JOURNAL OF VIROLOGY, June 1994, p. 3857–3867
0022-538X/94/$04.00+0

Vol. 68, No. 6

A Nonstructural *gag*-Encoded Glycoprotein Precursor Is Necessary for Efficient Spreading and Pathogenesis of Murine Leukemia Viruses†

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Received 2 December 1993/Accepted 8 March 1994

In addition to the Gag-Pol and Env precursors whose translation initiates at AUG codons, murine, feline, and simian type C oncoviruses also express glycosylated Gag-Pol precursors (glycoGag). glycoGag translation is initiated at CUG codons located upstream of the Gag AUG initiation codon. In contrast to Gag, glycoGag is translocated into the endoplasmic reticulum and is absent from virions. Since glycoGag has been described to be dispensable ex vivo, we investigated the in vivo effects of a glycoGag$^-$ mutation in the Friend murine leukemia virus (F-MuLV). F-MuLV induces severe early hemolytic anemia and subsequent erythroleukemia within 2 months after inoculation of newborn mice. We obtained a glycoGag$^-$ F-MuLV, strain H5, by inserting an octanucleotide linker downstream of the CUG codon leading to the reading of a stop codon in all reading frames upstream of the Gag AUG. F-MuLV H5 did not induce severe early hemolytic anemia, and latency of erythroleukemia was significantly increased most likely because of an approximately 1-week delay in the in vivo spreading. Accordingly, induction of recombinant polytropic viruses was also significantly delayed. Close examination of ex vivo spreading kinetics also showed a slower dissemination of F-MuLV H5. Western blot (immunoblot) performed after inoculation of newborn mice with this glycoGag$^-$ virus indicated the emergence of new glycoGag$^+$ viruses. PCR analyses with F-MuLV-specific primers demonstrated in vivo pseudoreversions restoring the glycoGag reading frame. Our results demonstrated that glycoGag expression is positively selected and essential for full spreading and pathogenic abilities.

Lentiviruses, spumaviruses, and oncoviruses from the human T-cell leukemia virus-bovine leukemia virus group express nonstructural proteins which are produced through additional splicings or alternative initiations of translation of the *env* subgenomic mRNA. Most of these proteins are not incorporated into virions, seem to be dispensable for viral replication, and are thought to be involved in the control of viral expression (7). As opposed to these viruses, which are designated as complex, most of the oncoviruses are designated as simple, in that they are believed to lack nonstructural regulatory proteins, although certain groups among the latter also encode a set of proteins which are not incorporated into virions. Thus, the Gross cell surface antigen (GCSA), initially identified antigenically, is membrane associated and is specifically expressed in cells infected with murine leukemia viruses (MuLV) (26) and is not present in the virions (10, 29). This antigen reacts with antibodies directed against the different *gag*-encoded viral proteins (MA, p12, CA, and NC) (11, 21, 22, 35, 36, 43, 46), and it has been shown that GCSA and the canonic Pr65^{gag} polyprotein precursor are two alternative translational products of the *gag* open reading frame which are processed via completely separate pathways (10, 13). The canonic Pr65^{gag} of MuLV anchors to the internal side of membranes through its N-terminal myristoyl moiety, whereas the GCSA is cotranslationally translocated into the endoplasmic reticulum as an alternative Pr75^{gag} precursor. After production of an intermediate gPr80^{gag} glycosylated molecule, this alternative Pr75^{gag} leads to the mature (externally oriented) plasma membrane-associated gp95^{gag} glycoprotein (10, 13, 35). gp95^{gag} can be cleaved to a gp85^{gag} form lacking NC-related sequences (22, 29). Other membrane-bound amino-terminal fragments (24, 29) and soluble fragments have also been described (10). The GCSA molecular mass is higher than that of the canonic Pr65^{gag}, because of both glycosylations and additional N-terminal peptidic sequences (11, 35–37). The origin of the GCSA-specific amino-terminal peptide, which has been suggested to function as a signal peptide, had been a matter of controversy. We have previously demonstrated that it is encoded by the *gag* open reading frame, from two in-frame alternative CUG initiation codons upstream from the canonical AUG initiation codon of Pr65^{gag} (31; unpublished data).

Many MuLV which encode GCSA have been described, and cells infected with several feline or simian type C oncoviruses also express on their surfaces *gag*-related glycoproteins (1, 25). All these viruses have an open reading frame upstream from the Gag initiator with similar genetic organization and whose deduced amino acid sequences share similar structural features. Moreover, it has been recently demonstrated that human immunodeficiency virus type 1-infected cells also express at their surfaces large glycosylated proteins recognized by several monoclonal antibodies directed against *gag*-encoded proteins of human immunodeficiency virus type 1 (18, 30, 38). Selection of these conserved glycosylated forms of Gag, which we will refer to as glycoGag, among various retroviral species suggests that these nonstructural proteins are likely to exert important functions during retroviral selection. Several MuLV

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† Dedicated to the memory of Gérard de Billy.

mutants lacking glycoGag have been derived. These mutants are replication competent, and ex vivo studies failed to reveal patent phenotypic differences with their wild-type counterparts (15, 37).

Nonstructural lentiviral proteins, although readily dispensable for ex vivo replication, have been shown to harbor functions poorly evaluated ex vivo and which appear to confer selective advantages in vivo (19). Since such dispensable coding features have never been evaluated in small rodent retroviral models, we searched to readdress the importance of glycoGag in vivo. For this purpose, we derived a glycoGag$^-$ mutant from strain 57 of the Friend-MuLV (F-MuLV), a retrovirus which induces both lytic and leukemogenic diseases of the erythroid lineage (27, 39, 42). In contrast to the previously reported ex vivo studies, we observed that the mutation had major effects on the in vivo phenotype. The mutant virus was attenuated for both the lytic and the leukemogenic diseases, and its in vivo dissemination was also clearly delayed early after infection. Ex vivo reassessment under sensitive conditions revealed a significant effect of the mutation on viral dissemination in cell cultures. Furthermore, we observed systematic phenotypic reversion of the mutation in vivo. Conservation of the cysteine-rich hydrophobic region among all the reverted sequences favored a role for this sequence in glycoGag functions.

MATERIALS AND METHODS

Plasmid construction. The plasmid pMUSA containing the wild-type F-MuLV 57 genome was a gift of R. Friedrich. pMUSA contains a permuted form of the proviral DNA (at position 2850 of the genome) cloned in the *Eco*RI site of vector pBR327. Numbering was made in reference to the first nucleotide of R as number 1.

To obtain the F-MuLV glycoGag$^-$ DNA, plasmid pMUSA was linearized by partial digestion with *Pst*I enzyme and ligated with the double-stranded octanucleotide 5′-CGCGTGCA-3′, which has *Pst*I sticky ends. Insertion of this linker removes the *Pst*I site and creates an *Mlu*I site. Among several recombinants plasmids, pMUSAC111 was characterized for one insertion of the linker in the correct *Pst*I site, at position 564 of the F-MuLV genome. Insertion of the linker was checked by DNA sequencing, using the dideoxy method. The *Sac*I fragments (nucleotide [nt] −30 to 2560) of pMUSA and pMUSAC111 containing the leader sequences and the *gag* genes of wild-type and mutant F-MuLVs, respectively, were introduced into vector pSP65 downstream of SP6 promoter, creating the plasmids pMUSAC 113 and 117.

In vitro transcription and translation. Plasmids pMUSAC 113 and 117 were linearized by *Bam*HI downstream from the 3′ end of the subcloned fragment. Capped RNAs were generated in vitro by SP6 RNA polymerase as instructed by the manufacturer (Promega) in the presence of m7GpppG (0.5 mM) in the transcription reaction mixture. RNA transcripts were quantified by ethidium bromide staining on agarose gel, which also allowed testing of their integrity. In vitro translation in rabbit reticulocyte lysate (Promega) was performed as indicated in the manufacturer's instructions, in the presence of [^{35}S]methionine (Amersham). Translation products were analyzed by electrophoresis in a sodium dodecyl sulfate (SDS)–8% polyacrylamide gel, and then dried gels were autoradiographed.

Tissue culture and viral stocks. Unless otherwise stated, *Mus dunni* (20) and NIH 3T3 fibroblasts were cultivated in Dulbecco's modified Eagle's medium with glutamine (2 mM), penicillin (50 IU ml^{-1}), streptomycin (50 mg ml^{-1}), and 10% heat-inactivated fetal calf serum. Cell supernatants were removed from subconfluent transfected NIH 3T3 fibroblasts or infected *M. dunni* fibroblasts 10 to 18 h after the replacement of the medium. These viral stocks were filtered (pore size, 0.45 mm) and stored in aliquots at −60°C. Viral stocks were titrated by using the focal immunofluorescence assay (FIA) previously described (41) with monoclonal antibodies discriminating among the envelope glycoproteins of F-MuLV and Moloney (M)-MuLV (4, 5, 14).

Construction of the glycoGag$^-$ H5 cell line. PMUSAC111 DNA (permuted glycoGag$^-$ provirus) was digested by *Eco*RI and religated at a DNA concentration of 100 μg/ml in order to obtain concatemers recreating the normal provirus. NIH 3T3 fibroblasts were cotransfected by 20 μg of pMUSAC111 concatemers per ml and 4 μg of vector pSV2neo per ml, by the calcium phosphate method (3). G418 (0.5 μg/ml) was added after 48 h. Two weeks after the transfection, when clones were visible, cells were trypsinized, diluted to a concentration of 2.5 cells per ml and transferred to 96-well microplate dishes (100 μl per well). Cells were grown for another 2 weeks before medium (10 μl) was collected from each well containing a clone and assayed for reverse transcriptase activity (16).

Viral titration by end point dilution. Filtered viral preparation were titrated by 10-fold dilutions from 10^{-2} to 10^{-8}. Each dilution (0.5 ml) was added in duplicate to 24-well tissue culture plates containing 2×10^4 NIH 3T3 cells per well. Cells were grown for a week, and then medium (10 μl) was collected from each well and assayed for reverse transcriptase activity.

Origin, infection, and clinical evaluation of mice. All experimentation was conducted on ICFW mice, an inbred line derived from Carworth Farms White outbred mice (47). Newborn mice at 1 to 2 days of age were inoculated intraperitoneally with 0.05 ml of viral stock. For studies of in vivo dissemination, all litters were inoculated at 1 day of age. In order to assess the induction of early hemolytic anemia (EHA), blood samples were taken under ether anesthesia from inoculated mice at 3-day intervals from 16 to 24 days of age by puncture at the retroorbital sinus with 20-ml heparinized capillary tubes (Drummond Scientific Company, Broomall, Pa.), and hematocrits, the volume of erythrocytes expressed as the percentage of blood volume, were determined. The mice were regularly examined for splenomegaly by palpation under ether anesthesia. Mice displaying gross organ enlargement and moribund animals were bled for the determination of hematocrits prior to sacrifice and autopsy. The diagnosis of erythroleukemia depended on the presence of splenomegaly and severe anemia, in the absence of lymphadenotrophy or thymotrophy.

Dissemination in vivo. *M. dunni* fibroblasts were plated at 5×10^4 cells per dish in 60-mm-diameter culture dishes. The following day, just prior to addition of splenocytes, the medium was replaced with 4 ml of fresh medium. Spleens from infected mice were removed at different ages and dispersed in complete Dulbecco modified Eagle medium. Spleen cell suspensions were washed once and adjusted to 10^7 live nucleated cells ml^{-1}. Serial 10-fold dilutions of splenocytes were prepared, and 1 ml of cells at 10^5, 10^4, and 10^3 ml^{-1} (for enumeration of ecotropic infectious centers) or 10^6, 10^5, and 10^4 ml^{-1} (for enumeration of recombinant polytropic infectious centers) was added to the fibroblast monolayers and incubated at 37°C. The splenocytes were removed the following day, and the fibroblasts were permitted to grow to confluency before quantitation of infectious centers by FIA with monoclonal antibodies which discriminate among the envelope glycoproteins of F-MuLV, M-MuLV, and polytropic viruses (4, 5, 14).

Western blot. After a 3-day cocultivation with splenocytes

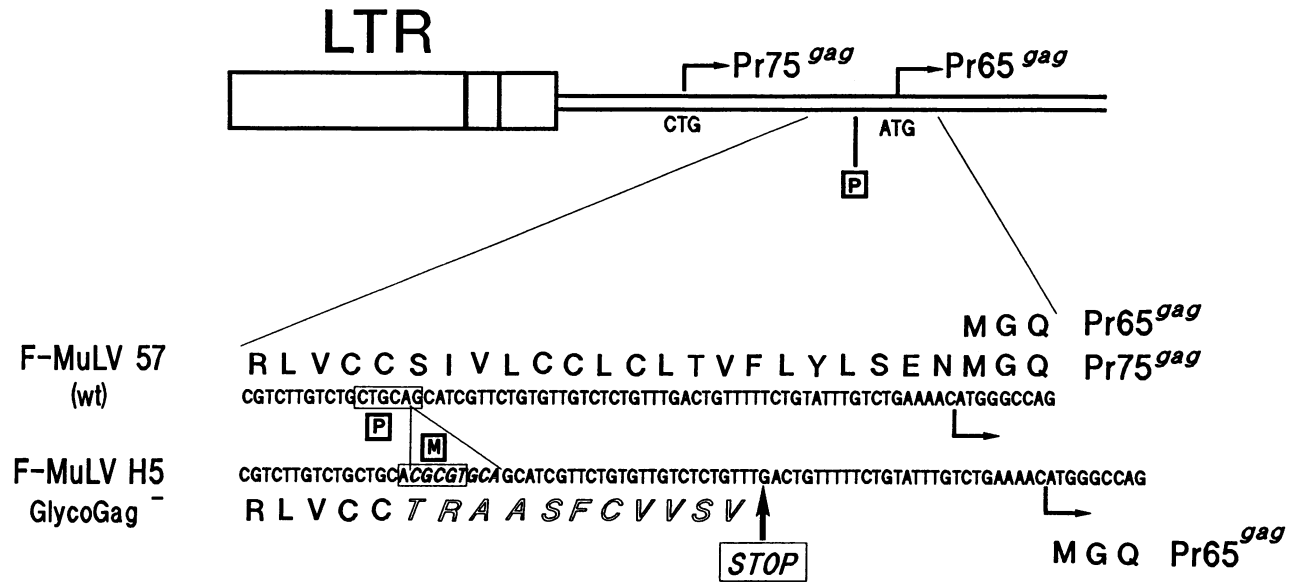

FIG. 1. Schematic representation of the mutated region. (Top) The major CTG initiation codon (nt 355) of the glycoGag precursor (Pr75^{gag}) and the ATG initiation codon (nt 619) of the Gag precursor (Pr65^{gag}) are indicated. (Bottom) Nucleotide and amino acid sequences resulting from the frameshifting mutation are indicated in italics. P, *Pst*I restriction site (nt 575); M, introduced *Mlu*I restriction site. Numbering is performed with the first nucleotide of R as 1. wt, wild type; LTR, long terminal repeat.

from infected mice (see above), a confluent *M. dunni* cell monolayer in a 60-mm-diameter dish was lysed in 300 μl of 2× SDS lysis buffer (135 mM Tris-HCl [pH 6.8], 6% SDS, 20% glycerol), the lysates were boiled for 10 min in the presence of 5% β-mercaptoethanol, and 20 μl was subjected to electrophoresis in a SDS–10% polyacrylamide gel and immediately transferred onto a nitrocellulose filter (BA-85, Schleicher & Schuell) with a high-intensity-field electrotransfer apparatus (Bio-Rad). Nitrocellulose filters were blocked by an overnight incubation at 4°C with TBS-Tween (10 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.05% Tween 20), 3% nonfat powdered milk, and 0.02% NaN_3. Filters were sequentially incubated with a rabbit polyclonal antiserum directed against the MuLV CAp30 (1/1,000) and peroxidase-coupled donkey anti-rabbit immunoglobulin G antibodies (Amersham Corp.) (1/1000) and intensively washed in TBS-Tween before revelation with the chemoluminescence ECL kit (Amersham Corp.) following the recommendations of the manufacturer. For Western blots (immunoblots) performed directly on splenocytes, 10^7 live nucleated cells were resuspended in 100 μl of phosphate-buffered saline, lysed in 300 μl of 2× lysis buffer, and treated as *M. dunni* cocultivation lysates.

PCR, cloning, and sequence analysis of the pseudoreverted region. Genomic DNA from splenocytes of infected mice was amplified by PCR with the oligonucleotide pair AC6 (nt 253 to 273) and C-gag (nt 631 to 651). PCR was conducted according to the recommendations of the GeneAmp PCR Reagent kit with AmpliTaq (Perkin-Elmer Cetus), with final concentrations of 200 mM nucleotides and 2 mM $MgCl_2$, in a volume of 100 μl. The genomic DNA was subjected to 32 cycles of amplification as follows: denaturation for 1 min at 96°C, annealing for 2 min at 39°C, and primer extension for 2 min at 72°C. These cycles were preceded by a 4-min denaturation at 96°C and followed by a 4-min extension at 72°C. Oligonucleotides (Genset, Paris) used for PCR were as follows: AC6, 5′-TGATTGATTTTATGCG CCTGC-3′; and C-gag, 5′-CAAACTTAAGGGGGTGGTAA C-3′. The products were digested with the restriction enzymes *Pst*I or *Mlu*I. After migration on a 1.6% agarose gel, the undigested bands were cut out and purified on SpinX columns (Costar) and reamplified by PCR under the same conditions. The PCR fragments, purified with the Gene Clean II kit, were blunted by treatment with the Klenow polymerase, kinased with the T4 polynucleotide kinase, and cloned in a dephosphorylated M13mp89 phage linearized by *Eco*RV. After preparation of single-stranded DNA, 10 clones derived from each PCR fragment were sequenced with the T7 sequencing kit (Pharmacia), by following the recommendation of the manufacturer and using either the M13 universal primer, 5′-GTAAAACGACGGC CAGT-3′, or the AC9 oligonucleotide (nt 421 to 437), 5′-CG TTTTGGACTCTTTGG-3′.

RESULTS

F-MuLV H5, a glycoGag$^-$ mutant. We derived a F-MuLV mutant which did not express any glycoGag molecule. Since it has previously been demonstrated that MuLV-infected cells express different forms of the glycoGag, which differ in their N-terminal sequences (35) because of initiation of translation at alternative CUG codons (unpublished observation), we did not choose to mutate the diverse putative initiation codons of translation. Rather, we inserted an octanucleotide *Mlu*I linker upstream from the Pr65^{gag} region, which resulted in a frameshift and in the elimination of a *Pst*I site in the specific glycoGag-coding sequence. This frameshift was predicted to lead to premature translational arrest in the glycoGag sequence whichever initiation codon is used, without altering the predicted coding sequence of Pr65^{gag} (Fig. 1). In vitro transcription and translation of the *gag* open reading frame of the wild-type plasmid yielded two translational products, a 65- and a 75-kDa protein characteristic of the wild-type precursors. Contrastingly, the glycoGag$^-$ plasmid gave rise only to the Pr65^{gag} precursor, with no protein detected in the 70- to 85-kDa range (data not shown).

A full-size glycoGag$^-$ F-MuLV plasmid was cotransfected in

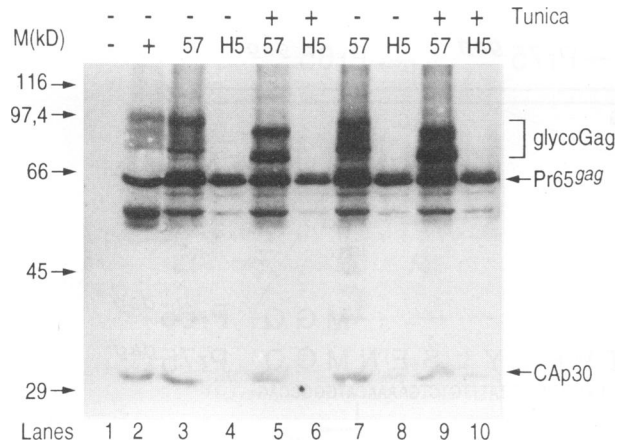

FIG. 2. Gag-related proteins produced by fibroblasts infected with wild-type F-MuLV 57 or glycoGag⁻ F-MuLV H5. *M. dunni* cells were infected with viral stocks derived from the H5 glycoGag⁻ NIH 3T3 clone (lanes 4, 6, 8, and 10) or a wild-type-infected NIH 3T3 clone (57) (lanes 3, 5, 7, and 9). Three days later, after tunicamycin (Tunica) treatment (lanes 5, 6, 9, and 10) or without treatment (lanes 1 to 4, 7, and 8), Gag-related protein expression was determined by Western blot as described in Materials and Methods, using an anti-CA antiserum. Lane 1, uninfected *M. dunni* cells; lane 2, control *M. dunni* cells chronically infected with F-MuLV 57. Migrations of glycoGag-specific bands (glycoGag), Gag precursor ($Pr65^{gag}$), and CA (CAp30) are indicated.

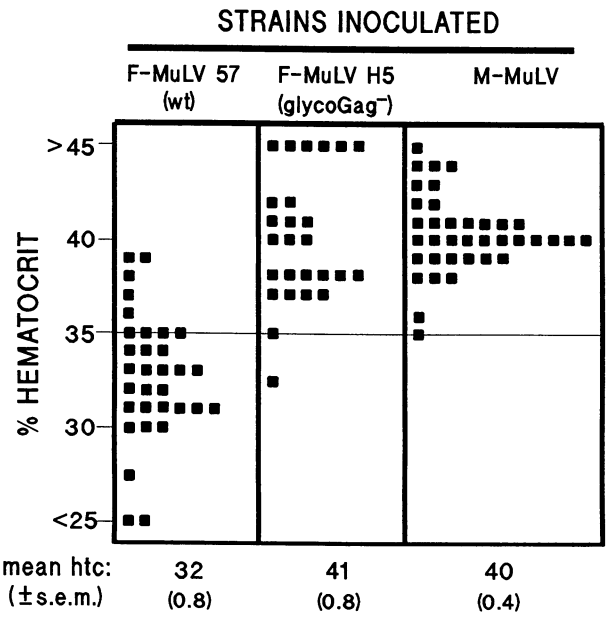

FIG. 3. The glycoGag⁻ mutation attenuates the capacity of F-MuLV for induction of EHA. Newborn ICFW mice were inoculated with the wild-type (wt) F-MuLV 57, the glycoGag⁻ F-MuLV H5, or the M-MuLV. Hematocrits (htc) determined between 19 and 21 days of age for individual mice are shown as closed squares. s.e.m., standard error of the mean.

NIH 3T3 fibroblasts with a Neo^r-encoding plasmid, and G418-resistant clones were tested for viral expression. The H5 clone expressed a high level of virus and was used for all the subsequent experiments. Gag-related protein expression was tested in this clone by immunoblot with an anti-CA antiserum. When compared with cells infected with the wild-type F-MuLV 57, several 80- to 95-kDa bands were lacking in the H5 clone, whereas all the other proteins were expressed (Fig. 2, compare lanes 4 and 8 with lanes 3 and 7). When treating the infected cells with tunicamycin before the assay, we observed that the bands which were present only in the wild-type-infected cells shifted to a lower apparent molecular mass. This result confirmed that bands present only in the wild-type virus corresponded to glycosylated forms of Gag. Contrastingly, the bands present in both the wild type and H5 mutant remained unchanged after treatment, indicating that they corresponded to unglycosylated $Pr65^{gag}$, CAp30, and incompletely processed Gag forms, whose expression was not modified in mutant-infected cells (Fig. 2, lanes 6 and 10).

Attenuated pathogenic effects of the glycoGag⁻ mutant of F-MuLV. As previously described for other glycoGag⁻ MuLV mutants (15, 37), the H5 clone produced high titers of replication-competent virus (F-MuLV H5) similar to those obtained with the wild-type F-MuLV 57. Mice inoculated as newborns with F-MuLV 57 developed a severe EHA characterized by mean hematocrit below 35%. Contrastingly, F-MuLV H5 produced only very mild EHA, with hematocrits at least as high as those observed with the non-severely hemolytic M-MuLV (Fig. 3) and F-MuLV B3 (42). F-MuLV also induces in ICFW mice an erythroleukemia (39, 42) whose latency depends on the age at inoculation. Although mice inoculated with F-MuLV H5 developed erythroleukemia, latency was markedly delayed when compared with that in F-MuLV 57-inoculated mice, and this delay was more marked in mice inoculated at older ages (Table 1).

Effects of the glycoGag⁻ mutation on in vivo and ex vivo viral dissemination. Since the glycoGag⁻ mutation might attenuate F-MuLV pathogenesis because of an intrinsic flaw impeding on the overall spreading of the virus, we compared in vivo dissemination of wild-type F-MuLV 57 and the glycoGag⁻ mutant. We observed a highly significant delay in the initial spreading of the mutant F-MuLV H5 (Fig. 4A). We also observed that induction of recombinant polytropic mink cell focus-forming (MCF) viruses was also significantly delayed in F-MuLV H5-inoculated mice compared with that in mice inoculated with F-MuLV 57 (Fig. 4B). We have recently demonstrated that vaccination of newborn mice with the weakly pathogenic F-MuLV B3, which has full spreading abilities (40), protects against subsequent challenges with the virulent F-MuLV 57 through interference to superinfection, a phenomenon highly dependent on viral dissemination (8). In agreement with a poor initial spreading of F-MuLV H5, we observed in this system that the glycoGag⁻ mutant failed to

TABLE 1. The glycoGag⁻ mutation delays onset of F-MuLV-induced erythroleukemia

Age (days) of inoculation[a]	Leukemia latency (mo [no. of animals])[b]	
	wt 57	glycoGag⁻ (H5)
1–2	1.8 (19)	3.6 (24)
7–10	1.7 (6)	3.8 (9)
12–13	3.0 (16)	5.0 (16)

[a] Mice were inoculated intraperitoneally (1- to 10 day-old mice) or at the retroorbital sinus (12- to 13 day-old mice) with 50 μl of the F-MuLV viral stocks; wild-type (wt 57) and glycoGag⁻ (H5) stocks titrated at 1.3×10^5 and 1.3×10^6 focus-forming units per ml, respectively.

[b] At least two different litters inoculated at different times were used for each series. All leukemias observed were erythroleukemias.

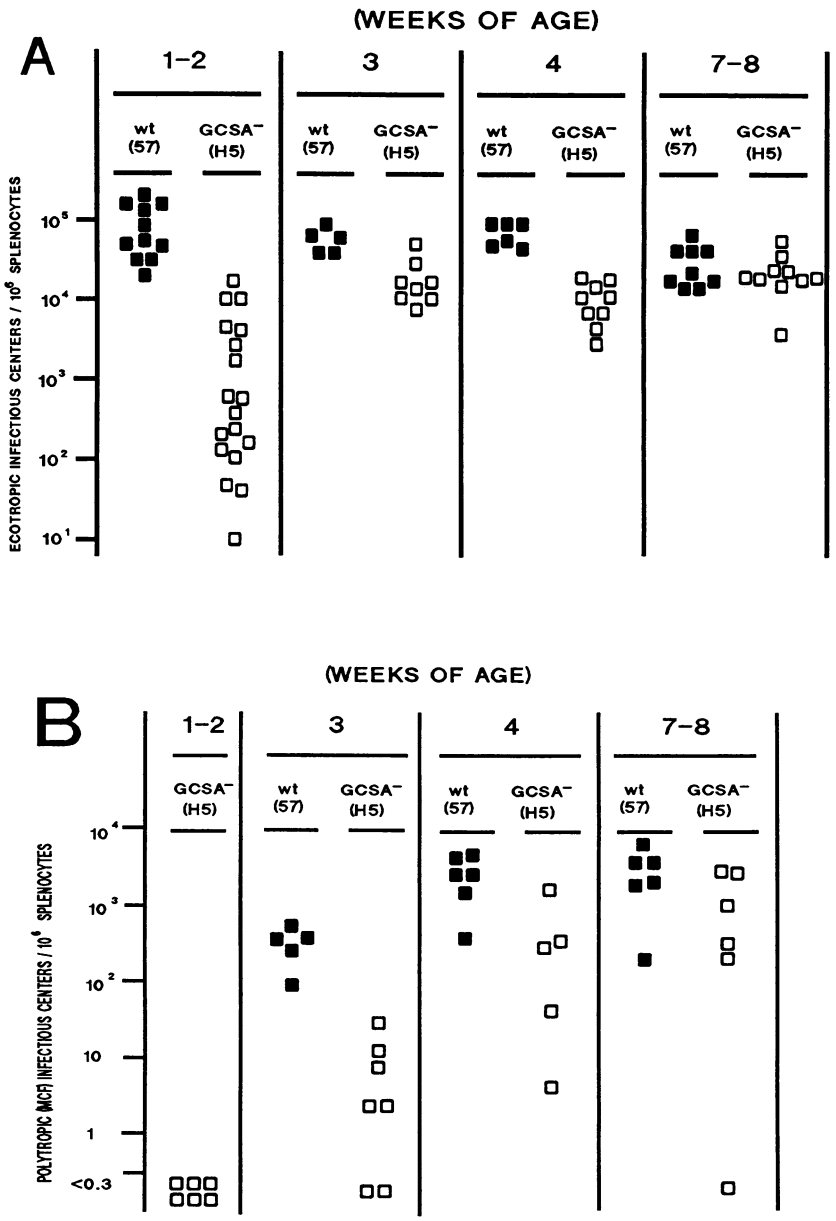

FIG. 4. Delayed in vivo dissemination and induction of polytropic recombinants of the glycoGag⁻ mutant F-MuLV H5 compared with that in wild-type (wt) F-MuLV 57. Mice inoculated as newborns with 50 μl of either the wild-type [wt (57), 6.5×10^3 focus-forming units per animal) or the glycoGag⁻ [GCSA⁻ (H5), 6.5×10^4 focus-forming units per animal) strain of F-MuLV were sacrificed at different ages. Splenic infectious centers were enumerated on *M. dunni* fibroblasts by FIA with monoclonal antibodies specific for ecotropic virus (H48) or polytropic viruses (H502 and H514). (A) Ecotropic infectious centers; (B) polytropic infectious centers.

interfere with F-MuLV 57 hemolytic effects (Fig. 5a) and M-MuLV dissemination (Fig. 5b).

These data on in vivo dissemination, which could at least partially explain the attenuated phenotype of F-MuLV H5, contrasted with previous studies which failed to point out gross defects in the ex vivo dissemination of other glycoGag⁻ mutants (15, 37). Therefore, we readdressed this question by using our F-MuLV H5 mutant. In agreement with these previous studies, no difference in the ex vivo dissemination was observed with a relatively high multiplicity of infection (MOI) equal to 1 or 10 (data not shown). On the contrary, we observed a significantly delayed initial spreading of F-MuLV H5 in culture infected at a lower MOI (less than 0.01) (Fig. 6).

In vivo phenotypic reversion of the glycoGag⁻ mutation. Because of the discrepancy between the initial spreading impairment of F-MuLV H5 and the normal levels of the late dissemination, we monitored cells infected with the glycoGag⁻ H5 mutant for potential reversions. Splenocytes of mice sacrificed at different times after inoculation were cocultivated with Dunni indicator cells and analyzed for Gag protein expression. As revealed with an anti-CA antiserum, we observed a progressive appearance of bands, which comigrated with the

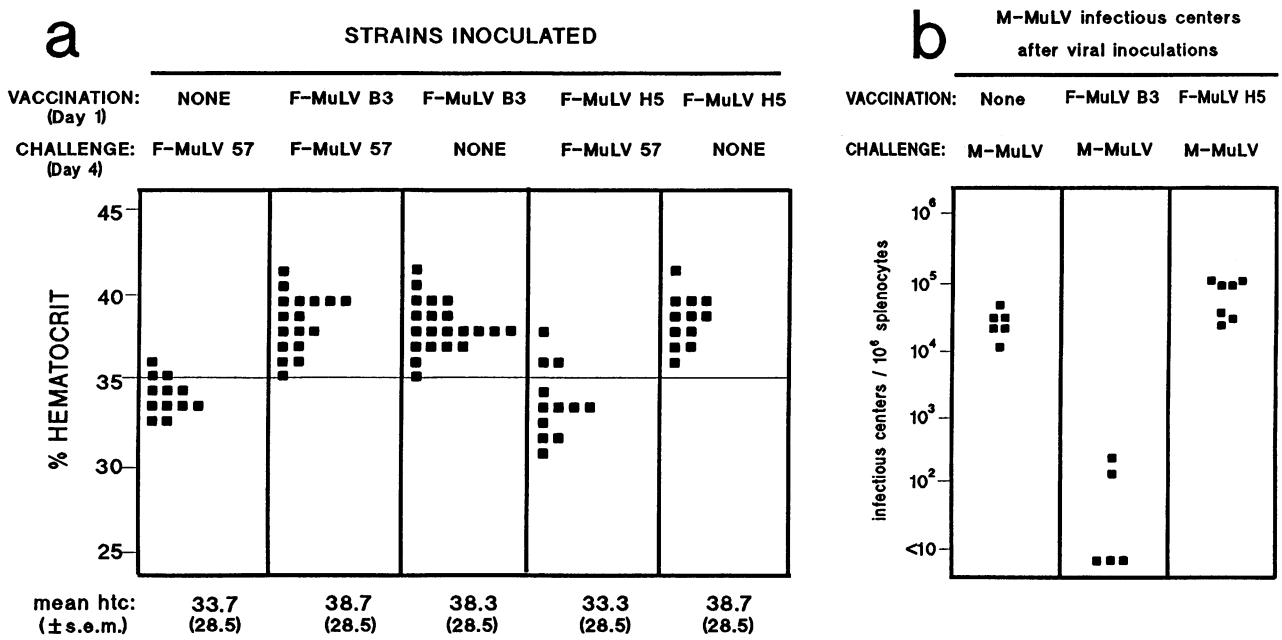

FIG. 5. The glycoGag⁻ F-MuLV H5 did not establish in vivo interference. Mice were vaccinated at 1 day of age with either of the attenuated F-MuLV H5 or the attenuated F-MuLV B3 and challenged 3 days later with F-MuLV 57 or M-MuLV. (A) Lack of protection by F-MuLV H5 against F-MuLV 57-induced EHA. Hematocrits (htc) from mice challenged with F-MuLV 57 were determined and are shown as described in the legend to Fig. 4. (B) Lack of efficient interference after F-MuLV H5 vaccination as measured by in vivo dissemination. Mice challenged with M-MuLV were sacrificed at 21 to 25 days of age, and the dissemination of M-MuLV was determined by FIA with an envelope-specific monoclonal antibody (generous gift of B. Chesebro). s.e.m., standard error of the mean.

glycoGag-specific bands of F-MuLV 57 controls and which were absent from Dunni cells infected by the F-MuLV H5 stock used for the inoculation of animals (Fig. 7A). In cocultivated Dunni cells treated with tunicamycin, these bands shifted to lower molecular weights, in a pattern identical to what was observed in F-MuLV 57 controls, confirming that they corresponded to the Gag-related glycoproteins (Fig. 7B). We also verified this reappearance of glycoGag directly on the splenocytes of F-MuLV H5-infected mice (Fig. 7C).

Since recombinant MCF viruses are induced after F-MuLV H5 inoculation, although with delayed kinetics, and since these viruses may potentially express glycoGag, it was possible that reappearance of glycoGag might be due to emergence of MCF viruses. To address this question, we inoculated F-MuLV H5 into DBA/2 newborn mice, which do not develop MCF virus infection after F-MuLV inoculation (2, 38a). We observed emergence of new glycoGag-specific bands in the absence of detection of MCF viruses (data not shown). In agreement with this result, glycoGag expression did not correlate with the level of MCF virus infection when splenocytes from individual ICFW mice were assayed for both glycoGag and MCF virus presence. We also observed phenotypic reversion of the glycoGag⁻ mutant in fibroblast cultures, although MCF virus induction has never been documented ex vivo. Therefore, reappearance of glycoGag was not due solely, if at all, to MCF virus emergence.

Sequence analysis of the viruses present upon phenotypic reversion of the glycoGag expression. We amplified the glycoGag-specific coding sequences from the genomic DNA of F-MuLV H5-infected mice splenocytes. Our conditions of PCR amplification were specific for the input virus, since no amplification product was observed when splenocytes of noninfected animals were used. The linker mutation has eliminated the *Pst*I site and introduced a new *Mlu*I site (Fig. 1). As expected, PCR amplification products from control F-MuLV 57 mice were readily digested by *Pst*I and resistant to *Mlu*I digestion (Fig. 8, lanes 2 and 3). Remarkably, all amplification

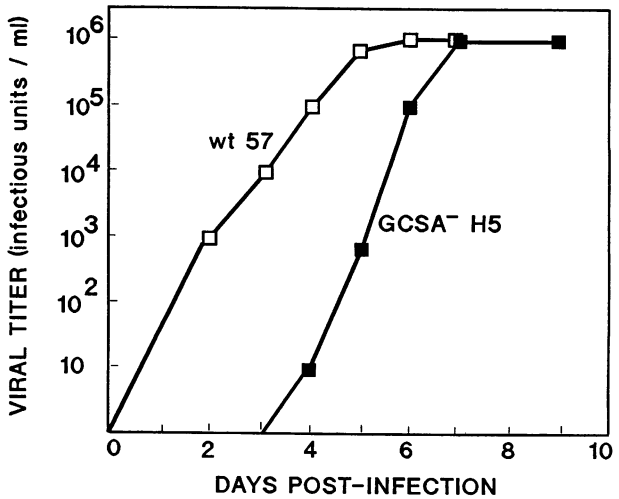

FIG. 6. Dissemination of the wild-type F-MuLV 57 (wt 57) and glycoGag⁻ F-MuLV H5 (GCSA⁻ H5) ex vivo. Twelve-well dishes containing 1.5×10^3 NIH 3T3 cells were infected by 10 focus-forming units (1 ml) of wt 57 or H5 virions. The medium was collected every day from 2 to 9 days postinfection, and infectious viruses were titrated by end point dilution, using a reverse transcription assay on NIH cells (see Materials and Methods).

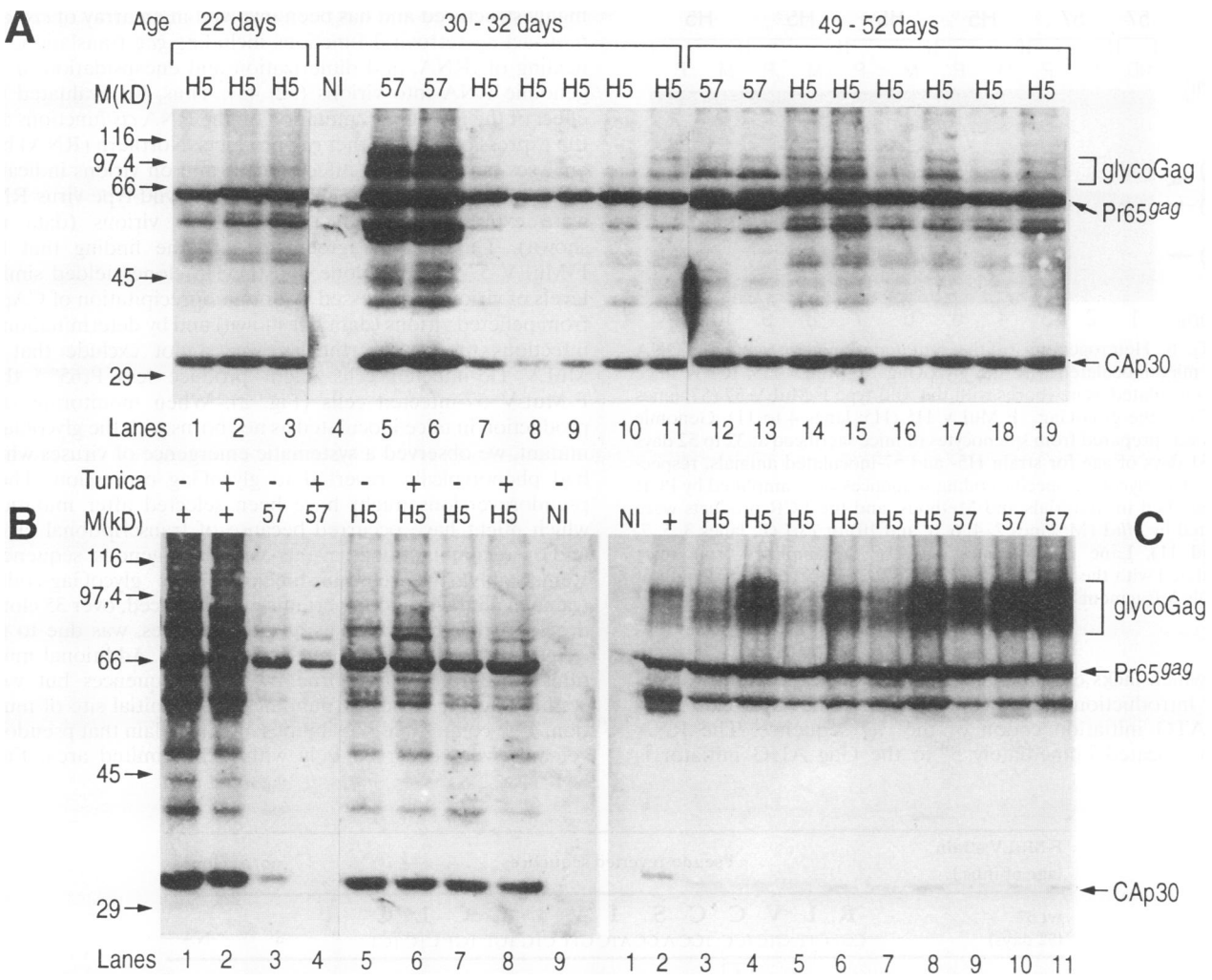

FIG. 7. Phenotypic reversion of the glycoGag mutant in vivo. Newborn ICFW mice were inoculated with the wild-type (57) or the glycoGag$^-$ (H5) F-MuLV. (A and B) Mice were sacrificed at different ages (30 days of age in panel B), and splenocytes were plated on *M. dunni* cell monolayers. After 3 days of cocultivation, the monolayers were lysed, and Gag-related protein expression was determined by Western blot with anti-CA antiserum without (panel A and panel B, lanes 1, 3, 5, 7, and 9) or after treatment with tunicamycin (Tunica; panel B, lanes 2, 4, 6, and 8). (C) Mice were sacrificed at 30 days of age, and Gag-related protein expression was determined directly on lysed splenocytes. NI, splenocytes or cocultivations derived from noninoculated animals. Control *M. dunni* cells infected ex vivo with F-MuLV 57 were used (panel B, lanes 1 and 2, and panel C, lane 2). Other indications are as described in the legend to Fig. 3.

products from F-MuLV H5-infected mice were only partially sensitive to *Mlu*I digestion although were still completely resistant to *Pst*I (Fig. 8, lanes 4 to 11), suggesting that the genomic DNA from F-MuLV H5-infected mice contained both mutant and pseudorevertant proviruses.

Since we could not exclude partial digestion of the PCR products, we further analyzed the sequences of two types of amplification products. First, clones were obtained from the total amplification materials directly derived from the infected mice. Second, clones were also derived from reamplified *Mlu*I-resistant DNA in order to enrich revertant DNA (Fig. 9). All of the PCR products obtained from independent amplifications from different mice inoculated with the glycoGag$^-$ mutant were heterogeneous in sequence. Contrastingly, all the PCR fragments cloned from the splenocyte DNA of mice inoculated with F-MuLV 57 had the wild-type sequence, indicating that heterogeneity observed in glycoGag$^-$ mutant materials was not due to artefactual misincorporations during the PCRs. The glycoGag-coding frame was restored in all the clones which differed from F-MuLV H5, indicating that revertants were selected on glycoGag expression. This restoration was always due to the exact deletion of the eight nucleotides which had been introduced in the wild-type F-MuLV 57, and although the majority of the clones exhibited additional point mutations, these mutations were always present in the immediate vicinity of the deletion.

DISCUSSION

Expression of nonstructural glycoproteins encoded in frame by the *gag* region of murine and feline oncoviruses and which are thought to be dispensable for viral replication was in fact necessary for complete in vivo spreading and pathogenic abilities of the F-MuLV. Expression of these proteins was indeed found to be positively selected in vivo. These results were obtained by using a glycoGag$^-$ mutant of F-MuLV, strain

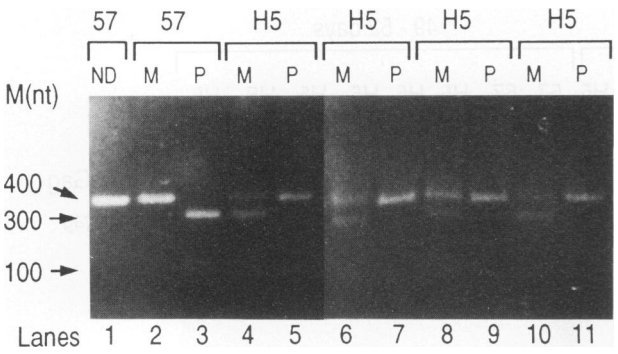

FIG. 8. Heterogeneity of the mutated region in genomic DNA from mice inoculated with the glycoGag⁻ F-MuLV H5. ICFW mice were inoculated as newborns with the wild-type F-MuLV 57 (57; lanes 1 to 3) or the glycoGag⁻ F-MuLV H5 (H5; lanes 4 to 11). Genomic DNA was prepared from splenocytes of mice sacrificed at 32 to 52 days and 51 days of age for strain H5- and 57-inoculated animals, respectively. The glycoGag-specific coding sequences were amplified by PCR as described in Materials and Methods, and the PCR products were digested by *Mlu*I (M; lanes 2, 4, 6, 8, and 10) or *Pst*I (P; lanes 3, 5, 7, 9, and 11). Lane 1, undigested DNA (ND) amplified from mice inoculated with the wild-type F-MuLV 57. The approximate length of the DNA fragments is indicated.

H5, which lacks expression of such Gag-related glycoproteins after introduction of an octanucleotide linker upstream from the ATG initiation codon of the *gag* sequence. The RNA region located immediately 5′ to the Gag AUG initiator is highly structured and has been involved in an array of *cis*- and *trans*-acting retroviral functions including *gag* translation, annealing of tRNA, and dimerization and encapsidation of the genomic RNA into virions (32, 45). Thus, we evaluated the effect of the glycoGag⁻ mutation on the RNA *cis* functions and the expression of the other *gag* products. Northern (RNA) blot analyses performed on infected cells and on virions indicated that similar levels of the glycoGag⁻ and wild-type virus RNA were expressed and encapsidated into virions (data not shown). This was in agreement with the finding that the F-MuLV 57-infected clone and the H5 clone yielded similar levels of virions, as assessed by immunoprecipitation of CAp30 from pelleted virions (data not shown) and by determination of infectious titers. Nevertheless, we cannot exclude that F-MuLV H5-infected cells might produce less $Pr65^{gag}$ than F-MuLV 57-infected cells (Fig. 2). When monitoring viral production in mice inoculated as newborns with the glycoGag⁻ mutant, we observed a systematic emergence of viruses which had phenotypically reverted to glycoGag expression. These pseudoreversions might have been selected after mutations which might have occurred because of transcriptional errors and/or recombinational events with endogenous sequences. Remarkably, the reestablishment of the glycoGag-coding frame in all the pseudorevertants we sequenced, over 35 clones derived from three animals at different ages, was due to the exact deletion of the octanucleotide linker. Additional mutations were frequently borne by these sequences but were confined to the close surroundings of the initial site of mutation. Two compatible possibilities might explain that pseudoreversions were observed only within this limited area. First,

F-MuLV strain (age of inoc.)	Pseudo-reverted sequences	no. of clones	
wt 57 (52 days)	R L V C C S I V L C C L C CGT CTT GTC TGC TGC A GC ATC GTT CTG TGT TGT CTC TGT	9	NA
glycoGag⁻ H5 (31 days)	CGT CTT GTC [I] *ATT* TGC A GC ATC GTT CTG TGT TGT CTC TGT	1	1/7
	CGT CTT GTC TGC TGC A GC ATC GTT CTG TGT TGT CTC TGC	1	6/11
	CGT CTT GTC [S] TCG TGC A GC ATC GTT CTG TGT TGT CTC TGT	1	
	CGT CTT GTC [A] *GCG* TGC A GC ATC GTT CTG TGT TGT CTC TGT	2	
	CGT CTT GTC [A] *GCG* TGC A GC ATC GTT CTG TGT TGT [P] CCC TGT	1	
	CGT CTT GTC [I] *ATT* TGC A GC ATC GTT CTG TGT TGT CTC TGT	1	
glycoGag⁻ H5 (51 days)	CGT CTT GTC TGC TGC A GC ATC GTT CTG TGT TGT CTC TGT	1	4/5
	CGT CTT GTC TGG [W] [F] TC A GC ATC GTT CTG TGT TGT CTC TGT	3	

FIG. 9. Pseudoreverted sequences present in mice inoculated with the glycoGag⁻ strain of F-MuLV. Splenocyte DNAs from one 52-day-old mouse inoculated with wild-type (wt) F-MuLV 57 and from two mice inoculated with glycoGag⁻ F-MuLV H5 sacrificed at either 31 or 51 days of age were used for analysis of F-MuLV-specific sequences after PCR amplification and cloning of the PCR fragments. Analyses of two independent PCR amplifications performed on the same DNA preparation from the 31-day-old mouse inoculated with glycoGag⁻ F-MuLV H5 are shown separately. The wild-type and the different pseudoreverted sequences found in each animal are shown with the amino acid and the nucleotide sequences corresponding to those of the wild type on top. All pseudoreverted sequences had a deletion corresponding to the 8-nt linker insertion which readily allowed alignment with the wild-type sequence. The arrow indicates the location of the linker insertion. All other nucleotide mutations in the pseudoreverted sequences are indicated in boldface and italics and are underlined, with the corresponding predicted amino acid. Nonsynonymous amino acid substitutions are indicated with a boxed letter, using the single-letter code. At the end of each line, the frequency at which the corresponding sequence occurred is indicated, and for each independent experiment the total number of pseudoreverted sequences (first number) and the total number of sequences analyzed (second number) are shown in the last column. NA, not applicable.

TABLE 2. Structural conservation of the mutated region in retroviruses encoding glycoGag[a]

Virus	Sequence
F-MuLV	WNRSRAARLVCCSIVLCCLCL-TVFLYLSENM
M-MuLV	WDRSRAARLVCCSIVLCCLCL-TVFLYLSENM
Akv	FRRNRAARLVCLSIVLSFVCS-LLFWTASKNM
FBR-MSV	FRRNRAARLICLSVILSFVCS-LLFWTVSKNM
FeLV-A	WYRSRAARLVIFCLVASFLVPCLTFLIAETVM
FeLV-B	WYRSRAARLVILCLVASFLVPCLTFLIAEAVM
SSV	ATRSRRRFWFLFVLFLISLCV-GVSLFEIFEM

[a] The sequences are aligned on the methionine initiation codon of $Pr65^{gag}$. The arginine-rich and hydrophobic regions are boxed individually; the cysteine residues are boxed. The alignment has been designed to emphasize the conserved distance between the arginine-rich region and the methionine initiator of $Pr65^{gag}$. Other alignments which emphasize the high conservation of two eight-residue-spaced cysteines within the hydrophobic region could be performed. Akv, ecotropic MuLV from AKR mice; FBR-MSV, FBR isolate of the murine sarcoma viruses; FeLV-A and -B, feline leukemia viruses subgroups A and B; SSV, simian sarcoma virus.

similar mutations occurring elsewhere and able to restore a glycoGag open reading frame might disrupt more dramatically either the nucleotidic secondary structures or the amino acids sequences specifically required for this region. Second, this restricted location for selected pseudoreversions might reflect recombinational events with endogenous sequences which share a highly conserved glycoGag-encoding region. Indeed, comparison of the predicted amino acid sequence encoded by this region in different murine, feline, and simian type C oncoviruses revealed conserved characteristics. Thus, at the same location from the AUG initiation codon of the Gag precursor, these viruses exhibit a cysteine-rich hydrophobic stretch of 20 to 23 amino acids which is immediately preceded by a small arginine-rich stretch of 6 to 7 amino acids (Table 2). Interestingly, the amino acid changes observed in the in vivo pseudorevertants, which were all located within the hydrophobic stretch, maintained or even increased the hydrophobicity of this region. In agreement with a function based on hydrophobicity, it has been previously reported that this region might act as a signal peptide for translocation into the endoplasmic reticulum (37). The rather long hydrophobic stretch and the upstream accumulation of basic amino acids would be unusual features for a cleavable signal peptide of type I glycoproteins although these features are reminiscent of the signal peptides of many retroviral Env precursors which are otherwise type I glycoproteins (12). However, the overall characteristics of the glycoGag-specific amino-terminal sequence seem to better fit with the description of the signal peptide-anchor domain of type II glycoproteins (28), i.e., glycoproteins with a cytoplasmic N-terminal and extracellular C-terminal orientation and an internal noncleavable signal peptide which acts also as an anchor domain. Numerous data support this hypothesis, such as the location of the actual glycosylation sites (11, 35, 36) or the association of the N-terminal—but not the C-terminal—proteolytically processed fragments of glycoGag with the cell surface (22, 24, 25, 29). We are investigating this question, and preliminary results indicate that glycoGag is a type II glycoprotein and that the conserved cysteine-rich hydrophobic amino acid stretch actually acts as a noncleavable signal peptide-anchor domain (1a).

Although importance of glycoGag was underlined by the in vivo model, we also found an effect of the glycoGag$^-$ mutation on the ex vivo dissemination. The apparent contradiction between our ex vivo results obtained with the F-MuLV and those of previous reports on the lack of defect of similar glycoGag$^-$ viruses (15, 37) is not likely to be due to the use of the M-MuLV in those previous reports, since we have also observed a lower efficiency of ex vivo spreading with a glycoGag$^-$ mutant of M-MuLV (not shown). This contradiction is more likely related to the fact that delay in the spreading of the glycoGag$^-$ mutant was apparent only when a low enough MOI was used. It is possible that higher MOI led to more rapid emergence of glycoGag$^+$ revertants, similar to what we observed in vivo. Delayed dissemination of the glycoGag$^-$ virus was more readily observed in vivo even at a high MOI, and inoculated animals did not develop severe EHA. Severe EHA induced after inoculation with the fully virulent wild-type strain of F-MuLV is no longer observed in 30-day-old animals, because hemolysis is masked by more efficient compensatory erythropoiesis in older animals, notably because of a stabilization of their growth rates (42). Therefore, the attenuated EHA observed in F-MuLV H5-inoculated mice might be due to a high level of viral dissemination in erythroid percursors being reached later, when compensatory erythropoiesis is not as crucial for the growth of the mice and is more efficient in correcting for hemolytic anemia. The more slowly spreading abilities of the F-MuLV H5 mutant are also likely to explain the late appearance of MCF viruses, since their induction, which occurs after recombinations between the inoculated F-MuLV and endogenous retroviral sequences, should be directly dependent on F-MuLV dissemination. Accordingly, we have previously reported that delayed induction of MCF viruses observed with certain MuLV chimeras was associated with a delayed dissemination (33). As MCF viruses have been implicated in certain MuLV-induced leukemogenic processes (6, 44), the delay in spreading of F-MuLV H5 and/or MCF viruses might also explain the longer latency of erythroleukemia induced by F-MuLV H5. Nevertheless, we have recently described chimeric viruses between F- and M-MuLV which also spread and induce MCF viruses with delayed kinetics closely similar to those observed for F-MuLV H5 but which retain full erythroleukemogenicity (33). Also, the strain B3 of F-MuLV, which has full spreading abilities and induces MCF viruses as rapidly as F-MuLV 57, induces erythroleukemia after even a much longer latency (27, 40, 42). It is therefore not excluded that alteration of glycoGag expression might specifically influence the leukemogenic effects of F-MuLV, independently from its effect on retroviral dissemination. Since early events following inoculation of F-MuLV seem to include rapid increases of cell proliferation in different hematopoietic lineages (9, 17, 23), it might be of interest to monitor a possible role of glycoGag in this type of phenomenon. In relation to such effects, we are currently examining the possible effects of glycoGag on ex vivo cell proliferation by using glycoGag-overexpressing vectors.

It seems paradoxical that virus-encoded glycoproteins such as glycoGag, expressed at the cell surface, are not incorporated in the virions which are formed by budding at the cell membranes. Whether glycoGag or its processed derivatives participate in the particle formation with concomitant incorporation of the envelope glycoproteins and the Gag precursor has yet to be evaluated. Nonstructural exported proteins in other enveloped viruses have also been described and include the precore protein (pre-C) of the hepadnaviruses, which share with the retroviruses the use of a reverse transcription step during the replication cycle. pre-C has features which are strikingly similar to those of glycoGag (34). Thus, pre-C, which has been described to be dispensable for viral replication, is encoded by the core protein open reading frame from an upstream in-frame initiation codon and is associated with cellular membranes and is present in secreted forms. In the

case of the hepatitis B virus, expression of the pre-C proteins, as HBe antigen, is thought to be related with certain pathological evolution which involves cell proliferation, such as fibrosis and hepatocarcinoma. The use of animal models will help in gathering additional information, not readily available from ex vivo studies, on the role of nonstructural and apparently dispensable regulatory viral glycoproteins.

ACKNOWLEDGMENTS

We are indebted to C. André for her help in cloning the PCR products; C. Berlioz for communication of unpublished results; G. de Billy, F. Pozo, and the animal care staff for excellent assistance; and S. Gisselbrecht for continuous support and helpful discussion.

A.C. is supported by a fellowship from the Fondation pour la Recherche Médicale.

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