Sequences Responsible for the Distinctive Hemolytic Potentials of Friend and Moloney Murine Leukemia Viruses Are Dispersed but Confined to the Ψ -gag-PR Region

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Received 18 March 1993/Accepted 15 June 1993

Friend and Moloney murine leukemia viruses (F- and M-MuLV) induce distinct diseases in hematopoietic tissues following inoculation of newborn mice of susceptible strains. F-MuLV induces erythroleukemia preceded by severe early hemolytic anemia; M-MuLV induces thymomas and only very mild hemolysis. The major viral determinant of severe early hemolytic anemia resides in the *env* gene, but sequences located outside this gene can modulate this effect. By means of genetic chimeras of F- and M-MuLV, we have found that although they are confined to the 5' portion of the *env* gene intron, sequences that determine the distinctive hemolytic potentials of F- and M-MuLV are widely distributed over a region spanning the RNA encapsidation domain, the *gag* gene, and the portion of the *pol* gene encoding the viral protease. Within this large region, two fragments of M-MuLV, a 1.3-kb region encoding the matrix, pp12, and capsid proteins and a 0.8-kb region encoding the nucleocapsid and the viral protease, were capable, individually, of partially attenuating the capacity of F-MuLV for induction of severe early hemolytic anemia. In association, these two fragments conferred complete attenuation. Moreover, a second pair of adjacent fragments within this large region appeared to behave cooperatively to confer complete attenuation; a 0.36-kb region roughly corresponding to the encapsidation domain, although not detectably altering hemolytic potential on its own, deepened the attenuation conferred by the adjacent 1.3-kb region. Whether capable of inducing severe early hemolytic anemia or not and despite different efficiencies of induction of recombinant polytropic viruses, all chimeric viruses retained the erythroleukemogenicity of the F-MuLV parent.

The murine leukemia viruses (MuLV) induce diverse pathologic changes in hematopoietic tissues. The cell type specificity of pathogenesis is highly dependent on the viral strain. Since these viral strains are genetically closely related, the study of their pathogenesis presents an opportunity to investigate how particular viral sequences, acting in *cis* or in *trans*, govern the interactions between virus and cells of a particular lineage. When inoculated into newborn mice of susceptible strains, Friend MuLV (F-MuLV) induces severe early hemolytic anemia (EHA) that is readily detectable 2 to 3 weeks after inoculation. This severe EHA may be distinguished from the erythroleukemia that appears at approximately 2 months of age, although both are characterized by anemia and splenomegaly, since severe EHA is attended by compensatory erythropoiesis in response to hemolysis (37). Moloney MuLV (M-MuLV) induces neither severe EHA nor erythroleukemia under the same conditions but rather induces thymomas appearing after approximately 3 months. A major determinant of the hemolytic potential of virulent strains of F-MuLV resides in the *env* gene—the substitution of a leucine for an isoleucine in a conserved sequence motif sufficing to attenuate the hemolytic potential (33). Nevertheless, the *env* gene of M-MuLV is competent for induction of severe EHA (34), and thus sequence polymorphism in other genomic regions must account for the specificity of F-MuLV for pathogenesis in the erythroid compartment.

Certain viral sequences responsible for the characteristic course of disease induced by F- and M-MuLV have already been localized. Short sequence motifs in the enhancer region of the long terminal repeat (LTR), recognized by *trans*-acting cellular transcription factors, are largely responsible for the tissue specificity of leukemic induction (3, 4, 38). We have shown that sequence differences outside this region, in a large R-U5-*gag-pol* fragment, determine the distinctive capacities of F- and M-MuLV for induction of severe EHA (34). Thus, although the capacities to induce erythroleukemia and severe EHA might both be expected to rely on a common tropism for the erythroid lineage, distinct sequences have been implicated in the two pathologies.

To identify sequences which differentiate their hemolytic potentials, we have made viral chimeras of F- and M-MuLV by exchanging allelic genomic fragments from the 5.1-kb R-U5-*gag-pol* region implicated in the induction of severe EHA. Since individual pathogenic effects may depend on the coexistence of multiple discrete genetic elements, we have initially attempted to attenuate the capacity of F-MuLV for induction of severe EHA by the introduction of M-MuLV sequences. Such an approach permits the identification of individual elements, whether strong or weak. Here, we show that although they are confined to the 5' part of the *env* intron, sequences which determine the characteristic hemolytic capacities of F- and M-MuLV are widely distributed in a region spanning the RNA encapsidation domain, the *gag* gene, and the portion of the *pol* gene encoding the viral protease. Within this large region, two fragments of M-MuLV were capable, individually, of partially attenuating

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the capacity of F-MuLV for induction of severe EHA. Moreover, two pairs of adjacent fragments within this region appeared to behave cooperatively to confer complete attenuation. These genetic exchanges did not alter the tissue specificity or latency of neoplastic transformation: all chimeric viruses were equally erythroleukemogenic. The capacities of the viral chimeras for dissemination in vitro and in vivo and for induction of polytropic viruses by recombination with endogenous sequences (12, 16) were also evaluated.

MATERIALS AND METHODS

Molecular constructions. Viral DNA chimeras of F-MuLV 57 (27) and M-MuLV 8.2 (32) were prepared from plasmids containing permuted versions of MuLV genomic DNA bearing a single LTR. F-MuLV 57 DNA was cloned in the *EcoRI* site of either pUC19A (34) or Bluescript, providing p57A and pBS/57, respectively; M-MuLV 8.2 was cloned in the *HindIII* site of pUC19B (34), yielding p8.2B. Since certain strategies for the reconstitution of complete retroviral genomic DNA in plasmid vectors seem to lead to deletion of retroviral sequences (7), the construction of chimeric viral genomes will be described in detail. The following nomenclature will be employed for the restriction enzyme fragments of M-MuLV introduced into F-MuLV genomic DNA (see Fig. 1): E (*SphI-ClaI*), contains most of the *env* gene; P (*BclI-SphI*), bears only *pol* sequences; U5G (*SacI-SacI*), contains the U5 region, all *gag* sequences, and the 5' portion of *pol* specifying the viral protease; U5SD (*KpnI-SpeI*), contains the U5 region and the 5' portion of the leader including the splice donor signal; G (*AflII-BclI*), encodes the proteins of the *gag* gene and the viral protease; Ψ MACA (*SpeI-BglII*), bears the encapsidation domain and *gag* sequences excluding sequences encoding the nucleocapsid; NCPR (*BglII-BclI*), encodes the nucleocapsid and viral protease; MACA (*AflII-BglII*), encodes the matrix, pp12, and most of the capsid; and Ψ (*SpeI-AflII*), roughly corresponds to the 351-nucleotide encapsidation domain defined by deletion analysis (21, 22). The derivation of the chimeric plasmid pFME, formerly pFMF, has been previously described (34). The chimeric plasmid pFMP/B was generated by ligation of the 5.9-kb *BclI-SphI* fragment of p57A and the 4.9-kb *BclI-SphI* fragment of p8.2B (P) which contains the vector pUC19B. The chimeric plasmid pFMU5G/A was generated by ligation of the 8.3- and 2.6-kb (U5G) *SacI-SacI* fragments of p57A and p8.2B, respectively. The 0.25-kb *KpnI-SpeI* fragment (U5SD), the 1.6-kb *SpeI-BglII* fragment (Ψ MACA), and the 0.83-kb *BglII-BclI* fragment (NCPR), as well as the 0.36-kb *SpeI-AflII* (Ψ) and 1.3-kb *AflII-BglII* (MACA) fragments, were prepared by digestion of p8.2B. To facilitate substitution of these fragments, the 2.8-kb *KpnI-EcoRI* fragment of p57A was subcloned in the corresponding restriction sites of pUC19. This subclone was digested with the appropriate enzymes for the insertion, like cassettes, of M-MuLV fragments U5SD, Ψ MACA, NCPR, Ψ , and MACA. The 2.7-kb *KpnI-BclI* chimeric fragment of these plasmids was then excised. pBS57 DNA was digested with either *NcoI* and *KpnI* or *NcoI* and *BclI*. Chimeric genomic DNA was reconstituted by ligation of the 4.4-kb *NcoI-KpnI* and 4.1-kb *BclI-NcoI* fragments of pBS57 with one of the 2.7-kb *KpnI-BclI* chimeric fragments bearing M-MuLV regions U5SD, Ψ MACA, NCPR, Ψ , and MACA, thereby generating plasmids pBS/FMU5SD, pBS/FM Δ G, pBS/FMNCPR, pBS/FM Ψ , and pBS/FM(MACA), respectively.

The plasmid pBS/FMG was constructed by ligation of the 4.4-kb *NcoI-KpnI* and 4.9-kb *BglII-NcoI* fragments of pBS/FMNC with the 1.9-kb *KpnI-BglII* fragment of pBS/FM(MACA). Constructions were verified by digestion of plasmid DNA with several discriminating restriction enzymes.

Tissue culture, transfection, and viral stocks. Unless otherwise stated, *Mus dunni* (20) and NIH 3T3 fibroblasts were cultivated in Dulbecco's modification of Eagle's medium (DMEM) with glutamine (2 mM), penicillin (50 IU ml⁻¹), streptomycin (50 μ g ml⁻¹), and 10% heat-inactivated fetal calf serum. Plasmid DNA (6 to 8 μ g) was digested with *EcoRI* to excise the permuted retroviral genomic DNA bearing a single LTR and transfected into NIH 3T3 cells without prior ligation according to the following adaptation of the method described by Golub et al. (15). Digested DNA was dissolved in 10 μ l of TE (10 mM Tris [pH 7.5], 1 mM EDTA) in a 1.5-ml Eppendorf tube. NIH 3T3 fibroblasts were adjusted to 5×10^6 cells ml⁻¹ in TBS (25 mM Tris HCl [pH 7.4], 137 mM NaCl, 5 mM KCl, 0.7 mM CaCl₂, 0.5 mM MgCl₂, 0.6 mM NaHPO₄). Two hundred microliters of the suspension of NIH 3T3 fibroblasts and 25 μ l of 1 mg of DEAE-dextran ml⁻¹ in TBS were added to the DNA solution. After gentle vortexing, the cells were incubated at 37°C for 40 min and were then transferred to medium and cultivated at 37°C. Transfection was monitored by focal immunofluorescence assay (FIA) (36) and assay of reverse transcriptase activity (14). 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 (0.45- μ m-pore-size filters) and stored in aliquots at -60°C. The identities of chimeric viruses were verified by digestion with discriminating restriction enzymes following amplification of viral genomic RNA by reverse transcription and polymerase chain reaction (PCR).

Reverse transcription and PCR. Genomic viral RNA from 400 μ l of viral stock was amplified by reverse transcription and PCR. The cDNA resulting from each stock was amplified with three pairs of oligonucleotides, pp and AC8, AC6 and AC8, and FMS15 and FMR24, and their products were digested with the restriction enzymes *HgaI*, *PvuI*, and *AflII* or *PvuI*, respectively. PCR was conducted according to the recommendations of the GeneAmp PCR Reagent Kit with AmpliTaq (Perkin-Elmer Cetus), with final concentrations of 200 μ M nucleotides and 2 mM MgCl₂, in a volume of 100 μ l. The cDNA was subjected to 30 cycles of amplification as follows: denaturation for 1 min at 92°C, annealing for 1 min at 40, 45, or 50°C for pairs of primers pp and AC8, AC6 and AC8, or FMS15 and FMR24, respectively, and primer extension for 1.5 min at 72°C. These cycles were preceded by a 5-min denaturation at 92°C and followed by a 5-min extension at 72°C. Oligonucleotides (Genset, Paris, France) used for PCR amplification in the R-U5-*gag* region were as follows: pp, 5'-CCCACCACCGGGAG-3'; AC6, 5'-TGATTGATTTATGCGCCTGC-3'; AC8, 5'-ACAACACAGAACGATGCTGC-3'; FMS15, 5'-CTGCTGAC(G/C)GGAGAAGAAAAC-3'; and FMR24, 5'-CTTAGGGGTCCAGGATTTTGGG-3'.

Origin, infection, and clinical evaluation of mice. All experimentation was conducted on ICFW mice, an inbred line derived from Carworth Farms White outbred mice (42). 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. To assess the induction of EHA, blood samples were taken under ether anesthesia from inoculated mice at 3-day inter-

vals from 16 to 24 days of age by puncture at the retroorbital sinus with 20 μ l of 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. Cell smears were prepared from the enlarged spleens of sacrificed animals and fixed in formaldehyde vapor. The smears were stained for nonspecific esterases by using the Sigma kit for detection of α -naphthyl butyrate esterase (181-B) in the presence and absence of NaF (1.5 mg ml⁻¹). The diagnosis of erythroleukemia depended on the presence of splenomegaly in the absence of lymphadenotrophy or thymotrophy, of severe anemia, and of a predominant population of splenocytes expressing nonspecific esterases (33, 34).

Dissemination in vivo. *M. dunni* and NIH 3T3 fibroblasts were plated at 5×10^4 cells per dish in 60-mm 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 7 and 21 days of age (6 and 20 days after inoculation) and dispersed in complete DMEM. 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 10^5 , 10^4 , and 10^3 cells ml⁻¹ (for enumeration of ecotropic infectious centers) or 10^6 , 10^5 , and 10^4 cells 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 (5, 6, 11). Infectious centers on *M. dunni* fibroblasts were quantitated for all viruses except the parental M-MuLV, for which infectious centers on both *M. dunni* and NIH 3T3 fibroblasts were quantitated, because *M. dunni* fibroblasts are less susceptible to infection by M-MuLV (20).

Dissemination in vitro. *M. dunni* and NIH 3T3 fibroblasts were plated at 5×10^4 cells per dish in 60-mm tissue culture dishes and incubated overnight. The medium was removed, and the cells were treated with 2 ml of 20 μ g of DEAE-dextran ml⁻¹ in DMEM with 5% fetal calf serum for 30 min at 37°C. Following removal of the DEAE-dextran, the cells were washed with 2 ml of DMEM without fetal calf serum and incubated for 15 min at 37°C with 0.5 ml of viral supernatant adjusted to either 2×10^4 or 2×10^3 focus-forming units ml⁻¹, thereby establishing multiplicities of infection of approximately 10^{-1} and 10^{-2} , respectively. A volume of 4.5 ml of medium was added to each plate. After 2 h, the supernatant was replaced with fresh medium. Beginning at 21 h after infection, 1 ml of cell supernatant was collected every 12 h and replaced with fresh medium until cell confluency. The supernatants were filtered (0.45- μ m-pore-size filters) and stored at -80°C until titration of ecotropic focus-forming units by FIA.

Statistical analyses. The means of hematocrits were compared by using the two-tailed *t* test. Differences were considered statistically significant when *P* values were less than 0.05.

RESULTS

Analysis of the pathogenesis of chimeric viruses produced by exchanging allelic DNA fragments of F- and M-MuLV has shown that some viral sequences responsible for the capacity of F-MuLV to induce severe EHA lie outside the U3 region and *env* gene (34). To map the determinants of severe EHA known to reside in the 5.1-kb R-U5-*gag-pol* region, we constructed chimeric viruses bearing M-MuLV sequences in place of F-MuLV sequences within this large region (Fig. 1). The chimeric viruses (except FMP and FMG) in the viral stocks used for inoculation of mice were verified by digestion with discriminating restriction enzymes following amplification of viral genomic RNA by reverse transcription and PCR. The cDNA of each viral chimera was amplified with three pairs of oligonucleotides (Fig. 1), and the three amplification fragments were digested with restriction enzymes which recognized sites in M-MuLV lacking in F-MuLV. For all chimeric viruses tested, only the amplification fragment spanning the introduced sequence of M-MuLV bore a restriction site absent from the allelic sequence of F-MuLV (data not shown). This precaution formally demonstrated the presence of the expected chimeric virus in the viral stocks used for inoculation.

Sequences of M-MuLV which attenuated the capacity for induction of severe EHA were distributed within a region spanning the encapsidation domain, the *gag* gene, and the 5' portion of the *pol* gene. Newborn ICFW mice were inoculated with the chimeric viruses, and hematocrits were compared with those accompanying infection by the parental F- and M-MuLV. The replacement of most of the *pol* gene of F-MuLV by M-MuLV sequences, as in FMP, did not significantly attenuate the lytic capacity of F-MuLV (Fig. 2). However, the replacement of the R-U5 region, the *gag* gene, and the region encoding the viral protease by M-MuLV sequences, as in FMU5G, markedly diminished the lytic capacity of F-MuLV to match that possessed by M-MuLV ($P < 0.001$). To localize the sequences residing in the 2.6-kb U5G region which differentiate the lytic potentials of F- and M-MuLV, we constructed chimeric viruses bearing contiguous fragments of M-MuLV extending across the U5G region within F-MuLV genomic DNA. The replacement of the R-U5 region and a portion of the 5' leader sequence bearing the splice donor site by M-MuLV sequences, as in FMU5SD, did not significantly affect the lytic potential of F-MuLV (Fig. 2). However, a fragment bearing the RNA encapsidation domain in association with the region encoding the matrix, pp12, and capsid proteins, as in FM Ψ MACA, conferred a completely attenuated phenotype ($P < 0.001$ when compared with F-MuLV 57) upon F-MuLV (Fig. 2). Partial although significant attenuation ($P < 0.001$) was observed for the chimeric virus bearing the nucleocapsid and the viral protease of M-MuLV, FMNCPR. Since viral chimeras bearing the *env* gene, FME (Fig. 2), or the U3 region of M-MuLV were previously shown to retain the severely lytic phenotype (34), the present results show that sequences of M-MuLV permitting attenuation of F-MuLV are confined to those specifying the encapsidation domain, the *Gag* proteins, and the viral protease.

Pairs of adjacent genomic regions of M-MuLV behaved cooperatively in enhancing attenuation of severe EHA. In an attempt to define the minimum fragment of M-MuLV sufficient to preclude induction of severe EHA, we constructed two chimeric viruses bearing contiguous portions of the Ψ MACA fragment of M-MuLV in the F-MuLV background. The selection of *Afl*II as restriction enzyme not only permit-

FIG. 1. Schematic representation of the genome of F-MuLV 57. Restriction enzyme sites used in the substitution of allelic genomic fragments of M-MuLV 8.2 are indicated. The major functional regions of the 5' extremity, where most of the genetic exchanges were carried out, are shown in the blowup (not drawn to scale). The locations and orientations of oligonucleotide pairs used for reverse transcriptase PCR, pp and AC8, AC6 and AC8, and FMS15 and FMR24, are indicated by boldface arrows. The exchanged fragments were designated as follows: U5G, *SacI-SacI*; U5SD, *KpnI-SpeI*; G, *AflII-BclI*; Ψ MACA, *SpeI-BglII*; Ψ , *SpeI-AflII*; MACA, *AflII-BglII*; NCPR, *BglII-BclI*; P, *BclI-SphI*. AUG, the canonical initiation codon for translation of the *gag* gene; SD, splice donor site; MA, CA, NC, PR, and RT, regions encoding the matrix, capsid, nucleocapsid, viral protease, and reverse transcriptase, respectively.

ted the division of regions with described noncoding (encapsidation domain) and coding (matrix, pp12, and capsid proteins) function but also permitted the isolation of a region (encapsidation domain) known to assume, as RNA, an independent and highly organized structural domain (41). The substitution of the small Ψ fragment (0.36 kb) of M-MuLV, as in FM Ψ , did not alter the pathogenesis characteristic of early infection by F-MuLV (Fig. 2). The replacement of the region encoding the matrix, pp12, and capsid proteins by M-MuLV sequences, as in FM(MACA), only partially attenuated the capacity of F-MuLV to induce severe EHA. Thus, neither the Ψ nor the MACA fragment of M-MuLV was sufficient to completely attenuate the lytic potential of F-MuLV, although when associated within the virus FM Ψ MACA, these regions conferred complete attenuation upon F-MuLV (Fig. 2).

Since a cryptic determinant (or determinants) in the Ψ region appeared to augment the capacity of the MACA region for attenuation of severe EHA, we were curious whether the Ψ region of M-MuLV was essential for complete attenuation and thus tested a second pair of adjacent regions exterior to the Ψ region. The viral chimera FMG, containing the MACA and NCPR fragments of M-MuLV capable individually of conferring only partial attenuation, possessed a completely attenuated hemolytic phenotype (Fig. 2). Thus, two pairs of adjacent fragments, Ψ plus MACA and MACA plus NCPR, appeared to behave cooperatively in completely attenuating the capacity for induction of severe EHA.

Chimeric viruses disseminated in vitro as rapidly as the hemolytic parental virus, F-MuLV 57. To determine whether the chimeric viruses retained the competence for dissemination of the F-MuLV parent in *M. dunni* and NIH 3T3 fibroblasts, we infected both cell lines at low multiplicities of infection (10^{-1} and 10^{-2}) with the chimeric viruses and performed titrations of viral supernatants collected at 12-h intervals as the infected cells grew to confluency. We observed that the chimeric viruses disseminated at least as

rapidly as the parental F-MuLV 57 virus in both *M. dunni* (Fig. 3) and NIH 3T3 (data not shown) fibroblasts, and with equal facility in the two cell lines. Therefore, both partially [FM(MACA) and FMNCPR] and completely (FM Ψ MACA) attenuated chimeric viruses had no intrinsic impediment to in vitro dissemination in two different cellular contexts.

Chimeric viruses with partially attenuated lytic capacities disseminated in vivo as rapidly as F-MuLV. To determine whether a correlation existed between the capacity to induce severe EHA and particular in vivo growth characteristics, such as rapid dissemination, we infected newborn mice with the chimeric and parental viruses and assessed the extent of viral dissemination in the spleens of infected mice at 7 and 21 days of age. Splenic infectious centers were enumerated after cocultivation of splenocytes with *M. dunni* fibroblasts, and also with NIH 3T3 fibroblasts in the case of the parental M-MuLV, FM Ψ , which induced severe EHA, as well as the two chimeric viruses with partially inhibited hemolytic capacities, FM(MACA) and FMNCPR, disseminated as readily as the parental F-MuLV virus upon infection of newborn mice (Fig. 4). The levels of infection established by these chimeric viruses and F-MuLV were similar, after both 7 and 21 days. Thus FM(MACA) and FMNCPR appeared to possess attenuated potentials for induction of severe EHA despite the apparent possession of a full capacity to disseminate in vivo.

Chimeric viruses with completely attenuated lytic potentials did not disseminate in vivo as rapidly as the parental F-MuLV. Three chimeric viruses fully attenuated in their capacity to induce severe EHA, FMU5G, FM Ψ MACA, and FMG, disseminated in vivo more slowly than the F-MuLV parent as assessed at 7 days (Fig. 4). At 21 days, however, FMU5G, FM Ψ MACA, and FMG almost overtook the level of infection established by the parental F-MuLV. Since the dissemination of FM Ψ MACA in vitro was indistinguishable from that of F-MuLV (Fig. 3), the murine host may apparently

FIG. 2. Attenuation of the capacity of F-MuLV for induction of EHA by the introduction of M-MuLV sequences. Newborn ICFW mice were inoculated with parental viruses, F-MuLV 57 or M-MuLV 8.2, or viral chimeras of these parental strains. Hematocrits determined between 19 and 21 days of age for individual mice are shown as closed squares. The vertical line at 35% hematocrit is meant as a visual aid to facilitate comparison. Other abbreviations are as described in the legend to Fig. 1. SEM, standard error of the mean.

FIG. 3. Dissemination of viral chimeras of F- and M-MuLV in vitro. *M. dunni* fibroblasts were infected with virus at a multiplicity of infection of approximately 10^{-1} . Cell supernatants were collected at regular intervals during viral dissemination and stored at -80°C until determination of viral titers by FIA. FFU, focus-forming units.

impose obstacles to dissemination which are not reproduced in murine fibroblasts.

All chimeric viruses possessed full erythroleukemogenic capacity despite possessing distinct capacities for induction of recombinant polytropic viruses. Chimeric viruses, like the F-MuLV parent, induced predominantly if not exclusively erythroleukemia within 2 months of inoculation, as assessed by determination of hematocrit, autopsy of leukemic mice,

and the demonstration of a predominant population of splenocytes expressing nonspecific esterases (Table 1). Thus, in agreement with the identification of the U3 region as the major determinant of the tissue specificity of leukemogenesis (3, 4, 38), neither the tissue specificity nor the latency of leukemic induction was altered by the introduction of R-U5, 5' leader, *gag*, and *pol* M-MuLV sequences into the F-MuLV background.

FIG. 4. Dissemination of viral chimeras of F- and M-MuLV in vivo. One-day-old mice were inoculated with the parental F-MuLV or viral chimeras of F- and M-MuLV. Splenic infectious centers were enumerated at 7 and 21 days of age on *M. dunni* fibroblasts by FIA with a monoclonal antibody specific for ecotropic virus (H48) and at 21 days of age with a monoclonal antibody specific for recombinant polytropic virus (H502), except in the case of mice infected with FMU5G, for which polytropic infectious centers were not enumerated. The titers of all viral stocks used for inoculation fell between 9×10^5 and $2 \times 10^6 \text{ ml}^{-1}$. $\blacktriangle$, ecotropic virus; $\diamond$, polytropic virus.

TABLE 1. Leukemogenesis of parental and chimeric viruses^a

Viral strain	EHA	Leukemia		
		Type	Incidence (%)	Latency (mos)
M-MuLV 8.2	Mild	THY	15/15 (100)	2.5
F-MuLV 57	Severe	EL	11/12 (92)	1.8
FMU5SD	Severe	EL	31/31 (100)	1.1
FMΨ	Severe	EL	58/61 (95)	2.2
FM(MACA)	Moderate	EL	35/37 (95)	2.2
FMNCPR	Moderate	EL	22/24 (92)	1.4
FMU5G	Mild	EL	11/11 (100)	1.8
FMΨMACA	Mild	EL	14/14 (100)	1.9
FMG	Mild	EL	12/13 (92)	1.7

^a Viral strains inducing mean hematocrits of <35, 36 to 37.5, and >38.5 in inoculated mice at 19 to 21 days of age were classified as causing severe, moderate, or mild EHA, respectively. The latency of leukemia was defined as the time at which 50% of the animals were leukemic or dead. THY, thymoma; EL, erythroleukemia.

To determine whether the leukemogenesis of the chimeric viruses reflected their capacity to generate recombinant polytropic viruses, polytropic splenic infectious centers were enumerated after cocultivation of splenocytes from infected mice at 21 days with *M. dunni* fibroblasts. Although the chimeric virus FMΨMACA generated significantly fewer recombinant polytropic viruses at 21 days than the other chimeric viruses and the F-MuLV parent (Fig. 4), all chimeras induced erythroleukemia as rapidly as the parental F-MuLV virus (Table 1). Thus, a strict dependency between the latent period of erythroleukemia and the facility for induction of polytropic viruses as measured by FIA was not observed.

Whereas a chimeric virus which disseminated poorly at 7 days, FMΨMACA, only weakly induced recombinant polytropic viruses at 21 days, the chimeric viruses which disseminated in vivo as rapidly as the parental F-MuLV virus, FMΨ, FM(MACA), and FMNCPR, efficiently induced polytropic viruses (Fig. 4). Thus, a correlation did appear to exist between the kinetics of dissemination of chimeric ecotropic viruses and the generation of recombinant polytropic viruses.

DISCUSSION

We have found that sequences which determine the distinctive hemolytic potentials of F- and M-MuLV, although confined to the intron of the *env* gene, are distributed over a large region spanning the RNA encapsidation domain, the *gag* gene, and the region encoding the viral protease. Moreover, we observed that adjacent fragments within this large region of M-MuLV behaved coordinately in suppressing the capacity of F-MuLV for induction of severe EHA.

Following the appearance of severe EHA, F-MuLV induces erythroleukemia, with a latent period of approximately 2 months. The induction of recombinant polytropic viruses is thought to play an important role in the leukemogenesis of MuLV, although the contribution of such viruses is not always readily demonstrated. For viral chimeras of F- and M-MuLV, a correlation appeared to exist between the kinetics of ecotropic viral dissemination and induction of polytropic virus. It seems likely that rapid early dissemination of ecotropic viruses promotes the recombinational events required for induction of polytropic viruses by furnishing a larger number of infected cells or by establishing a higher multiplicity of infection. We observed, however, that

the latencies of erythroleukemia induced by several chimeric viruses were indistinguishable, despite distinct kinetics of ecotropic and polytropic viral dissemination. Similarly, we have shown that two ecotropic viruses which induce erythroleukemia after very different latent periods disseminated in vivo and generated polytropic viruses with equal proficiency (35). Thus, the variation in the facility of some viruses for rapid dissemination and induction of recombinant polytropic viruses has no bearing on their capacity for erythroleukemogenesis, whereas rapid early dissemination appears essential for induction of severe EHA (7).

Since the envelope glycoprotein is likely to mediate the molecular events responsible for the induction of severe EHA (33, 37), the attenuation of lytic capacity by the replacement of sequences exterior to *env* may be attributable to perturbation in the expression of the viral envelope. Mounting evidence attests to the physiological importance of the interaction between the retroviral envelope glycoprotein and the polypeptide products of the *gag* gene and the viral protease (2, 28, 43). Alteration of such interaction could conceivably affect either the qualitative or quantitative expression of the envelope glycoprotein and might impinge upon the stability of the plasma membrane.

Accessory genetic determinants of a pathology mediated directly by the viral envelope need not, however, act in *trans*. *cis*-acting sequences which augment the expression of the envelope glycoprotein at the cell surface might be expected to influence its cytopathic effect. We have shown, for example, that the capacity of F-MuLV for induction of severe EHA, which depends on particular *env* sequences, is influenced by the enhancer region of the viral LTR (33). The influence of this *cis*-acting sequence is likely to concern its role in determining the rate of proviral transcription according to the cellular context (1, 40). The enhancer region is not the only viral regulatory element to influence the quantitative expression of the viral envelope; in avian retroviruses, the maintenance of sufficient genomic RNA and the appropriate molecular ratio of mRNAs encoding the products of either *gag* and *pol* or *env* genes is ensured not only by the maintenance of suboptimal 3' splice acceptor sites but also by sequences in the *gag* gene of the *env* intron which inhibit splicing of the primary retroviral transcript (13, 19, 23, 24). An intronic regulatory element of the Rous sarcoma virus, the negative regulator of splicing, has been located in the region encoding the capsid protein, and its influence has been suggested to depend on the formation of a particular secondary structure (22). Regarding murine leukemia viruses, Hwang et al. (19) have shown that an intronic sequence of 560 nucleotides overlapping the regions encoding the capsid and nucleocapsid is essential for efficient splicing of retroviral RNA transcribed from expression vectors based on M-MuLV (19). As for the sequences responsible for the distinctive hemolytic capacities of F- and M-MuLV, their wide distribution within the *env* intron and the apparent cooperativity of adjacent sequences are consistent with the intervention of a noncoding function of these sequences in the pathogenesis of MuLV. We are now investigating whether such intervention might concern modulation of splicing of the proviral transcript in cells of the erythroid lineage.

Particular retroviral pathogenic effects are frequently governed by multiple discrete regions of the retroviral genome (18, 26). Several models of retroviral pathogenesis exist in which a cytopathic effect mediated by the envelope glycoprotein is believed to be modulated by determinants residing in a second region. Sequences in the envelope glycoprotein

of human immunodeficiency virus type 1 (HIV-1) are critical to the cytopathic effects of HIV-1 in vitro, both dependent on and independent of the formation of syncytia (39); however, the amino-terminal portion of the matrix protein of HIV-1 (9) or the *pol* gene and the 3' part of the *gag* gene (17), respectively, influence these cytopathic effects. Also, in the neuropathogenesis of the wild mouse ecotropic virus (Cas-BrE), the *env* gene has been shown to be a major determinant of neurovirulence (10, 29, 30). Nevertheless, the substitution of a 0.53-kb R-U5-5' leader fragment of a nonneurovirulent strain of F-MuLV sufficed to dramatically reduce the incubation period of spongiform neurodegenerative disease (31). The influence of the R-U5-5' sequences appeared to concern, in part, the kinetics of early viral dissemination or replication in peripheral tissues, ultimately determining both the viral load in the central nervous system and the latency of neurodegenerative disease (8). These R-U5-5' sequences of F-MuLV with profound influence on neuropathogenesis do not overlap the genomic fragments that we identify as differentiating the hemolytic capacities of F- and M-MuLV. Thus, the models of neuropathogenesis and severe EHA would appear to implicate distinct sequences of F-MuLV—whose role might concern the modulation of the pathogenic effect of the retroviral envelope—in pathogenesis specific to particular cellular contexts, neurological or erythroid.

Genomic changes which attenuate pathogenesis may simply impinge upon the efficacy of the basal viral replicative cycle or may alter viral behavior in particular cellular compartments. Partial attenuation of severe EHA was apparently conferred by MACA and NCPR fragments of M-MuLV with no evidence of diminished in vivo dissemination, since the dissemination of the chimeric viruses FM-(MACA) and FMNCPR matched that of the F-MuLV parent in 7-day-old mice. It is unlikely that such partial attenuation was due to a delay in dissemination manifest very early in infection and imperceptible at 7 days of age, since the extent of dissemination established even by inoculation of 7-day-old mice with the F-MuLV parent suffices for induction of severe EHA (7). Thus, the influence of genetic determinants of EHA residing in the MACA and NCPR regions is not exerted by altering the overall efficiency of viral replication.

The complete attenuation of severe EHA conferred by FMU5G and FM Ψ MACA accompanied delayed in vivo dissemination. It is thus possible that the capacities of FMU5G and FM Ψ MACA to induce severe EHA are attenuated, at least in part, because of a defect in viral dissemination. However, since we have shown that the dissemination of FM Ψ MACA in vitro is indistinguishable from that of F-MuLV, the basis for the restricted dissemination of FM Ψ MACA in vivo must reflect interaction between the virus and murine host not faithfully reproduced in the fibroblast model, and possibly a disadvantage manifested only in certain cell types. Moreover, in combination with the *env* gene of M-MuLV, the 5G and Ψ MACA regions of M-MuLV appeared to enhance induction of promonocytic leukemia (25). The implication of these regions in both enhancement and suppression of pathogenesis, depending on the cellular compartment, suggests a role in the tissue specificity of viral pathogenesis. The study of sequences of MuLV involved in erythroid, promonocytic, or neurological disease might be of use in the elucidation of cellular factors which influence the expression of the retroviral replicative cycle in specific cellular contexts.

ACKNOWLEDGMENTS

We are indebted to Bruce Chesebro for the generous gift of monoclonal antibodies and to Anne-Catherine Prats for the oligonucleotide pp. We thank Marie-Laure Chaix-Baudier and Sentob Saragosti for assistance with reverse transcription and PCR amplification of viral RNA and also Michel Bouillot, Caroline Denesvre, Iris Angermann, and Nathalie Neyroud for assistance with certain molecular constructions. We are grateful to Sylvie Gisselbrecht for her insight and encouragement and to Jean-Jacques Kupiec for his critical reading of the manuscript.

J.R. was supported by a fellowship from the Fondation pour la Recherche Médicale (Paris, France) and a Poste Vert from the Institut National de la Santé et de la Recherche Médicale. Part of this work was supported by awards from the Philippe Foundation, Inc., to A.C. and M.S.

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