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A Weakly Pathogenic Rauscher Spleen Focus-Forming Virus Mutant that Lacks the Carboxyl-Terminal Membrane Anchor of Its Envelope Glycoprotein

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A mutant Rauscher spleen focus-forming virus (mutant 4-3) that causes mild splenic erythroblastosis in mice has a 44-base-pair deletion in the 3′ region of its envelope glycoprotein (*env*) gene. The encoded glycoprotein terminates prematurely, lacks a hydrophobic membrane anchor, and has a shortened intracellular lifespan. An active site for causing erythroblast proliferation may occur in the undamaged amino-terminal domain of the *env* glycoprotein.

The Rauscher and Friend erythroleukemia viruses are independently isolated complexes of two components. One component is a replication-competent murine leukemia virus (MuLV), and the second component is a replication-defective highly pathogenic spleen focus-forming virus (SFFV) that causes rapid formation of erythroid colonies in the spleens of susceptible mice (2, 3, 8 11). Infection of adult mice with the viral complexes causes an initial hyperplasia of erythroid cells, followed 3 to 8 weeks later by the emergence and dissemination of increasingly malignant erythroleukemia cells (17, 27). Consequently, these diseases are similar to progressive natural cancers, which evolve from premalignant to malignant stages (12).

Recent evidence indicates that the SFFV envelope glycoprotein (*env*) gene is required for causing the proliferation of erythroblasts and for subsequent leukemic progression (15, 16, 21). DNA sequence analyses of the *env* genes of several strains of SFFVs (1, 4, 7, 28) have shown them to be structurally related to the hybrid- or recombinant-type *env* genes of dual-tropic MuLVs (5, 6, 10, 26). However, the Rauscher (R) and Friend (F) SFFV *env* genes are smaller (1, 4, 7, 28) and encode glycoproteins with apparent M_r values of 54,000 (gp54) and 55,000 (gp55), respectively (9, 20, 23). Their smaller sizes are caused by a 585-base pair (bp) deletion and by insertion of a single base pair which produces a frameshift and a premature termination codon (1, 4, 7, 28). Recently, we have described genetic and structural evidence that the xenotropic- and ecotropic-related portions of the gp54s-gp55s may fold into separate domains connected by a flexible, proline-rich linker (C. Machida, R. Bestwick, B. Boswell, and D. Kabat, manuscript in preparation). These structural features of the glycoproteins are all diagrammed in Fig. 1.

We previously isolated spontaneous SFFV *env* gene mutants and found that they were all either nonpathogenic or only weakly pathogenic in adult NIH/Swiss mice (16, 21). The R-SFFV mutant 4-3 seemed particularly interesting because it caused a mild splenomegaly despite a substantial *env* gene abnormality. To more carefully analyze the role of gp54 and gp55 in leukemogenesis, we have now isolated a molecular clone of the 4-3 proviral DNA. We report here the restriction map and the nucleotide sequence of the *env* gene region containing the 4-3 mutation.

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The cloning strategy was described previously (4). The circular unintegrated form of R-SFFV 4-3 proviral DNA was cleaved at its single *Eco*RI site and was ligated to the *Eco*RI arms of Charon 3A. One recombinant clone, lambda:4-3, was found to contain insert DNA of two sizes, 8.3 and 7.7 kilobase pairs. The two insert DNAs were subcloned into the *Eco*RI site of pBR322 and given the designations pBC 4-3:9 (8.3-kilobase pair insert) and pBC 4-3:10 (7.7-kilobase pair insert). The restriction maps of these two plasmids are nearly identical to that of wild-type R-SFFV, which is shown in Fig. 1A. The pBC 4-3:9 clone contains an additional *Kpn*I and *Sst*I site when compared with pBC 4-3:10. Furthermore, the additional pBC 4-3:9 fragment generated by digestion with either *Kpn*I or *Sst*I is 500 to 600 bp and hybridizes to a long terminal repeat-specific hybridization probe from R-SFFV (data not shown). For these reasons, we conclude that the 500- to 600-bp difference between the two 4-3 molecular clones is due to an additional long terminal repeat. Both clones lack a *Hin*dIII site and a *Hin*dII site at map positions 1.1 and 5.0, respectively. These results indicate that mutations exist in the *gag* and *pol* genes of the 4-3 SFFV, consistent with previous findings (16). Several lines of evidence suggest that the *gag* and *pol* mutations in the 4-3 mutant are not responsible for its reduced leukemogenic activity. First, wild-type F-SFFV contains numerous deletions in its *gag* and *pol* genes (7). Second, mutations in the SFFV *env* genes and losses of pathogenicity cosegregate during virus cloning experiments (16, 21). Third, pathogenically active revertants can be isolated from the SFFV mutants, and these contain second-site mutations in their *env* genes (Machida et al., in preparation).

Previous studies (16) indicated that a small deletion occurred in the 4-3 mutant genome in a region between the *Eco*RI site in the *env* gene and the *Kpn*I site in the 3′ long terminal repeat. A careful analysis of the restriction sites and fragment sizes from this region indicated that 4-3 lacks a *Stu*I site normally located in the 3′-terminal one-quarter of the *env* gene (Fig. 1B). Our DNA sequencing efforts were therefore concentrated in this region and indicated that a 44-bp deletion had occurred in the 4-3 *env* gene (Fig. 1C and D). The deletion would predict that translation of the 4-3 *env* mRNA terminates prematurely and that the resulting glycoprotein lacks the membrane anchor that occurs at the carboxyl terminus of gp54. The M_r of the truncated polypeptide predicted from the DNA sequence is in agreement with

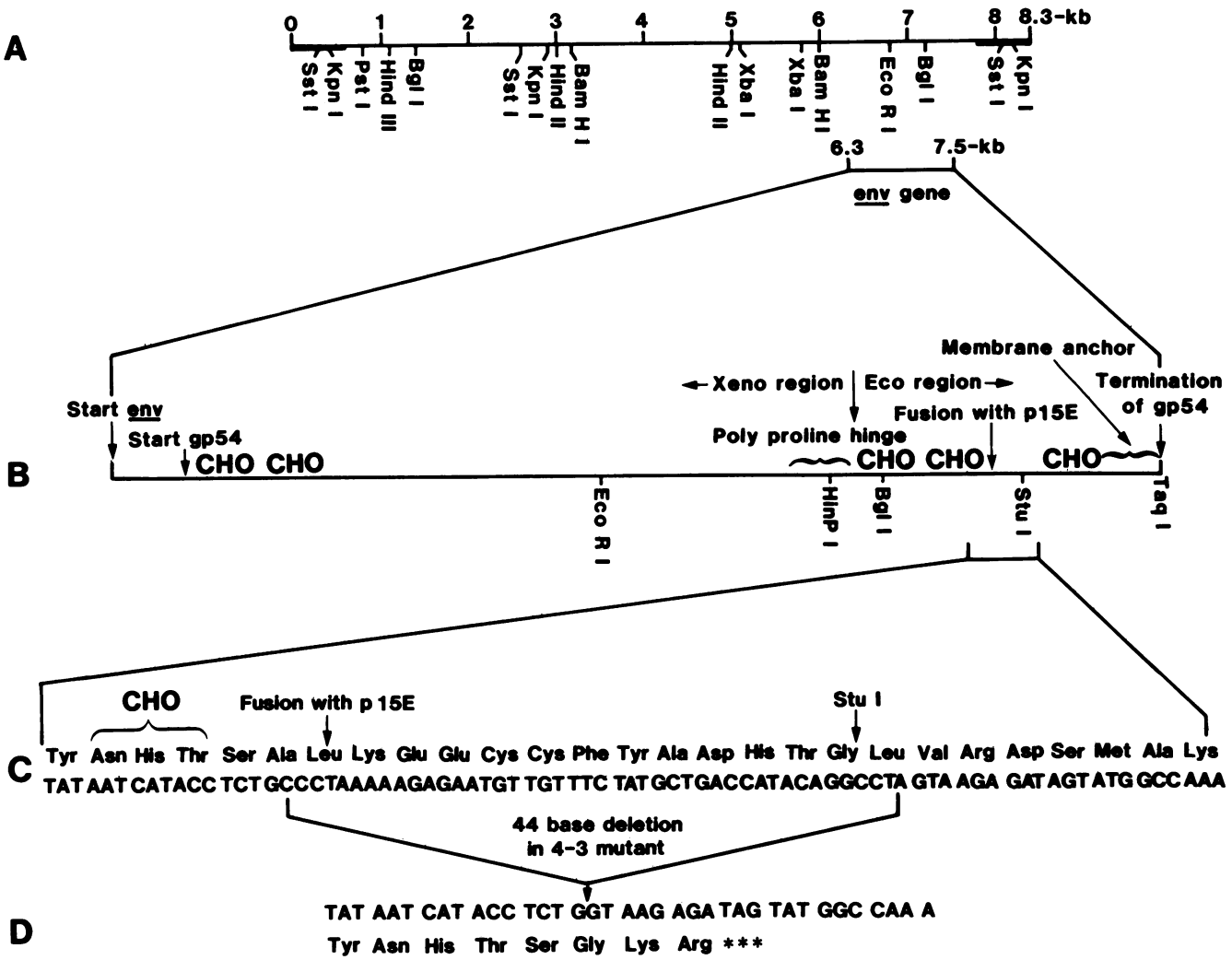

FIG. 1. Restriction map and nucleotide sequence of the R-SFFV mutant 4-3 as compared with wild-type R-SFFV. All map distances are shown in kilobase pairs. (A) Colinear restriction map of wild-type R-SFFV. As described in the text, the 4-3 mutant has lost the *Hin*dIII and *Hin*dII sites at map positions 1.1 and 5.0, respectively. (B) Envelope glycoprotein gene of R-SFFV, showing some of the structural features of the glycoprotein above the line and several restriction sites located within the *env* gene below the line. There are additional *Hin*PI, *Stu*I, and *Taq*I sites within the *env* gene which have been omitted from this map because they are irrelevant to this presentation. The structural features of the glycoprotein include potential carbohydrate addition sites (CHO), the leader peptide of the *env* precursor (the region between "start *env*" and "start gp54"), a proline-rich putative hinge-type sequence ("poly proline hinge"), the site of a 585-bp deletion that results in a fusion of p15E with gp70-derived amino acids ("fusion with p15E"), the recombination site between ecotropic-derived and xenotropic-derived sequences (Xeno region | Eco region), the hydrophobic membrane anchor ("membrane anchor"), and the site of the early termination of the p15E coding sequences ("termination of gp54"). (C) A further enlargement of that region of the R-SFFV *env* gene that contains the 4-3 mutation. (D) The DNA sequence of the 4-3 mutant, showing the effect of the 44-base deletion on the predicted amino acid sequence. DNA sequence analysis was done with M13 clones and by dideoxynucleotide sequencing as previously described (18, 24). An M13 subclone of pBC 4-3:10 extending from the *Taq*I site to the *Hin*PI site yielded the critical sequence information. A substantial portion of the rest of the 4-3 *env* gene sequence was also determined, including the entire 3'-terminal half (data not shown). Only a few single base pair differences were found that resulted in either no difference or conservative differences in the predicted amino acid sequence.

the M_r of 38,000 previously measured for the unglycosylated polypeptide chain (16). Moreover, the nucleotide sequence confirms previously described protein fragmentation results (16) which indicated that the 4-3 mutation affects the carboxyl-terminal ecotropic domain of the SFFV glycoprotein but not the amino-terminal xenotropic domain.

To understand the mechanism by which the 4-3 deletion was generated, we performed diagonal dot matrix analysis of the *env* gene region surrounding the deletion. The results indicated the presence of a small, imperfect, inverted repeat immediately flanking the site of the deletion (Fig. 2). The presence of this inverted repeat is suggestive of a mechanism similar to those involved in illegitimate recombination in other systems including the C_μ to C_δ immunoglobulin gene class switch (13, 25). It is also noteworthy that the 4-3 deletion overlaps the 585-bp deletion (the gp70/p15E fusion site in Fig. 1B) that is characteristic of all SFFVs studied to date (1, 4, 7, 28). It is possible that this region of the *env* gene is a target for recombinational mechanisms that result in deletions.

Previous studies have shown that a small proportion of the wild-type R-SFFV gp54 glycoprotein occurs on the surfaces

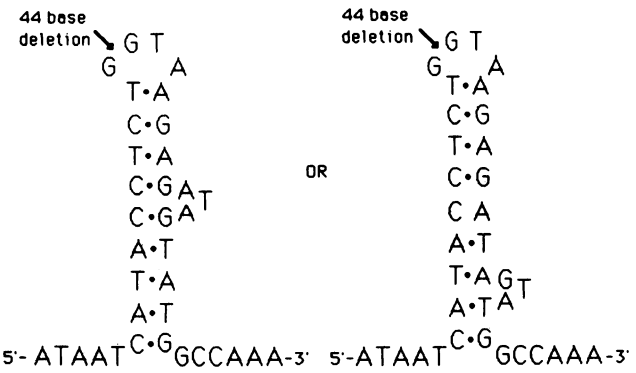

FIG. 2. Inverted repeats flanking the site of the 4-3 mutant deletion. The sequence is the same for both structures, with the left structure forming 9 bp and the right structure forming 8 bp.

of infected cells, whereas the majority occurs in the rough endoplasmic reticulum (14, 19, 22, 23). However, the mutant 4-3 glycoprotein may be absent or in only low concentrations on cell surfaces (16), in agreement with the structural evidence described here. The gp45 glycoprotein encoded by mutant 4-3 also has a shortened intracellular half-life of 50 min, compared with at least 5 h for gp54. Thus, L-[^{35}S]methionine-labeled gp45 loses its radioactivity more rapidly during a cold chase with nonradioactive methionine (Fig. 3). We presume that gp45 is degraded intracellularly because it does not accumulate in large amounts in the extracellular medium.

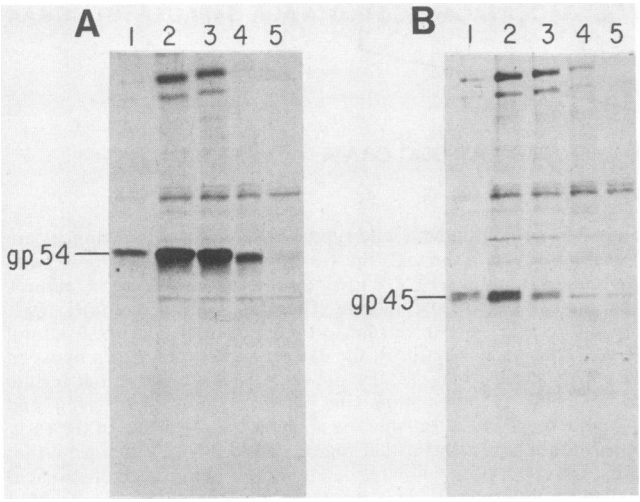

FIG. 3. Kinetic analysis of the envelope glycoproteins encoded by R-SFFV (A) and mutant 4-3 (B). R-SFFV- and 4-3-infected NIH/3T3 cells were pulse-labeled with 100 μCi of L-[^{35}S]methionine for various time periods and chased with the addition of unlabeled methionine. The cells were subsequently lysed, and the viral proteins in the lysates were immunoprecipitated with monospecific antiserum to MuLV gp70^{env}. Immunoprecipitated proteins were electrophoresed in 10 to 20% polyacrylamide gels containing 0.1% sodium dodecyl sulfate. The gels were then processed for fluorography. Fluorograms were scanned by densitometry to determine the intracellular half-lives of the glycoproteins. All of the biochemical methods used have been previously described (9, 23). Labeling and electrophoresis conditions for samples in A and B were identical. A minor nonspecific component migrated slightly faster than gp45. Lanes: 1, 10-min pulse; 2, 30-min pulse; 3, 30-min pulse followed by a 1-h chase; 4, 30-min pulse followed by a 6-h chase; 5, 30-min pulse followed by a 24-h chase.

Previous studies (16) have shown the 4-3 mutant to be weakly pathogenic in adult NIH/Swiss mice. The mutant reproducibly causes a mild splenomegaly (ca. 50% enlargement in weight) with no foci. This mild disease was not substantially amplified, even after four serial passages of the mutant virus in mice (16), indicating that the reduced pathogenicity is not due to low titers of virus injected or to contamination with a pathogenic SFFV. To more carefully analyze this weak pathogenicity, we examined the histology of spleen sections. The results indicated a substantial increase in erythroblast proliferation in the red pulp (Fig. 4) without effacement of lymphoid follicles. The erythroblast

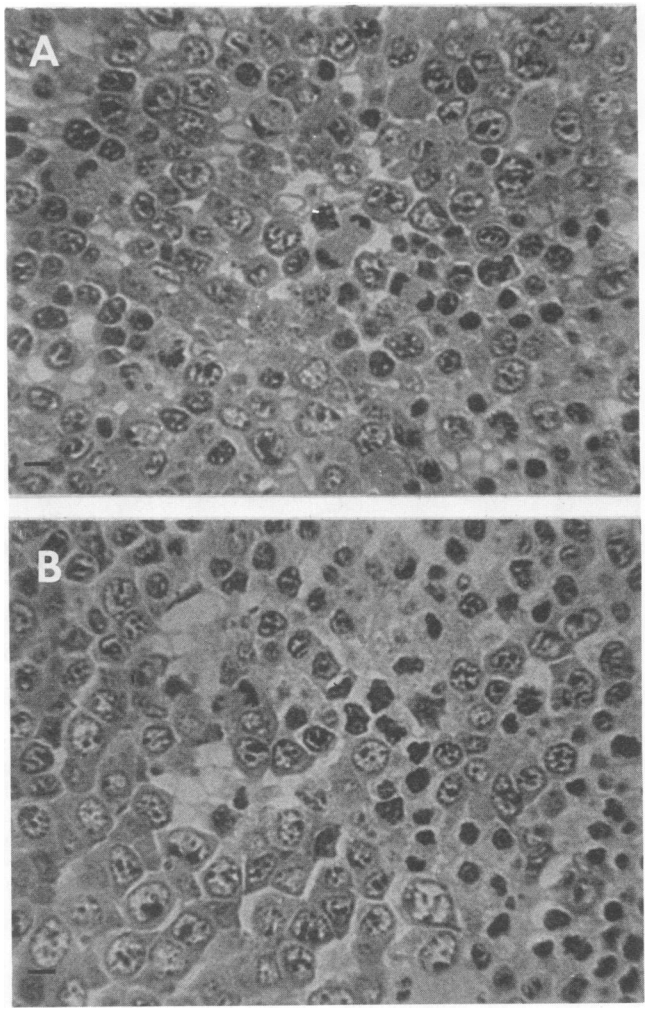

FIG. 4. Histological examination of spleens infected with R-SFFV (A) or mutant 4-3 (B). Adult (4- to 6-week-old) female NIH/Swiss mice were injected intravenously with virus complexes containing R-MuLV and wild-type R-SFFV or R-MuLV and mutant R-SFFV. Animals were sacrificed at 3 weeks postinfection; enlarged spleens (0.63 g, wild-type complex; 0.31 g, mutant complex) were removed and fixed in Bouin solution. Spleens from uninfected mice and mice infected with R-MuLV alone were 0.12 to 0.16 g (16). Sections were stained with hematoxylin-eosin and examined by light microscopy. Bars = 15 μm. The regions of erythropoiesis are increased in both spleens. They are characterized by immature proliferating cells with large nuclei amid differentiating progeny cells with nuclei that are moderately to extremely pycnotic. Spleens from normal or R-MuLV-infected mice contain only relatively infrequent small erythropoietic islands.

proliferation was indicated by mitotic figures. We conclude that the 4-3 mutant causes a relatively mild erythroproliferative disorder which is histologically similar to the more extensive disease caused by wild-type R-SFFV. In addition, cells were obtained from spleens infected with mutant 4-3 and were subsequently labeled with L-[^{35}S]methionine to analyze *env* gene expression. These cells synthesized the MuLV *env* gene product gPr90 and its processed derivatives gp70 and p15E in addition to the mutant 4-3 gp45 glycoprotein; no other *env*-related molecules were observed in these cells (unpublished data).

We describe elsewhere (Machida et al., in preparation) a structural model for the SFFV glycoprotein. According to that model, the xenotropic-related and ecotropic-related regions of gp55 occur within independently folded amino-terminal and carboxyl-terminal globular domains that are connected by a proline-rich flexible linker. The carboxyl-terminal domain of gp55 contains a hydrophobic membrane anchor that affixes gp55 to cell membranes. Based on the evidence described here, we propose that the amino-terminal xenotropic-related domain of the SFFV glycoprotein may contain an active site for causing erythroblast proliferation. According to this model, the reduced pathogenic activity of the truncated gp45 glycoprotein may derive from its shortened intracellular lifespan and from its reduced concentration at the critical subcellular target site.

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