A Novel Subgenomic Murine Leukemia Virus RNA Transcript Results from Alternative Splicing

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Here we show the existence of a novel subgenomic 4.4-kb RNA in cells infected with the prototypic replication-competent Friend or Moloney murine leukemia viruses (MuLV). This RNA derives by splicing from an alternative donor site (SD') within the capsid-coding region to the canonical envelope splice acceptor site. The position and the sequence of SD' was highly conserved among mammalian type C and D oncoviruses. Point mutations used to inactivate SD' without changing the capsid-coding ability affected viral RNA splicing and reduced viral replication in infected cells.

The retroviral life cycle requires that significant amounts of RNA remain unspliced and perform several functions in the cytoplasm. Thus, the full-length RNA serves as the viral genetic material that will be encapsidated in viral particles and as the mRNA encoding structural and enzymatic proteins required for viral replication. Simple retroviruses are defined as those viruses which produce one single-spliced env RNA from this full-length precursor RNA, whereas complex retroviruses are characterized by the production of multiple spliced RNA species (18). Because cis-acting and coding functions in the viral genome frequently overlap, most of the studies on RNA splicing and transport regulation in murine leukemia viruses (MuLV) have been conducted with extensively reshaped retroviral vectors that are replication defective (9, 14, 17). Besides these models with vectors, usage of MuLV canonical or cryptic splice sites remain unclear.

In the present work, performed on cells infected ex vivo with the closely related replication-competent Friend and Moloney MuLVs, we report for the first time the production of a large subgenomic transcript in a simple retrovirus. This novel RNA was generated from an alternative splice donor site, SD', which is conserved among mammalian simple retroviruses, in combination with the env splice acceptor site. Mutations of SD' in Friend and Moloney MuLVs affect the general splicing pattern of viral RNAs and reduce viral replication. Identification of this novel MuLV subgenomic RNA provides new insights into simple retrovirus functions.

MATERIALS AND METHODS

Mutagenesis and proviral constructs. The parental prototypic Friend MuLV strain 57 (16) and Moloney MuLV strain 8.2 (23) were used as permutated simple retrovirus functions.

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RESULTS

Identification of a novel MuLV RNA transcript. Total cellular RNA extracted from Dunni cells infected with Friend MuLV was examined by Northern blot hybridization using a probe complementary to the 3′ end of the Friend MuLV sequence. This yielded two expected major bands corresponding to the full-length genomic transcript (8.3 kb) and the single-spliced env RNA (3 kb) (Fig. 1). However, we noted an additional faint band, below the 28S RNA, corresponding to a potential 4- to 5-kb transcript. This faint band was more readily detected in the FDV mutant of Friend MuLV, which was obtained after introduction of theFriendMuLV vector sequences (3, 14). An SPFLV vector was derived after insertion of the Friend SacI8273-RulL13 homologous fragment in pGEM. The 274-nt Friend riboprobe was obtained after linearization of SPFLV with EcoRI. These probes should both yield a 214-nt genomic and a 205-nt spliced fragment after RNase digestion, as previously described (14). RNase protection products were quantified by the PhosphorImager.

Extensive amplifications (30 cycles) were also performed to obtain sufficient amounts of DNA for sequencing. Amplified DNA was sequenced on an automated sequencer (ABI PRISM 377; Perkin-Elmer) with the dye terminator cycle sequencing ready reaction kit (ABI PRISM) by following the recommendations of the manufacturer and using an oligonucleotide complementary to nt 5601 to 5620 of MuLV. For RNase protection assays, 15 µg of total RNA were generally used. Riboprobe transcription was performed with linearized template plasmids according to standard procedures (3). The 274-nt Moloney riboprobe was generated from the SPMLV plasmid linearized with EcoRI and includes 5′ and 3′ non-MuLV vector sequences (3, 14). An SPFLV vector was derived after insertion of the Friend SacI8273-RulL13 homologous fragment in pGEM. The 274-nt Friend riboprobe was obtained after linearization of SPFLV with EcoRI. These probes should both yield a 214-nt genomic and a 205-nt spliced fragment after RNase digestion, as previously described (14). RNase protection products were quantified by the PhosphorImager.

Effects of SD′ mutations on viral replication. To evaluate the influence of the SD′ sequence on the MuLV life cycle, we generated replication-competent MuLV mutants with distinct point mutations in this sequence. It is important to note that all the mutations maintained the wt Gag amino acid sequence. In the aforementioned FDV mutant, the two mutated nucleotides also maintained the potential parental base-pairing of SD′ with the U1 snRNA consensus (Fig. 2). We generated two other Friend mutants, F1 and F2, and one Moloney mutant, M1, in which putative mismatches with U1 snRNA were introduced (Fig. 2). The Gag and Env precursor proteins and cleaved products were detected in all mutants, as assessed by immunoblotting (not shown). The infectivities of the mutant and wt strains were determined by monitoring cell surface envelope glycoprotein expression on newly infected cells by using the quantitative FIA (25) (Fig. 4), as well as by measuring the RT activity of the various supernatants (not shown). The former technique is a measure of protein production from the spliced env RNA while the latter reflects the production of RT by the unspliced gag-pol RNA. These two tests yielded similar results, indicating a significant reduction (ranging from 7- to 100-fold) in the infectivity observed with the different SD′ mutants (Fig. 4). The F1 and M1 mutants, which displayed the lowest potential base pairing with U1 snRNA, exhibited the lowest infectious ability. Interestingly, the replication capacity of the FDV mutant,
which maintained the parental U1 snRNA base pairing, was the least altered.

Effects of SD' mutations on MuLV splicing. To evaluate whether the observed reduction in MuLV titers following mutation of the SD' sequence was associated with an alteration in the alternative splicing efficiency, we next examined the splicing patterns of these mutants in infected cells. Semi-quantitative RT-PCR was performed with total RNA extracted from de novo-infected Dunni cells. Full-length RNA, single-spliced SD' RNA, alternatively spliced RNA, and total MuLV transcripts were monitored with different combinations of oligonucleotide pairs (Fig. 5A). Although similar results were obtained when the initial RT reaction was performed with a specific MuLV or an oligo(dT) primer, we preferred the latter because it allowed amplification of the total cellular RNA independently of infection efficiency. The RT-PCR band obtained with the F1, F2, and M1 mutants migrated slightly slower than that detected with wt MuLV or FDV (Fig. 5A). Sequencing of the amplified product in F1, F2, and M1 revealed the recruitment of another product in F1, F2, and M1 mutants migrated slightly slower than that detected with wt MuLV or FDV (Fig. 5A). Sequencing of the amplified product in F1, F2, and M1 revealed the recruitment of another product in F1, F2, and M1 mutants migrated slightly slower than that detected with wt MuLV or FDV (Fig. 5A). Sequencing of the amplified product in F1, F2, and M1 revealed the recruitment of another product in F1, F2, and M1 revealed the recruitment of another product in F1, F2, and M1. Reverse transcriptions were performed with oligo(dT), and PCRs were performed with oligonucleotides.

FIG. 3. Amplification of a new alternatively spliced MuLV transcript by RT-PCR. (A) Schematic structures of the unspliced genomic and alternatively spliced (SD') RNA, including the canonical (SD), acceptor (SA), and alternative (SD') splice sites. Nucleotides are numbered starting from the first nucleotide of R according to the Friend MuLV sequence. Also noted are the gag gene components, including the matrix (MA), capsid (CA), and nucelocapsid (NC). Arrows refer to the approximate positions of primers used for RT with the oligo(dT) primer and for PCR amplification. (B) RT-PCR was conducted on total RNA samples extracted from mock-infected cells (lane 3) or cells infected with either Friend (lane 4) or Moloney virus (lane 6). Reverse transcriptions were performed with oligo(dT), and PCRs were performed with oligonucleotides s1450 and a5620 as described in the Materials and Methods. The alternatively spliced SD' RNA yielded an amplified product of 276 bp. After 30 cycles, amplified samples were loaded onto an agarose gel and stained with ethidium bromide. Lanes 1 and 2, 1-kb and 100-bp ladders, respectively.

For comparison purposes, the ratio of the amount of each transcript to the total level of MuLV RNA was determined and normalized to the ratio of the corresponding wt MuLV (Fig. 5B). Differences were evaluated by using the two-tailed Student t test and considered significant when probability values (P) were <0.05. Although semiquantitative RT-PCR does not allow the precise quantification of small differences in RNA expression, this method revealed dramatic differences in the relative amounts of alternatively spliced RNA species between mutant and wt MuLV. We observed a significant increase in the SD' RNA level in cells infected with the FDV mutant (P < 0.001), in agreement with the initial observation made on Northern blots (Fig. 1). Also, the F1 and F2 Friend mutants harbored a much higher level of SD' RNA than the equivalent M1 Moloney mutant (Fig. 5), indicating that elements, additional to a functional alternative SD' site, influenced SD' usage.

Since the F1 and F2 mutations appeared to decrease levels of the canonically spliced env RNA (Fig. 5B), a more direct quantification of env RNA levels was performed by using the RNase protection assay. Total cellular RNA was hybridized to a riboprobe that spans the MuLV canonical splice donor site (SD) at position 214 (Fig. 6A). RNase digestion yielded a 214-nt protected fragment derived from the unspliced RNA and the SD' or SD' alternatively spliced RNA species and a shorter 205-nt long fragment corresponding to the canonical SD-spliced env transcript (Fig. 6A). A reproducible decrease in env RNA levels was observed for all mutants, albeit to different extents, when compared to the respective wt strains (P < 0.04) (Fig. 6B and C).

Conservation of a potential alternative splice donor site in the capsid of mammalian simple retroviruses. gag sequences of replication-competent MuLV, feline, porcine, and simian C- and D-type retroviruses were aligned. This showed that a 5' splice site consensus sequence, corresponding to a conserved putative SD' site, is present approximately 100 nt upstream of the major homology region (MHR) (4) in the capsid-coding gene (Fig. 7). Interestingly, the GU dinucleotide immediately adjacent to the cleavage site, which is present in almost all mammalian pre-mRNA introns (10), was strictly conserved among these viral sequences.
We have identified a new 4.4-kb RNA transcript produced by two prototypic strains of MuLV. This novel RNA results from splicing between an alternative splice donor site, SD9, located in the capsid-coding region, and the canonical env splice acceptor site. Although in vivo studies of Moloney MuLV-induced promonocytic leukemia have shown that the recruitment of an SD9 sequence produces a rearranged truncated c-myb gene upon insertional mutagenesis (8, 21), this is the first report demonstrating that a second spliced RNA is produced during the life cycle of a replication-competent simple retrovirus. Moreover, we found that an SD9 sequence is present at a similar position in the capsid-coding gene of all the replication-competent MuLV, feline, porcine, and simian C-type retroviruses we examined. This conserved feature also extended to the more distantly related simian D-type oncoviruses. It will be interesting to determine whether an additional homologous subgenomic transcript is also present in these...
retroviruses, as already suggested in a previous report with the gibbon ape leukemia virus (5).

This new 4.4-kb RNA was weakly detectable by Northern blot analysis of RNA from cells infected with the wt Friend strain, suggesting a low level of production and/or a potential instability of the mature SD' transcript. Since the synonymous mutations introduced in the FDV mutant flanked the SD' site and increased the SD' RNA level, it is likely that potential local RNA structures also modulate splicing at this site.

Production of two spliced RNAs from a single precursor and maintenance of the full-length RNA pool require incomplete splicing. According to our results, production of the alternatively spliced SD' RNA might play an important role in splicing regulation in MuLV simple retroviruses. Thus, when SD' splicing was maintained (wt and FDV), cryptic splicing at SD' was not detected and mutations in Friend-MuLV that activated the alternative SD' or cryptic SD' were accompanied by a decreased production of the canonical env spliced RNA (FDV, F1, and F2). However, a strict competition model between these sites cannot account for the splicing pattern observed in the Moloney MuLV strains, since a significant impairment of alternative splicing in the M1 mutant did not lead to increased levels of the env RNA. Furthermore, usage of these potentially competing sites did not strictly correlate to U1 base pairing. For instance, although both SD' and the canonical SD site harbored two potential mispairings with U1, an SD' RNA might be directly involved in the increased level of infectious retroviral RNA. Additionally, usage of these putative SD' sites in the capsid-coding region of a series of replication-competent mammalian types C and D retroviruses. The 5' splice donor site consensus sequence (5'SS) is shown on top, with the potential splicing cleavage site indicated by a vertical line. All sequences were located approximately 100 nt upstream of the capsid major homology region. Numbering is according to the Friend-MuLV 57 sequence. Lower-case letters indicate mismatches between the 5' SS consensus and the viral sequence. Abbreviations and strains correspond to the following retroviruses. (i) MuLVs: Friend-MuLV, strain 57; Moloney-MuLV, strain 8.2; RadLV, radiation leukemia virus; Cas-Br-E, Lake Casitas brain E neurotropic virus; WN55, the N- and B-tropic clones of the WN1892 isolate; and AKV, from clone AKR 623 of endogenous virus from the AKR mouse strain. All of the above are ecotropic MuLVs. MCF, clone MCF1233 of the polytropic mink cell focus-inducing viruses. (ii) Feline leukemia viruses: FeLV-A and FeLV-B, strains A and B. (iii) Primate simple retroviruses: simian type C retroviruses include BaEV, baboon endogenous virus, and GaLV, gibbon ape leukemia virus. Simian type D retroviruses include MPMV, Mason-Pfizer monkey virus, and SRV-Pc, a baboon simian retrovirus-like isolate. (iv) Porcine endogenous virus: a human-tropic C-type porcine endogenous retrovirus.

FIG. 6. Quantification by RNase protection of the canonical env transcript level in MuLV-infected cells. (A) Friend and Moloney transcripts were detected by RNase protection with the uniformly labeled antisense SPLFLV or SPMLV probes, respectively (see Materials and Methods), which overlap the canonical SD site. Hybridization of the 274-nt probe to viral RNA species that are not spliced at the canonical SD site (full length, SD', and SD'' RNA) yields a protected 214-nt fragment, while canonically spliced env RNA yields a protected 205-nt fragment. (B) RNase protection assays were carried out with 15 ng of total cellular RNA (lanes 2 to 6, 8, 9, and 13) and RNA extracted from 10% total cellular RNA (lanes 1 to 5, 7, 10, and 12). The positions of the probe and the protected fragments corresponding to canonically spliced env RNA (SD) as well as the noncanonically spliced RNAs (non SD) are indicated by arrows. The size markers (lane 14) consist of end-labeled X174 HaeIII DNA fragments. (C) Quantification of RNase protection assays. For each series, the percentage of env-protected fragments versus the total of protected signals is represented (± standard of the mean). Each value corresponds to the average of at least three measurements performed on different RNA preparations.

FIG. 7. Conservation of the SD' site. Sequence alignment of a putative SD' site in the capsid-coding region of a series of replication-competent mammalian types C and D retroviruses. The 5' splice donor site consensus sequence (5'SS) is shown on top, with the potential splicing cleavage site indicated by a vertical line. All sequences were located approximately 100 nt upstream of the capsid major homology region. Numbering is according to the Friend-MuLV 57 sequence. Lower-case letters indicate mismatches between the 5' SS consensus and the viral sequence. Abbreviations and strains correspond to the following retroviruses. (i) MuLVs: Friend-MuLV, strain 57; Moloney-MuLV, strain 8.2; RadLV, radiation leukemia virus; Cas-Br-E, Lake Casitas brain E neurotropic virus; WN55, the N- and B-tropic clones of the WN1892 isolate; and AKV, from clone AKR 623 of endogenous virus from the AKR mouse strain. All of the above are ecotropic MuLVs. MCF, clone MCF1233 of the polytropic mink cell focus-inducing viruses. (ii) Feline leukemia viruses: FeLV-A and FeLV-B, strains A and B. (iii) Primate simple retroviruses: simian type C retroviruses include BaEV, baboon endogenous virus, and GaLV, gibbon ape leukemia virus. Simian type D retroviruses include MPMV, Mason-Pfizer monkey virus, and SRV-Pc, a baboon simian retrovirus-like isolate. (iv) Porcine endogenous virus: a human-tropic C-type porcine endogenous retrovirus.

The CDV mutant, which harbors a high level of SD' RNA, lost the SD' transcript. Since decreased levels of infection observed with the mutants resulted from synonymous mutations in the SD' region, this was the first demonstration of a cis-acting effect of the capsid region in the context of a replication-competent retrovirus. The severe drop observed in replication abilities of the F1, F2, and M1 mutants could result from a negative effect of the newly produced SD' RNA, loss of the SD' RNA, or alteration of the splicing balance. Nevertheless, results obtained with the FDV mutant, which harbors a high level of SD' RNA and a slightly altered viral titer, suggest that SD' usage is required for a balanced splicing profile and optimal replication.

In addition, the coding capability of the SD' RNA might be directly involved in the increased level of infectious retroviral
particles. The alternatively spliced SD’ RNA was polyadenylated, as shown by its presence in the poly(A)^+ RNA fraction (not shown), and could be reverse transcribed with an oligo(dT) primer. Also, the SD’ RNA presents a large coding potential, with multiple translation initiation codons; these include initiation codons used in the glycoprotein gag, gag, and env open reading frames, which are active in the full-length or canonically spliced mRNA, as well as the initiation codon in the capsid that is used in the chimerical gag-pol transcripts (19, 26). These many putative initiation codons, placed in the context of new gag and env open reading frames, provide the potential for new MuLV translational products. Identification of this novel alternatively spliced MuLV RNA at a site that is highly conserved among types C and D mammalian oncoviruses provides new avenues of investigation on the influence of new RNA species in replication of simple retroviruses.

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