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Mutational Analysis of the Envelope Gene of Moloney Murine Leukemia Virus

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The *env* gene products of Moloney murine leukemia virus are required for binding and entry of the virus into the target cell. Thirty-three linker insertion mutations were constructed throughout the *env* gene of Moloney murine leukemia virus. Twenty of the mutations were located in the surface protein (SU), and the remaining thirteen were located in the transmembrane protein (TM). The viability of the viruses containing these *env* gene mutations was determined by performing transient transfections and screening for the release of reverse transcriptase. Eleven viable mutants were isolated, nine in SU and two in TM. Three of the viable mutants were temperature sensitive. Four of the viable mutants were clustered in the carboxy terminus of SU. The *env* gene products of transfected cell lines which produced viable virus were analyzed. Our results indicated two regions of SU important for the stability of the SU/TM heteropolymer and one region important for the interaction of the *env* gene products with the viral core.

Retroviruses, in particular murine leukemia viruses (MuLVs), are frequently used as vehicles for introducing genes into cells. Despite their wide usage, many aspects of the mechanism of entry of the virus remain unknown. Entry of the virus into the cell involves the interaction of the viral envelope glycoproteins with a specific cell surface receptor. After binding, the virus is internalized, allowing for replication and establishment of the integrated provirus (37).

The envelope proteins (Env) are translated from a spliced message on the rough endoplasmic reticulum as precursor proteins and are transported to the cell surface through the Golgi apparatus. During the transport, the Env proteins are modified. The putative signal sequence at the amino terminus is removed, glycosylation occurs, and the molecule is cleaved by a cellular protease into two subunits, the surface protein (SU) and the transmembrane protein (TM) (18). An additional proteolytic cleavage of TM (Pr15E) removing 16 amino acids from the carboxy terminus (R peptide) occurs at or around the time of budding by a virus-encoded protease, yielding the processed TM, p15E (7, 14). SU and TM are incorporated into virions as heteropolymers and remain associated through disulfide bonds as well as noncovalent interactions (18, 30).

Murine retroviruses have been classified on the basis of competitive interference assays as ecotropic, amphotropic, xenotropic, polytropic, and 10A1 (18, 31). These viruses each bind to a unique host cell receptor; the receptor for the ecotropic virus has been cloned and is the cationic amino acid transporter (1, 20, 39). Amino acid sequence alignments of the different MuLV Env proteins show the largest divergence in the amino terminus of SU (9, 21, 26, 27, 36). Truncation studies of the ecotropic virus show that this region is involved in binding to the host receptor (15). Chimeric proteins between the various isolates have proven more complicated, indicating that regions of SU may interact (2, 28).

Sequence scanning of SU and TM has identified several domains or motifs. For MuLVs, these include a proline-rich region within SU, clustering of N-linked glycosylation sites at the carboxy terminus of SU, and a hydrophobic stretch followed by a heptad repeat within the extracellular domain of TM (18, 27). Clearly, specific amino acids or domains must be required for the binding of SU to its receptor, for the SU/TM association, for the association of TM with the membrane, for the formation of syncytial plaques, and possibly for the binding of the core virion particle to the membrane containing the *env* gene product. In this study, linker insertion mutagenesis of the *env* gene of Moloney MuLV (M-MuLV) was used to define functional domains of the proteins.

MATERIALS AND METHODS

Cell lines. Rat1, NIH 3T3, and XC cells were grown in Dulbecco's modified Eagle's medium (GIBCO-BRL) supplemented with 10% newborn calf serum (HyClone). These cell lines were maintained at 37°C in a 5% CO_2 atmosphere.

Plasmids and linkers. Plasmid pNCA-C expresses the M-MuLV provirus and was described previously (5). The *env* gene of M-MuLV was cloned into the *Escherichia coli* expression vector pATH 10 (GenBank accession no. M33-623). pATH 10 was cut with *Eco*RI, blunt ends were generated by digestion with mung bean nuclease (GIBCO-BRL), and a 12-mer *Not*I linker (New England Biolabs no. 1127) was inserted, generating pATH 10-*Not*I. *in*6215a (32) was digested with *Not*I and *Sac*I, and the 2.47-kb fragment was isolated by glass powder and ligated to pATH 10-*Not*I digested with *Not*I and *Sac*I. The resulting plasmid, pNS, was used for mutagenesis (see below). For the linker insertion mutagenesis, two 12-mer *Eco*RI linkers were used (a [CCGGAATTCCGG; New England Biolabs no. 1019] and b [CGCCGAATTCGG; synthesized in this department]).

Mutagenesis. Mutagenesis of the *env* gene was performed on pNS. The plasmid was partially digested with either *Alu*I, *Eco*RV, *Hae*III, *Hin*cII, *Msp*I, *Nla*IV, or *Rsa*I, and the full-length linear DNA was isolated. Linker a or b was ligated into the partially digested plasmid (T4 DNA ligase; Bethesda Research Laboratories), using an approximately 100-fold molar excess of linker, and transformed into *E. coli* HB101. Plasmid DNA was then screened for the presence of the *Eco*RI site. For mutations made at the *Alu*I, *Eco*RV,

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*Hin*cII, and *Rsa*I sites, the positions of the inserts were mapped by analyzing for the loss of one of the restriction sites. These positions were verified by performing an *Acc*I-*Eco*RI double digestion. Mutations created at the *Hae*III, *Nla*IV, and *Msp*I sites were mapped by performing a double digestion with *Hin*cII and *Eco*RI. These positions were verified by using *Acc*I and *Eco*RI. The DNA fragments containing mutations within the first 1,180 bp of *env* were reconstructed into the M-MuLV provirus, using the 1,184-bp *Sca*I-*Nsi*I fragment. Plasmids with mutations within the next 618 bp were reconstructed by using the *Nsi*I-*Cla*I fragment. In the case of *in*7705-12a and *in*7748-12a, the 792-bp *Nsi*I-*Nhe*I fragment was used for reconstruction. Mutations were sequenced on both strands, using the dideoxy sequencing method (Sequenase; U.S. Biochemical). Mutants were numbered according to the sequence determined by Shinnick et al. (35).

Transfections. Plasmid DNA was isolated by CsCl gradients (24). Each provirus containing a unique mutation was transfected into 10^5 Rat1 cells per 60-mm-diameter dish at 32 or 37°C, using the DEAE-dextran method (25).

RT assay. Supernatant was collected from the transfected cells periodically for approximately 40 days. The supernatant was assayed for the release of reverse transcriptase (RT) into the medium (12).

Hirt assay. NIH 3T3 cells were infected in the presence of Polybrene (8 μg/ml) with 2 ml of filtered (0.45-μm-pore-size Acrodisc) supernatant from the transfected cell lines. Cells were incubated for 2 days at 32°C and lysed, and the low-molecular-weight DNA was isolated (17). Samples were treated with equal volumes of phenol and then of chloroform and were precipitated with ethanol.

Southern blots. Low-molecular-weight DNA isolated from the transfected cell lines (*in*5884-12b, *in*6239-12a, *in*6696-12b, *in*6839-12a, and *in*6995-12a) was digested with *Nhe*I and *Eco*RI, transferred to nitrocellulose, and probed (34) with ^{32}P-labeled pNCA-C (Prime-It; Stratagene).

PCR. Low-molecular-weight DNA isolated from the transfected cell lines was amplified by the polymerase chain reaction (PCR; GeneAmp; Perkin-Elmer/Cetus). Primers 2101 (GCAACACTGCCTGGAAC) and 2103 (GAGTTGA CTCAAACAG) were used to amplify *in*6898-12a and *in*7153-12a. Primers 2102 (GGCCAAACCCCGTTCTG) and 4091 (CTCTTTTATTGAGCTCGGG) were used to amplify *in*7705-12a and *in*7748-12a. All primers were synthesized in this department.

XC assays. XC assays were performed on all of the transfected cell lines. Medium was removed from transfected cells, and the cells were UV irradiated for 10 s. The UV-treated cells were then overlaid with XC cells and allowed to grow at either 32°C for 2 days or 37°C for 1 day. The cells were fixed with 37% formaldehyde and stained with hematoxylin (Sigma) staining solution (33).

Metabolic labeling of transfected cells. To detect virion proteins, producer cells were washed twice with phosphate-buffered saline (PBS), incubated with Hanks' balanced salt solution for 30 min, and labeled as described previously (10). To label the proteins in the total supernatant, the cells were washed with PBS and incubated in 5 ml of medium without methionine or serum in the presence of 250 μCi of Translabel (ICN) per 10-cm-diameter plate for 12 h. Cells that were pulse-chase labeled for intracellular protein content were washed and incubated in medium without methionine for 20 min. These cells were then incubated for an additional 2 h in the presence of 150 μCi of Translabel per 10-cm-diameter plate. Radioactive medium was removed, and the cells were incubated for an additional 3 h in complete medium.

Immunoprecipitation. Immunoprecipitation of viral and intracellular proteins was performed as previously described (10). The antisera used were MLV antiserum 81S000107 (National Cancer Institute) and anti-p15E monoclonal antibody 42-114 (30). The protein complex was precipitated with Pansorbin (Calbiochem), washed twice with phospholysis buffer (PLB; 1% Triton X-100, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 0.01 M sodium phosphate [pH 7.5], 0.1 M NaCl), resuspended in 2× protein sample buffer (22), and separated on an SDS-polyacrylamide gel. For immunoprecipitations using 42-114, 5 μl of rabbit anti-rat immunoglobulin G serum was added 1 h prior to addition of the Pansorbin. To immunoprecipitate the proteins from the total supernatant, the supernatant was collected from the labeled cells and filtered through an Acrodisc (0.45-μm pore size). One milliliter of supernatant plus PLB (final concentration of PLB, 1×) was incubated with 10 μl of normal goat serum on ice for 2 h. Pansorbin (50 μl) was added, and the sample was incubated for 1 h on ice. The sample was centrifuged, and the precleared supernatant was transferred to a new tube and incubated overnight on ice with 4 or 5 μl of antiserum.

RESULTS

Detection of viable virus. Thirty-three linker insertion mutants were constructed (Table 1). The effect of the mutation on the viral life cycle was assayed through transient expression of the virus, using the DEAE-dextran method of transfection (25). The viability of each mutant was determined by assaying for the release of RT into the medium (12).

Of the 33 mutants, 11 were positive for the release of RT at 32°C (Table 1): 2 in the amino terminus of SU (*in*5884-12b and *in*6239-12a), 3 in the proline-rich region (*in*6566-12a, *in*6584-12a, and *in*6696-12b), 4 in the carboxy terminus of the SU (*in*6839-12a, *in*6898-12a, *in*6995-12a, and *in*7042-12b), and 2 in cytoplasmic tail of TM (*in*7705-12a and *in*7748-12a). Each mutant was transfected at least twice. Mutants *in*7705-12a, *in*7042-12b, and *in*6584-12a spread noticeably more slowly than the wild-type virus. These mutants became positive for RT activity 15, 25, and 15 days later, respectively, than the wild type (data not shown).

Supernatant from the transfected cells was used to infect NIH 3T3 cells, and the low-molecular-weight DNA from the NIH 3T3 cells was collected by the Hirt method (17). The DNA was analyzed by using restriction enzymes and Southern blotting (*in*5884-12b, *in*6239-12a, *in*6696-12b, *in*6839-12a, and *in*6995-12a) or amplified by PCR and then analyzed (*in*6898-12a, *in*7153-12a, *in*7705-12a, and *in*7748-12a). Of the nine mutations screened for the maintenance of the *Eco*RI linker, only one, *in*7153-12a, was negative (Table 1). The presence of the *Eco*RI site, however, does not eliminate the possibility that second-site mutations occurred elsewhere in the linker or in the genome.

At 37°C, only 8 of the 11 mutants were positive for the release of RT (Table 1). Thus, three of the mutations have a temperature-sensitive (*ts*) phenotype: two in the proline-rich region (*in*6566-12a and *in*6584-12a) and one in the cytoplasmic tail of TM (*in*7705-12a). Mutants *in*6898-12a and *in*7042-12b spread more slowly than the wild type at 37°C. Mutant *in*6898-12a was transfected twice and became positive for RT activity 4 and 18 days later than the wild type. Mutant *in*7042-12b was transfected three times at 37°C but became positive for RT activity only once, 13 days after the wild type.

Syncytium formation with XC cells. Each cell line created

TABLE 1. Summary of mutations

Mutation[a]	Site[b]	Amino acids inserted[c]	RT assay[d]		XC assay[e]		*Eco*RI site[f]
			32°C	37°C	32°C	37°C	
*in*5884-12b	*Msp*I	Ala Glu Phe Glu	+	+	+	+	+
*in*5990-12a	*Hae*III	Pro Glu Phe Arg	−	−	−	−	
*in*6084-12a	*Nla*IV	Gly Ile Pro Ala	−	−	−	−	
*in*6235-12b	*Msp*I	Ala Glu Phe Gly	−	−	−	−	
*in*6239-12a	*Hae*III	Pro Glu Phe Arg	+	+	++	+	+
*in*6274-12a	*Nla*IV	Arg Asn Ser Gln	−	−	−	−	
*in*6316-12b	*Msp*I	Ala Glu Phe Gly	−	−	−	−	
*in*6451-12b	*Msp*I	Ala Glu Phe Gly	−	−	−	−	
*in*6511-12b	*Msp*I	Ala Glu Phe Gly	−	−	−	−	
*in*6540-12a	*Nla*IV	Thr Gly Ile Pro*	−	−	−	−	
*in*6566-12a	*Nla*IV	Pro Glu Phe Arg	+	−	++	−	ND
*in*6584-12a	*Hae*III	Pro Glu Phe Arg	+[s]	−	++	−	ND
*in*6696-12b	*Msp*I	PrA Asn Ser Ala	+	+	++	+	+
*in*6839-12a	*Rsa*I	Pro Glu Phe Arg	+	+	++	+	+
*in*6898-12a	*Hinc*II	Arg Asn Ser Gly*	+	+[s]	+/−	+/−	+, +
*in*6995-12a	*Nla*IV	Pro Glu Phe Arg	+	+	++	+	+
*in*7040-12a	*Rsa*I	Pro Glu Phe Arg	−	−	−	−	
*in*7042-12b	*Msp*I	Ala Glu Phe Gly	+[s]	+[s]	+/−	+/−	ND
*in*7141-12a	*Alu*I	Arg Asn Ser Gly	−	−	−	−	
*in*7153-12a	*Hae*III	Arg Asn Ser Gly	−[g]	−	−[g]	−	−[g]
*in*7188-12b	*Msp*I	Pro Asn Ser Ala	−	−	−	−	
*in*7198-12a	*Hinc*II	Arg Asn Ser Gly*	−	−	−	−	
*in*7278-12a	*Hae*III	Gly Ile Pro Ala	−	−	−	−	
*in*7298-12a	*Alu*I	Pro Glu Phe Arg	−	−	−	−	
*in*7312-12a	*Rsa*I	Arg Asn Ser Gly	−	−	−	−	
*in*7404-12a	*Nla*IV	Gly Ile Pro Gly*	−	−	−	−	
*in*7506-12a	*Hae*III	Gly Ile Pro Ala	−	−	−	−	
*in*7552-12a	*Hinc*II	Arg Asn Ser Gly	−	−	−	−	
*in*7624-12a	*Nla*IV	Arg Asn Ser Gly	−	−	−	−	
*in*7636-12a	*Rsa*I	Arg Asn Ser Gly	−	−	−	−	
*in*7661-12a	*Nla*IV	Pro Glu Phe Arg	−	−	−	−	
*in*7705-12a	*Eco*RV	Arg Asn Ser Gly	+[s]	−	+	−	+
*in*7748-12a	*Alu*I	Pro Glu Phe Arg	+	+	++	++	+

[a] The number of the mutation represents the first nucleotide of the insert and the length and sequence of the linker. The nucleotide sequence is according to Shinnick et al. (35).

[b] The restriction site in which the linker was inserted.

[c] In addition to the amino acids inserted, four mutations (*) had an amino acid changed immediately preceding or following the insertion: *in*6540-12a, Ile to Val; *in*6898-12a, Phe to Leu; *in*7198-12a, Leu to Phe; and *in*7404-12a, Gly to Ala.

[d] Mutants delayed in the appearance of RT activity are indicated (s).

[e] The amounts of syncytia produced by the mutants were compared with the amount produced by the wild-type virus. ++, greater than 70%; +, between 35 and 70%; +/−, 1 to 35%; −, less than 1%.

[f] The *Eco*RI site was maintained (+), not maintained (−), or not determined (ND) after passage of virus at 32°C. Virus isolated at 37°C for mutant *in*6898-12a maintained the *Eco*RI site.

[g] Mutant *in*7153-12a was positive one of the three times it was transfected at 32°C. The viable virus was XC ++, and sequence analysis indicated it had lost the linker and had the wild-type M-MuLV sequence.

by transfection of Rat1 cells with mutated M-MuLV was assayed for syncytium formation. When XC cells are placed in contact with cells infected with an ecotropic MuLV, syncytium formation occurs (33). The region of the *env* gene responsible for XC plaque formation has not been determined. The amount of syncytia produced by each transfected cell line was counted and compared with the amount of syncytia produced by a cell line transfected with wild-type M-MuLV (Table 1).

All of the cell lines that were positive for the release of RT produced various amounts of syncytia. The mutants determined to be nonviable by RT activity produced less than 1% of the amount of syncytia produced by the wild type. Interestingly, two cell lines (*in*6898-12a and *in*7042-12b) produced less than 35% of the amount of syncytia produced by the wild type (XC +/−) at 32 and 37°C. Qualitatively, these syncytia contained fewer nuclei than did syncytia produced by the wild type. These cell lines also spread more slowly than the wild type at 37°C. When the *ts* mutants were shifted to the nonpermissive temperature (37°C) and maintained for 2 weeks, the amount of syncytia produced remained the same.

Analysis of SU. Viral proteins associated with the virus or in the supernatant were immunoprecipitated with various antibodies. The SU protein recognizes the cell surface receptor and is anchored to the virus by TM through disulfide bonds and noncovalent interactions (38). A structural change in SU or a disruption of the noncovalent or disulfide bonds could disrupt or weaken the SU/TM heteropolymer, allowing SU to shed into the medium. Viable virus should contain a complete complement of viral proteins. Figure 1A is an autoradiogram of immunoprecipitations of viral proteins of the viable mutants at 32°C with total MuLV antisera. All of the viable mutants produced similar amounts of CA (capsid) protein in comparison with the wild type. However, the amount of *env* gene product produced varied considerably. When the ratio of SU to CA of the mutants was compared with the ratio of SU to CA of wild type, four mutants, *in*6566-12a (Fig. 1A, lane 3), *in*6584-12a

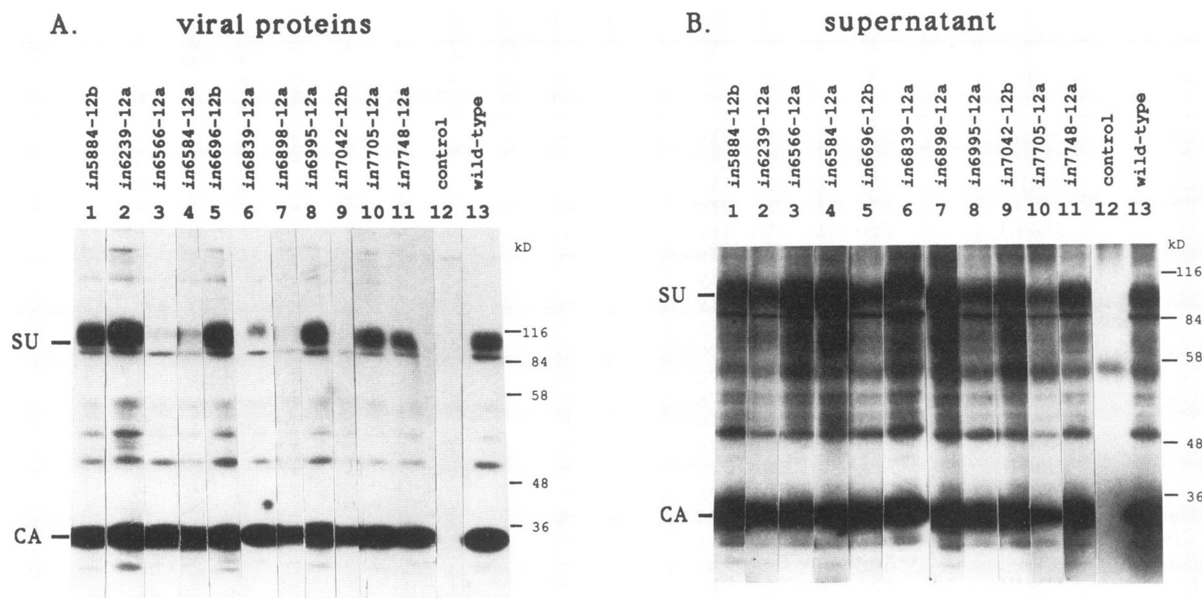

FIG. 1. Analysis of SU at 32°C. (A) Producer cells were labeled overnight, and the supernatant was centrifuged through a sucrose cushion to pellet the virus. The virus was resuspended, and the viral proteins were immunoprecipitated with antisera to total MuLV and electrophoresed on an SDS-polyacrylamide gel (see Materials and Methods). (B) Producer cells were labeled for 12 h. The supernatant was collected, and the viral proteins were immunoprecipitated with antisera to total MuLV (see Materials and Methods). Migration of molecular mass standards is indicated at the right. Samples are indicated above the lanes.

(lane 4), *in*6898-12a (lane 7), and *in*7042-12b (lane 9), had a ratio much lower than that of the wild type. *in*6566-12a and *in*6584-12a were the *ts* mutants in the proline-rich region. *in*6898-12a and *in*7042-12b were the XC +/− mutants. Mutants *in*5884-12b (lane 1), *in*6239-12a (lane 2), *in*6696-12b (lane 5), *in*6995-12a (lane 8), *in*7705-12a (lane 10), and *in*7748-12a (lane 11) had amounts of SU comparable to wild-type amounts. Mutant *in*6839-12a (lane 6) had an intermediate amount of SU.

The amount of SU on the virus could be reduced as a result of several factors, including transcription, splicing, incorrect transport, or protein stability. Alternatively, the lack of SU on the virus could be due to a disruption of the interaction between SU and TM. A disruption of the SU/TM interaction would result in SU falling off the virus and/or the cell into the medium. To examine this possibility, an immunoprecipitation was performed on the total supernatant of the producer cell lines (Fig. 1B). The total supernatant includes the SU that has fallen off of the virus and the cell and the SU that is associated with the virus. Compared with the wild type, the four mutants with a lower SU-to-CA ratio associated with the virus, *in*6566-12a, *in*6584-12a, *in*6898-12a, and *in*7042-12b, have a much higher SU-to-CA ratio in the supernatant (Fig. 1B, lanes 3, 4, 7, and 9, respectively). These results suggest that the SU protein is shedding from the virus and/or the cell into the supernatant.

The effects of shifting the mutations from the permissive to the nonpermissive temperature were examined. The *ts* and XC+/− mutants were maintained at 37°C for 2 weeks and then analyzed. Figure 2A presents the results of the immunoprecipitations of the viral proteins with antibody to total MuLV. No protein comigrating with the wild-type SU was detected with the *ts* mutants in the proline-rich region, *in*6566-12a and *in*6584-12a. Two proteins bracketing the wild-type SU protein were detected (Fig. 2A, lanes 1 and 2, respectively). The nature of these two bands were examined. Immunoprecipitations with antibodies specific to Env and Gag revealed that the proteins in lanes 1 and 2 of Fig. 2A were Gag related (Fig. 2C). The bands indicated by the arrowheads were precipitated by antisera to the Gag protein p30 (Fig. 2C, lanes 1 and 4) and antisera to total MuLV (lanes 3 and 6) but not antisera to the Env protein gp70 (lanes 2 and 5). No SU was detected on the virus of the XC +/− mutants, *in*6898-12a and *in*7042-12b (Fig. 2A, lanes 3 and 4, respectively). The *ts* mutant in TM, *in*7705-12a (Fig. 1A, lanes 10), had a ratio of SU to CA comparable to the wild-type ratio at 32°C, but at 37°C there was very little SU associated with the virus (Fig. 2A, lane 5). Analysis of the total supernatant of the *ts* and XC +/− mutants at 37°C suggests that SU is being produced, processed, and transported to the cell surface (Fig. 2B).

Analysis of TM. The effect that the mutations had on the association of TM with the virus was examined. Since the TM protein is required to anchor the Env proteins to the virion, several possibilities could be predicted. First, TM may associate with the virion in the absence of SU. Alternatively, TM lacking SU may no* be able to associate with virions or may have a short half-life on the cell. The timing of the dissociation of SU may also affect the association of TM with the virion. Finally, mutations in TM may directly block the association of Env proteins with the virion. To address these possibilities, the expression of TM was characterized for each of the transfected cell lines producing viable virus.

A representative autoradiogram of an immunoprecipitation performed on viral proteins at 32°C with antisera to p15E is shown in Fig. 3A. In the virion, the virus-encoded protease cleaves Pr15E to produce the processed form of TM, p15E (7, 14). In the wild-type virus, the ratio of Pr15E to p15E is approximately 1:1 (Fig. 3A, lane 13). The *ts* mutants in the proline-rich region, *in*6566-12a and *in*6584-12a (lanes 3 and 4), and the XC +/− mutants, *in*6898-12a and *in*7042-12b (lanes 7 and 9), had less TM compared with the

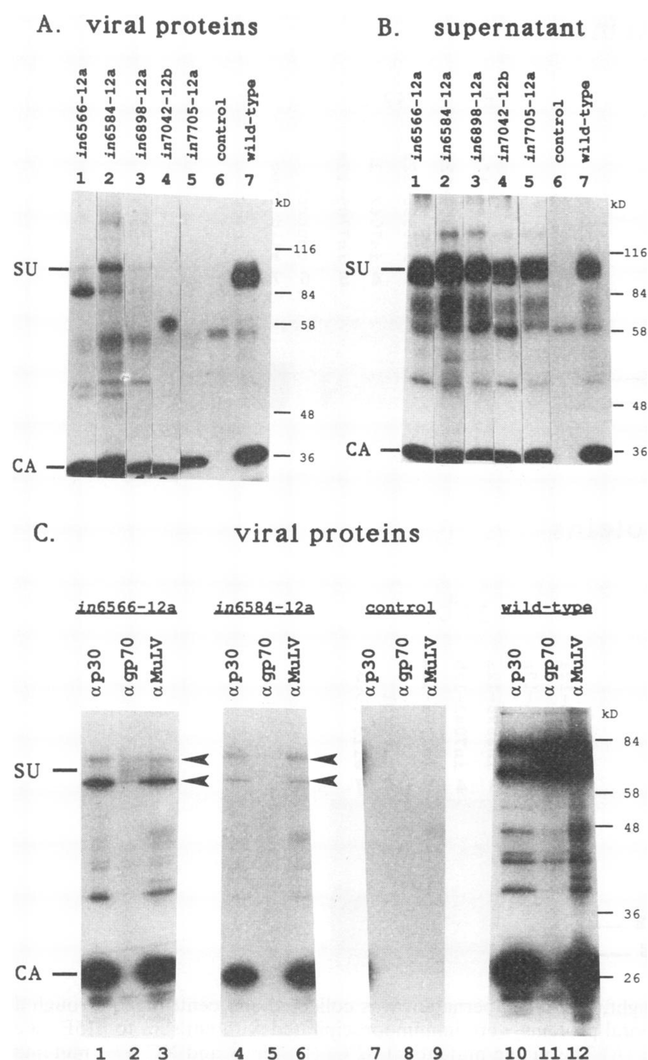

FIG. 2. Analysis of SU at 37°C. The *ts* and XC +/− mutants were shifted to the nonpermissive temperature and maintained for approximately 2 weeks, and the viral proteins were analyzed. (A and C) Producer cells were labeled overnight, and the supernatant was collected and centrifuged through a sucrose cushion to pellet the virus. The virus was resuspended, and the viral proteins were immunoprecipitated with antisera (see Materials and Methods) to total MuLV (A) and to total MuLV, gp70 (SU), and p30 (CA), as indicated (C). (B) Producer cells were labeled for 12 h. The supernatant was collected, and the viral proteins were immunoprecipitated with antisera to total MuLV. Migration of molecular mass standards is indicated at the right. Samples are indicated above the lanes.

wild type. These mutants did contain CA in amounts comparative to that of the wild type (Fig. 1A, lanes 3, 4, 7, and 9, respectively). The four-amino-acid insertion was evident in mutant *in*7705-12a (lane 10) in that p15E migrates more slowly than the wild type. This mutated TM appeared to favor processing; 100% of the Pr15E was cleaved to p15E. Mutants *in*5884-12b (lane 1), *in*6239-12a (lane 2), *in*6696-12b (lane 5), *in*6995-12a (lane 8), and *in*7748 (lane 11) had amounts of TM comparable to the wild-type amount. Mutant *in*6839-12a (lane 6) had an intermediate amount of TM.

The amounts of TM associated with the virus of the *ts* and XC +/− mutants at 37°C are shown in Fig. 3B. The *ts* mutants in the proline-rich region, *in*6566-12a and *in*6584-12a (lanes 1 and 2), had TM associated with the virus. The XC +/− mutants, *in*6898-12a and *in*7042-12b (lanes 3 and 4), had very little TM associated with the virus, as did the *ts* mutant in the cytoplasmic tail of TM, *in*7705-12a (lane 5).

A pulse-chase experiment was performed on the producer cell lines, and the cells were lysed to analyze the cell-associated TM proteins (Fig. 3C). The cells were labeled for 2 h and then chased for 3 h to allow time to complete the processing events. All of the *ts* and XC +/− mutants were producing a sufficient amount of TM compared with the wild type at 32°C.

Figure 3D shows the results of an immunoprecipitation of the cellular TM proteins at 37°C. The *ts* mutants in the proline-rich region, *in*6566-12a and *in*6584-12a (lanes 1 and 2), were producing a sufficient amount of TM, and virus-associated TM was also detected (Fig. 3B, lanes 1 and 2). In the cells producing the XC +/− mutants, *in*6898-12a and *in*7042-12b, cellular TM was less abundant at 37°C than at 32°C (Fig. 3C and D, lanes 3 and 4). A time course experiment revealed that cell-associated TM of the XC +/− mutants reached a maximum level after a 1-h chase and the wild-type cell-associated TM reached a maximum level after a 2-h chase. The cell-associated TM of mutant *in*7042-12b was less stable than the wild-type TM over the time period examined (data not shown). The *ts* mutant in the cytoplasmic tail, *in*7705-12a, had an amount of cellular TM comparable to the wild-type amount at 37°C (Fig. 3D, lanes 5 and 7). Despite the availability of TM, the protein did not associate with the virus (Fig. 3B, lane 5), hence identifying a region of Env required for the assembly of the *env* gene products with the virion.

DISCUSSION

Linker insertion mutagenesis was used to identify functional domains of the Env proteins. The data presented in this study suggest three phenotypes: the *ts* mutants in the proline-rich region, which are XC ++ and shed SU; the XC +/− mutants, which shed SU; and the *ts* mutant in the cytoplasmic tail of TM, which is XC + and has no Env proteins associated with the virions at the nonpermissive temperature.

***ts* mutants in the proline-rich region.** Virus containing the SU and TM envelope proteins buds from the cell during the course of a wild-type viral life cycle. At 32°C, mutants *in*6566-12a and *in*6584-12a had a ratio of SU to CA in the supernatant higher than that associated with the virus. At 37°C, the two mutants had no SU associated with the virus. Both mutants *in*6566-12a and *in*6584-12a had TM associated with the virus at 32 and 37°C. The amount of XC syncytia produced by these mutants was comparable to the wild-type amount. Previous studies using chimera of amphotropic, polytropic, and xenotropic MuLVs have shown that the proline-rich region is not needed to recognize the receptor and suggest that the proline-rich region is required to stabilize the receptor-specific structure at the surface of the viral particle (2). Additional chimeric studies suggest the possibility that the amino and carboxy termini of SU may interact (28). The insertion of the four-amino-acid linker could result in a slight structural change in the surface protein that ultimately weakens the SU/TM interaction. This change leads to large amounts of the SU protein being shed into the media.

XC +/− mutants. At 32°C, the SU-to-CA ratio of the XC +/− mutants, *in*6898-12a and *in*7042-12b, was much higher in the supernatant than the SU-to-CA ratio associated with the virus. At 37°C, there was no SU or TM associated with the virus, but SU could be detected in the medium. The level

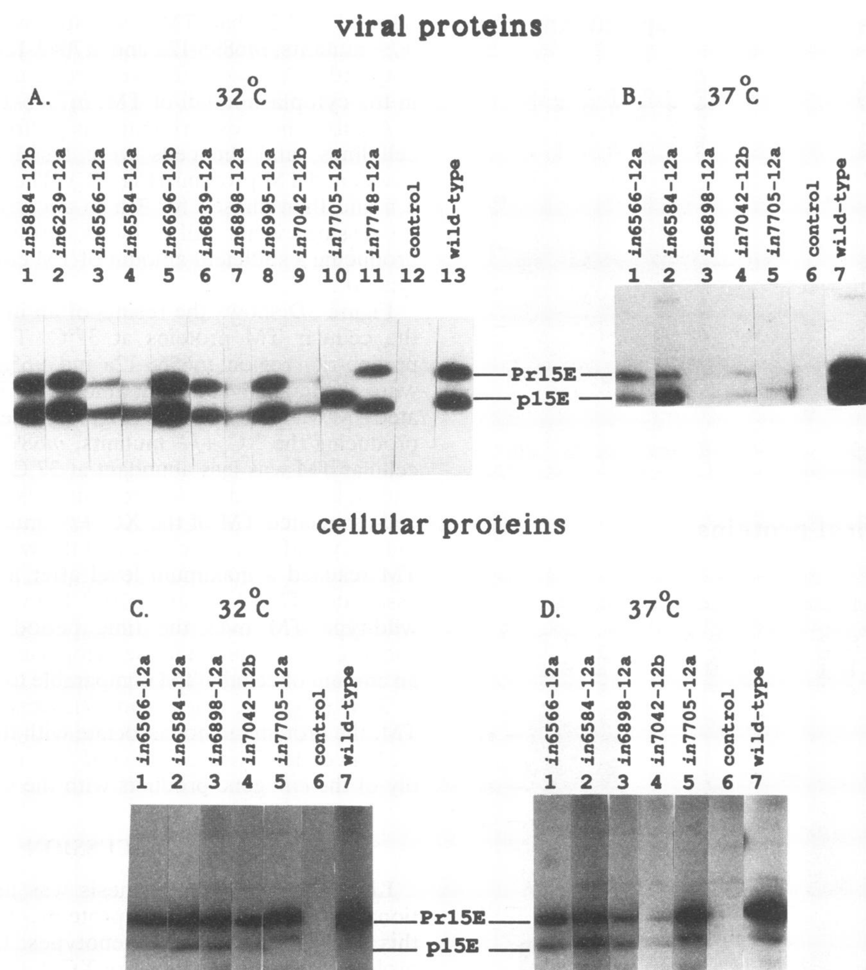

FIG. 3. Analysis of TM. (A and B) Producer cells were labeled overnight, and the supernatant was collected and centrifuged through a sucrose cushion to pellet the virus. The virus was resuspended, and the viral proteins were immunoprecipitated with antisera to p15E (see Materials and Methods). Pr15E is precursor TM; p15E is processed TM. (A) The viable mutants at 32°C; (B) the *ts* and XC +/− mutants shifted and maintained at the nonpermissive temperature. (C and D) Intracellular pulse-chase of the TM proteins at 32°C (C) and 37°C (D). The producer cells of the *ts* and XC +/− mutants were labeled for 2 h and chased for 3 h. The cells were lysed, and the cell lysates were centrifuged. The intracellular proteins were immunoprecipitated from the supernatant with antisera to p15E.

of wild-type cell-associated TM reached a maximum after a 2-h chase, while the maximum level of cell-associated TM for the XC +/− mutants was reached after 1 h. Further, the half-life of the TM of *in*7042-12b was shorter than that of wild-type TM (data not shown). Helseth et al. have presented similar data for human immunodeficiency virus type 1 (HIV-1), suggesting that the transmembrane glycoprotein of HIV-1 (gp41) is rapidly degraded following loss of association with HIV-1 SU (16).

The difference between the *ts* mutants in the proline-rich region and the XC +/− mutants could be the stage of the virus life cycle at which SU is shed. Later shedding (*in*6566-12a and *in*6584-12a) would allow wild-type levels of infection and syncytium formation. Shedding of most of SU immediately following budding could result in slower infection rates and low levels of syncytium formation, as seen with *in*6898-12a and *in*7042-12b.

Interestingly, a deletion mutation within the immunosuppressive peptide (7208-7312) of TM which has recently been described for Mason-Pfizer monkey virus has a similar phenotype. This region is conserved among a divergent number of retroviruses (3).

***ts* mutant in the cytoplasmic tail.** At 32°C, mutant *in*7705-12a produced wild-type levels of SU and TM associated with the virus. At 37°C, no SU was associated with the virus, but SU was found in the supernatant. There was a trace amount of TM associated with the virus at 37°C, but intracellular labeling showed that close to wild-type levels of TM were being produced. However, after maintenance of the cells in culture for 5 months, Env proteins associated with the virus were detected at 37°C. This finding may be due to a second-site mutation and is currently being examined.

Analysis of early-passage *in*7705-12a virus suggests that the cytoplasmic tail of TM is responsible for the interaction of the Env proteins with the viral core. These results agree with the data of Granowitz et al. (13), who performed a study in which additional mutants in this area of M-MuLV were constructed. Mutant *in*8125-2 (two nucleotides, GC, were inserted after nucleotide 8125, equivalent to *in*7676-2) encodes an Env protein with a carboxy terminus truncated two amino acid

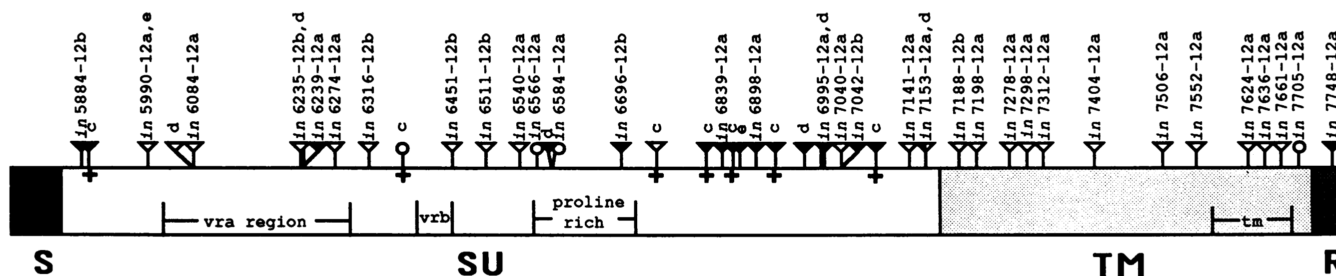

FIG. 4. Summary of envelope mutations from this study and from other published mutagenesis studies (6, 10, 23). The envelope gene product is divided into four regions: S (signal sequence), SU, TM, and R (R peptide). The variable regions vra and vrb and the proline-rich region are indicated (2). The region of TM which spans the membrane is indicated (tm). Linker insertion mutations from these studies are shown with the positions of the mutations and the linker used (a or b). c, Mutations of glycosylation sites (+) (10); d, linker insertion mutations described by Lobel and Goff (23); e, linker insertion mutations described by Colicelli et al. (6). Y, viable mutations; ♀, *ts* mutations; Y, lethal mutations.

residues into the cytoplasmic tail. Failure of the Env proteins to associate with virions is most likely due to loss of its cytoplasmic tail. Another Env protein mutant, *dl*8122-21 (equivalent to *dl*7673), missing seven residues near the cytoplasmic face of the membrane, is also not associated with virions (13). Mutations within the matrix protein of Mason-Pfizer monkey virus can have a profound effect on the amount of TM cytoplasmic domain cleavage that occurs in the maturing virion (4). Truncation of the cytoplasmic domain of HIV-1 gp41 by only six amino acids results in a significant reduction in the amount of virion associated glycoprotein (8), whereas other deletional studies reported have no effect on association of the Env proteins with virions (11). Mutational analysis of the Rous sarcoma virus *env* gene product indicates that the tail of TM is not required in the assembly of infectious virus (29). The Env of mutant *in*7705a is processed much more efficiently than is wild-type Env. The effect of this increased cleavage on the morphology of the virus is of interest.

Summary of mutants. Figure 4 is a combination of data for the linker insertion mutants discussed above and other mutants and information found in the literature (2, 6, 10, 23). Sequence alignments of MuLV indicate regions of SU that are highly variable and highly conserved (9, 21, 26, 27, 36). Variable regions a and b (vra and vrb) are the regions most variable between the envelopes of MuLVs (2). Battini et al. (2) used chimera studies in which they exchanged the vra, vrb, and proline-rich regions of the amphotropic, polytropic, and xenotropic envelopes. Their results suggest that the vra region recognizes the receptor, while downstream regions such as the vrb and proline-rich regions are important for stabilization of the receptor-specific structure on the surface of the virion (2). Our data present a viable mutant, *in*6239-12a, in the vra region. M-MuLV has the largest vra region among the MuLVs compared, and these data may suggest that not all of M-MuLV's vra region is required for recognition of the cell surface receptor.

The facts that the mutants in the proline-rich region, *in*6566-12a and *in*6584-12a, are *ts* and that at the nonpermissive temperature the SU/TM heteropolymer is unstable agree with the suggestion that this region may be important only for structural stability. A mutant in this region, *in*7030-9 (equivalent to *in*6581-9), that is viable has been found by Lobel and Goff (23). It is interesting that these mutants are in a region where the prolines are quite ordered (a proline every third amino acid) compared with the rest of the proline-rich region. This 11-amino-acid region is conserved among related ecotropic, amphotropic, xenotropic, and polytropic MuLVs. Mutant *in*6566-12a disrupts this proline order. Mutant *in*6584-12a maintained this order but increased the distance between the last proline in the ordered structure and the next proline in the proline-rich region. A viable mutant, *in*6696-12b, was found at the carboxy terminus of the proline-rich region, and the proline at the site of this insertion was conserved.

Viable mutants were clustered in the carboxy terminus region of SU. This region is highly conserved among MuLV envelopes (9, 21, 26, 27, 36). From these studies, the carboxy terminus is probably involved in SU/TM noncovalent interactions. The regions of disulfide bonding between SU and TM have also been mapped to the carboxy terminus of SU (30). The two XC +/− mutants are separated by 48 amino acids and may indicate several regions of SU forming contact with TM. The viable mutant *in*6839-12a could also be a region of contact, since it had low levels of SU associated with the virus. The high number of viable mutations may reflect the redundancy of the SU/TM interaction. Mutations at one site may be compensated for by interactions at the other sites. Two other viable mutants in this region, *in*7407-9 and *in*7442-9 (equivalent to *in*6958-9 and *in*6993-9, respectively), have been described by Lobel and Goff (23). Glycosylation mutants at the carboxy terminus are also viable (10). The clustering of viable mutations at the carboxy terminus of SU may also reflect the mechanism of viral entry. The Env protein spikes of MuLV are thought to open upon binding to its receptor to expose its fusion domain located within the extracellular domain of TM (19). For this process to occur, the carboxy terminus of SU would need to be flexible. The viable mutants in this region could suggest this flexible region.

Mutant *in*7153-12a was transfected twice at 37°C and three times at 32°C. *in*7153-12a tested positive for the release of RT once at 32°C. When the low-molecular-weight DNA was extracted from the cells and analyzed, *in*7153-12a did not maintain the *Eco*RI site. Lobel and Goff also presented a viable mutant with an identical insertion in this site but did not determine whether the mutant maintained the *Eco*RI site (23). Mutants *in*5884-12b, *in*6239-12a, *in*6696-12b, *in*6995-12a, *in*7705-12a, and *in*7748-12a maintained the *Eco*RI site at 32°C. Mutant *in*6898-12a maintained the *Eco*RI site at 32 and 37°C. This finding does not eliminate the possibility that a second-site mutation occurred elsewhere in the linker or in the genome.

The TM of the MuLVs is also conserved, but the extracellular and transmembrane domains are intolerant of insertions. In this study, 11 of the 13 mutants that were mapped to TM

were nonviable. One *ts* mutant in TM, *in*7705-12a, was located in the cytoplasmic tail. Our data indicate that in M-MuLV, this region is important for the association between the Env proteins and the viral core. One viable mutant in TM, *in*7748-12a, mapped to the R region. The R region is partially cleaved by the virus-encoded protease in the virus (7, 14). The significance of this processing is unknown.

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