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Precise Identification of Endogenous Proviruses of NFS/N Mice Participating in Recombination with Moloney Ecotropic Murine Leukemia Virus (MuLV) To Generate Polytropic MuLVs

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Polytropic murine leukemia viruses (MuLVs) are generated by recombination of ecotropic MuLVs with *env* genes of a family of endogenous proviruses in mice, resulting in viruses with an expanded host range and greater virulence. Inbred mouse strains contain numerous endogenous proviruses that are potential donors of the *env* gene sequences of polytropic MuLVs; however, the precise identification of those proviruses that participate in recombination has been elusive. Three different structural groups of proviruses in NFS/N mice have been described and different ecotropic MuLVs preferentially recombine with different groups of proviruses. In contrast to other ecotropic MuLVs such as Friend MuLV or Akv that recombine predominantly with a single group of proviruses, Moloney MuLV (M-MuLV) recombines with at least two distinct groups. In this study, we determined that only three endogenous proviruses, two of one group and one of another group, are major participants in recombination with M-MuLV. Furthermore, the distinction between the polytropic MuLVs generated by M-MuLV and other ecotropic MuLVs is the result of recombination with a single endogenous provirus. This provirus exhibits a frameshift mutation in the 3′ region of the surface glycoprotein-encoding sequences that is excluded in recombinants with M-MuLV. The sites of recombination between the *env* genes of M-MuLV and endogenous proviruses were confined to a short region exhibiting maximum homology between the ecotropic and polytropic *env* sequences and maximum stability of predicted RNA secondary structure. These observations suggest a possible mechanism for the specificity of recombination observed for different ecotropic MuLVs.

Vertebrate species harbor a very large number of endogenous retroviral elements in their genomes that have originated from the infrequent insertion of exogenous viruses into the germ line. It is estimated that ~8% of the human genome is composed of retrovirus sequences and that the number of distinct retroviral elements approaches the total number of structural genes identified in the genome (35). Endogenous retroviruses or their gene products are differentially expressed in tissues and in certain physiological or pathological conditions (3, 13, 46, 51, 57, 69); however, the control and consequences of such expression are poorly understood. It has been suggested that endogenous retrovirus expression may be involved in human pathogenesis, including neoplasms (40, 41) and autoimmune disorders (44), as well as normal physiological processes such as placental development (43).

One of the more extensively investigated groups of endogenous retroviruses are those participating in recombination with exogenous ecotropic retroviruses in mice giving rise to host range variants. Ecotropic murine leukemia viruses (MuLVs) are infectious for murine cells but not cells of other species. Upon infection of mice, these MuLVs frequently recombine with members of a group of endogenous proviruses to generate polytropic MuLVs (18, 19, 28, 30, 54, 55). Recombination involves substitution of the *env* gene sequences encoding the receptor-binding region of the ecotropic MuLV with analogous *env* gene sequences of the endogenous proviruses (1, 4, 7, 9, 16, 18, 19, 34, 52, 62). The recombinant MuLVs utilize a different cell-surface receptor for infectious entry and are capable of infecting murine cells as well as cells of other species, including humans.

The generation of polytropic viruses is intimately involved in a number of proliferative diseases in mice including lymphocytic leukemia induced by Moloney MuLV (M-MuLV) (5, 12, 64), erythroleukemia induced by the Friend MuLV (F-MuLV) (55), and spontaneous leukemia observed in highly leukemic mouse strains (6, 28, 59, 61, 62, 65). In addition, polytropic MuLVs may contribute to neurological disorders (48).

There are about 20 to 40 endogenous proviruses in different inbred strains of mice that bear very close homology to the sequences found in polytropic recombinant viruses; however, the participation of the proviruses in recombination with ecotropic viruses is not random. Polytropic MuLVs belong to one of two antigenic subclasses based on their reactivity to one of two monoclonal antibodies (MAbs), termed Hy 7 and MAb 516 (39). Reactivities to the antibodies are mutually exclusive and signal recombination with distinct structural groups of endogenous proviruses. The ecotropic viruses Akv and F-MuLV

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generate predominantly Hy 7-reactive polytropic MuLVs with few MAb 516-reactive viruses, whereas infection with M-MuLV results in approximately equal numbers of Hy 7- and MAb 516-reactive polytropic viruses (37, 38). Thus, M-MuLV recombines with a distinct structural group of endogenous proviruses reactive with MAb 516.

There are a number of possible mechanisms for the specificity of recombination with different ecotropic MuLVs, ranging from tissue-specific expression of the proviruses to specific interactions of the ecotropic MuLVs with the endogenous proviruses or their RNA transcripts. To gain an understanding of this specificity, it is first necessary to obtain a more complete description of the phenomenon, namely, the precise identification of those proviruses that actually participate in recombination. Efforts to precisely identify the endogenous origin of polytropic MuLVs have been hampered by the very close homology of the *env* gene sequences of the endogenous proviruses and the rate of divergence of these sequences by random mutation once they are incorporated into a replicating MuLV (31, 32, 33, 58). In our previous study, we found that nearly all of the endogenous proviruses of NFS/N mice were distinguishable by sequence microheterogeneity (22). Thus, if the divergence of polytropic MuLV sequences could be minimized, comparisons to *env* gene sequences in recombinant polytropic MuLVs should identify which of the proviruses participate in recombination. In the present study, we utilized procedures to minimize the number of replication cycles prior to sequence analyses of the recombinant viruses and were able to unambiguously identify the endogenous proviral origin of numerous polytropic MuLVs derived after inoculation of M-MuLV. We found that a surprisingly small number of endogenous proviruses are major participants in recombination with M-MuLV and that recombination with a single provirus accounts for the difference in the polytropic MuLVs generated after inoculation of M-MuLV compared to other ecotropic viruses. Furthermore, examination of the recombination patterns of the polytropic MuLVs suggested a mechanism that may account for this specificity. Additional findings suggest that the polytropic MuLVs, once generated, may continue to recombine with one another and evolve to more virulent forms in the host.

MATERIALS AND METHODS

Mice. NFS/N mice were maintained as an inbred strain at Rocky Mountain Laboratories, Veterinary Branch. All animals were treated in accordance with National Institutes of Health regulations and the guidelines of the Animal Care and Use Committee of Rocky Mountain Laboratories.

Isolation of recombinant polytropic MuLVs. Polytropic MuLVs were isolated from NFS/N mice that had been inoculated within 24 h of birth with the ecotropic virus M-MuLV$_{1387}$ (20). The infected mice were sacrificed 3 to 5 weeks after inoculation, the spleens and thymuses were dissociated, and serial dilutions of cells from the tissues were distributed as infectious centers on NIH 3T3, *Mus dunni* (36), or mink (ATTC CCL64) cell cultures as previously described (18). At confluence, the monolayers were examined for infection by a focal immunofluorescence assay (56). The assay was used in conjunction with MAb 516 (8) or Hy 7 (10) to detect polytropic MuLVs corresponding to the two major antigenic subclasses of polytropic isolates (39) and MAb 538 (37) to detect M-MuLV. Infected cells from individual foci developed with Hy 7 or MAb 516 were subcultured and expanded to obtain virus stocks. Care was taken to exclude uninfected cells from the subcultures, to minimize virus spread during expansion. The viruses were biologically cloned by endpoint dilution to obtain cultures infected with a single polytropic MuLV and lacking ecotropic M-MuLV. In some instances, genomic DNA lambda libraries were constructed from infected *M. dunni* cells after cocultivation with tissues of M-MuLV-infected mice, and the libraries were screened for polytropic proviruses as previously described (22). *M. dunni* cells lack endogenous polytropic proviruses (31, 36); thus, positive clones obtained from the libraries correspond to newly integrated polytropic proviruses.

PCR and reverse transcription-PCR (RT-PCR) analyses and DNA sequencing. DNA from lambda clones containing integrated proviruses from infected *M. dunni* genomic libraries was prepared as previously reported (22) and used as the template for PCRs. An approximately 900-bp product that encompassed sequences encoding the receptor-binding region of the surface glycoprotein (SU) protein was generated with a forward primer termed FORPOLY (GCAGTAC AACGAGAGGTCTGG) and a reverse primer termed REVPOLY (GGGTCA AAGAGAACCGGGTCAC). In some instances where the ecotropic-polytropic recombination boundary was 3′ to the polytropic forward primer, an ecotropic forward primer termed FORECO (CTGGTCCAGCACGAAGTCTGG) was employed. PCRs were conducted using *Pfu* Turbo DNA polymerase (catalog no. 600153; Stratagene), Platinum Pfx DNA polymerase (catalog no. 11708-021; Invitrogen), or the puReTaq Ready-To-Go PCR system (catalog no. 27-9558-01; Amersham) according to the manufacturer's instructions.

RNA from virions released from infected cultures was used for RT-PCR analysis. The tissue culture medium from infected cultures was collected at 24-h intervals and sedimented in an SW 28.1 Beckman ultracentrifuge rotor at 25,000 rpm for 90 min. The pelleted virions from 200 to 500 ml of medium were resuspended in 0.1 M NaCl, 0.01 M Tris HCl, 0.001 M EDTA, and the RNA purified with the QIAamp Viral RNA Mini Kit (catalog no. 52906; QIAGEN) with the following modifications of the manufacturer's instructions. Lysis of the sample with buffer AVL was done in the absence of carrier RNA, and elution of the RNA from the spin column was accomplished with two successive elutions, each with 60 μl of distilled water DNase and RNase free (catalog no. 10977-015; Invitrogen). The second elution typically increased the total yield of RNA from 50 to 80%. Synthesis of cDNA was performed using the SuperScript First-Stand Synthesis system for RT-PCR (catalog no. 11904-018; Invitrogen) according to the manufacturer's instructions with the primer REVPOLY at 70 pmol and 1 μg of virion RNA per reaction mixture. PCR was performed on the cDNAs with the primers FORPOLY and REVPOLY with Platinum Pfx DNA polymerase to generate the ~900-bp product. Primers used for sequencing the ~900-bp PCR and RT-PCR products included FORPOLY, REVPOLY, MIDFORPOLY (AGCTAATGCTACCTCCCTCCTG), MIDREVPOLY (ACATTGAAGACC TGATGAGGG), and 329MX33RC (CAGTCTCATCCCAGTCGTCC). Primers used to for sequence analysis of additional *env* gene regions included 510MX33 (CCCTTAAGCGAGGAAACACCCCTCGG), 897MX33RC (GCTT GGTAGGCTCCATCTACCAGGT), 820MX33 (CATGCTCCCCAGGCCTCC TC), 1203MX33RC (AGCCCGGTGTTGCAAGCCC), and 1645MX33RC (CT ACGCCAGTGTGGTCCGCG). Sequence analyses were performed using an ABI model 377 DNA sequencer by the DNA Sequencing and Synthesis Facility at Iowa State University or by an ABI model 3700 DNA sequencer at Rocky Mountain Laboratories.

Alignments, homology, and free-energy plots. Sequence alignments were performed with the CLUSTAL_X sequence alignment program (63). Homology at each nucleotide was calculated as the percent homology of a 31-base sequence alignment that included 15 bases to the 3′ and 5′ sides of the nucleotide. Free-energy computations of predicted RNA conformations of the overlapping 251-bp *env* gene sequences were done with the Mfold program (version 3.1) (71). The free energy of conformation at a particular position was plotted as the free energy of the RNA sequence, which included 125 bases to the 3′ and 5′ sides of the nucleotide.

Nucleotide sequence accession numbers. The sequences in the report have been deposited in GenBank under accession no. AY714500 through AY714525.

RESULTS

Minimizing sequence heterogeneity of polytropic MuLV nucleotide sequences due to random mutation. We recently characterized the endogenous polytropic proviruses in the NFS/N mouse genome (22). It was found that nearly all of the proviruses were distinguishable by base differences within an 893-base region, encompassing sequences encoding the receptor-binding domain that is acquired during recombination by all polytropic MuLV isolates. An average divergence of 2.2% was observed among the sequences in this region. In the present study, we obtained numerous polytropic MuLVs from M-MuLV-infected NFS/N mice, utilizing isolation procedures designed

TABLE 1. Identification of endogenous polytropic proviruses of NFS/N mice that recombine with M-MuLV to generate polytropic MuLVs

Recombinant polytropic MuLV	NFS/N endogenous polytropic provirus[a]																					
	Polytropic (PT)													Modified polytropic (mPT)						Intermediate polytropic (iPT)		
	NC1	NC2	NC3	ND1	NE1	NF1	NG1	NH1	NI1	NN1	NP1	NQ1	NS1	NA1	NA2	NK1	NL1	NM1	NR1	NB1	NJ1	NO1
Hy 7 reactive																						
M-RV 5A[b]											+											
M-RV 7A			+																			
M-RV 8A											+											
M-RV 8B[c]			+																			
M-RV 10A[b]									+													
M-RV 10B											+											
M-RV 10C			+																			
M-RV 12A			+																			
M-RV 14A											+											
M-RV 15A											+											
M-RV 15B[d]												+										
MAb 516 reactive																						
M-RV 1A[c]																				+		
M-RV 2A																				+		
M-RV 2B																						
M-RV 7B[e]														+								
M-RV 8C																				+		
M-RV 9A																				+		
M-RV 9B[c]																				+		
M-RV 9C[c]																						
M-RV 10D																				+		
M-RV 10E																				+		
M-RV 11A[b]																				+		
M-RV 12B																				+		
M-RV 13A[a]																				+		

[a] Designations of the NFS/N proviruses correspond to the original lambda clone in which they were identified.
[b] Recombinant MuLVs differing from their parental provirus by two point mutations within the 893-base 3′ *pol*/5′ *env* sequence.
[c] Recombinant MuLVs differing from their parental provirus by one point mutation within the 893-base 3′ *pol*/5′ *env* sequence.
[d] This recombinant MuLV contains sequences of NQ1 as well as another endogenous provirus that cannot be unambiguously identified.
[e] The 3′ portion of the polytropic substitution in this MuLV is identical to NB1, whereas the 5′ portion is identical to NC1, NC2, and NC3.

to limit the number of virus replication cycles and thereby minimize random mutations. Comparisons of the nucleotide sequence of the 893-base regions of the polytropic virus isolates to those of the endogenous proviruses identified the endogenous parent of each polytropic MuLV (Table 1). This included polytropic MuLVs of the Hy 7-reactive and MAb 516-reactive antigenic subclasses, both of which are generated as major populations in M-MuLV-infected mice (37, 38). In most cases (14 of 24 isolates), the polytropic sequence determined in the recombinant viruses exactly matched the sequence of their respective endogenous parents. Several other recombinant viruses differed from their endogenous parent by a single base change within the 893-base sequence, and three polytropic MuLVs differed from their endogenous parents by two base changes in this region. In each instance, the base differences were unique in that none of the specific changes were observed in any other recombinant virus or endogenous polytropic proviral sequence and were almost certainly the result of random mutation during active replication of the recombinant viruses. The rate of mutation of an MuLV has been determined to be approximately 1.4×10^{-5} (42); thus, a sequence of ~900 bases would, on the average, acquire a point mutation every 70 or 80 replication cycles. The frequency of mutation we observed (11 in ~21,000 bases examined) agreed closely to what would be expected from the mutation rate of MuLVs and the estimated number of replication cycles (30 to 50) undergone by the viruses before sequence analyses.

Two additional polytropic MuLVs arose from multiple recombination events and therefore exhibited multiple base differences compared to any single endogenous provirus (M-RV 7B and M-RV 15B). These viruses are considered in a separate section of the results.

Three endogenous polytropic proviruses give rise to nearly all recombinant polytropic MuLVs in M-MuLV-infected NFS/N mice. In contrast to other ecotropic MuLVs such as Akv and F-MuLV, which generate predominantly Hy 7-reactive polytropic recombinant viruses, M-MuLV generates both Hy 7-reactive and MAb 516-reactive polytropic MuLVs in approximately equal amounts (17, 23, 37, 38). Three distinct structural subgroups of endogenous polytropic proviruses have been described previously (22, 58) and have been termed the polytropic (PT), modified polytropic (mPT), and intermediate polytropic (iPT) subgroups. Hy 7-reactive polytropic MuLVs correspond to recombinants with the PT subgroup of endogenous polytropic proviruses, while MAb 516-reactive polytropic MuLVs correspond to recombinants with either the mPT or iPT subgroups (22). Surprisingly, we found that nearly all of the polytropic MuLVs generated by M-MuLV were derived by recombination with only three endogenous polytropic proviruses. Nine of the 11 Hy 7-reactive polytropic MuLVs were derived from one of two PT subgroup proviruses, NC3 (4 MuLVs) or NP1 (5 MuLVs). Even more striking was the observation that 12 of the 13 MAb 516-reactive polytropic MuLVs were derived from a single iPT subgroup provirus,

NB1. Thus, the prominent population of MAb 516-reactive recombinant viruses characteristic of M-MuLV infection can be ascribed to the recombination of M-MuLV with this particular provirus. It is noteworthy that NB1, while it possesses an intact receptor-binding region, does not encode a functional SU or transmembrane protein as a result of a frameshift mutation in the 3′ half of the SU-encoding sequences (22).

Other proviruses also participate in recombination with M-MuLV to yield polytropic MuLVs; however, they were identified at a much lower frequency. These include two PT subgroup proviruses, NI1 and NQ1, and one mPT subgroup provirus, NA1. It seems likely that additional proviruses may also contribute sequences to the polytropic MuLVs at a low frequency.

M-MuLV and endogenous polytropic proviruses recombine in a short, highly homologous region of the *env* gene. The sequence analyses above were extended to include the sites of recombination between the proviruses and M-MuLV within the *env* gene of the recombinant viruses. Surprisingly, we found that almost all of the junctions between ecotropic and polytropic sequences in the *env* genes of the recombinants were confined to a short region in the SU-encoding sequences. The region lies immediately to the 3′ side of the sequences encoding the proline-rich hinge region of the SU protein and corresponds to the region of highest homology between the ecotropic and polytropic *env* gene sequences and very near a region of high predicted secondary structural stability in the M-MuLV *env* gene (Fig. 1). Within this region, the recombination sites of several different viruses were indistinguishable from one another. This was particularly striking for recombinants with the NB1 provirus (Fig. 1A). As noted earlier, NB1, the most frequent endogenous participant in recombination, contains a frameshift mutation disrupting the translation of the carboxyl end of the Env polyprotein. This suggests that recombination of NB1 with M-MuLV must occur on the 5′ side of the mutation to yield a replication-competent MuLV. We found this to be true in all recombinants of M-MuLV with this provirus (Fig. 1A).

A polytropic MuLV can be derived by recombination with more than one endogenous polytropic-like provirus. Two polytropic MuLV isolates (M-RV 7B and M-RV 15B) exhibited multiple base differences when compared to any of the endogenous proviruses (Table 1). Comparison to the closest related provirus revealed that the differences were not randomly distributed but rather were localized to particular regions of the 893-base sequence. Alignments of subsections of the viral sequences with those of the proviruses revealed that each of the viruses was derived by recombination of M-MuLV with two different endogenous proviruses. M-RV 7B was found to be a recombinant with a provirus of the PT subgroup and a provirus of the iPT subgroup (Fig. 2). The iPT subgroup parent (NB1) of M-RV 7B could be identified unambiguously; however, the region contributed by the PT subgroup parent was indistinguishable from three distinct PT proviruses (NC1, NC2, and NC3). A second polytropic virus isolate, M-RV 15B, exhibited polytropic sequences identical to the PT provirus, NQ1, except for 2 bases flanking a short 14-base sequence near the middle of the polytropic *env* sequences. It is conceivable that these base differences were due to point mutations. However, in contrast to the point mutations we have identified in other MuLVs, which are not found in any other virus isolates or proviruses, the bases in M-RV 15B differing from NQ1 are common to numerous endogenous proviruses. This strongly suggests that they were the result of a second recombination event. Alignments with the remaining polytropic proviruses identified 14 different proviruses, any one of which could have recombined to yield M-RV 15B.

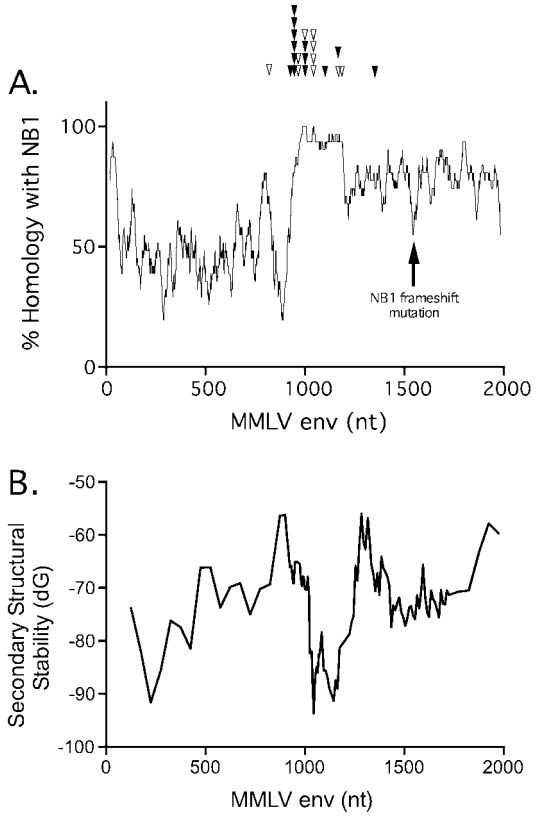

FIG. 1. Recombination sites of M-MuLV with polytropic proviruses: sequence homology and stability of RNA secondary structure. (A) Sequence homology between the *env* genes of M-MuLV and the NB1 provirus was calculated at each nucleotide position for a 31-base sequence composed of 15 bases to the 3′ and 15 bases to the 5′ of the nucleotide. The arrowheads above the graph indicate the points of recombination between M-MuLV and the different endogenous polytropic provirus. Recombination points between two sequences of different origin are defined by sequence differences and cannot be precisely determined if the point of recombination lies within a region of sequence identity. Here, the point of recombination was taken as the midpoint of the region of recombination determined for each virus. Points of recombination of polytropic MuLVs derived from the NB1 provirus are indicated by black arrowheads, while points of recombination of polytropic MuLVs derived from other proviruses are indicated by white arrowheads. The position of the frameshift mutation present in the NB1 provirus is indicated by the arrow below the graph. (B) The free energy of predicted RNA structures for overlapping 251-base sequences of the M-MuLV *env* gene was computed using the Mfold program, version 3.1 (71), and the free energy of the most stable structure for each sequence (lowest free energy) was plotted at the midpoint of each sequence.

Our analyses of polytropic virus isolates suggest that approximately 10% of the viruses examined were derived by multiple recombination events with more than one endogenous provirus. It is quite possible that this is an underestimation of the actual frequency of such events, considering the close homology of the endogenous proviruses, particularly those of the PT

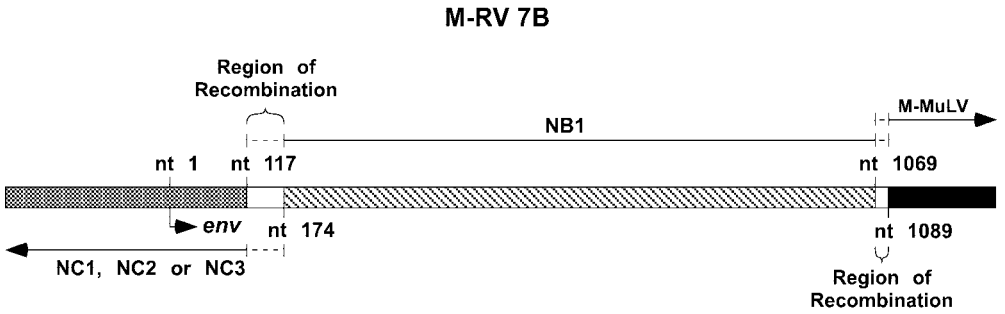

FIG. 2. A polytropic MuLV derived by recombination with two distinct endogenous proviruses. The bar diagram represents the sequenced regions of the polytropic MuLV, M-RV 7B, extending from the 3′ end of the *pol* gene into the *env* gene to include the 3′ recombination boundary between the polytropic and ecotropic sequences. The origin of the sequences and their boundaries are indicated above or below the bar diagram as well as by different fills in the diagram. Regions derived from ecotropic M-MuLV are indicated by black filling. Regions derived from polytropic-like proviruses are indicated by grey or crosshatched filling. The regions where recombination occurred are indicated by white filling and are labeled. These are regions in which the precise point of recombination between two sequences of different origin cannot be defined because of sequence identity in the region. Nucleotides (nt) are numbered from the beginning of the *env* gene.

and mPT subclasses. These subgroups exhibit long regions of sequence identity to one another, and recombination within an identical region would be undetectable.

Multiple polytropic MuLVs are generated in an individual mouse. It is possible that the double recombinants were formed by sequential recombination in which the polytropic MuLV generated after the initial recombination event subsequently underwent recombination with a second endogenous provirus or its transcript. Alternatively, two different polytropic viruses may have been generated and thereafter infected the same cell, where they recombined with one another to generate the double recombinant. The latter mechanism predicts the simultaneous replication of multiple recombinant viruses in a single animal. Our earlier studies suggested that at least two different polytropic recombinant viruses were detectable in nearly all M-MuLV-infected mice. This was based on the presence of both Hy 7- and MAb 516-reactive subclasses of viruses in individual animals (37, 38) and our subsequent finding that the antigenic subclasses correspond to different subgroups of endogenous polytropic-like proviruses (22). However, the extent of polytropic MuLV heterogeneity in a single animal was not known. To address this question, we isolated and characterized multiple polytropic MuLVs derived from a single mouse. Of the five polytropic MuLVs derived from this mouse, only two (M-RV 10D and M-RV 10E) were derived from the same endogenous provirus (NB1) (Fig. 3). These two viruses differed in their sites of recombination at both the 5′ and 3′ ecotropic and/or polytropic sequence boundaries. Thus, each of the five polytropic MuLVs derived from this animal were derived by different recombination events.

DISCUSSION

The unambiguous identification of endogenous polytropic-like proviruses gives rise to polytropic recombinant MuLVs. The identity of the endogenous proviruses participating in recombination to generate polytropic MuLVs has been considered in numerous studies (31, 32, 33, 58); however, the precise identification of those proviruses has been difficult. There are numerous very closely related proviruses in the genomes of inbred mice that are potential donors of the envelope sequences found in polytropic viruses. Proviral sequences diverge very slowly when replicating as components of the mouse ge-

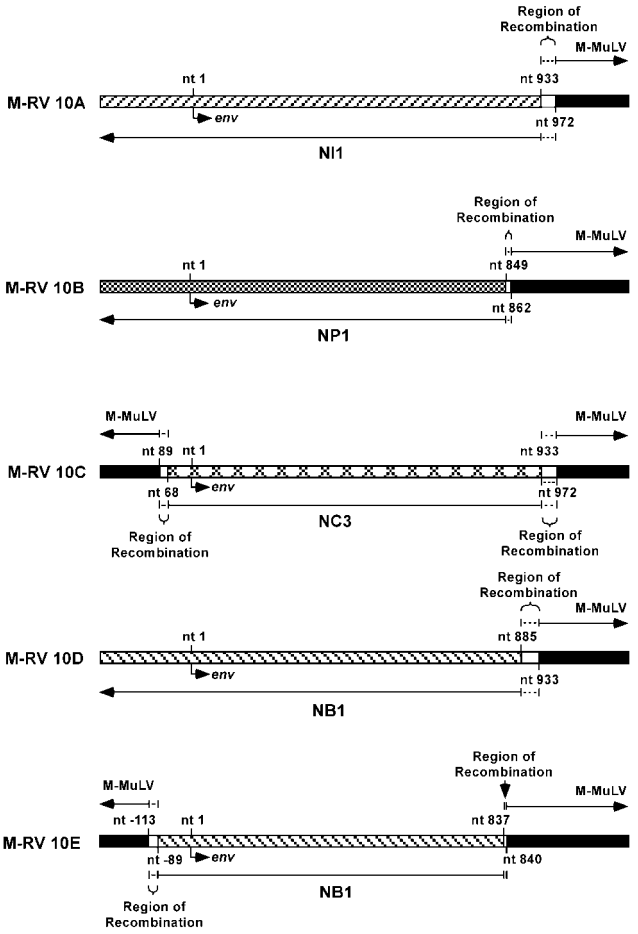

FIG. 3. Polytropic MuLVs derived from different recombination events in an individual mouse. Structures of the polytropic MuLV genomes are represented by the bar diagrams and include RNA sequences extending from the 3′ *pol* region through the point of recombination between polytropic and ecotropic sequences in the *env* gene. Areas of the bar with black filling indicate ecotropic M-MuLV regions, and shaded or hatched areas represent sequences derived from polytropic proviruses. Regions in which recombination occurred are indicated by the white filling. Sequences within these regions are identical in the ecotropic and polytropic genomes, precluding the exact determination of the recombination site. Nucleotides are numbered from the beginning of the *env* gene.

nome; however, upon incorporation into a replicating retrovirus their rate of divergence increases by several orders of magnitude (26). In a recent study, we were able to distinguish nearly all of the endogenous polytropic proviruses of NFS/N mice by microheterogeneity in a ~900-base sequence of their genome that was incorporated into the recombinant viruses (22). In the present study, utilizing procedures to minimize the number of replication cycles undergone by the recombinant viruses, we were able to unambiguously identify the proviral parents for all of the polytropic MuLV isolates examined.

Selectivity of endogenous proviruses contributing to polytropic MuLVs. Our analyses indicate that the overwhelming majority of polytropic MuLVs detected after infection with M-MuLV were derived by recombination with only three endogenous proviruses. At present, it is unclear what factors influence this selectivity. Among the possible factors is replicative selection of the recombinant viruses. Replicative selection could be the result of inefficient spread of certain recombinant viruses or less subtle effects, such as the generation of recombinant viruses that are replication defective. Although replicative selection likely influences the types of polytropic MuLVs that are detected in infected mice, its effect may be dampened by the extensive pseudotyping of the polytropic MuLV genomes within ecotropic virions (37).

A second possible factor that influences the selectivity of the proviruses participating in recombination is the availability of the proviral transcripts for recombination with the ecotropic MuLV. The finding that only three proviruses were major contributors to the polytropic MuLVs found in M-MuLV-infected mice could reflect the relative abundance of their RNA transcripts. Endogenous proviruses appear to be expressed in a tightly controlled manner in mice, as well as in humans (3, 13, 46, 51, 57, 69), but the identity of the transcripts and the mechanism of control are poorly understood. It is possible that high levels of transcription of the endogenous polytropic proviruses are limited to the three proviruses identified as major participants in recombination. In contrast to other ecotropic MuLVs, infection by M-MuLV results in a major population of polytropic MuLVs derived from a single provirus reactive with MAb 516. Ecotropic MuLVs exhibit distinct in vivo tropisms for replication in different tissues (14, 24, 53); thus, the different types of recombinants observed after infection with different ecotropic MuLVs could simply reflect differences in the endogenous proviruses expressed in those tissues in which the ecotropic MuLV is propagating. In this regard, Akv, the ecotropic MuLV of AKR/J mice, induces thymic lymphomas similar to M-MuLV and exhibits a similar temporal pattern of replication in the spleen and thymus (17, 24). With both M-MuLV-infected mice and AKR/J mice, polytropic recombinant viruses are initially detected in the spleen. However, in contrast to mice infected with M-MuLV, predominantly Hy 7-reactive recombinants are generated in AKR/J mice (17, 23). Although it cannot be excluded that the two viruses exhibit subtle differences in the cellular subpopulations in which they are propagating, the observations suggest that the different types of recombinants generated by M-MuLV and Akv are not the result of tissue tropism differences. Furthermore, studies with chimeric viruses between M-MuLV and F-MuLV indicate that sequences encoding the nucleocapsid protein (NC) and a portion of the protease (NCPR sequence) strongly influence recombination of the MuLVs to generate MAb 516-reactive polytropic MuLVs (38). The NCPR sequences, however, have no obvious effect on tissue tropism or the pathology induced by the viruses. Thus, it seems unlikely that the different polytropic MuLVs generated by M-MuLV compared to other MuLVs are entirely the result of replication in different tissues.

It is possible that the selectivity of proviruses involved in recombination is the result of events that influence the recombination process between the ecotropic MuLV and polytropic proviruses. The prevailing mechanism proposed for retroviral recombination involves the formation of a virion RNA heterodimer consisting of one transcript of each parental genome (29, 60). Recombination is thought to occur by a copy choice mechanism, involving template switching during RT of the heterodimer (11). The selectivity of proviruses involved in recombination could be manifested at various processes occurring prior to or during recombination. These include possible changes in transcription levels of the proviruses, the ability to form or package heterodimeric RNAs, or the ability to efficiently transcribe the heterodimers upon infection.

A "hot spot" for recombination between M-MuLV and endogenous polytropic proviruses. We found that the points of recombination in the *env* genes of M-MuLV and the polytropic proviruses are highly constrained to a small region of the SU-encoding sequences, coinciding with the highest degree of homology between the two genes, as well as the highest predicted secondary structural stability in the M-MuLV *env* gene (Fig. 1). This finding may have important implications with regard to the mechanism of recombination and possibly to the specificity of recombination of the endogenous proviruses with different ecotropic MuLVs. Accurate template switching during M-MuLV RT has been reported to be severely compromised under conditions of limited homology (47). Thus, recombination at the peak of highest homology may reflect the requirement of the newly synthesized negative-strand DNA to correctly anneal to the second RNA strand. It is unlikely that strand switching during RT of a heterodimer could proceed accurately in a region of low homology. In this regard, no recombination points were observed within the receptor-binding region of the SU-encoding sequences which exhibited low homology (Fig. 1A). It is also possible that the hot spot for recombination reflects, to some extent, the replicative selection of viruses. The recombinants encode chimeric SU proteins, some of which may function better than others. In this regard, any recombination that did occur in the nonhomologous receptor-binding region of the SU protein may result in the disruption of receptor-binding activity and the elimination of such recombinants.

Template switching during recombination is frequently envisioned as a forced copy choice mechanism involving a break in the RNA encountered during RT, requiring switching of templates to continue synthesis (11). However, breaks would be expected to occur randomly along the RNA, and it is not obvious that compensation by template switching would be restricted to regions of near-sequence identity. Moreover, comparisons of the sequences of M-MuLV and the endogenous polytropic-like proviruses reveal other regions of their SU genes that are highly homologous (Fig. 1A), including substantial regions (ca. >20 bases) that are identical in sequence.

Template switching by the polymerase can occur not only when random breakage is encountered, but also at defined

points along the genome that exhibit complex secondary and possibly tertiary structures (15, 68, 70). The observation that recombination between M-MuLV and the polytropic proviruses occurs most frequently near the region of maximal homology and secondary structural stability may reflect a region of complex structure, resulting in a higher occurrence of template switching. The NC protein facilitates transcription through regions of complex RNA structures during RT, thereby decreasing the frequency of strand switching (and recombination) in these regions (67, 70). Subtle differences in the NC protein markedly alter the interactions of the protein with nucleic acids as well as the frequency of strand switching (27, 67, 70). Thus, different NC proteins (e.g., M-MuLV NC and F-MuLV NC proteins) may differ in their ability to facilitate transcription through complex RNA structures. As noted earlier, the provirus NB1, which gives rise to the major MAb 516-reactive polytropic MuLV recombinants with M-MuLV, contains a frameshift mutation in the 3′ region of the SU protein-encoding sequences. Thus, if the NC protein of F-MuLV was unable to promote transcription through a troublesome region on the 3′ side of the mutation and required template switching to proceed, the resulting recombinants would be replication defective. Conversely, if the M-MuLV NC protein could facilitate the successful negotiation of this region, transcription would proceed past the mutation until another region of difficulty was reached. Recombination in that region would yield a replication-competent MuLV. This hypothesis is consistent with the different types of polytropic MuLVs observed after infection with M-MuLV and F-MuLV and the finding that this difference is strongly influenced by the NCPR sequence.

Potential for in vivo evolution of polytropic MuLVs. Several examples exist of highly virulent viruses that are the result of multiple recombination and/or deletion events. The highly oncogenic mink cell focus-forming viruses of AKR/J and HRS/J mice are polytropic MuLVs that have undergone additional recombination to acquire a xenotropic MuLV long terminal repeat (49, 59) and are proximal agents in the transcriptional activation of proto-oncogenes (45, 50). The spleen focus-forming viruses which cause an acute erytholeukemia in adult mice appear to be the result of several deletion and/or recombination events involving the deletion of a polytropic MuLV and the acquisition of a short xenotropic *env* sequence (2, 21, 66). Similarly, FMCF98, a neurovirulent polytropic MuLV (48) (GenBank accession number AF133256) appears to have sequences derived from a polytropic provirus and a xenotropic MuLV. Recombination between viruses in an infected individual giving rise to a more virulent form appears to have been recently documented in a human HIV infection in which the emergence of the recombinant was associated with a relapse in the disease (25).

The finding that some of the polytropic viruses are recombinants with more than one endogenous sequence indicates that the viruses continue to undergo recombination subsequent to their generation. Furthermore, there is a heterogeneous array of recombinant viruses generated in an individual animal that could recombine with one another to yield new variants. Pseudotyping within ecotropic virions circumvents polytropic viral interference and increases the likelihood of coinfection of the same cell, a prerequisite for recombination. Pseudotyping may also serve to maintain defective viruses in the population, possibly including viruses that carry defects inherent to the endogenous polytropic provirus parent, thereby increasing the available pool of sequences that might contribute to new variants. These observations suggest a high potential for the rapid evolution of polytropic MuLVs in M-MuLV-infected mice. Evolution of polytropic MuLVs might be reflected in polytropic MuLVs that are the proximal agents of transformation in M-MuLV-induced tumors. In light of these considerations, a more detailed structural analysis of polytropic MuLVs integrated near proto-oncogenes in M-MuLV-induced leukemia is warranted.

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