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Mutational Analysis of the N-Linked Glycosylation Sites of the SU Envelope Protein of Moloney Murine Leukemia Virus

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The role of the N-linked glycosylation sites in the major envelope glycoprotein, SU (gp70), of Moloney murine leukemia virus has been examined. By using site-specific oligonucleotide-directed mutagenesis, each of the seven glycan addition sites has been individually eliminated. Mutations resulting in the loss of a single glycosylation site produced, intracellularly, stable precursor SU-TM proteins which were 4 to 5 kDa smaller than the wild-type virus SU-TM protein. Mutant Δ1,4,7, a trimutant lacking three N-linked glycan addition sites, resulted in a viable, infectious virus with a stable SU-TM protein approximately 12 to 15 kDa smaller than the wild-type SU-TM protein. Five of the seven single-site mutations resulted in viable virus as judged by the release of reverse transcriptase in transient-expression assays and XC syncytium assays. Mutations at two of the sites resulted in a detectable phenotype. Virus mutated at position 2 was temperature sensitive in Rat2 cells; viable virus was produced at 32°C but not at 37°C. Virus mutated at position 3 was noninfectious and yielded virions lacking detectable mature SU protein. The mutation results in the block of transport of the protein to the cell surface and assembly into virion particles.

The entry of a retrovirus into the host cell involves the interaction between the viral envelope gene product and the host cell surface receptor. Murine leukemia viruses (MuLV) have been classified on the basis of their host range as ecotropic, amphotropic, polytropic, and xenotropic. Ecotropic viruses replicate only in mouse or rat cells. Interference assays show that the different classes of MuLV recognize their own specific host receptor (26, 27). The ecotropic MuLV receptor has been identified (1) and encodes a cationic amino acid transporter molecule (13, 38).

Moloney MuLV (M-MuLV) is an ecotropic virus. The viral *env* gene is initially expressed as a precursor protein, which is proteolytically cleaved to yield two virion-associated proteins, the surface (SU) and transmembrane (TM) proteins. The precursor SU-TM protein is extensively glycosylated. The number of glycosylation sites varies within the ecotropic murine retroviruses as well as between the related polytropic, amphotropic, and xenotropic viruses. There are seven N-linked glycosylation sites within SU of M-MuLV. In related MuLVs, all of these sites are glycosylated (29). The N-linked glycosylation site in TM is not utilized because of an adjacent proline (2). One O-linked glycosylation site has also been identified (23).

In the transport through the endoplasmic reticulum and Golgi apparatus to the cell surface, several distinct processing intermediates have been described. First the initial glycosylated product containing the core oligosaccharides is detected as an 80-kDa protein ($gPr80^{env}$). This is followed by processing of the core structure, in which a 90-kDa intermediate ($gPr90^{env}$) is detected and is largely resistant to endoglycosidase H. After the addition of fucose in the Golgi apparatus, the protein is proteolytically cleaved to yield the products SU (gp70) and TM (20). These products are then assembled with the virion core structure. Additional processing of the cytoplasmic tail of TM also occurs.

Although there is no universal function for carbohydrates (7, 24), roles for oligosaccharides have varied from intracellular transport (17) to virus-receptor interactions (39). Chemical addition of lactose has been successful in redirecting MuLV to infect via a novel receptor (19). Early studies on MuLV with tunicamycin and other chemical inhibitors have shown that glycosylation is essential for the transport and processing of the *env* gene products (28, 32). The significance of its specific N-linked glycans, however, is just beginning to be understood (12). To examine the role of the N-linked glycans in the life cycle of the virus, we used oligonucleotide-directed mutagenesis to generate mutants of M-MuLV that lacked specific N-linked glycosylation sites. Five of these seven mutants resulted in the release of viable infectious virus, as judged by the release of reverse transcriptase in transient-expression assays and formation of XC syncytia. Mutant Δ2, a temperature-sensitive mutant, was viable at both 32 and 37°C in NIH 3T3 cells but only at 32°C in Rat1 and Rat2 cells. A mutation altering position 3 was lethal to the virus.

MATERIALS AND METHODS

Cells, viruses, and infections. NIH 3T3, Rat2, and Rat1 cells were grown in Dulbecco modified Eagle medium (GIBCO) supplemented with 10% (vol/vol) calf serum (HyClone). Wild-type M-MuLV was derived from clone pNCA-C, a full-length M-MuLV provirus in which the *Cla*I site of pBR322 of pNCA (4) has been removed. Mutants of M-MuLV were tested for replication competence by the XC assay (31) and by the release of reverse transcriptase into the medium (9) after introduction of cloned DNA (0.25 μg/10^5 cells) into cells by the DEAE-dextran method (18). Producer Rat1 or Rat2 cell lines expressing mutant genomes were established after either dimethyl sulfoxide-Polybrene-mediated (11) or calcium phosphate-mediated (3, 40, 41) cotransformation with pSV2neo DNA (34). Individual colonies were selected for growth in the presence of the drug G418 (400 μg/ml; GIBCO) and assayed for release of reverse transcriptase activity (9). Mutant Δ3 colonies were assayed for

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the presence of the envelope by Western immunoblotting with envelope antiserum (no. 79S000771; National Cancer Institute). Mutants Δ3, Δ5, and the trimutant Δ1,4,7 producer cell lines were expressed in Rat1 cells; mutants Δ1, Δ2, Δ4, Δ6, and Δ7 were expressed in Rat2 cells. Virus (5 ml) used for infection was collected after 12 h from 10-cm plates. Infections of Rat1 and Rat2 cells were carried out in the presence of Polybrene (8 μg/ml).

Mutagenesis and mutant reconstruction. Each of the seven asparagine codons was individually altered, eliminating the potential N-linked glycosylation sites. The 3,335-bp *Hin*dIII-*Sac*I fragment of pNCA-C was subcloned into M13mp18, and this was designated pHS. Mutagenesis was carried out by the method of Kunkel et al. (14, 15). Mutagenic oligonucleotide primers contained novel restriction sites, which were used for the screening of the resultant plasmids (pHS1 to pHS8) (see Table 1). Viral genomes bearing the individual mutations Δ1, Δ2, Δ4, Δ6, and Δ7 were reconstructed by exchanging the 2,780-bp *Hin*dIII-*Cla*I fragment with the wild-type sequence in pNCA-C (pNCA-CΔ1, pNCACΔ2, pNCACΔ4, pNCACΔ6, and pNCACΔ7). For the DEAE-dextran transient-expression assay, mutant Δ2 was further reconstructed. The *Bst*EII-*Bam*HI fragment (nucleotides 6372 to 6986 on the viral DNA) was isolated and subcloned into pSCB150-18 (30). The *Hin*dIII-*Cla*I 2,780-bp fragment bearing the mutation was isolated and subcloned into pNCA-C, replacing the wild-type sequence. The *Bst*EII-*Bam*HI fragment was sequenced in its entirety. The predicted two nucleotide changes originally encoded by the mutagenic oligonucleotides (see Table 1) were the only differences from the wild type detected. Mutants Δ3 and Δ5 were reconstructed into the viral genome by digesting pHS3 and pHS5 with *Bam*HI and *Nsi*I (New England BioLabs). The 517-bp fragment was subcloned into pSCB150-18 (30). These plasmids were then digested with *Hin*dIII-*Cla*I, and the 2,780-bp fragment was subcloned into pNCA-C, creating the mutant proviruses pNCA-CΔ3 and pNCA-CΔ5. The 517-bp region between the *Bam*HI and *Nsi*I restriction sites was sequenced in its entirety. The only nucleotide which differed from the wild-type sequence was that encoded by the mutagenic oligonucleotide (see Table 1). Mutant Δ7 was further reconstructed by subcloning the 620-bp *Nsi*I-*Cla*I fragment of pNCA-CΔ7 into pNCA-C. This plasmid was then digested with *Hpa*I (New England BioLabs), and the 1,379-bp fragment was again ligated into pNCA-C. Mutant Δ1,4,7, a trimutant lacking three N-linked glycan addition sites, was constructed by using pNCA-CΔ7, pNCA-CΔ4, and pNCA-CΔ1, consisting of the pNCA-CΔ7 *Sph*I-*Nsi*I (9,170-bp), pNCA-CΔ4 *Bam*HI-*Nsi*I (517-bp), and pNCA-CΔ1 *Bam*HI-*Sph*I (1,400-bp) fragments. All pNCA-C mutants were subsequently screened for the presence of the new restriction sites (see Table 1).

Antisera, cell labeling, and immunoprecipitations. Goat anti-Env serum and goat anti-Gag serum (no. 79S000771 and 77S000158, respectively) were obtained from the National Cancer Institute. Rabbit anti-Pol serum was raised against fusion proteins produced in *Escherichia coli* (37). To detect intracellular proteins, cells (on 10-cm plates) were rinsed and incubated in 3 ml of Hanks' buffered saline (GIBCO) for 20 min and then labeled with Trans-label ([^{35}S]Met and [^{35}S]Cys) (100 μCi per plate; ICN) for 40 min. For pulse-chase labeled intracellular proteins, the supernatants were removed after the 40-minute labeling period. The cells were then incubated for 2, 4, 6, or 8 h in 10 ml of Dulbecco modified Eagle medium supplemented with 10% calf serum. The cells were lysed in 1× PLB (10 mM Na_2HPO_4-NaH_2PO_4

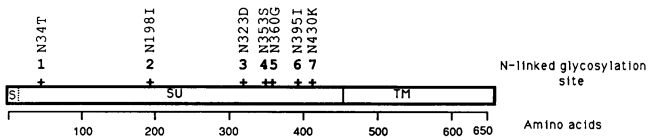

FIG. 1. Schematic diagram of the M-MuLV envelope gene product. Proteolytic processing of the precursor protein releases the signal peptide (S), the surface protein (SU) and the transmembrane protein (TM). Additional proteolytic processing of TM occurs at the C terminus (not shown). The positions of the seven N-linked glycosylation sites (Asn-X-Ser/Thr) are indicated (+) at the top of the SU protein. The sites are numbered sequentially from the N to the C terminus of the protein and are used to name the specific mutations (Table 1). Specific amino acid substitutions are indicated above each glycosylation site.

[pH 7.5], 100 mM NaCl, 0.1% sodium dodecyl sulfate [SDS], 0.5% deoxycholate, 1% Triton X-100; 3 ml) precleared with normal goat serum (GIBCO) (10 μl of normal goat serum, 700 μl of supernatant) and immunoprecipitated with antiserum (3 μl of anti-Env, 4 μl of anti-Gag, or 4 μl of anti-Pol) as previously described (37). To detect virion proteins, the producer cell lines (on 10 cm plates) were incubated in Hanks' buffered saline (40 min), labeled with Trans-label (150 μCi/plate; ICN) in 5 ml of Dulbecco modified Eagle medium (without methionine) for 2 h, and subjected to continued labeling overnight in 2.5% dialyzed calf serum (GIBCO). The virions were collected, pelleted through a 25% sucrose cushion, and immunoprecipitated as described previously (37). Molecular weight markers were obtained from Sigma. Immunoprecipitated proteins were analyzed on SDS-polyacrylamide gel electrophoresis (PAGE) gels by the method of Laemmli (16).

Treatment with endoglycosidase H or endoglycosidase F–*N*-glycosidase F. When indicated, samples were treated with either endoglycosidase H or endoglycosidase F–*N*-glycosidase F (Boehringer Mannheim) prior to SDS-PAGE. Endoglycosidase H reaction mixtures (10 to 50 μl) contained 25 mM sodium acetate (pH 5.2), 1.2 mM phenylmethylsulfonyl fluoride, 1 μg of bovine serum albumin, and 1 mU of endoglycosidase H. Envelope proteins subjected to endoglycosidase F–*N*-glycosidase F treatment were boiled for 2 min in sample buffer containing 2% SDS. Reaction mixtures (final volume, 10 to 50 μl) contained 25 mM sodium acetate (pH 5.2), 1 μg of bovine serum albumin, 1.2 mM phenylmethylsulfonyl fluoride, and 50 mU of endoglycosidase F–*N*-glycosidase F. *n*-Octyl-β-D-glucopyranoside was added in a 1:1 (vol/vol) ratio with SDS. SDS varied between 0.14 and 0.86%, depending on the volume of protein fraction in the sample buffer added. Digestions were carried out at 37°C for at least 16 h.

RESULTS

Mutagenesis of the M-MuLV *env* gene N-linked glycosylation sites. The region of the M-MuLV *env* gene which encodes the SU protein contains seven N-linked glycosylation sites (Asn-X-Ser/Thr). The relative positions of these sites are indicated in Fig. 1. The glycosylation site indicated as position 2 in M-MuLV is unique to the ecotropic viruses. Each of the individual N-linked glycosylation sites was abolished by the substitution of the Asn by an alternative amino acid. The oligonucleotide mutagenesis is summarized in Table 1. In addition, a construct which contained the three mutations at positions 1, 4, and 7 was generated.

TABLE 1. Mutagenic oligonucleotide primers

Mutant[a]	Site[b]	Oligonucleotide sequence[c]	Restriction site[d]	Amino acid[e]
1	6359	CATCAAGTgTATAcTATCACCTGG	GTATAC (*Acc*I)	N34T
2	6821	CAGTAAACAAtAtTCTCACCTCTG	AATATT (*Ssp*I)	N198I
3	7201	CAAGCCCTCgACCTCACC	TCGA (*Taq*I)	N323D
4	7298	GTACCTACTCgAgCCATACCTCTG	CTCGAG (*Xho*I)	N353S
5	7318/9	GCTCCAGCCggCTGCTCCGTG	GCCGGC (*Nae*I)	N360G
6	7418	GCCCTATGcAtTACCACCCAG	ATGCAT (*Ava*III)	N395I
7	7527	CCATACTGAAgCTTACCACTG	AAGCTT (*Hin*dIII)	N430K

[a] Mutants 1 to 7 are as indicated in Fig. 1.
[b] The positions of the mutations in the DNA sequence of M-MuLV are indicated.
[c] The oligonucleotides used in the mutagenesis are shown. Base changes are indicated by lowercase letters. Nucleotides in bold result in amino acid substitutions.
[d] The restriction enzyme sites generated in the mutagenesis are shown.
[e] Mutagenesis resulted in the substitution of the Asn (N) residues by the amino acids indicated. Numbering refers to the amino acid position in the SU protein.

Analysis of mutant viability. Complete M-MuLV proviral genomes containing the various mutations were constructed and introduced into NIH 3T3 (Fig. 2A and B) and Rat2 (Fig. 2C and D; data not shown) cells by the DEAE-dextran method. Transient expression of the plasmid allows for the release of viral particles and the subsequent reinfection of cells. Viability of these mutant viruses was assayed by the release of reverse transcriptase into the supernatant medium with time. In NIH 3T3 cells, only the glycosylation mutation at position 3 was found to be nonviable (Fig. 2A and B, lanes 4). Mutations at position 1 (lanes 2), 2 (lanes 3), 4 (lane 5), 5 (lanes 6), and 7 (lanes 8), as well as in the trimutant Δ1,4,7 (lane 9), spread with a similar time course to that of wild-type virus (lane 10). All cell lines which were positive for reverse transcriptase formed syncytia when overlaid with XC cells (data not shown).

Mutational analyses of proteins have frequently revealed temperature-sensitive phenotypes (5, 25, 36). Transient expression of the glycosylation mutants in NIH 3T3 cells resulted in similar results when the cells were maintained at 32°C (Fig. 2A) and 37°C (Fig. 2B). Interestingly, in Rat2 cells, the mutation at position 2 resulted in a temperature-sensitive phenotype. Mutant virus Δ2 was viable at 32 but not 37°C (Fig. 2C and D, lanes 2). Identical results were found in Rat1 cells (data not shown).

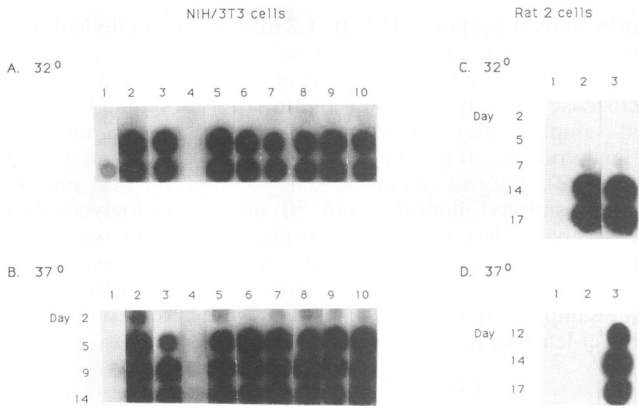

FIG. 2. Time course of infection of N-linked glycosylation mutants. (A and B) NIH 3T3 cells. (C and D) Rat2 cells. Plasmid DNA was introduced into cells by the DEAE-dextran method, allowing transient expression of the virus. Supernatant medium (10 μl of 10 ml) from cells maintained at 32°C (panels A and C) or at 37°C (panels B and D) was collected and assayed for the presence of the virally encoded reverse transcriptase. The number of days after introduction of the DNA is indicated on the left. (A and B) Lanes: 1, NIH 3T3 cells, mock infection; 2, mutant Δ1; 3, mutant Δ2; 4, mutant Δ3; 5, mutant Δ4; 6, mutant Δ5; 7, mutant Δ6; 8, mutant Δ7; 9, mutant Δ1,4,7; 10, pNCA-C, wild-type M-MuLV. (C and D) Temperature sensitivity of mutant Δ2 in Rat2 cells. Lanes: 1, Rat2 cells, mock infection; 2, mutant Δ2; 3, pNCA-C, wild-type M-MuLV.

Analysis of intracellular proteins. Stable producer cell lines were generated in Rat1 or Rat2 cells for each of the glycosylation mutants, and the *env* gene products were examined. Cells were metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine for 40 min and immunoprecipitated with antiserum raised against the *env* gene product (Fig. 3). The wild-type precursor *env* gene product migrated as a species of approximately 86 kDa, corresponding to the intermediate gPr80^{env}. Each of the mutant proteins in which one glycosylation site was removed resulted in a protein species that was on average 4 kDa smaller than the wild-type envelope protein (Fig. 3A, lanes 3 to 9). The protein in which three N-linked glycosylation sites were removed (Δ1,4,7) was on average 11 kDa smaller than the wild-type protein (Fig. 3A, lane 10). To determine whether these mobility shifts were in fact a result of the loss of glycan, the proteins were treated with endoglycosidase H (Fig. 3B) and endoglycosidase F–*N*-glycosidase F (Fig. 3C). In Fig. 3B and C, untreated wild-type product (lanes 16), the Δ1,4,7 trimutant (lanes 15), and an exemplary mutant with one site removed (lanes 14) were included for comparison. Treatment of all the mutants (lanes 2 to 10) with either glycosidase yielded a protein product whose migration was identical with that of the wild-type protein (lanes 11). The endoglysidase H and endoglycosidase F products shifted the mobility of the protein by approximately 22 and 26 kDa, respectively, corresponding to the loss of 7 glycan additions. This difference in mobility reflects the specificities of the enzymes; endoglycosidase F–*N*-glycosidase F removes the entire oligosaccharide, whereas endoglycosidase H leaves a terminal *N*-acetylglucosamine residue attached to the asparagine. The higher mobility of the mutant protein observed in Fig. 3A is therefore due to the absence of specific glycans.

Producer cell lines were also tested for the other viral gene products. Immunoprecipitation with *gag*-specific antibodies detected a major p65 precursor protein as well as the minor glycosylated protein, gp80; *pol*-specific antibodies detected a protein of approximately 200 kDa representing the unprocessed Gag-Pol precursor protein (data not shown).

Analysis of viral proteins. The protein products associated with viral particles were examined by immunoprecipitation and SDS-PAGE analysis (Fig. 4). Viral SU proteins could be

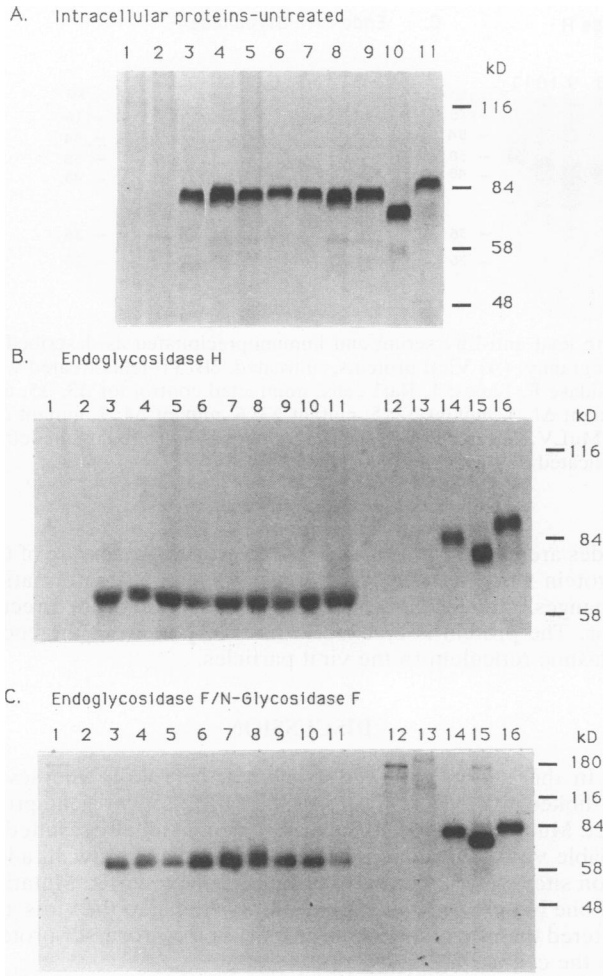

FIG. 3. Analysis of intracellular proteins. Cells were metabolically labeled with [^{35}S]Cys and [^{35}S]Met (Trans-label) immunoprecipitated with goat anti-Env serum (see Materials and Methods). Proteins were analyzed by SDS-PAGE and fluorography. Lanes: 1 and 12, Rat1 cells, uninfected control for Δ3, Δ5, and Δ1,4,7; 2 and 13, Rat2 cells, uninfected control for Δ1, Δ2, Δ4, Δ6, and Δ7; 3, mutant Δ1; 4, mutant Δ2; 5 and 14, mutant Δ3; 6, mutant Δ4; 7, mutant Δ5; 8, mutant Δ6; 9, mutant Δ7; 10 and 15, mutant Δ1,4,7; 11 and 16, pNCA-C. (A) Untreated control. (B) Lanes 1 to 11, proteins treated with endoglycosidase H; lanes 12 to 16, untreated controls. (C) Lanes 1 to 11, proteins treated with endoglycosidase F–*N*-glycosidase F; lanes 12 to 16, untreated controls. The migrations of the prestained molecular mass markers are indicated at the right of each panel.

detected in mutants Δ1 (lane 3), Δ2 (lane 4), Δ4 to Δ7 (lanes 6 to 9, respectively), and Δ1,4,7 (lane 11) and the wild-type M-MuLV (lane 12). Mutant Δ3 viral particles possessed no envelope protein (Fig. 4A, lane 5). A protein product at M_r = 85,000 is detectable; however, this is unlikely to be an SU product because it is present in control lanes (lane 2) and is insensitive to treatment with both endoglycosidase H (Fig. 4B) and endoglycosidase F–*N*-glycosidase F (Fig. 4C). The lack of envelope protein associated with mutant Δ3 viral particles would result in nonviable virus, as observed above for the DEAE-dextran transient-expression assay.

The mobilities of the viral SU proteins varied greatly and did not directly correspond to the simple lack of a glycan. Some of the variability may be due to differences in the glycosylation pathways of the closely related Rat1 and Rat2 cells. However, within one cell type, variability is clearly seen. Mutant Δ4 (Rat2 cells; Fig. 4A, lane 6) and mutant Δ6 (Rat2 cells; Fig. 4A, lane 8) both lack a single glycosylation site yet differ in mobility by approximately 15.5 kDa. This variability may be caused by altered levels of processing of the viral envelope proteins. This was examined further by treatment with endoglycosidase H (Fig. 4B) and endoglycosidase F–*N*-glycosidase F (Fig. 4C).

The oligosaccharides associated with the viral SU protein are predominantly complex; however, endoglycosidase H-sensitive glycans have been reported (21, 22). Treatment of the wild-type M-MuLV produced in Rat2 cells with endoglycosidase H caused a small shift in the SU protein (Fig. 4B, lane 11). The sensitivities of the mutants to endoglycosidase H digestion varied; the products shifted in mobilities and appeared more heterogenous and diffuse (Fig. 4B, lanes 3 to 10). Digestion of all the mutant SU products (Fig. 4C, lanes 3 to 10) with endoglycosidase F–*N*-glycosidase F yielded products which migrated identically to the wild-type product (Fig. 4C, lane 11). The variation in mobility of the SU products therefore is a result of heterogeneity in the oligosaccharides. This heterogeneity has little effect on the virus, because as all of the mutants which contained envelope on their viral particles were viable.

The absence of SU protein released into the medium in mutant Δ3 could simply reflect the low level of production of viral particles from this cell line. To eliminate this possibility, we isolated viral particles and immunoprecipitated the individual proteins. Figure 5 shows the presence of the CA (capsid) protein encoded by the *gag* gene within the viral particle. CA was detected in all of the cell lines, including the mutant Δ3 producer line. The amount of CA detected varied from one line to another. Mutant Δ3 clearly contained at least as much CA as the other viable viruses did. Reverse transcriptase and integrase were also detected in the viral particles (data not shown). Mutant Δ3 therefore releases viral particles which lack the SU protein.

Pulse-chase analysis of intracellular proteins. Analysis of mutant Δ3 indicates that the protein is made intracellularly, yet no protein is detected associated with the virus and the virus is nonviable. Processing of the intracellular precursor involves multiple steps including proteolytic cleavage and modification of the oligosaccharides. The fate of the intracellular protein was examined by pulse-chase analysis. Proteins were metabolically labeled for 40 min, the radioactivity was removed, and the cells were incubated with medium containing calf serum for 2, 4, 6, and 8 h. To determine whether the mutant Δ3 protein could be correctly proteolytically processed, the intracellular *env* protein was immunoprecipitated before (Fig. 6A) and after (Fig. 6B) complete removal of the oligosaccharides by endoglycosidase F–*N*-glycosidase F. In the absence of endoglycosidase F–*N*-glycosidase F, wild-type precursor protein was detectable at the 2-h time point (Fig. 6A, lane 3), as was a heterogeneous population of faster-migrating products, representing processed proteins. These processed products are a combination of the proteolytic and oligosaccharide modifications. Digestion of these wild-type *env* gene products with endoglycosidase F–*N*-glycosidase F would remove all the glycans, revealing the level of proteolytic processing (Fig. 6B). After 2 h the endoglycosidase F–*N*-glycosidase F treated wild-type *env* gene product appeared as two species, a 69-kDa protein and a second protein that is approximately 17 kDa smaller. These products correspond to the predicted molecular mass, based on the primary sequence, of the precursor SU-TM

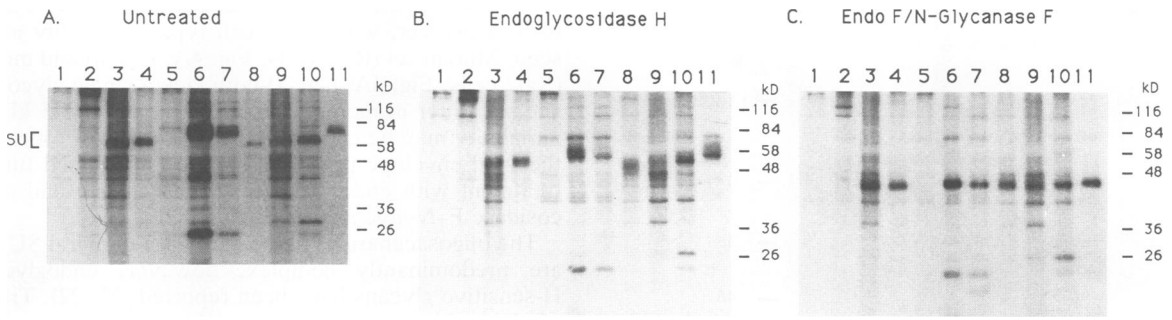

FIG. 4. Analysis of virion proteins. Viral particles were incubated with goat anti-Env serum and immunoprecipitated as described in Materials and Methods. Proteins were analyzed by SDS-PAGE and fluorography. (A) Viral proteins, untreated. (B) Proteins treated with endoglycosidase H. (C) Proteins treated with endoglycosidase F–*N*-glycosidase F. Lanes: 1, Rat1 cells, uninfected control for Δ3, Δ5, and Δ1,4,7; 2, Rat2 cells, uninfected control for Δ1, Δ2, Δ4, Δ6, and Δ7; 3, mutant Δ1; 4, mutant Δ2; 5, mutant Δ3; 6, mutant Δ4; 7, mutant Δ5; 8, mutant Δ6; 9, mutant Δ7; 10, mutant Δ1,4,7; 11, pNCA-C, wild-type M-MuLV. The position of the SU proteins is indicated on the left of panel A. The migrations of the prestained molecular mass markers are indicated on the right of each panel.

protein and the proteolytically processed SU domain, respectively. After 4 h no precursor product could be detected in the wild-type cells; most of the species had been proteolytically processed (Fig. 6B, lane 5). Intracellularly, the mutant Δ3 precursor product was initially expressed at high levels (lanes 2) and remained abundant even after 4 and 6 h (Fig. 6A, lanes 4 and 6, respectively). Mutant Δ3 migrated faster than the wild-type protein, as seen previously, owing to the absence of the glycan. At 2 h no processed products were detected (lanes 2). Two minor processed forms appeared during the 8-h chase; their migration differed greatly from that of the wild-type protein. Endoglycosidase F–*N*-glycosidase F treatment of the mutant Δ3 protein revealed no correctly proteolytic processed products up to 8 h postchase (Fig. 6B, lanes 2, 4, 6, and 8). A low level of processed proteins whose mobility is vastly different from that of the wild-type protein could be detected and probably represents the cellular degradation of the accumulated protein. Proteolytic processing of the precursor protein occurs within the Golgi apparatus at a position downstream of fucose addition (20). The results of these studies show that mutant Δ3 is correctly synthesized and six high-mannose oligosaccharides are initially attached. The proteolytic processing of the protein does not occur, because of either conformation changes of the protein or cellular localization of the precursor. The protein is blocked in its transport from the endoplasmic reticulum to the viral particles.

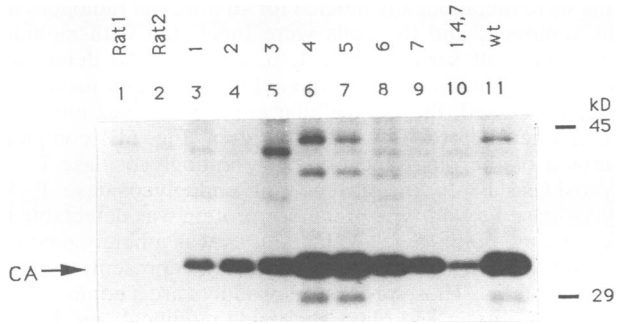

FIG. 5. Analysis of virion proteins. Virus particles were incubated with goat anti-Gag serum and immunoprecipitated as described in Materials and Methods. Lanes: 1, Rat1 cells, uninfected control; 2, Rat2 cells, uninfected control; 3, mutant Δ1; 4, mutant Δ2; 5, mutant Δ3; 6, mutant Δ4; 7, mutant Δ5; 8, mutant Δ6; 9, mutant Δ7; 10, mutant Δ1,4,7; 11, pNCA-C, wild-type M-MuLV. The position of migration of the CA (capsid) protein is indicated on the left; the migrations of the molecular mass markers are shown on the right.

DISCUSSION

In this study, we have examined the role of the seven N-linked glycosylation sites of the M-MuLV *env* gene product. Mutation of six of the seven sites individually resulted in viable virus. Proteins in which three N-linked glycan addition sites were altered also produced viable virus. Mutation of one N-linked glycosylation site was lethal to the virus and altered the processing and transport of the precursor protein to the cell surface and virus.

Recently, a similar analysis of the N-linked glycosylation site has been performed with Friend MuLV (12). There are eight N-linked glycosylation sites in the SU protein of Friend MuLV, compared with the seven sites of M-MuLV. One N-linked glycosylation site, at position 4, was lethal to Friend MuLV. This is homologous with position 3 of M-MuLV and results in a similar phenotype. In the studies of Friend MuLV, all of the mutations were designed to substitute the Asn with an Asp. The mutagenesis scheme we used resulted in the substitution of the Asn with a variety of amino acids. Only the mutation at position 3 resulted in the Asn-to-Asp substitution and yielded the lethal phenotype. The mutant viruses which were viable sustained the loss of the glycan as well as the insertion of a random amino acid, including lysine at position 7. It is possible that the lethality at position 3 is not simply due to the loss of the glycan. The specific substitution of Asp or a local conformational change may cause the phenotype. For human immunodeficiency virus, mutation of a single glycosylation site blocked infectivity (42). However, mutations of neighboring amino acids with the glycosylation site intact also resulted in a similar phenotype (42). Additional mutagenesis at and around position 3 of M-MuLV is required to resolve this question.

The amino-terminal two glycosylation sites are within the region of SU which is involved in binding to the ecotropic receptor (10). The temperature sensitivity of the glycosylation site Δ2 in Rat2 cells with respect to that in NIH 3T3 cells is therefore intriguing. We have performed this assay six times with identical results. Temperature sensitivity was

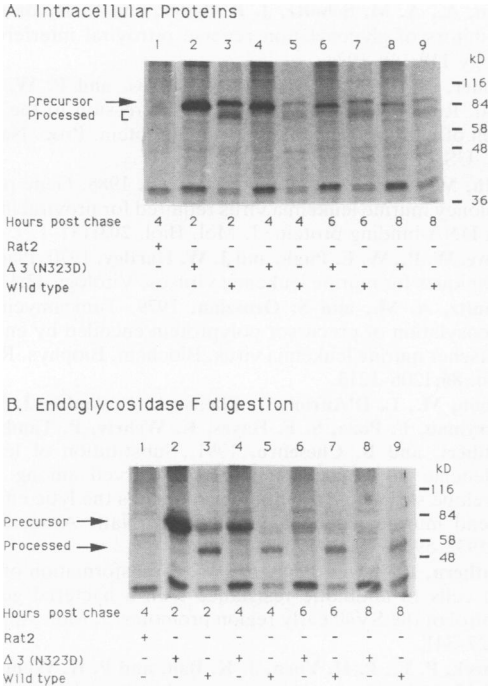

FIG. 6. Pulse-chase analysis of intracellular proteins. Cells were pulse-labeled with [^{35}S]methionine for 40 min, the radioactivity was removed, and cultures were incubated with unlabeled methionine for various times (see Materials and Methods). (A) After the lysate had been precleared, proteins were immunoprecipitated with goat anti-Env serum. (B) Part of the sample was treated with endoglycosidase F–*N*-glycosidase F. Lanes: 1, Rat2 cells; 2, 4, 6, and 8, mutant Δ3; 3, 5, 7, and 9, pNCA-C wild-type M-MuLV. The hours postchase are indicated at the bottom of each panel. The positions of the precursor and the processed *env* gene products are indicated at the left. The migrations of the molecular mass markers are shown at the right.

found in Rat1 cells as well, indicating that there was nothing unusual about the Rat2 cells. The degree of homology between the mouse and rat ecotropic receptors has not been examined. Differences in the receptors may be sensitive to small changes in the structure of the envelope protein around this region.

The migration of the processed SU proteins associated with the virus varied. Part of the heterogeneity may correspond to differences in cell-specific glycosylation. Alternatively, the variations may be a result of alternative processing of the oligosaccharides. With either mechanism, the net result is SU proteins that differ in their oligosaccharide content. The virus, however, is functional despite this large variation in its glycans. It is unlikely that a specific oligosaccharide conformation is required for the binding of the virus with and its entry into the target cell.

Although the six glycosylation sites are not required for the virus to function in tissue culture, they may have a function in the mouse. Glycosylation is known to mask antigenic regions of the virus. G_{IX} is an antigenic determinant expressed on thymocytes of some mouse strains that corresponds to the loss of a glycosylation site of the envelope gene of an endogenous retrovirus (29). The G_{IX} marker is equivalent to the position 7 glycosylation site of MuLV. Glycosylation of this site masks the antigenic determinant. The effect of expression of the mutant Δ3 protein in vivo is also unknown. Accumulation of unprocessed *env* gene products has been correlated with the neurovirulence of the *ts*1 mutant of M-MuLV (35).

Four of the six nonessential glycosylation sites map to the C terminus of the SU protein. Recent data in our laboratory have shown that this region is also tolerant of multiple linker insertions. The C terminus of SU appears to be flexible and has less stringent structural requirements than do other regions of the protein.

The mutation at position 3 blocks the processing of the precursor protein. Under conditions where a large amount of wild-type protein has become endoglycosidase H resistant, very little endoglycosidase H-resistant Δ3 protein could be detected (data not shown). A large percentage of the mutant is therefore blocked in its transport out of the endoplasmic reticulum. Indirect immunofluorescence of the cells expressing the Δ3 mutant indicates the intracellular accumulation of the protein (data not shown).

Virus lacking glycosylation sites 1, 4, and 7 is still viable. Whether the position 3 glycosylation site alone is sufficient for the correct processing and transport of the protein remains to be examined. Friend spleen focus-forming virus retroviral *env* gene products contain this region, however, and are still blocked in the transport from the endoplasmic reticulum (6).

Critical domains essential for the correct folding or dimerization of the precursor protein may be located at or near glycosylation site 3. Incorrect disulfide dimerization is shown to block the release of Friend spleen focus-forming virus protein from the rough endoplasmic reticulum (8). Glycosylation site 3 is within 8 amino acids of a highly conserved CWLCL sequence (33), which could function directly in protein oligomerization. In Friend MuLV, a conservative Leu-to-Ile substitution within the CWLCL region has been found to attenuate the lytic effect of the virus (33).

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