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A Small Region of the Ecotropic Murine Leukemia Virus (MuLV) *gag* Gene Profoundly Influences the Types of Polytropic MuLVs Generated in Mice

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The vast majority of recombinant polytropic murine leukemia viruses (MuLVs) generated in mice after infection by ecotropic MuLVs can be classified into two major antigenic groups based on their reactivities to two monoclonal antibodies (MAbs) termed Hy 7 and 516. These groups very likely correspond to viruses formed by recombination of the ecotropic MuLV with two distinct sets of polytropic *env* genes present in the genomes of inbred mouse strains. We have found that nearly all polytropic MuLVs identified in mice infected with a substrain of Friend MuLV (F-MuLV$_{57}$) are reactive with Hy 7, whereas mice infected with Moloney MuLV (Mo-MuLV) generate major populations of both Hy 7- and 516-reactive polytropic MuLVs. We examined polytropic MuLVs generated in NFS/N mice after inoculation with Mo-MuLV–F-MuLV$_{57}$ chimeras to determine which regions of the viral genome influence this difference between the two ecotropic MuLVs. These studies identified a region of the MuLV genome which encodes the nucleocapsid protein and a portion of the viral protease as the only region that influenced the difference in polytropic-MuLV generation by Mo-MuLV and F-MuLV$_{57}$.

Mice inoculated with exogenous ecotropic murine leukemia viruses (MuLVs) or mice expressing endogenous ecotropic MuLVs frequently generate polytropic viruses which have been implicated in the induction of a number of proliferative diseases in mice (1, 3, 15, 16, 18, 21, 22, 25, 30). These viruses, also termed dualtropic or mink cell focus-forming MuLVs, utilize a cellular receptor different from that used by ecotropic MuLVs and exhibit a distinct host range (22, 38). Ecotropic MuLVs infect only murine cell lines, while polytropic MuLVs infect murine cell lines as well as lines from several other species. Polytropic MuLVs are generated by recombination between the inoculated ecotropic MuLV and endogenous retroviral sequences resulting in a replacement of the 5′ region of the ecotropic *env* gene (4, 6, 7, 10, 12–14, 41, 42), which determines the specificity for different MuLV cellular receptors (2, 33, 38).

We have previously reported that nearly all polytropic MuLV isolates can be classified as members of two major antigenic subclasses defined by their reactivity with two monoclonal antibodies (MAbs) termed Hy 7 and 516 (26). These two MAbs react with mutually exclusive epitopes, both of which have been mapped to the same amino acid in the first third of the SU protein and very likely reflect the existence of two discrete families of endogenous polytropic sequences. In this study we have found that the populations of polytropic MuLVs generated after infection of NFS/N or IRW (inbred Rocky Mountain White) mice with Moloney MuLV (Mo-MuLV) (44) are strikingly different than those generated after inoculation with a substrain of F-MuLV (F-MuLV$_{57}$) (31) and that this difference is determined by a small region encompassing the sequences encoding the viral nucleocapsid (NC) protein and a portion of the viral protease (PR).

Different antigenic subclasses of polytropic MuLVs are generated after neonatal inoculation with Mo-MuLV or F-MuLV$_{57}$. Our initial analyses examined polytropic MuLVs generated in mice after inoculation with Mo-MuLV or F-MuLV$_{57}$. Quantification of the two antigenic subclasses of polytropic MuLVs in mixed populations was accomplished by a focal immunofluorescence assay (46) with MAbs Hy 7 (11) and 516 (8), specifically reactive with each of the two polytropic MuLV subclasses. Viruses were quantified as infectious centers (ICs) of splenocytes and thymocytes from infected NFS/N mice as well as from mice of a second inbred strain, IRW (9). Endogenous ecotropic or polytropic MuLVs are not expressed in uninfected mice of either strain.

Following infection of NFS/N or IRW mice by Mo-MuLV, most animals exhibited substantial proportions of both antigenic polytropic subclasses, although a large range in the relative levels of Hy 7- and 516-reactive MuLVs was observed among individual mice (Fig. 1A and B). In contrast, mice infected with F-MuLV$_{57}$ consistently exhibited predominantly Hy 7-reactive polytropic viruses, with several mice expressing this antigenic subclass exclusively (Fig. 1C and D).

The 5′ region of the ecotropic MuLV genome influences the types of polytropic MuLVs generated in infected mice. Mo-MuLV and F-MuLV$_{57}$ infect the spleen with approximately equal efficiencies starting about 3 to 4 days after inoculation. In contrast, infection of the thymus by F-MuLV$_{57}$ or by chimeric viruses containing the F-MuLV$_{57}$ transcriptional regulatory sequences resident in the long terminal repeat occurs only after a substantial delay and is variable among different mice (17). Several of the chimeras examined in this study failed to consistently express a sufficiently high level of viruses in the thymus to accurately assess. Accordingly, we have evaluated polytropic MuLVs generated after inoculation of the chimeric

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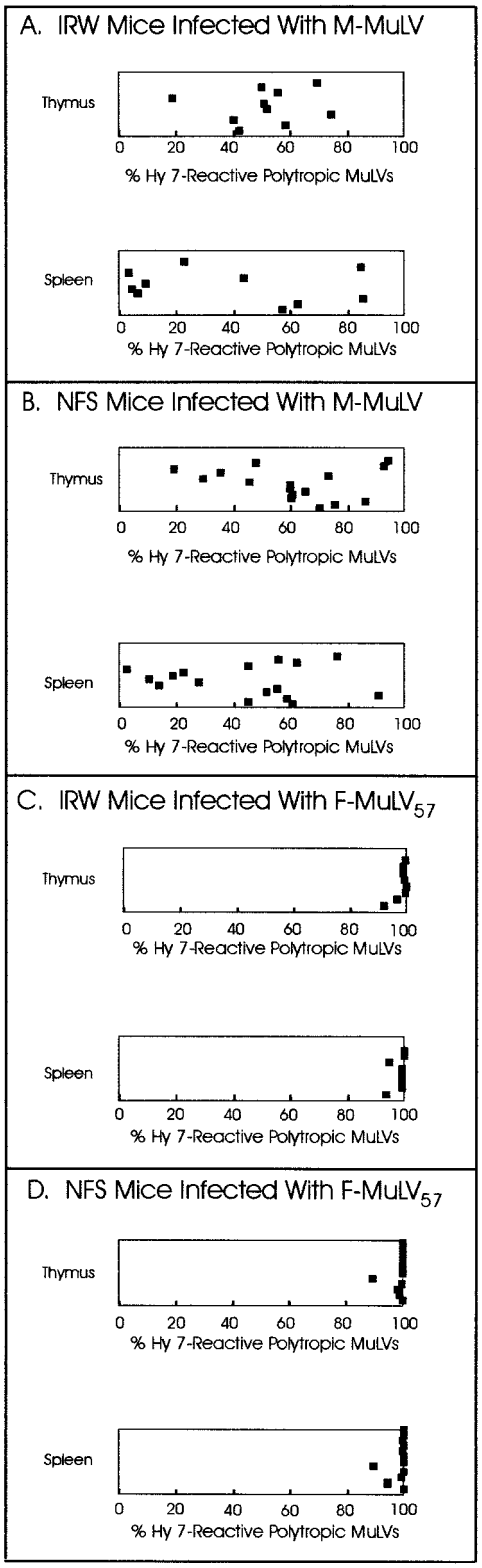

FIG. 1. Expression of polytropic MuLV antigenic subclasses in NFS/N and IRW mice after inoculation with F-MuLV$_{57}$ or Mo-MuLV. Mice were inoculated neonatally and sacrificed at 4 to 8 weeks of age. IC assays were conducted on thymocytes and splenocytes as previously described (26). For determination of the percentage of polytropic MuLVs reactive with MAb Hy 7, the total polytropic MuLV population was taken to be the sum of Hy 7-reactive and 516-reactive polytropic MuLVs. Each data point represents the result obtained for a single animal.

MuLVs by assays of polytropic viruses from splenocytes 4 to 8 weeks after neonatal inoculation.

Analyses of polytropic MuLVs generated after infection by chimeric viruses in which the U3 region of the long terminal repeat, the *env* gene, or the *pol* gene of F-MuLV$_{57}$ had been replaced by the analogous gene sequences from Mo-MuLV revealed no significant differences from those generated in mice inoculated with F-MuLV$_{57}$ (Fig. 2B to 2D). These results indicated that these regions, which encode a number of functions, such as transcriptional regulation, tissue tropism, viral genome replication, integration, and receptor binding, do not determine the difference in the polytropic MuLV subclasses generated by F-MuLV$_{57}$ and Mo-MuLV. In contrast, mice infected with the chimeric virus FMU5G, in which the R, U5, and *gag* regions of F-MuLV$_{57}$ were replaced by analogous Mo-MuLV sequences, exhibited a profile of polytropic MuLVs clearly different from that of F-MuLV$_{57}$-infected mice (Fig. 2E). Mice infected with this chimera generated substantial proportions of both Hy 7- and 516-reactive polytropic MuLVs, similar to that observed after Mo-MuLV infection. As was the case with mice infected with Mo-MuLV, large variations in the percentages of the two antigenic subclasses of polytropic MuLVs were observed among individual mice.

Determinants which influence the relative levels of polytropic MuLV subclasses in infected mice map to the nucleocapsid/protease-encoding sequences of the inoculated ecotropic MuLV. FMU5G contains, in addition to the structural *gag* gene, sequences which are involved in genomic RNA packaging, dimer formation, mRNA splicing, and transcriptional initiation (20, 27, 29, 35–37, 39). To define more closely the sequences of FMU5G which influence the generation of the two subclasses of polytropic MuLVs, chimeric viruses containing smaller substitutions within the U5G region were examined (Fig. 3). Mice infected with chimeric MuLVs corresponding to F-MuLV$_{57}$ containing M-MuLV packaging sequences (FMΨ) or Mo-MuLV packaging sequences along with the Mo-MuLV sequences encoding the matrix and capsid proteins (FMΨMACA) exhibited predominantly Hy 7-reactive polytropic MuLVs similar to F-MuLV$_{57}$ (Fig. 3B and C). In contrast, polytropic MuLVs generated in mice infected with the chimera containing Mo-MuLV sequences encoding the nucleocapsid protein and the viral protease (FMNCPR) (Fig. 3D) differed markedly from those observed in mice infected with F-MuLV$_{57}$, indicating the presence of determinants in this region which strongly influence the generation of the polytropic MuLV antigenic subclasses.

The NCPR region of F-MuLV$_{57}$ may facilitate the generation of Hy 7-reactive polytropic MuLVs in infected mice. The difference in the relative levels of polytropic MuLV subclasses observed in mice inoculated with MuLVs containing Mo-MuLV NCPR sequences from those in mice infected with MuLVs containing F-MuLV$_{57}$ NCPR region could be the result of a difference in the levels of expression of one or of both subclasses of polytropic MuLVs. A comparison of the IC titers in splenocytes from mice infected with viruses containing the F-MuLV$_{57}$ NCPR region with the titers in splenocytes from mice infected with viruses in which this region was derived from Mo-MuLV indicated that mice infected with the former viruses exhibit a significantly higher level of Hy 7-reactive polytropic MuLVs ($P < 0.001$) (Fig. 4). These results suggest that the NCPR sequences of F-MuLV$_{57}$ facilitate the generation of an Hy 7-reactive recombinant(s). The emergence of a new population of Hy 7-reactive MuLVs could result from amplification of a single species of polytropic MuLV or, alternatively, from coordinate amplification of a family of Hy 7-reactive MuLVs. The depressed levels of MAb 516-reactive

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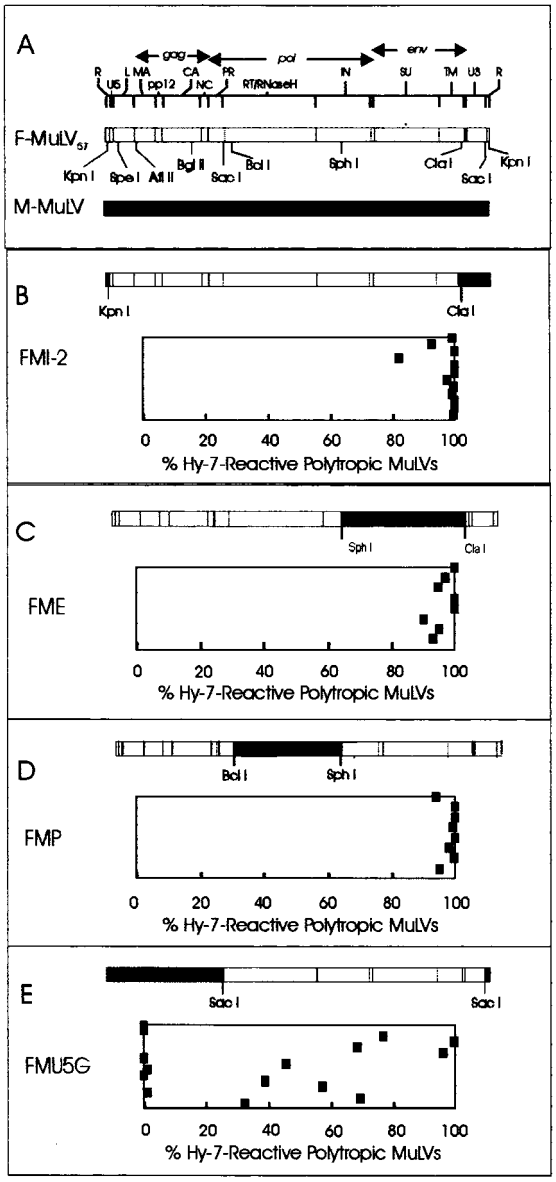

FIG. 2. Expression of polytropic MuLV antigenic subclasses in NFS/N mice inoculated with chimeric viruses corresponding to F-MuLV$_{57}$ in which the transcriptional regulatory sequences (FMI-2), the *env* gene (FME), the *pol* gene (FMP), or the *gag* gene (FMU5G) was replaced by Mo-MuLV sequences. NFS/N mice were inoculated neonatally, and polytropic-MuLV expression in the spleens was evaluated as described for Fig. 1. (A) Schematic depicting the ecotropic viral RNA genomes of F-MuLV$_{57}$ and Mo-MuLV (M-MuLV). The locations of the viral gene boundaries in the ecotropic viral genomes are indicated above the F-MuLV$_{57}$ diagram. The restriction endonuclease cleavage sites are indicated at the bottom of the F-MuLV$_{57}$ diagram and closely approximate the sites on the analogous proviral DNA used to construct the F-MuLV$_{57}$–Mo-MuLV chimeras. (B to E) Schematics of chimeric MuLVs and the percentages of Hy 7-reactive polytropic MuLVs detected in splenocytes. The chimeric MuLV FMI-2 was originally described by Chatis et al. (5). All other chimeras have been previously reported (40). Each data point in the graphs represents a determination for an individual mouse.

polytropic MuLVs in mice infected with viruses containing the F-MuLV$_{57}$ NCPR region compared to the levels in mice infected with ecotropic MuLVs possessing the Mo-MuLV NCPR sequences (Fig. 4) may be the result of interference by an emerging Hy 7-reactive population. In this regard we have observed that coinoculation of F-MuLV$_{57}$ or Mo-MuLV with polytropic MuLV isolates results in nearly complete suppression of de novo polytropic MuLV expression in mice, presumably through viral interference mediated by the exogenously administered polytropic MuLV (24).

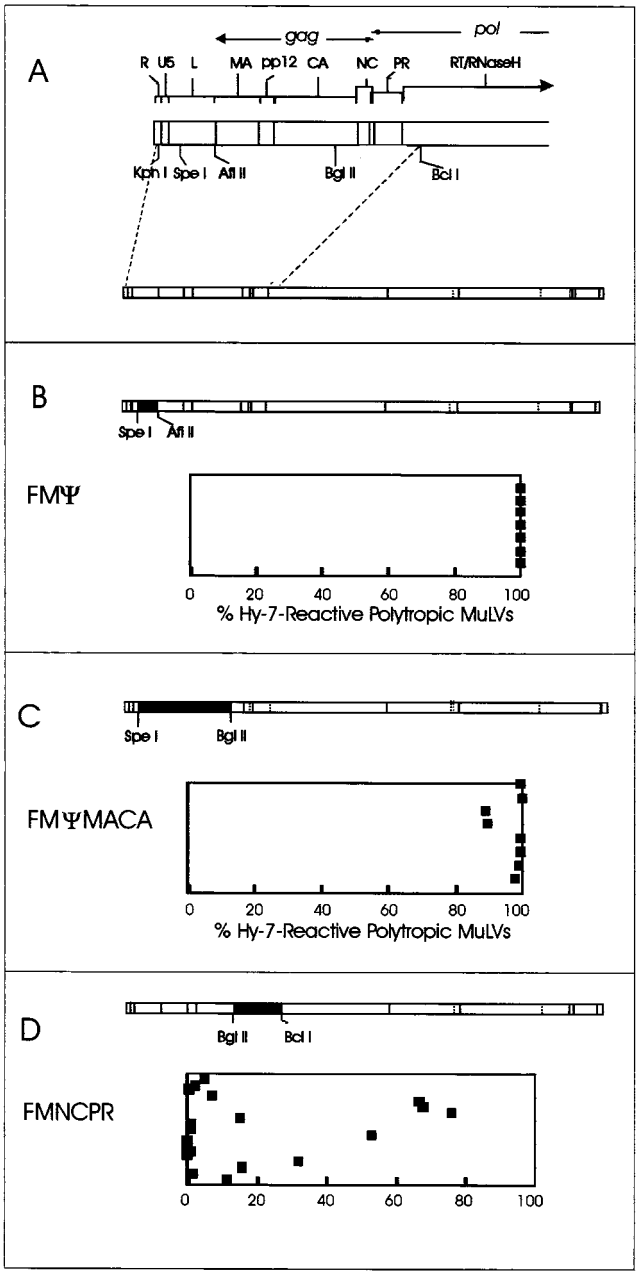

FIG. 3. Expression of polytropic MuLV antigenic subclasses in NFS/N mice inoculated with chimeric viruses corresponding to F-MuLV$_{57}$ in which sequences encoding the RNA packaging region (FMΨ), the packaging region plus the MA and CA protein (FMΨMACA), or the NC protein and PR (FMNCPR) have been replaced by Mo-MuLV elements. NFS/N mice were inoculated neonatally, and polytropic-MuLV expression in the spleens was evaluated as described for Fig. 1. (A) Schematic indicating the region of the viral genome encompassing the substitutions in the chimeras. The locations of the genetic elements and the restriction sites used in the construction of the chimeras are indicated in the enlarged view. (B to D) Schematics of the chimeric MuLVs and the percentages of Hy 7-reactive polytropic MuLVs detected in splenocytes. The region of the viral genome in corresponding to F-MuLV$_{57}$ or Mo-MuLV is indicated by white bars or black bars, respectively. The chimeras have been previously reported (40). Each data point in the graphs represents a determination for an individual mouse.

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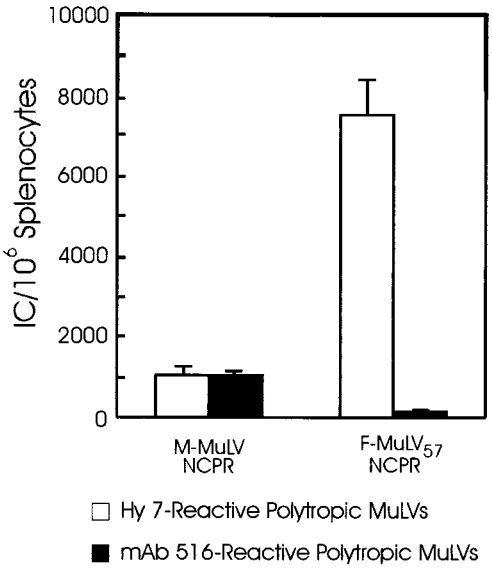

FIG. 4. Polytropic-MuLV titers expressed in NFS/N mice inoculated with ecotropic MuLVs. Inoculated MuLVs contained the F-MuLV$_{57}$ NCPR sequences (F-MuLV$_{57}$, FME, FMP, FMU5G, FMΨ, and FMΨMACA) (55 mice) or the Mo-MuLV NCPR sequences (M-MuLV, FMU5G, and FMNCPR) (51 mice). Average titers of the polytropic subclasses are indicated. The error bars indicate the standard errors of the means.

Sequence difference that may influence the difference in polytropic MuLVs generated by F-MuLV57 and Mo-MuLV. From the analyses of FMU5G (Fig. 2E) and FMNCPR (Fig. 3D) it appears that critical sequences which influence the types of polytropic MuLVs generated after inoculation of the viruses are between the *Bgl*II restriction endonuclease site near the 3′ end of the CA gene sequences and the *Sac*I site near the 3′ end of the PR gene sequences. A comparison of the nucleotide sequences of F-MuLV$_{57}$ (accession no. J02192) and Mo-MuLV (accession no. J02255) in this region reveals differences that result in nine different amino acids. These include two differences in the carboxyl-terminal region of the CA protein, five differences in the NC protein, and two differences in the portion of the PR protein encoded by these sequences.

The carboxyl-terminal region of the CA protein of Mo-MuLV differs from that of the CA protein of F-MuLV$_{57}$ at residue 252 (threonine in Mo-MuLV versus alanine in F-MuLV$_{57}$) and residue 257 (lysine in Mo-MuLV versus arginine in F-MuLV$_{57}$). Although the threonine/alanine difference alters the hydrophobicity of this region, these differences in the CA protein do not suggest a mechanism which might influence the types of polytropic MuLVs generated by recombination.

Most of the viral PR is encoded by the sequences between the *Bgl*II and *Sac*I sites. The PR catalyzes cleavage of the polyprotein precursor to the proteins encoded by the *gag* gene as well as cleavage of a 16-amino-acid carboxyl-terminal peptide from the TM protein (32, 45). Since all polytropic MuLVs possess an altered *env* gene compared to that of their ecotropic MuLV parents, changes in PR function could conceivably affect processing of the TM protein and perhaps influence the propagation of recombinant viruses. The two differences in the PR between Mo-MuLV and F-MuLV$_{57}$ are at residue 71 (lysine in Mo-MuLV versus arginine in F-MuLV$_{57}$) and residue 96 (asparagine in Mo-MuLV versus histidine in F-MuLV$_{57}$). Neither of these residues is in the catalytic site or the substrate binding pocket of the PR (28, 32); however, this does not exclude the possibility that these differences affect PR function.

The NC protein is encoded entirely by Mo-MuLV sequences in the two chimeras (FMU5G and FMNCPR) that differed from F-MuLV$_{57}$ with regard to the types of polytropic MuLVs generated in infected mice. If the effect of the NCPR region is at the level of generation of polytropic MuLV recombinants, the NC protein seems an attractive candidate. This protein is involved in the dimerization of viral genomic RNA, annealing of the tRNA^{Pro} primer, and in vivo proviral synthesis and has recently been reported to influence the specificity as well as the efficiency of viral genomic RNA packaging (20, 29, 35–37, 39, 47, 48). The Mo-MuLV NC differs from that of F-MuLV$_{57}$ at residue 4 (valine in Mo-MuLV versus isoleucine in F-MuLV$_{57}$), residue 8 (lysine in Mo-MuLV versus arginine in F-MuLV$_{57}$), residue 19 (serine in Mo-MuLV versus proline in F-MuLV$_{57}$), residue 23 (arginine in Mo-MuLV versus histidine in F-MuLV$_{57}$), and residue 37 (lysine in Mo-MuLV versus arginine in F-MuLV$_{57}$). A region critical for the functions of the NC protein is a centrally located zinc finger and flanking regions containing several basic amino acids (20, 23, 29, 39, 47). Residue 37 lies within the zinc finger, and residue 23 is in the amino terminus-flanking region (19, 43). The lysine/arginine difference at residue 37 is conservative with regard to stearic and charge considerations. However, the histidine/arginine difference at residue 23 in the flanking region may be notable. At physiological pH virtually all arginine residues bear a positive charge, in contrast to less than 5% of histidine residues. Although the effects of mutations at this precise position have not been reported, replacement of other basic amino acids with neutral residues in the flanking regions have marked effects on infectivity and packaging of viral genomic RNAs (23, 39). Recombination may require the formation and reverse transcription of heterodimeric RNAs. It is conceivable that the differences in the generation of polytropic MuLV by F-MuLV$_{57}$ and Mo-MuLV are a result of selective formation and/or packaging of heterodimers mediated by the NC protein.

The NCPR region of a second F-MuLV, FB29, is nearly identical to that of F-MuLV$_{57}$ (34). Infection of NFS/N mice with FB29 frequently results in a higher proportion of Hy 7-reactive than 516-reactive recombinants in the spleen; however, more MAb 516-reactive polytropic MuLVs are detected in FB29-infected mice than in mice infected with F-MuLV$_{57}$ (26). It is possible that regions of the genome other than the NCPR region in other ecotropic MuLVs may influence the types of polytropic MuLVs generated. Although our discussion has focused on differences in the proteins encoded by the retroviral sequences which affect the generation of polytropic MuLVs, it is also possible that the effect is a result of differences in the nucleic acid sequences rather than the proteins encoded by these sequences. Further studies which include site-directed mutagenesis of the NCPR region, determination of the heterogeneity of Hy 7-reactive polytropic SU sequences in F-MuLV-induced recombinants, and identification and analyses of the polytropic progenitor sequences present in the mouse genome may help elucidate the mechanism(s) by which the NCPR sequences influence the generation of polytropic MuLVs.

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