Functional Analysis of the Murine Sarcoma Virus RNA Packaging Sequence

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We investigated the features of the Moloney murine sarcoma virus leader sequence necessary for RNA packaging function by using a deletion analysis approach. We found that sequences that extend beyond those characterized genetically in previous reports are important for optimal packaging efficiency. A fragment covering a minimum of four potential stem-loop structures is required for the shortest packaging element compatible with gene transfer. Our results reveal the extent to which each of the segments of the packaging sequence contribute to packaging efficiency.

The assembly of infectious retroviral particles requires the selective encapsidation of two unspliced viral genomic RNA molecules (11, 35). This process, termed RNA packaging, depends upon specific sequences in the unspliced viral RNA and in the Gag polyprotein (2, 6, 19, 32, 36). The cis-acting signals involved in retroviral RNA packaging include the essential encapsidation sequences () located in the genomic RNA between the first splice donor site and the gag initiation codon (35). There are additional regions of the genome that facilitate encapsidation in the presence of and these are located proximal to the major subgenomic splice donor and at the 5’ end of the gag coding sequence (7, 25, 26).

Packaging of retroviral RNA involves dimerization via RNA-RNA contacts as well as association with the Gag polyprotein. A palindromic element, called the kissing loop, and stretches of guanines located within the 5’ untranslated leader sequence of the RNA genome have both been suggested to be key elements for RNA dimerization (3, 10, 12, 13, 15, 18, 23, 27, 31, 34, 38–40). Dimerization might be linked to efficient packaging, although some retroviral packaging can occur in the absence of significant dimerization, as mutants that package monomeric genomic RNA have been observed (9, 10, 12, 17, 22, 34, 38).

The Moloney murine leukemia virus (MoMLV) packaging signal has provided an excellent system to study the determinants of retroviral packaging (reference 35 and references therein). In vivo and in vitro studies showed that a critical fragment of the MoMLV packaging signal lies within 520 nucleotides located at the 5’ end of the MoMLV genome (5, 13, 34). Moloney murine sarcoma virus (MoMSV) is a replication defective virus that was derived from MoMLV and in which most of the pol and env open reading frames have been replaced by that of the c-mos gene. In MoMSV the encapsidation signal has been localized to a 333-nucleotide sequence located downstream of the 5’ splice donor site, corresponding to a portion of the MoMLV sequence. This region has been previously shown to produce virus titers comparable to those of wild-type MoMLV when inserted into a gene transfer vector (1). The sequences of MoMLV and MoMSV are almost identical with the exception of a 50-nucleotide highly divergent fragment located within the sequence of MoMLV. In MoMSV, this 50-nucleotide fragment is replaced by a 32-nucleotide sequence. This MoMSV fragment is virtually identical to virus-like (VL30) sequences, and this hybrid MoMLV-VL30 sequence is likely to have resulted from the recombination of MoMLV with the endogenous virus-like retrotransposon (21). VL30 can copackage with the retroviral genome, and therefore, recombination between these elements can occur (20, 45).

Deletion analysis of sequences included in predicted stem-loops E, F, and G. The sequence of MoMLV folds into several stem-loop structures (28, 29, 42). When a subset of these stem-loops, named A, B, C, and D, were individually deleted, the virus titer and level of RNA packaging were reduced 5- to 10-fold but not abolished, indicating that none of these elements is necessary for RNA packaging (29). Previous genetic studies have not focused on the contribution to RNA packaging of sequences located 3’ of the D stem-loop (bp 374 to 544), which were proposed to fold into additional stem-loop structures (42). Similar stem-loop structures are predicted for the corresponding fragment of the MoMSV sequence (bp 827 to 934 of the MoMSV clone 124 sequence, GenBank accession no. J02263), and we named those stem-loops E, F, and G.

To investigate the role of these sequences in RNA packaging, we carried out deletion mutagenesis of the proposed stem loops E, F, and G and tested these mutant viruses in a gene transfer assay (Fig. 1). A linker containing two different sites replaced the MoMLV packaging sequence in plasmid pMFGpuro (37). A MoMSV packaging vector carrying the wild-type sequence was generated by amplifying the MoMSV sequence from plasmid PNL6 (nucleotides 799 to 1132, BalI-PstI fragment of plNL6, accession no. M63653, corresponding to nucleotides 657 to 989 of the MoMSV clone 124 sequence, accession no. J02263) and cloning it into the modified pMFGpuro. To generate the deletion mutants, portions of the deleted sequences were amplified by using the PCR-based splice-overlap extension method as described previously (32) and cloning them into the modified pMFGpuro. The gene transfer assay was carried out by infecting CHO cells
according to previously published procedures (30) and by evaluating the number of puromycin-resistant colonies 9 days after infection. Infectious titers of wild-type and mutant viruses were normalized according to equal amounts of reverse transcriptase (RT) activity in the supernatants used in infectivity assays.

To evaluate the contribution to packaging of the entire sequence predicted to fold into stem-loops E, F, and G, we generated a mutant in which the DNA fragment that includes these stem-loops was deleted (bp 382 to 489, mutant ΔE–G) (Fig. 1). This deletion resulted in a virus titer that was approximately 50-fold lower than that of the wild type, indicating that this sequence is required for high efficiency of viral packaging, although it is not necessary for virus viability.

Mutant viruses carrying a deletion of the sequences included in each of the potential stem-loops showed a reduction in virus titer of 11- to 14-fold (mutants ΔE, ΔF, and ΔG) (Fig. 1), implying that each of these sequences contributes significantly to high-efficiency packaging. This level of reduction is similar to the reduction observed by Mougel et al. when stem-loop A, B, C, or D was deleted individually (29), indicating that the sequences included in stem-loops E, F, and G contribute to a similar degree to the packaging process and are critical to optimal RNA packaging and virus titer.

It was previously reported that when the 333-bp MoMSV fragment that we have investigated was compared to its homologous fragment from MoMLV in a gene transfer assay, the titer from the MoMSV virus was 10-fold higher (1). The difference was attributed to a highly divergent fragment that in MoMSV is part of the sequence that we assigned to stem-loop F and that shows high homology to sequences of VL30 (1).

However, we were not able to detect significant titer differences between the two viruses carrying the MoMSV and MoMLV packaging sequences when these sequences were inserted in our gene transfer vector and transfected individually (data not shown). These data suggest that the sequences included in stem-loop F, proposed for the divergent fragment of MoMLV, are functionally equivalent to the sequences present in MoMSV (8, 14). It is possible that the different context of these sequences might have played a role in the different outcome of the experiment reported by Adam and Miller (1) or, alternatively, that the differences in packaging efficiency of the two sequences can only be revealed in competition experiments (20, 41).

Identification of the minimal Ψ domain by gene transfer analysis. Previous studies were not able to define conclusively the MoMLV or MoMSV elements sufficient for minimal packaging function. Stem-loops B, C, and D are virtually identical in MoMLV and MoMSV. Insertion of the 205-nucleotide fragment covering stem-loops B, C, and D into a 712-nucleotide fragment derived from the human immunodeficiency virus type 1 (HIV-1) leader sequence permits packaging of this hybrid Ψ sequence into MoMLV particles at 75% of the level allowed by the wild-type MoMLV packaging sequence (28). The extent of packaging function provided by this fragment in the absence of HIV-1 leader sequences was not established. In this study, Mougel and Barklis reached the conclusion that stem-loops B, C, and D are crucial for encapsidation and comprise the packaging core element for MoMLV. It is nevertheless unclear whether these structures can support RNA packaging and virus replication in the absence of heterologous packaging elements that may have been provided by HIV or whether additional MoMLV elements might be required.

To identify the shortest MoMSV Ψ fragment compatible with RNA packaging and virus viability in a gene transfer assay, we generated a series of deletion mutants which included only certain parts of the MoMSV Ψ region and evaluated their infectious titers in CHO cells (Fig. 2). Stem-loops C and D were previously described as critical elements of the Ψ sequence of MoMLV (16, 28, 29). The contribution of these sequences to packaging was evaluated in mutants ΔC–D, ΔB–D, ΔC–E, ΔC–F, and ΔC–A’ (Fig. 2). None of these viruses was infectious. Construct C–A’ contains 70% of the packaging signal, and the mutant virus derived from it is unable to support virus replication. Thus, additional sequences upstream of stem-loop C are required to yield infectious virus and the sequences included in stem-loops C and D are not sufficient to provide...
positive gene transfer. When two overlapping fragments that include sequences assigned to stem-loops A, B, C, and D (bp 212 to 374) or of stem-loops B, C, D, and E (bp 280 to 399) were investigated (mutant A-D or B-E) (Fig. 2), each corresponding virus yielded a titer of approximately 10^2 CFU/ml. One virus, carrying the fragment that covers stem-loops B, C, and D (bp 280 to 374), was noninfectious. Since the addition of either stem-loop B to construct C-E or stem-loop E to construct B-D appears in these experiments to be crucial to yield infectious virus, we generated a construct that contained only sequences of stem-loops B and E (mutant B'/H11001E). The virus from this construct was noninfectious. This suggests that sequences located at the 5' and 3' ends of C-D can provide packaging function when they are linked to the C-D sequence and therefore, like C and D, are necessary but not sufficient. The addition of the sequences located 5' of stem-loop B or in stem-loop E to the sequence that covers stem-loops B, C, and D leads to virus titers that are very similar, suggesting that the contribution of these sequences to function is equivalent. When additional sequences are added to this core sequence, the titers of the corresponding viruses increase, indicating that each of the additional stem-loops can contribute to packaging function (mutants B-F, A-E, A-F). Only one portion of the sequence previously assigned to stem A is present in the packaging sequences that extend to nucleotide 212 in mutants A-D, A-E, and A-F. Therefore, it is more likely that one or both of the two stem-loops located 3' of A and 5' of the B stem-loop, suggested by Tounekti et al. (42), are important for the phenotype of these mutants.

Our data indicate that multiple structural elements are required in vivo for packaging function. Although sequences in stem-loop B can be deleted from the optimal sequence without losing virus viability (29), they are required when a shorter sequence is evaluated. We found that Ψ fragments that included sequences assigned to stem-loops C and D (or B, C, and D) did not permit successful gene transfer when included in a viral genome. The smallest sequence compatible with virus viability includes sequences that have the potential to fold into at least four stem-loop structures.

**Encapsidation of mutant viral RNAs.** As the efficiency of gene transfer depends upon the virus infecting the target cell and integrating its genome into the cellular genome, the efficiency of gene transfer correlates well with the efficiency of viral RNA packaging. Because gene transfer also depends on successful reverse transcription and integration, viruses whose mutations affect these processes might have an infectious titer lower than the actual amount of packaged RNA.

To investigate whether our mutations affected steps other than RNA packaging, we determined the levels of viral genomic RNA present in particles by RT-PCR (32, 33). The results suggest that the MoMSV deletion mutations that we analyzed modulated viral infectivity through a reduction in the amount of encapsidated viral RNA, as we found a good, although imperfect, correlation between the amount of incorpo-
rated RNA and the viral titer of the mutants (Fig. 1, 2, and 3B). It is unlikely that other steps of the virus life cycle, such as reverse transcription, were significantly altered in these mutated viruses.

When stem-loops E, F, and G were deleted individually, the amount of incorporated viral genomic RNA was approximately 20% of that of the wild-type virus (mutants ΔE, ΔF, ΔG) (Fig. 3). When these three stem-loops were simultaneously deleted in mutant ΔE-G, the genomic RNA incorporation was reduced to 7% of that of the wild-type virus (Fig. 3). These data support the conclusion that the lower virus titer observed for these mutants is due to decreased genomic RNA incorporation and indicate that these sequences contribute significantly to optimal RNA incorporation.

We did not detect measurable amounts of RNA in any mutants that were noninfectious (C-A’, B-D, C-E, C-F, and C-D) or in the constructs that yielded the lowest viral titer (B-E and A-D; 10^2 CFU/ml) when the RT-PCR assay was carried out under our standard conditions (data not shown). This result indicated that these mutants incorporate less than 6% of viral RNA. When the total number of PCR cycles was increased, we could detect levels of RNA incorporation around 3% (which is our lowest standard) for mutants B-E and A-D and below 3% for mutants C-A’, B-D, C-E, C-F, and C-D (Fig. 3A). The RNA incorporation data for mutants B-D and C-D further confirm the findings of the infectivity assay and indicate that the Ψ sequences included in these mutants do not support incorporation of significant levels of RNA.

RNA incorporation for MoMLV mutant viruses carrying B-D and C-D motifs was reported to be 74 and 48%, respectively, by Mougel and Barklis (28). In our experiments, RNA packaging for both B-D and C-D was less than 3% of the RNA incorporation observed for the wild-type mutants. The discrepancies between the data of Mougel and Barklis and those...
reported here could be due to differences in the two experimental systems. Our gene transfer vector includes only MoMSV virus sequences. In the experiments described by Mougel and Barklis, the BCD and CD motifs were surrounded by HIV-1 sequences derived from the HIV-1 5’ leader sequence which is involved in HIV-1 packaging (28). Stem-loop E was also included in the HIV BCD and HIV CD constructs. Furthermore, 67 nucleotides located at the 5’ end of stem-loop B, predicted to fold into additional stem-loops (42), were also included in HIV BCD. MoMLV and HIV-1 cannot cross package their RNAs. However, MoMLV and HIV-1 RNAs are able to form heterologous dimers (24). It is possible that in the experiments carried out with a MoMLV–HIV-1 chimeric packaging signal, the HIV-1 sequences fold into elements that, although not sufficient for RNA packaging when tested alone, can provide packaging function together with the B, C, and D sequences. In our case, there were no sequences in addition to B, C, and D to support packaging function, and efficient RNA packaging and gene transfer could not be achieved with these sequences alone.

To exclude the possibility that the reduced RNA incorporation by packaging mutants results from decreased intracellular transcription of the viral RNA, reverse transcriptase PCR was carried out on cellular RNA with primers specific for the MoMSV genomic RNA. We found that all of the mutants produced similar amounts of intracellular viral RNA and that, therefore, the reduced rate of packaging of the mutants results from reduced packaging efficiency (data not shown).

Phylogenetic analysis, computer modeling, and genetic analysis were also used in the investigation of the minimal elements involved in the packaging of avian leukemia-sarcoma virus (4). In that study, the term minimal was used to indicate the smallest fragment that has a level of RNA incorporation similar to the wild type and not, as we use it here, to indicate a fragment compatible with the minimal level of function in a gene transfer assay. In avian sarcoma virus, an element composed of three stem-loops could provide a level of RNA incorporation only threefold lower than the wild-type sequence. In our case, a larger RNA fragment that spans at least seven stem-loops appears to be necessary for optimal RNA incorporation. The deletion of each of the seven stem-loops has a significant impact on the same parameters, reducing RNA incorporation fivefold or more (reference 29 and this report). Therefore, 82 nucleotides are sufficient for avian sarcoma virus wild-type RNA incorporation levels while a much larger fragment seems necessary in MoMSV for the same level of RNA incorporation efficiency. It is unclear why elements that are so different in their structural features carry out the same function in these different viruses. It is possible that differences in the conformation of the proteins they interact with might play a role in determination of the critical RNA structure.

In summary, the results obtained from the deletion analysis extend our understanding of the MoMSV Ψ sequence and indicate that this sequence is composed of multiple elements, all of which are necessary for optimal packaging efficiency. This is also true for the elements contained in the 3’ portion of the Ψ sequence that had not been analyzed previously. A fragment that is likely to fold into at least four stem-loop structures provides the minimal sequence that is compatible with RNA packaging and gene transfer.

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REFERENCES


