Substitution of leucine for isoleucine in a sequence highly conserved among retroviral envelope surface glycoproteins attenuates the lytic effect of the Friend murine leukemia virus

(erythroleukemia/hemolytic anemia/long terminal repeat/retroviral pathogenesis)

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ABSTRACT Friend murine leukemia virus is a replication-competent retrovirus that contains no oncogene and that exerts lytic and leukemogenic properties. Thus, newborn mice inoculated with Friend murine leukemia virus develop severe early hemolytic anemia before appearance of erythroleukemia. To identify the retroviral determinants regulating these effects, we used chimeric infectious constructions and site-directed point mutations between a virulent Friend murine leukemia virus strain and a naturally occurring variant attenuated in lytic and leukemogenic effects. We found that severe hemolytic anemia was always associated with higher numbers of blood reticulocytes with budding retroviral particles. Furthermore, a remarkably conservative leucine to isoleucine change in the extracellular SU component of the retroviral envelope was sufficient to attenuate this lytic effect. Also, this leucine at position 348 of the envelope precursor protein was located within the only stretch of five amino acids that is conserved in the extracellular SU component of all murine, feline, and primate type C and type D retroviral envelopes. This observation suggested an important structural function for this yet undescribed conserved sequence of the envelope. Lastly, we observed that lytic and leukemogenic effects were attenuated by a deletion of a second repeat in the transcriptional enhancer region of the viral long terminal repeats of the variant strain.

Neoplastic transformation by retroviruses lacking oncogenes depends mostly on enhancer sequences present in the U3 region of their long terminal repeats (LTRs) (1–7). In contrast, lytic effects have been mapped mostly to the env region (8–16). In the absence of virus-encoded oncogenes, retroviral pathogenesis results from an intricate array of events that, in general, are directly dependent on these two regions. Beyond their direct role in the replication cycle of the virus, the U3 and env regions have been shown to influence the cell types involved during the pathogenic processes (1, 2, 5, 7). To identify and better understand the respective role(s) of the different retroviral regions in cell-specific lytic and leukemogenic effects, we have used the replication-competent Friend murine leukemia virus (Fr-MuLV). The Fr-MuLV provides a unique tool for such studies, since it exerts cytolytic and leukemogenic effects in erythroid cells, leading to a severe early hemolytic anemia (EHA) followed by a late erythroleukemia in newborn-inoculated mice of susceptible strains (10). A 2.5-kilobase (kb) pol-env fragment from Fr-MuLV strain B3 could attenuate the EHA induced by the virulent Fr-MuLV strain FB29, whereas sequences in the LTR or gag of Fr-MuLV B3 increased the latency of erythroleukemia (10). Fr-MuLV FB29 and B3 are independent viral isolates and diverge markedly. In contrast, the virulent strain Fr-MuLV 57 and the attenuated variant B3 were cloned from the same original stock (17–19), and these two strains had very few detectable differences and similar overall replication abilities in vitro and in vivo (19). Therefore, in the current experiments we constructed chimeric viruses exchanging env and LTR fragments between the attenuated strain B3 and the virulent strain 57 of Fr-MuLV to reduce the number of sequence differences to account for the differences in pathogenic effects. The present results obtained with such constructs as well as with site-directed point mutants indicated the following. (i) A remarkably conservative Leu → Ile substitution in the extracellular SU component of the envelope was sufficient to attenuate the hemolytic effect; this substitution enabled us to identify a unique stretch of five amino acids of SU that is conserved among type C and type D retroviruses, including all murine, feline, and primate isolates. (ii) Loss of one direct repeat sequence in U3 altered both the hemolytic effect and latency of leukemia.

MATERIALS AND METHODS

Oligonucleotides. Fr-MuLV 57 and B3 sequencing was performed with synthetic oligonucleotides derived from the sequences of Fr-MuLV 57 (20, 39) and the Bluescript plasmid (Stratagene). We used 23 oligonucleotides 20–25 base pairs (bp) long, located =170 bases apart and covering both strands of the pol-env region. The env–LTR regions were sequenced using 4 oligonucleotides derived from both strands. Oligonucleotides used for PCR amplification in env were as follows: MS3, ACCGCTGCCACGTAAAGGCTCCCGG; MS8, CGCCAGCAGGAACCGGAGACAGT; MS16, GGCAGGAGCAGTTAGCTGGGCGCAG; and MS21, GCCCTTTGAGACAGGAAAGGAA. For site-directed mutagenesis we used the following: 57Ser, GGGATTTCGAGCGTTGAAAC; 57Ile, ACTAGGCATAATCCAGC; 57TCB; B3Pro, GGAGTTTGGGAGCGTTGAAAC; and B3Leu, ACTAGGCGATACCCGACTCT.

Molecular Clones and Constructions, Transfections, and Viral Stocks. Infectious clones of Fr-MuLV 57 (18) and B3 (19) were recloned in permuted forms with one copy of the LTR in the pUC19A plasmid (21), which allowed substitution of fragments in a cassette manner. We derived chimeric

Abbreviations: Fr- and Mo-MuLV, Friend and Moloney murine leukemia viruses; LTR, long terminal repeat; EHA, early hemolytic anemia.

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constructions using a pol Sph I–Xba I fragment and a pol–env Xba I–Cla I fragment of clone B3 and the env–U3–R Cla I–Kpn I fragment of either clone B3 or 57 (Fig. 1). In clone 57, these pol, pol–env, and env–U3–R fragments comprised 495 bp, 2069 bp, and 621 bp, respectively (20, 39, 40). All steps, including production and titration of infectious viral stocks, were performed as described (10, 21).

**Mice, Viral Inoculation, and Animal Follow-Up.** Eighteen to 36-hour-old ICFW and IRW mice were inoculated and followed for EHA and leukemia as reported (10, 19, 21). EHA was evaluated from three sequential bleedings performed at 3-day intervals between 16 and 24 days of age.

**Electron Microscopy.** The percentage of circulating reticulocytes with budding retroviral particles in 18- to 22-day-old IRW mice was determined by electron microscopy (10).

**Sequence Analysis, Mutagenesis, and PCR.** We sequenced both strands of the pol–env–U3–R Sph I–Kpn I fragment of clones B3 and 57 and compared them to that published for clone 57 (20, 39, 40). We used the Sanger method (22) and dITP to resolve compression effects.

Site-directed mutagenesis of the 57 clone at position 19 or 1042 of the env coding region was performed using oligonucleotide 57Ser or 57Ile, respectively, and oligonucleotides B3Pro and B3Leu were used for mutations of clone B3. All steps leading to isolation and characterization of the mutants were performed as described (23). An Sph I–Cla I mutated envelope fragment was reconstituted after ligation of an Sph I–HincII fragment 1785-bp mutated fragment and an HincII–Cla I 778-bp wild-type fragment and inserted into the desired infectious molecular clone.

The presence of the expected point mutation after transfection and animal inoculation was assessed by PCR analyses performed on genomic DNA prepared from splenocytes of 10-day-old ICFW mice inoculated as newborns. PCR surrounding position 19 of the env coding region was performed using oligonucleotides MS3 and MS21 (305 bp apart), and oligonucleotides MS8 and MS16 (139 bp apart) were used for PCR surrounding position 1042. Amplification was performed in a Perkin–Elmer/Cetus DNA thermal cycler using one cycle of 6 min at 94°C, 2 min at 45°C, and 2 min at 72°C; this was followed by 29 cycles of 1 min at 94°C, 2 min at 45°C, and 2 min at 72°C. Aliquots (10 μl) of the amplified products were treated as described (23) except that prehybridization was performed for at least 1 hr at 37°C and hybridization was carried out at 37°C for at least 1 hr. Membranes were then washed five times at room temperature and once at 50°C in 6× SSC/0.1% SDS (1× SSC = 150 mM NaCl/15 mM sodium citrate, pH 7.0), and a further wash was performed at the desired temperatures before 1 hr of exposure at −80°C.

![FIG. 1. Differences between Fr-MuLV 57 and B3 in env. Viruses were sequenced from the Sph I to the Kpn I sites. The nucleotide and the deduced amino acid sequences are shown in the vicinity of the two differences observed in the env region. Numbering starts at the initiator codon of the envelope signal peptide. SP, signal peptide; SU/gp70, extracellular surface component of the envelope; TM/p15E, transmembrane component.](image)

**RESULTS**

**Use of Molecular Constructs to Map Induction of EHA.** Infectious Fr-MuLV clones were constructed exchanging various portions of the pol, env, and LTR regions between strains 57 and B3. These viruses were then compared for their ability to induce EHA. The results indicated that Fr-MuLV 57 substituted by Fr-MuLV B3 at either a 2.0-kb pol–env (Xba I–Cla I) fragment or a 0.6-kb env–U3 (Cla I–Kpn I) fragment (Fig. 1) reduced the severity of anemia to the level observed with Fr-MuLV B3 itself (Fig. 2, constructs 16 and 57B3L). Therefore, both of these regions appeared to be involved in induction of severe EHA.

**DNA Sequencing and Site-Directed Mutagenesis to Define Effects on EHA.** To identify more precisely the sequence changes responsible for the reduction in EHA, we sequenced Fr-MuLV B3 and 57 in these regions. The results for 2564 bp of pol–env, including the Xba I–Cla I fragment, indicated that Fr-MuLV B3 and Fr-MuLV 57 differed at only two bases in this region (Fig. 1). The first difference was a C → T transition located in the region that overlaps between the pol and env
coding regions, at position 19 of the env sequence. In the pol reading frame, both corresponding codons (TCC and TCT) encoded a serine, whereas in the env reading frame, this C→T transition changed the CCA (proline) codon of Fr-MuLV 57 into a TCA (serine) codon in Fr-MuLV B3. This codon corresponded to the seventh amino acid of the signal peptide of the envelope protein. The second difference was a T→A transversion at position 1042 of the envelope coding frame. This caused a change at codon 348 from leucine in Fr-MuLV 57 to isoleucine in Fr-MuLV B3 (Fig. 1).

To identify which of these env mutations was involved in the differences in EHA, we introduced these mutations into the virulent strain Fr-MuLV 57 by using molecular constructions and site-directed mutations. As shown in Fig. 2, we found that a change of the proline codon in the Fr-MuLV 57 env signal peptide into a serine codon did not alter severity of EHA (construct 50), whereas changing the leucine at codon 348 in Fr-MuLV 57 into an isoleucine codon significantly reduced severity of EHA (construct 52). Similarly, construct 16, which included both mutations, also had a reduced EHA effect. Therefore, the difference at the envelope codon 348 was sufficient to account for the attenuation of EHA associated with the strain B3 env region.

The effect of the env–U3 (Cla I–Kpn I) fragment of Fr-MuLV B3 on attenuation of EHA was also analyzed by sequencing and comparison to Fr-MuLV 57. The results indicated that there was no difference between the env coding regions in this fragment, but there were two changes in the U3 region, a large deletion and a single nucleotide transition (Fig. 5). Most striking was the deletion observed in the Fr-MuLV B3 U3 region, which contained only one of the two repeats found in Fr-MuLV 57 (Fig. 5). The G→A nucleotide transition was located downstream from the repeats. In light of previous findings by others showing that deletion of one of the two direct repeat structures present in Fr-MuLV 57 and other MuLVs reduced the viral pathogenicity (5, 6, 24), it is most likely that the absence of a second repeat in Fr-MuLV B3 was sufficient to attenuate the pathogenicity of this virus.

Since either the envelope or U3 of Fr-MuLV B3 was able to attenuate induction of severe EHA by Fr-MuLV 57, we also examined the requirements for restoring induction of severe EHA by Fr-MuLV B3. We found that neither the leucine at position 348 nor the Fr-MuLV 57 U3 alone (Fig. 2, construct 53 or B357L, respectively) was sufficient to restore induction of severe EHA in this strain. However, the combination of both determinants appeared to be necessary and sufficient to restore this effect (Fig. 2, construct 54).

Association Between Severe EHA and Retroviral Budding in Reticulocytes. Severe EHA is associated with a high percentage of circulating reticulocytes with C-type budding particles (10), suggesting a direct correlation between hemolysis and viral expression in reticulocytes. Therefore, we examined the presence of reticulocytes with budding C-type particles after inoculation of some of the constructs and mutants used in the present study. The highest numbers of infected reticulocytes were observed after inoculation of the virulent strain 57 or either of the mutants that induced severe EHA (Table 1, constructs 50 and 54). Significantly reduced numbers of infected reticulocytes were observed after inoculation of the attenuated mutants even when high viral doses were inoculated (Table 1). The range of percentages of infected reticulocytes did not overlap between severe and attenuated EHA-inducing strains of Fr-MuLV. Thus, severe EHA was associated with a higher level of virus particle expression in circulating reticulocytes.

In Vivo Stability of env Point Mutations. To verify whether the point mutations we introduced were still present after in vitro and in vivo replication, we used four of the Fr-MuLV strains mutated in the envelope codon 7 or 348, strains 50, 52, 53, and 54 (Fig. 2). By using PCR analyses on splenocyte DNA obtained at the time of onset of EHA and hybridization with specific oligonucleotides (see Materials and Methods), we observed that viruses spreading in the spleen harbored the corresponding mutation(s) (data not shown).

Influence of the U3 Region on Latency of Leukemia. Approximately 1.5 months after EHA occurred, animals inoculated with Fr-MuLV 57 developed erythroleukemia. By comparing Fr-MuLV strains B3 and FB29, we have previously seen that latency of induction of erythroleukemia was strongly influenced by sequences in the LTR or gag region (10). To map this effect more precisely, the constructs described above were also studied for leukemogenicity. The eight virus constructs studied segregated into two groups with either short latency (1.6–3 months) or long latency (8–11 months). These results showed that the presence of the U3 region from Fr-MuLV 57 was associated with low latency (Table 2). The different combinations of amino acids at the envelope codons 7 and 348 did not affect latency. Further...
Newborn-inoculated ICFW mice were followed for gross organ enlargement by regular palpation under ether anesthesia. Latency was defined as the time when >50% of the animals were leukemic or dead. Severity of EHA (S, severe; M, mild) was taken from data in Table 1. FFU, focus-forming units.

more, the severe EHA was not required for induction of erythroleukemia with short latency (Table 2, constructs 16, 52, and B357L).

**DISCUSSION**

It was most interesting to find that a single leucine to isoleucine substitution in the envelope codon 348 of Fr-MuLV could dramatically alter the hemolytic effect and the number of reticulocytes with budding retroviruses. This change might appear to be too conservative to have such a strong influence on a pathogenic effect. However, dramatic biological changes have been described in the case of another rather conservative (Val → Ile) substitution in the envelope of a neurovirulent MuLV (9). Similar conservative changes have been shown to alter significantly the biological properties and crystal structure of T4 phage lysozyme (25).

The leucine that we identified to be crucial for the lytic effect was located directly C terminal of the hypervariable proline-rich region of the extracellular SU component of the envelope. This leucine was included within a stretch of six contiguous amino acids (CWLCU) that was rigorously conserved among all murine and feline exogenous and endogenous retroviruses for which sequences were available (Fig. 4). In addition, the first four or five of these amino acids were also found at a similar location in all primate type C and type D retroviruses and one avian retrovirus (Fig. 4). Interestingly, no other amino acid sequence with comparable conservation was found in the SU of these retroviruses. Since the corresponding nucleotide sequences were significantly divergent (data not shown), and since no cis-acting regulatory sequences have been described in this region, the amino acid change we observed in this region was most likely responsible for the differences observed in the lytic activity. Other lytic effects have been described for some retroviruses harboring this conserved determinant. These include syncytiotic lysis of human lymphoblastoid cell lines by the baboon endogenous virus (41), env-dependent degenerative diseases of the central nervous system in mice (9, 15, 30), and hematopoietic syndromes in cats (12, 13). It would be of interest to evaluate the importance of the conserved stretch of amino acids identified in the present report in the pathogenic effects of murine, feline, avian, and primate retroviruses.

Recently, a correlation has been shown between altered envelope processing and neurovirulence of a mutant of Mo-MuLV (9) and immunodeficiency-inducing ability of a strain of feline leukemia retrovirus (14). However, we have observed no obvious differences in the envelope processing of the attenuated and virulent strains B3 and 57 of Fr-MuLV in murine fibroblasts (unpublished observations). Therefore, the precise mechanism by which the switch in a single envelope amino acid influences severity of EHA is not known. This envelope effect might act by altering the efficiency of infection of erythroid cells, but Fr-MuLV B3 and Fr-MuLV 57 do not differ in their overall efficiency of infection as observed either in vivo on mouse spleens or in vitro on fibroblast cell lines (19). Therefore, if such an effect occurs in vivo, it might be specific for viral receptors present on the cells of the erythroid lineage. Alternatively, envelope protein expression might decrease the longevity of infected erythroid cells. We have previously documented a decrease in the half-life of erythroid cells in mice infected with a virulent Fr-MuLV (10). Antibody-mediated lysis is unlikely

![Figure 4](image-url)
to be the mechanism of destruction because the mice are tolerant of Fr-MuLV after neonatal inoculation, and no antiviral antibodies can be detected. However, cells expressing viral envelope protein could be directly cleared by the reticuloendothelial system, and Fr-MuLV 57 envelope might be more readily recognized than Fr-MuLV B3 envelope. Furthermore, interaction of retroviral envelopes with their cellular receptors or other cellular components might interfere with normal physiological functions, as described for the envelope of the murine spleen focus-forming virus and the erythropoietin receptor (42), and this might result in direct cytopathic effects that might be different in the case of Fr-MuLV strains 57 and B3.

The present results showed that the U3 region also was important in regulating severity of the lytic effect induced by Fr-MuLV. The U3 sequence of Fr-MuLV B3 lacked the second repeat found in the transcriptional enhancer region of Fr-MuLV 57 (7, 20, 43, 44). There was also a single base substitution downstream from these repeats. However, the lack of the second repeat in Fr-MuLV B3 is likely to be the more important change because deletion of one repeat has been associated with decreased enhancer activity and leukemogenicity in several MuLVs (5, 6, 24, 43). In Fr-MuLV 57, the presence of these direct repeats might increase EHA by increasing the level of viral transcription leading to higher levels of envelope protein expression in erythroid cells. In contrast to these results, no effects of the U3 region on EHA were noted in our previous experiments comparing Fr-MuLV B3 with another virulent strain, Fr-MuLV FB29 (10). However, FB29 differs significantly from Fr-MuLV 57 throughout the viral genome, and variations in other regions might explain the differences in results observed.

Differences in the type of leukemia induced by the erythroleukemogenic Fr-MuLV 57 and the lymphomagenic Mo-MuLV have been found to depend on sequences in U3 (1, 2, 5, 7). In addition to the envelope and U3 regions, other Fr-MuLV sequences have also been noted to be important for induction of EHA. In our previous experiments comparing EHA induced by Fr-MuLV and Mo-MuLV we found that Mo-MuLV lacked sequences in the U5– gag-pol region that were important for inducing severe EHA (21). Since exchange of the U5– gag-pol region between Fr-MuLV strains B3 and 57 appeared to have no effect on EHA (construct 54), it seems likely that Fr-MuLV B3 and 57 have appropriate U5– gag-pol sequences for induction of EHA. Therefore, data from present and previous studies indicate that variation in three different regions can influence severity of EHA without altering overall replicating abilities (10, 21). These include the conserved structural envelope determinant identified in the present report, transcriptional enhancer sequences in U3, and U5– gag-pol sequences yet to be identified. Study of the mechanisms of action of each of these regions should contribute to better understanding of the molecular basis of complex pathogenic processes induced by retroviruses.

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