with monoclonal antibody 42-114 (48) and required the use of secondary goat anti-rat immunoglobulin G antibody for efficient binding to Pansorbin (Calbiochem).

RESULTS

Generation of chimeric ecotropic and amphotropic envelope proteins. A series of 22 reciprocal chimeric envelope genes were generated between M-MuLV and the amphotropic 4070A isolate, enabling the definition of functional domains of the SU/TM proteins. The chimeras were constructed within a full-length M-MuLV provirus, providing a uniform background for the long terminal repeats, the *gag* genes, and the *pol* genes.

Figure 1 summarizes the chimeric constructs and their general structural features (6). The positions of crossover were created by generation of common restriction sites within relatively conserved amino acid sequences. Junction 1 is prior to the first cysteine loop of the VRA region. Junction 2 spans the first hypervariable region, VRA. Position 3 spans VRA plus the second hypervariable region, VRB. The junction of position 4 is within the ordered proline or hinge region (H) (15). Position 5 is 3′ to the proline-rich region (PPP). The junctions 6 and 7 are within the carboxyl terminus of SU, which is a highly conserved region between the ecotropic and amphotropic proteins. Position 8 is within the fusion peptide at the N terminus of TM protein. Early retroviral vector studies had substituted ecotropic and amphotropic *env* genes through the *Cla*I site within the TM protein (position 9) and demonstrated the production of viable virus with the corresponding change in the host range (11). This was the outer boundary of the fragment exchanges.

Constructs were also generated in which two crossover events occurred. In these, approximately the first 72 amino acids up to the junction of position 1 (corresponding to the first 42 amino acids of the processed M-MuLV SU protein) were moved into the corresponding chimeras at positions 4, 5, and 6 (EAE1/4, EAE1/5, EAE1/6, AEA1/4, AEA1/5, and AEA1/6).

Viral spreading of the EA and AE chimeric series. DNAs introduced into cells by DEAE-dextran are transiently expressed. Constructs which express viable virus are able to spread throughout a culture plate and are assayed by the release of reverse transcriptase in the medium supernatants (14). The chimeric clones were transfected into both NIH 3T3 and D17 cells. Cells of the mouse fibroblast line NIH 3T3 express both the amphotropic and ecotropic receptors. D17 is a dog cell line that expresses the amphotropic receptor but is not infected by ecotropic virus. After the transfection, cells were maintained for a period of at least 40 days.

Figure 2 shows the results of the transient expression experiments with the EA series with NIH 3T3 cells. Lanes 13 and 14 show the mock transfection (no DNA). The time course of the wild-type M-MuLV (lanes 12 and 16) and virus expressing the amphotropic 4070A *env* gene (lane 11) indicates that the reverse transcriptase within the virus is detected by day 6. EA1 (lane 1), EA2 (lane 2), and EA3 (lane 3) showed no viral spread. EA4 was the first construct in the series which was viable (lane 4) and spread with a time course similar to that of the wild-type virus. It is composed of the first 231 amino acids of the processed ecotropic M-MuLV *env* gene (through P-264). Additional viable chimeric viruses included constructs EA5 (lane 5), EA6 (lane 6), EA7 (lane 7), and EA8 (lane 15). Reverse transcriptase activity was readily detectable between 8 and 13 days for EA5, EA6, and EA7. EA8's spread was identical to that of wild-type ecotropic virus, appearing positive at day 4 (shown from an independent transfection). EA6 was always the slowest virus in this assay, reproducibly delayed 7 to 9 days compared with the wild type. Exchange of the N terminus of 4070A through position 1 into EA4, EA5, and EA6, yielding AEA1/4, AEA1/5, and AEA1/6, respectively, inactivated the proteins (compare lanes 4 and 8, 5 and 9, and 6 and 10).

Transfection of the EA series into D17 cells resulted in viability only for the control MuLV expressing the 4070A amphotropic *env* gene (junction 9) (data not shown). This indicates that the ecotropic host range determinants are within the N-terminal half of the SU proteins.

Figure 3 shows the results of a transient expression of the AE series in NIH 3T3 (Fig. 3A) and D17 (Fig. 3B) cells. In NIH 3T3 cells, AE1, AE2, and AE3 were found to be nonviable (Fig. 3A, lanes 1, 2, and 3, respectively). The first construct along the sequence showing viability is AE4 (lane 4), and it consists of the first 209 amino acids of the (processed) amphotropic 4070A *env* sequence (through P-239). All constructs with junctions 3′ to this position were viable. These include AE5, AE6, AE7, and AE8 (Fig. 3A, lanes 5, 6, 7, and 8, respectively). The effect of substitution of the N-terminal domain was examined. Although the parental AE4 and AE5 were viable (Fig. 3A, lanes 4 and 5, respectively), the mixed constructs EAE1/4 and EAE1/5 failed to spread. Quite interestingly, construct EAE1/6 (Fig. 3A, lane 11) was found to be viable. This construct encodes the amphotropic 4070A *env* sequence from amino acids D-73 through K-362. All of the positive constructs showed stable reverse transcriptase activity at day 4 in the NIH 3T3 cell line.

The AE series was also assayed for viral spread on the D17 cell line (Fig. 3B). D17 cells provided more variable results than NIH 3T3 cells. The constructs which were viable included

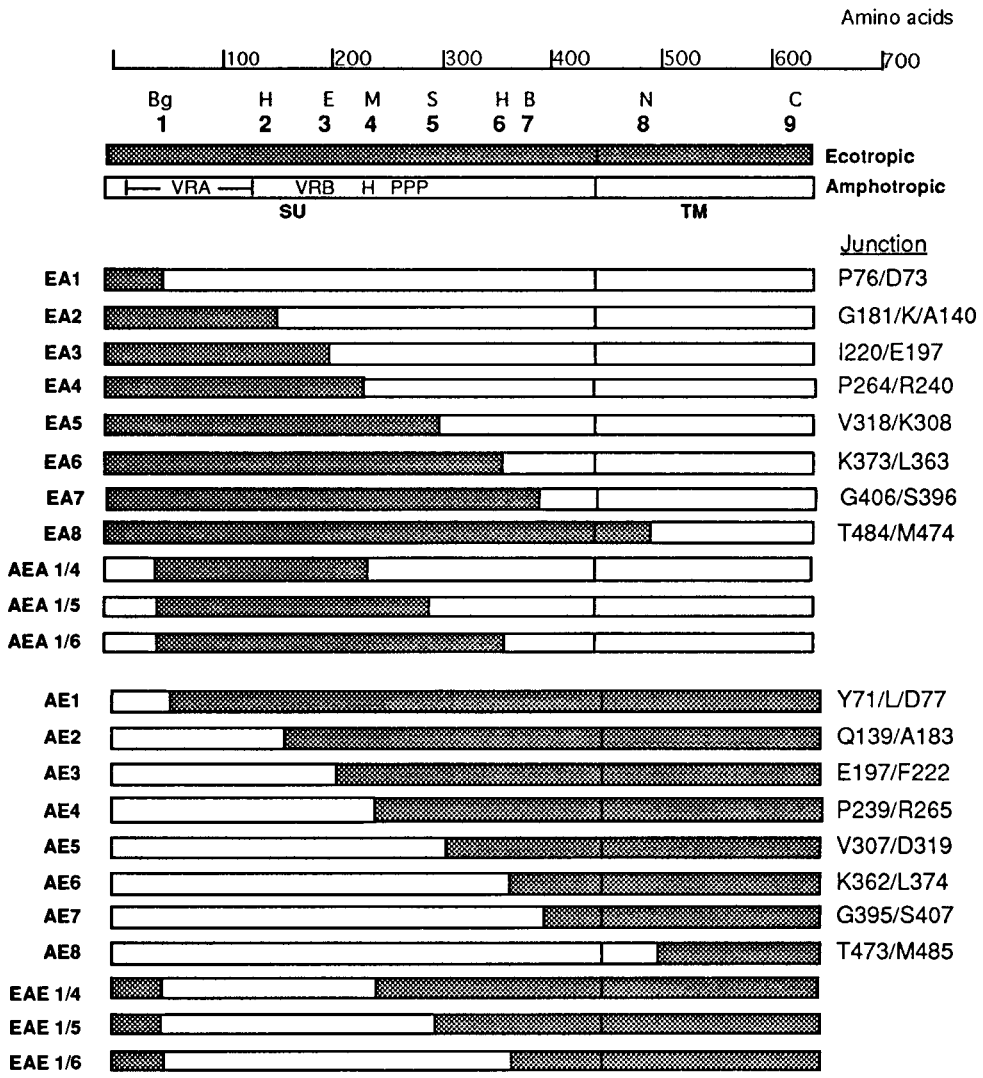

FIG. 1. Generation of chimeric ecotropic and amphotropic envelope proteins. Twenty-two chimeras of the ecotropic M-MuLV (shaded) and amphotropic 4070A (open) envelope proteins were generated. The constructs are named by the order of the envelope genes from the 5′ to the 3′ terminus. Positions of crossover were created by generating common restriction sites within relatively conserved amino acid sequences. The junctions are numbered 1 to 9, and the relative positions are shown above the ecotropic box. Restriction sites at each junction are indicated above the junction number. Bg, *Bgl*II; H, *Hin*dIII; E, *Eco*RI; M, *Mlu*I; A, *Acc*I; B, *Bam*HI; N, *Nco*I; C, *Cla*I. General features of the SU protein (6) are indicated in the amphotropic SU protein: VRA, VRB, the hinge (H), and polyproline (PPP). The amino acid positions of the corresponding envelope proteins at the junction are shown to the right of each construct. Double-crossover junctions would be identical to the individual junctions and are not indicated.

AE4, AE6, AE7, and AE8 (Fig. 3B, lanes 1, 3, 4, and 5, respectively). The exchange of the N terminus through position 1 had the same effect on the spreading in this cell line that it did on the NIH 3T3 cell line; EAE1/6 (lane 6) was found to be viable, whereas EAE1/4 and EAE1/5 were not (data not shown). Constructs AE6, AE7, and EAE1/6, although viable, did spread more slowly than the wild-type ecotropic, amphotropic, AE4, and AE8 envelopes, in which reverse transcriptase activity was detected after 5 days. Reverse transcriptase activity for AE6 (lane 3), AE7 (lane 4), and EAE1/6 (lane 6) was detected between 14 and 19 days posttransfection. AE1, AE2, and AE3 were tested five times in transient transfection assays and were consistently found to be nonviable (data not shown).

The results of the transient expression experiments with the AE series indicated that the AE5 chimera was capable of spreading in NIH 3T3 cells but not in D17 cells. The inability of AE5 to spread in transient transfection assays in D17 cells was checked six times with DNA concentrations ranging between 2.5 and 5.0 μg. In order to determine the cause of this difference, viral stocks isolated from chronically infected NIH 3T3 cells were used to infect D17 cells. The AE5 chimeric virus was shown to be infectious on D17 cells. After the initial burst of reverse transcriptase activity immediately after infection, AE5 spread with slower kinetics of infection than that of the wild-type 4070A-MuLV construct. Reverse transcriptase activity lagged approximately 3 to 5 days behind that of the amphotropic virus and the AE4 chimera (data not shown). The ability to infect the D17 cells could only be detected in the presence of a high titer of infecting virus. The AE5 construct was therefore hindered in its ability to enter the canine cells.

The host range of MuLV maps to the amino terminus of *env*. The receptor utilized for entry of the chimeric envelopes was identified by viral interference assays. Env proteins expressed in the infected cell associate with the cellular receptor for the

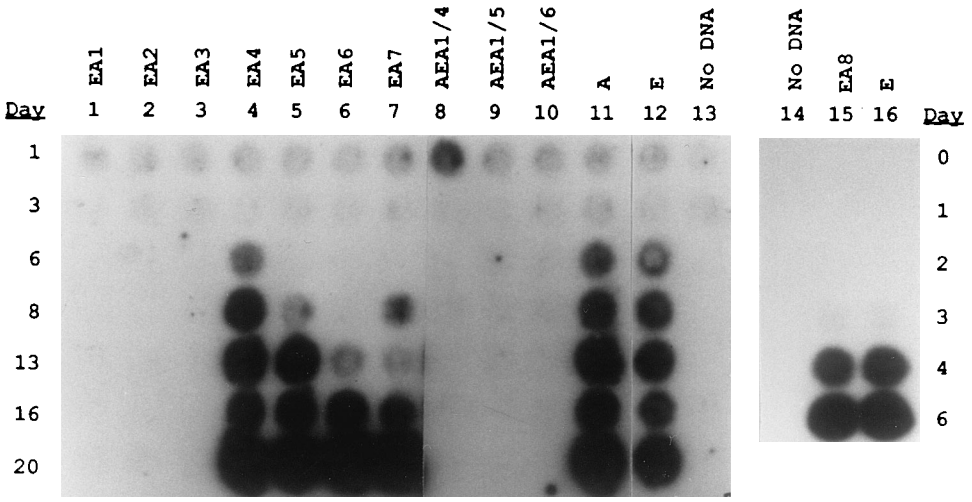

FIG. 2. Time course of infection of chimeric EA *env* series in NIH 3T3 cells. Plasmid DNAs expressing the individual chimeric EA envelope proteins within the MuLV provirus were introduced into 10^6 NIH 3T3 cells per 100-mm-diameter plate by the DEAE-dextran method, allowing transient expression of the virus. Supernatant medium was assayed for the presence of the virally encoded reverse transcriptase as described in Materials and Methods. The number of days after introduction of the DNA is indicated to the side. The various constructs are indicated at the top of each lane. A, amphotropic 4070A; E, ecotropic M-MuLV. Lanes 14 to 16 show the results from an independent transfection.

virus and competitively block receptor sites, rendering the cell immune to superinfection by another virus bearing a similar envelope (60).

NIH 3T3 virus producer cells expressing the viable AE and EA series were challenged with ecotropic and amphotropic viruses. The challenge viruses were isolated from helper cells ΨCRE and ΨCRIP containing the MFG-NB vector, expressing a nucleus localized *E. coli lacZ* gene. Forty-eight hours after the challenge, the cells were stained with X-Gal and the number of foci with blue-stained nuclei was determined. Chimeric envelope proteins which successfully block superinfection produce no stained cells. The results of these experiments are summarized in Table 1.

The receptor binding domains, as defined by viral interference, were located at the amino terminus of the SU protein. The AE series contained increasing N-terminal extensions of amphotropic 4070A. None of the viable AE series, which contained the N terminus of 4070A SU protein, could be infected by the amphotropic *lacZ* pseudotype virus but were infected by the ecotropic *lacZ* pseudotype virus. Conversely, the NIH 3T3 cells expressing the EA series were not infected by the ecotropic *lacZ* pseudotype and were infected by the amphotropic virus packaging the *lacZ* gene. EAE1/6, which contained the ecotropic sequence through position 1, followed by the amphotropic VRA, VRB, and polyproline region, interfered with the amphotropic virus. This indicates that the first 42 amino acids of the processed amphotropic SU protein (through F-72) is not a determinant for host receptor usage on NIH 3T3 cells.

The chimeric envelope proteins are stably expressed. In order to verify the production and size of the chimeric envelope proteins, producer cells were metabolically labeled and the proteins were subjected to immunoprecipitation. Analysis of intracellular proteins with anti-SU antibodies (Fig. 4) allowed for the identification of the envelope precursor SU/TM polyprotein (Pr85). Full-length precursor products with a size of approximately 95 kDa were detected for AE5, AE6, AE7, AE8, and EAE1/6 (Fig. 4A, lanes 2 to 6, respectively). Based on the primary sequence, the AE4 protein is predicted to be approximately 1.3 kDa smaller than the other chimeras in this

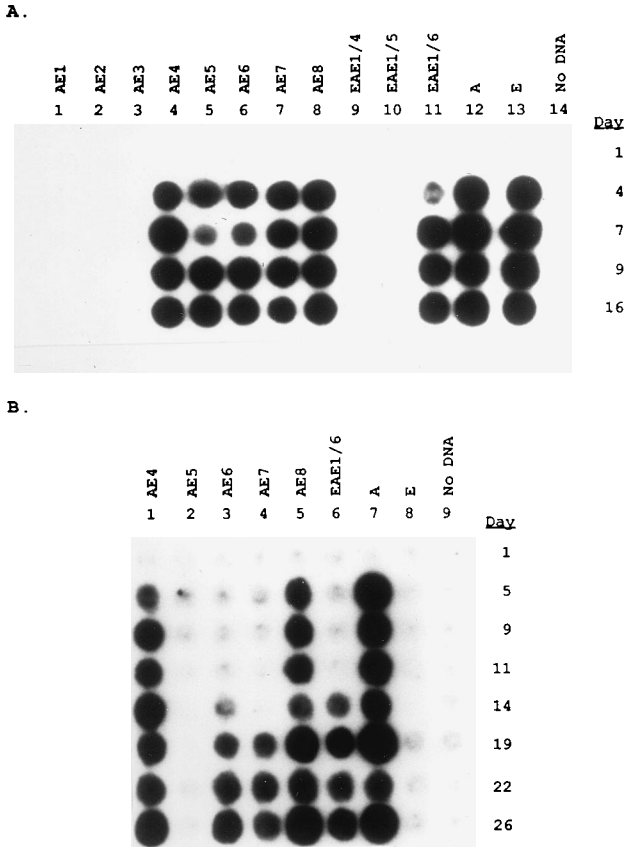

FIG. 3. Time course of infection of chimeric AE *env* series. (A) NIH 3T3 cells. (B) D17 cells. Aliquots of medium after transient introduction of proviral DNA were assayed for reverse transcriptase activity as described in Materials and Methods. A total of 2.5 μg of DNA was introduced into 10^5 cells per 60-mm-diameter plate. Samples are indicated at the top of each lane. A, amphotropic 4070A; E, ecotropic M-MuLV. The number of days postintroduction of the DNA is indicated to the right.

TABLE 1. Summary of characteristics of viruses bearing chimeric ecotropic and amphotropic envelope proteins

Virus	Viral spread[a]		Interference[b]		XC syncytium formation[c]
	NIH 3T3	D17	Ecotropic	Amphotropic	
EA1	−	−	ND[d]	ND	ND
EA2	−	−	ND	ND	ND
EA3	−	−	ND	ND	ND
EA4	+	−	+	−	+
EA5	+	−	+	−	+
EA6	+	−	+	−	+
EA7	+	−	+	−	+
EA8	+	−	ND	ND	+
AEA1/4	−	−	ND	ND	ND
AEA1/5	−	−	ND	ND	ND
AEA1/6	−	−	ND	ND	ND
AE1	−	−	ND	ND	ND
AE2	−	−	ND	ND	ND
AE3	−	−	ND	ND	ND
AE4	+	+	−	+	−
AE5	+	−[e]	−	+	−
AE6	+	+	−	+	−
AE7	+	+	−	+	−
AE8	+	+	−	+	−
EAE1/4	−	−	ND	ND	ND
EAE1/5	−	−	ND	ND	ND
EAE1/6	+	+	−	+	−

[a] Reverse transcriptase activity released (+) or not released (−) into media.
[b] Superinfection interference of pseudotype virus bearing *lacZ*. +, challenge infection blocked, with no X-Gal-positive cells. For the AE series, the titer of the challenge amphotropic virus produced from ΨCRIP was 9.9×10^5. For the EA series, the titer of the challenge ecotropic virus produced from ΨCRE was 2×10^4. −, challenge infection proceeds, with X-Gal-positive cells present. For the AE series, the titer of the challenge ecotropic virus produced from ΨCRE was 4.7×10^6. The AE series resulted in 3.3×10^6 to 4.3×10^6 X-Gal-positive-staining foci when challenged with ecotropic virus. For the EA series, the titer of the challenge amphotropic virus produced from ΨCRIP was 3.3×10^5. All of the EA series produced between 3.1×10^5 and 3.6×10^5 X-Gal-positive staining foci when challenged with amphotropic virus.
[c] Syncytia formed with XC cells. Producer cells (NIH 3T3) were UV irradiated and overlaid with 10^5 XC cells. +, syncytia detected. The number of syncytia for any of the EA series and wild-type M-MuLV was between 3×10^5 and 9×10^5, averaging 12 to 32 nuclei per syncytium.
[d] ND, not determined.
[e] AE5 virus isolated from NIH 3T3 cells could infect D17 cells.

series; it contains the smaller VRA of amphotropic virus and the smaller proline region of the ecotropic M-MuLV. Experimentally, the migration of AE4 (Fig. 4A, lane 1) showed it to be approximately 5 to 6 kDa smaller than AE5 (Fig. 4A, lane 2). The full-length products of EA5, EA6, and EA7 migrated as 89-kDa proteins (Fig. 4B, lanes 2 to 4, respectively). In a similar fashion, the EA4 protein is predicted to be 1.3 kDa larger than the other chimeras in the EA series and to consist of the larger VRA of the M-MuLV ecotropic virus and the larger proline region of the amphotropic virus. Experimentally, the migration of EA4 (Fig. 4B, lane 1) showed it to be 4 to 5 kDa larger than EA5. The higher molecular masses of AE5-8 versus AE4 and of EA4 versus EA5-8 parallel the presence of the amphotropic polyproline region. This mobility shift cannot be due to differences in O-linked or N-linked glycosylations. No *O*-glycosidase-sensitive glycans were detected on the precursor SU/TM protein. Removal of all the N-linked glycans by *N*-glycosidase F maintained the 4- to 5-kDa difference in the apparent molecular mass between AE4 and AE8 and EA4 and EA8 (data not shown). It is possible that the primary sequence or some other posttranslational modification alters the mobility of the protein. The amphotropic SU protein (Fig. 4A and B, lanes 7) contains eight N-linked glycosylation sites, versus the seven sites in the M-MuLV ecotropic SU protein (Fig. 4A, lane 8, and B, lane 6), and thus could compensate for the difference in the molecular mass on the basis of the primary sequence of the wild-type controls.

The supernatants from metabolically labeled virus producer cells were collected and filtered, and virus was isolated after being pelleted through a sucrose cushion. Viral proteins were visualized by immunoprecipitation with goat anti-SU (α-SU) serum (Fig. 4C and D) or goat anti-capsid protein (α-CA) serum, or the rat anti-TM (α-TM) monoclonal antibody (data not shown) (48). The mobilities of the SU proteins are consistent with that found with the intracellular precursors; the AE4 SU protein migrates faster (Fig. 4A, α-SU, lane 1) and the EA4 protein migrates slower (Fig. 4B, α-SU, lane 1) than the corresponding proteins in their series. The CA protein is an integral component of the viral core and is therefore a measure of total viral particles. After reverse transcriptase-positive cell lines were established, all of the viral particles contained proportional levels of SU, TM, and CA proteins (data not shown), indicating the envelope proteins can associate with the core particles.

Restriction analysis with PCR-amplified Hirt viral DNA. Passage of virus through cells frequently results in recombination with endogenous viral sequences. In order to examine the possibility of a recombinatorial event that would grant viability, the viral DNA was checked for the initial junction restriction site. Supernatants were collected from all chimeric virus producer cells. NIH 3T3 cells were infected, and low-molecular-mass DNA was purified (17). This DNA was amplified by PCR with *env* primers that would span between 600 and 2,900 bp of *env* sequence. Restriction analysis testing for the presence of the junction restriction site was performed with these amplified viral DNAs. All of the viable chimeric envelope genes tested had maintained the restriction site which generated the crossover position (data not shown). This indicates that no gross structural changes had occurred during the passage of the virus. However, the possibility of second-site mutations resulting in minor changes cannot be excluded.

Syncytium formation phenotype maps at the amino terminus of SU. Ecotropic and amphotropic viruses differ in their ability to form syncytia with XC cells. Ecotropic virus forms large, multinucleated syncytia (33, 54), whereas amphotropic virus is reported not to form syncytia with XC cells (33, 59). To further analyze the viral chimeric series, the viruses' ability to cause fusion with XC cells was tested. This was performed both with UV-irradiated producer cells and by cocultivation (data not shown) with the XC indicator cell line.

Figure 5 shows the syncytia resulting from the UV-irradiated producer cells overlayed with XC cells. The control, mock-infected NIH 3T3 cells overlaid with the indicator XC cells are shown at the bottom left. No syncytia can be detected. The 4070A producer NIH 3T3 cells overlaid with XC cells are shown at the bottom middle. As reported previously (33), the MuLV expressing amphotropic 4070A *env* produces few, if any, multinucleated cells above the level in control NIH 3T3 cells. The M-MuLV producer NIH 3T3 cells overlaid with XC cells are shown at the bottom right. The ecotropic M-MuLV produced large syncytia containing, on average, 12 nuclei. Syncytia occurred throughout the plate. Syncytia formed with producer cells of the EA series with XC cells are shown at the top of the figure. EA4, EA5, EA6, EA7, and EA8, like the parental ecotropic virus, produced large, multinucleated cells. In most

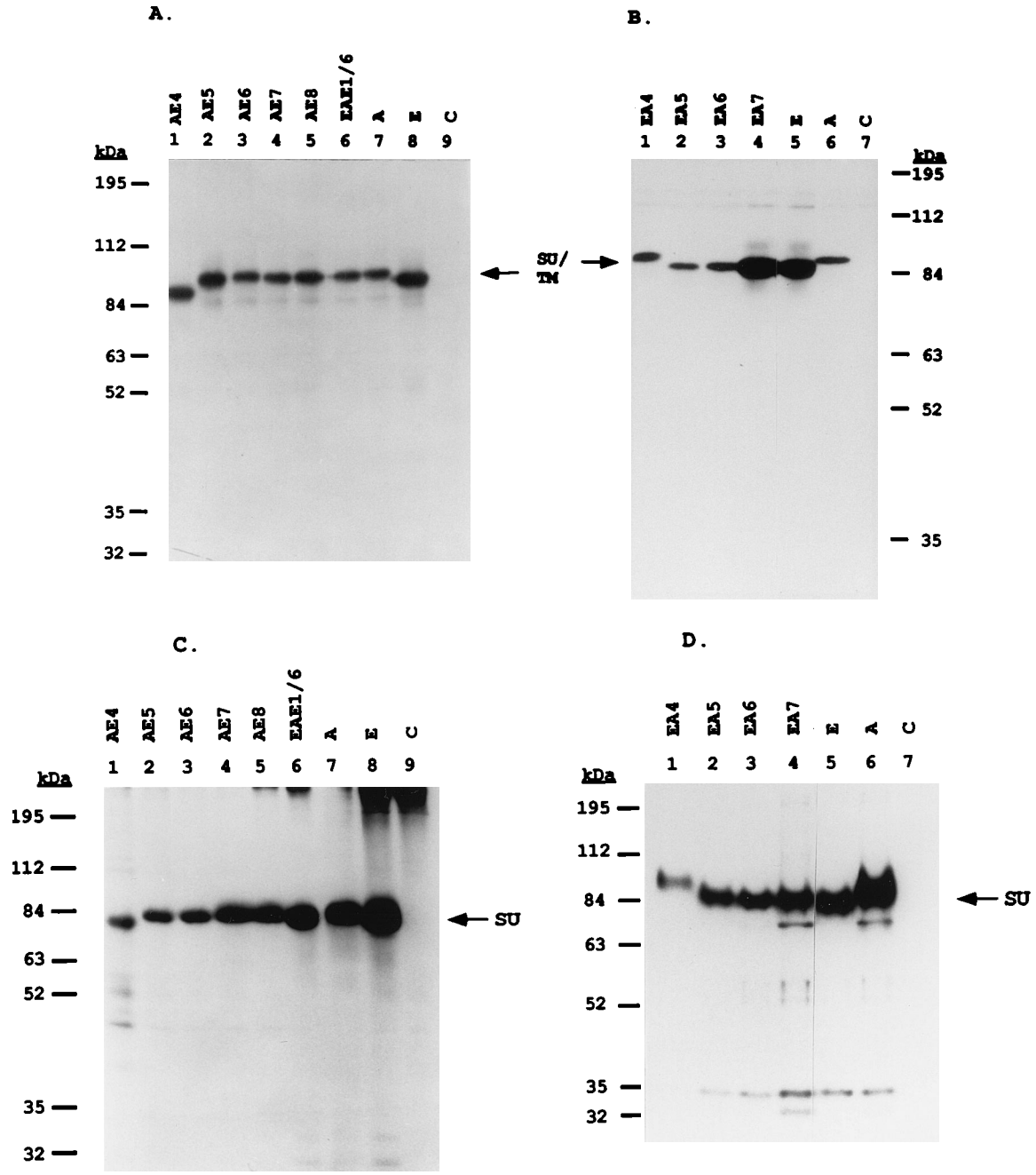

FIG. 4. Analysis of intracellular and virus-associated proteins. Cells were metabolically labeled and immunoprecipitated with 5 μl of antiserum recognizing SU (79S-842) as described in Materials and Methods. Samples underwent sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were subjected to fluorography. (A and B) Intracellular proteins. (C and D) Virus-associated proteins. (A and C) AE series which yielded infectious virus. Lane 7, 4070A amphotropic virus (A); lane 8, ecotropic M-MuLV (E); lane 9, uninfected control cells (C). (B and D) EA series which yielded infectious virus. Lane 5, ecotropic M-MuLV (E); lane 6, amphotropic 4070A (A); lane 7, uninfected NIH 3T3 control (C). The positions of migration of molecular mass markers are indicated to the side of each panel. The positions of the precursor SU/TM protein (A and B) or processed SU (C and D) are indicated.

experiments, the number of nuclei per syncytium produced with EA4 outnumbered those of the wild-type ecotropic M-MuLV, containing up to 72 nuclei per syncytium.

The syncytia of the AE series are shown in the middle of the figure. The products of overlying XC cells on the AE producer cells appear identical to that of the parental amphotropic 4070A envelope. No syncytia were detected in either the 4070A or the AE series.

In general, the characteristics of the syncytia segregated with the N-terminal domain of SU (Table 1). Large, multinucleated cells were detected in all constructs which contained the first 231 amino acids of the processed ecotropic M-MuLV (through P-264). These results indicate that the domains of the *env* gene product that determine the level of syncytium formation are located at the N terminus of SU and segregate with the host range determinants.

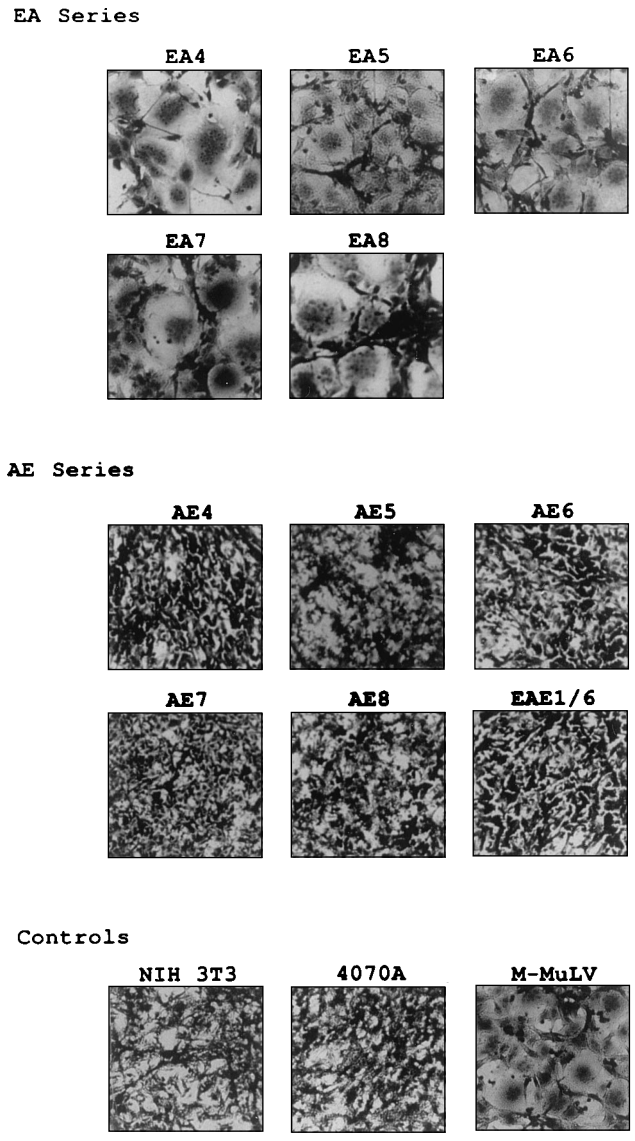

FIG. 5. Syncytium formation with rat XC cells. Viral producer cell lines were UV irradiated and overlaid with XC cells as described in Materials and Methods. The constructs are labeled above each panel. Objective magnification, ×50.

DISCUSSION

The five classes of MuLVs each display their own characteristic requirements for productive infection of the host cell. These variables include receptor choice, pH dependence of entry, and the ability to form syncytia with XC cells. In this report, the domains and determinants which are required for two classes of MuLVs, the amphotropic and ecotropic viruses, were examined; the results are summarized in Table 1. Chimeric envelope genes were generated in the context of a complete provirus, and the ability of the virus to spread through multiple rounds of infection was examined. The minimal domain required for the ecotropic host range of M-MuLV encoded amino acids 1 to 231 of the processed SU protein (through P-264). For the amphotropic 4070A isolate, the host range determinants are defined between amino acids D-73 and P-239 of the processed SU protein.

For both the ecotropic and amphotropic chimeras, the C-terminal junction which produced the minimal receptor binding domain from these studies was located within the N terminus of the polyproline region. This region contains an ordered proline repeat conserved among all classes of MuLV (GPRI/VPIGPNP). Linker insertions within the ordered prolines were temperature sensitive and resulted in the dissociation of the SU protein from the virus particles (15). Previous studies mapping the functional receptor binding domains for ecotropic and amphotropic viruses contained junctions or truncations within 15 amino acids (C terminal) of junction 4 (5, 6, 16, 37, 39). The ability to reproducibly obtain functional receptor binding with constructs which terminate in this region supports the definition of the outer limit of the domain providing efficient binding (5) and entry.

The results in this study differ from those reported by Morgan et al. (37). The minimal receptor binding domains for ecotropic and amphotropic viruses which were detected from our studies corresponded to chimeras which contained the complete VRA and VRB through the hinge or ordered proline region. Although our approach created junction points within conserved regions and maintained the integrity of the cysteine loops, no functional envelope proteins were obtained with junctions prior to the hinge region. Two truncations were reported previously to be viable (37, 39). Construct AE4 of Morgan et al. appears to maintain an amphotropic host range. This construct contains an amphotropic sequence terminating within VRB joined to ecotropic sequence downstream of VRB and generates a deletion in which the terminal cysteine plus approximately eight amino acids of the VRB are removed. This implies that the VRB cysteine loop structure is not essential. The amphotropic/ecotropic construct we have described utilizes a junction at position 3 within two bases of the ecotropic junction described by Morgan. However, rather than delete the amphotropic cysteine of VRB, this region was maintained. Although the approach we have outlined appears more conservative, this construct (AE3) was found to be nonviable. In a similar manner, Morgan et al. described a construct named AE9, which contains the first 88 amino acids of ecotropic M-MuLV comprising the first cysteine loop joined to amphotropic sequence within the second cysteine loop. This apparent deletion construct has low levels of ecotropic host range. Chimeras from this study which maintain both the first and second cysteine loop structures (junctions at position 2) were nonviable. Perhaps the deletions created by Morgan et al. in generating the chimeras alleviate some structural restraint within the context of the whole SU protein. It is rather surprising that the intact SU did not behave in a similar fashion.

The domain required for amphotropic host range has also been examined with chimeras between amphotropic, polytropic, and xenotropic viruses and 10A1 (6, 42). These viruses are more homologous to each other and provide different results from the ecotropic/amphotropic chimeras. Chimeric junctions for exchange of VRA segments were generated at the amphotropic *Afl*II site located approximately 14 amino acids downstream of junction 2 in this study. The junction at which VRB regions were exchanged was at the *Eco*RI site, identical to junction 3 in this study. The *Eco*RI site is not conserved in the ecotropic M-MuLV and was introduced into the M-MuLV *env* gene to facilitate the creation of the chimera. From these studies, it was concluded that amphotropic host range requires the VRA and VRB domains through the *Eco*RI site to form a stable structure and thus allow viral entry. Constructs which had the amphotropic VRA alone were not active. The VRB region did not define receptor binding as judged by interference, but the exchange of the VRB region did decrease the titer of the virus enough that infection in D17 cells was not

detected. In the present study, the ecotropic and amphotropic chimeras at junction 3 (AE3 and EA3) were both found to be nonviable. We have sequenced the AE3 construct in its entirety to eliminate the possibility of a second-site mutation causing the defect in virus viability. Although the N terminus of amphotropic virus was sufficient for viral entry when joined to either the 10A1, polytropic, or xenotropic C-terminal half of SU protein (6, 42), the virus was nonviable when associated with the ecotropic SU C terminus. The amino acid sequence between junctions 3 and 4 is highly conserved among the amphotropic/polytropic/xenotropic family. Between junctions 3 and 4, there are approximately 14 amino acids which are distinct to the ecotropic virus. These individual amino acids may be critical to the function and/or tertiary folding of the receptor binding domain. These results extend the observations regarding the complex interrelationships among different portions of the SU protein.

The N-terminal 42 amino acids of the processed amphotropic SU protein are not essential for host range determination. Exchange of the ecotropic N terminus into construct AE6 resulted in viable virus (EAE1/6) which displayed the interference pattern of an amphotropic virus. Interestingly, the exchange of this region into two other viable chimeras, AE4 and AE5 (EAE1/4 and EAE1/5, respectively), inactivated the virus. These results cannot be explained by a simple binary interaction between domains 1 and 4, 1 and 5, or 4 and 5. The transient expression assay measures the functional viral entry and infection. Although both EAE1/4 and EAE1/5 contain the receptor binding domain, the inactivity of these constructs in transient expression assays might reflect the more complex subunit interactions necessary for productive infection.

Although a chimera with the amphotropic host range could tolerate the presence of the ecotropic N-terminal 45 amino acids of the processed SU protein (EAE1/6), no construct with the ecotropic host range was identified in which the amphotropic N-terminal 44 amino acids could be substituted. One noticeable difference within the first 45 amino acids of the processed SU protein between the ecotropic and amphotropic *env* genes is the N-linked glycosylation sites. The position of the first N-linked glycosylation site is conserved between the ecotropic and amphotropic envelopes (N-45 of MuLV Env; N-40 of 4070A Env). Site-directed mutagenesis of this site within the ecotropic MuLV has indicated that the sites can be eliminated without affecting viral function (12, 22). A second N-linked glycosylation site is present in the N terminus of the amphotropic SU 15 amino acids downstream of the conserved site (N-55 of 4070A Env). Exchange of the N terminus of amphotropic SU into ecotropic SU therefore results in the introduction of an additional N-linked glycosylation site. This could interfere with the folding and tertiary structure of the SU/TM complex. Preliminary studies in which the second N-linked glycosylation site was introduced by site-directed mutagenesis into the M-MuLV *env* gene indicate that the virus, in fact, is not viable (12a).

The approach utilized identifies which constructs result in viable virus and cannot address the basis of the defective phenotypes. To address this, stable producer lines have been generated for several of the AE series. Minimally, it can be said that AE1 and AE2 produce stable precursor SU/TM proteins intracellularly and that AE1 can associate with viral particles (data not shown).

Although the time course of infection of AE5 in NIH 3T3 cells paralleled that of the wild-type virus, this was not found in canine D17 cells. The transient expression assay of AE5 was repeated six times, and no viral spread was ever detected. The AE5 virus isolated from NIH 3T3 cells could infect D17 cells. This difference of infection versus transient expression may reflect the titer of the virus and the receptor interaction. Virus transiently expressed which does not spread rapidly would be diluted through every passage in tissue culture. Variations in the amphotropic receptor from D17 cells may also be a determinant. Recently, it was noted that amphotropic SU protein which was shed into conditioned media could not bind the canine D17 receptor as efficiently as that from other cell lines (5).

In this study, multiple viable chimeras have been identified with junctions throughout the C terminus of the SU protein and within the TM protein. Although the C terminus of the SU protein is highly conserved between ecotropic and amphotropic envelopes, the junctions within this region appear to have a slightly delayed time course with respect to that of wild-type virus. This is seen most consistently with chimeras with the junction at position 6. Linker insertion mutagenesis of SU protein has identified two positions within the C terminus of SU protein in which insertion of four amino acids affects the SU and TM protein interactions and results in a delay in the viral spread at 37°C (15). One of these mutations (*in*6898-12a) maps within one amino acid of junction 6. In a similar manner, differences in the primary sequence of the ecotropic and amphotropic SU/TM may affect the association of these proteins in the chimeras and may result in the delay detected. Analysis of the Hirt DNA indicated that the junction site is maintained in these constructs. However, the possibility that the delay is indicative of a reversion due to second-site mutations cannot be eliminated and is currently being examined.

In many virus-cell systems, the presence or absence of syncytium formation reflects the pH requirements involved in viral envelope-mediated fusion. (33, 67). Viruses, such as influenza virus, that fuse with the cell membrane only when inside an acidic endosome can induce cell-to-cell fusion when provided with an acidic environment (7, 8, 58). Evidence from various systems has indicated that the ability of ecotropic MuLV to form syncytia and the pH dependence of viral entry are independent processes. M-MuLV is not capable of fusing at the cell surface when provided with low pH; passage through the endosomal compartment is still required (39). XC cells are unique in that ecotropic infection occurs in a pH-independent manner. Even though both amphotropic and ecotropic MuLV enter XC cells in a pH-independent fashion, only ecotropic MuLV causes a large multinucleated syncytium with XC cells (33). A single point mutation within the VRA of Friend-related MuLV TR1.3 has been identified which induces syncytium formation without affecting the pH dependence of entry (43). Entry of M-MuLV into NIH 3T3/DTras cells is inhibited by the lysosomotropic agent chloroquine without affecting the syncytium (68). It was postulated that different epitopes on the viral envelope glycoprotein may be involved in viral entry and M-MuLV cell-to-cell fusion. The results of these studies indicate that the N terminus of the ecotropic SU protein encoding the receptor binding domain contains a major determinant for the cell-to-cell fusion with XC cells. Within this region is the amino acid which can activate fusion in TR1.3 (43).

The receptor binding domain can indirectly regulate fusion. Genetic determinants for fusion have been identified within the TM protein (19). The ecotropic MuLV is capable of inducing syncytium under quite varied conditions. These include mutation of the cytoplasmic tail of TM protein (49, 51), point mutations in SU protein (43), infection in the presence of amphotericin B (44), and infection of transformed cells (68), including rat XC cells (54). Post-receptor binding, dynamic changes within the SU/TM complex would be needed to position the fusion peptide and the viral and cellular membranes in

proximity. The activation or trigger of these processes is not well understood. The tertiary structure of the host receptor-viral envelope complex can have a direct influence on the range of conformational changes which are feasible. The trigger for the ecotropic MuLV envelope-cationic amino acid transporter complex can be distinct from the amphotropic MuLV envelope-phosphate symporter receptor complex. The ability of specific mutations to regulate this process can be envisioned. Mutations which increase the binding may provide longer contact time for membranes to associate. Models in which SU is required to be released to expose the TM fusion peptide would be stimulated by mutations which favor conformational changes. Alternatively, the ecotropic receptor binding domain may encode a protease recognition site not conserved in amphotropic 4070A. Cleavage of the protein could trigger the activation of fusion (2, 3, 33). The lipid composition of the membranes can also regulate cell-specific fusion (23, 68).

Initial mapping of the determinant for pH dependence of MuLV entry indicates that it is not within the N-terminal region of SU protein up to the polyproline hinge (39). Further definition of the factors which control the pH dependence requires viable chimeras between the ecotropic and amphotropic virus with junctions within the C terminus of the SU and TM proteins. The set of 11 viable chimeras identified in this study should prove an invaluable tool for this analysis.

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