Efficient replication, integration, and packaging of retroviral vectors with modified long terminal repeats containing the packaging signal

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ABSTRACT

Retroviral vectors were modified to contain packaging (Ψ) signals of varying lengths (nucleotides 211 – 355, 211 – 565, or 211 – 1039 of MoMuLV RNA) between the U3-r and U5 sequences of their 5′ long terminal repeat (LTR). For the vector MoTN-PR3, containing the full length 211 – 1039 nucleotide-long Ψ signal within the 5′ LTR, replication, integration, and packaging were almost as efficient as for the original unmodified vector. This result confirmed that the 211 – 1039 nucleotide-long sequence from the MoMuLV RNA is sufficient and necessary to allow efficient packaging of RNAs. In addition, an important site was revealed where insertion of foreign DNA sequences of up to 829 nucleotides can be made within the 5′ LTR, between U3-r and U5 sequences, without affecting viral replication, integration, or packaging.

INTRODUCTION

Retroviral vectors contain 5′ and 3′ LTRs made of U3-r-U5 sequences (1). The 5′ LTR is followed by a primer binding site (PBS) and a packaging (Ψ) signal. Both LTRs contain promoter sequences; thus, 2 RNAs can potentially be transcribed from the proviral DNA: viral RNA from the 5′ LTR and cellular RNA from the 3′ LTR. However, since only the viral RNA contains the Ψ signal, only this RNA can be packaged. If the Ψ signal was also part of the cellular transcripts, then one would expect that these RNAs could also be packaged. The goal of this work was to develop retroviral vectors that contain the Ψ signal as part of their LTR, such that any cellular RNAs transcribed from the 3′ LTR might also get packaged and secreted into the medium within a viral particle.

In order to design such vectors, it is crucial to establish: **(i)** where within the viral LTRs can large DNA insertions be tolerated without affecting viral replication, and **(ii)** what constitutes the minimal Ψ signal that, when moved to a site within the viral LTR, would allow packaging without affecting viral replication.

With respect to the first question, the SuIII$^+$ tyrosine suppressor tRNA gene (220 bp) has been successfully cloned in a retroviral 3′ LTR within U3 promoter sequences (2,3). However, when a packaging signal was moved within this U3 region, little or no packaging was observed (4). Thus, this position was not suitable for insertion of a Ψ signal.

With respect to the second question, previous studies have identified the Ψ signal to be located between nucleotides 215−1039 of MoMuLV RNA (4−9). Nucleotides 215−355 of MoMuLV RNA seem to be crucial for packaging, since deletion of these sequences decreased the viral RNA packaging rate significantly (5). When the 215−565 nucleotide-long fragment of MoMuLV RNA was moved to a region near the 3′ LTR, viral packaging could still occur (4). The inclusion of nucleotides 215−1039, however, resulted in an increased vector titer due to more efficient packaging (8). Inclusion of nucleotides 215−563 of MoMuLV RNA into non-retroviral vectors revealed that this packaging signal is sufficient and can allow packaging (9). However, this signal does not seem to be efficient since the packaging rates of vectors containing nucleotides 215−1015 of Murine sarcoma virus were significantly higher (9).

It remained to be shown whether a Ψ signal could be moved within an LTR and still allow packaging. As well, the ideal size of the MoMuLV Ψ signal to be used for this purpose remained to be determined.

In this paper, retroviral vectors were generated in which the normal LTR containing the U3-r-U5 sequences was modified to contain Ψ signals of different lengths. These signals were inserted downstream of the U3 promoter sequence between the r and U5 sequences, such that the vector RNA and any cellular RNA transcribed from the 3′ LTR promoter should both contain the Ψ signal. The design and construction of these vectors are described, and their ability to replicate, integrate, and package has been assessed.

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MATERIALS AND METHODS

Materials

*Bam*HI, *Nde*I, *Pvu*II, *Sph*I, and *Sst*I were purchased from BRL, and *Hind*III was purchased from New England Biolabs. Calf intestinal alkaline phosphatase and 5-bromo-4-chloro-3-indolyl-β-D-galactoside were obtained from Boehringer-Mannheim. T4 DNA ligase, T4 polynucleotide kinase, DNA polymerase I Klenow fragment, and *Xho*I were obtained from Pharmacia. Isopropyl-thio-β-D-galactoside was obtained from PL Biochemicals Inc. Bovine calf serum and Geneticin (G418) were purchased from Gibco.

Cells

NIH/3T3 fibroblasts and the Psi-2 mouse ecotropic packaging cell line (5) were cultured in α-MEM medium supplemented with 10% calf serum at 37°C in a humidified atmosphere with 6% CO_2.

Recombinant DNA techniques

Unless stated otherwise, all recombinant DNA techniques were performed as described in (10). DNA fragments suitable for cloning were purified and concentrated using Elutip-d chromatography.

Site specific *in vitro* mutagenesis was carried out essentially as described in (11) with the following modifications: BW313 cells were grown overnight in 2YT medium without thymidine and deoxyadenosine supplementation, and uridine was used at a final concentration of 0.5 μg/ml. The oligonucleotides containing the reverse-complement of the following sequences:

5'- CCCTCTTGCA GTTGCATCTG GCCAGCAACT TATC-3',

5'- ACACCCGGCC GCAACTCCGA CTTGTGGTCT CGCTGTTCCT TGGGAGGGTC TCCTCTGAGT GATTCACTAC CCGTCAGCGG GGGTCTTTCA TTTGGGGGCT CGTCCGGGAT CCTGGGAGAC GTCCC-3',

5'- GCGTCTTGTC TGCTGTCCGA CTTGTGGTCT CGCTGTTCCT TGGGAGGGTC TCCTCTGAGT GATTGACTAC CCGTCAGCGG GGGTCTTTCA TTTGGGGGCT CGTCCGGGAT cCAGCATCGT TCTGTG-3', and

5'- TCCTTCTCTA GGCGCTCCGA CTTGTGGTCT CGCTGTTCCT TGGGAGGGTC TCCTCTGAGT GATTGACTAC CCGTCAGCGG GGGTCTTTCA TTTGGGGGCT CGTCCGGGAT cCAAACCTAA ACCTCA-3'

were used for *in vitro* mutagenesis to obtain ΔPR, PR1, PR2, and PR3 vectors, respectively.

The 3′ end of the U5-PBS sequence to be inserted contained the sequence GGAT and the 5′ end of the DNA where the U5-PBS sequence was to be inserted to create the PR1 vector, provided 2 Cs, thus creating a *Bam*HI restriction site (GGATCC). In the case of PR2 and PR3 vectors, only 1 C was provided by the site of insertion; an additional c (represented above in lower case) was therefore included in the oligonucleotides used for *in vitro* mutagenesis to create the *Bam*HI restriction site.

After *in vitro* mutagenesis, correct clones were identified by restriction enzyme analysis and were then confirmed by DNA sequencing using the dideoxynucleotide chain termination procedure (12) and with M13mp19 single-stranded templates as described in (13).

Vector Constructions

The 2.2 kb *Sst*I-*Hind*III fragment of pUCMoTN, containing part of the 5′ LTR and the Ψ signal, was subcloned into M13mp18. Using site-specific *in vitro* mutagenesis techniques (11), a 142 bp deletion was made to span nucleotides 69−210 inclusive of MoMuLV RNA, thereby removing the U5-PBS-SD sequences in the vector ΔPR. This vector was then mutagenized *in vitro* in three separate instances to reinsert the 95 bp U5-PBS DNA sequence (nucleotide 69−163 inclusive of MoMuLV RNA) at three different locations within the Ψ signal. Insertions after nucleotides 355, 565, and 1039 produced PR1, PR2, and PR3 vectors, respectively.

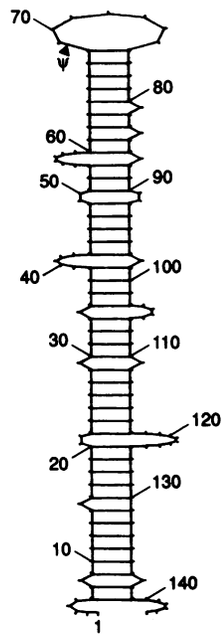

Fig. 1. Secondary structure prediction of r-U5 region (145 nucleotides) of the MoMuLV RNA. This structure was predicted using Zucker's computer program (18). The energy contained within this structure was calculated to be −35.6 kcal. The triangle indicates the position where Ψ signal was inserted.

The vector pUCMoTN was digested with *Xho*I and *Sst*I, and the resulting 3.8 kb *Xho*I-*Sst*I fragment and the 4.32 kb *Sst*I-*Sst*I fragment were then ligated to the 1.45 kb *Xho*I-*Sst*I fragment (ΔPR) or the 1.55 kb *Xho*I-*Sst*I fragment (PR1, PR2, and PR3) in order to obtain pUCMoTN-ΔPR, pUCMoTN-PR1, pUCMoTN-PR2, and pUCMoTN-PR3.

Transfection and Infection

Six hours after 2×10^5 cells were seeded on 60 mm tissue culture dishes, the cultures were transfected with 1 μg of plasmid DNA (14,15). Twenty-four hours later, cells were washed and cultured in α-MEM medium containing 10% calf serum and G418 (200 μg/ml). The selective medium was changed every 4−5 days and the number of colonies were counted after 14 days. Vector particles were obtained by filtering the culture medium through 0.2 μm filter. Virus titer was obtained by infecting NIH/3T3 cells as described (16).

RESULTS AND DISCUSSION

Three retroviral vectors were designed containing varying lengths of the Ψ signal between the U3-r and the U5 sequences of the 5′ LTR. These vectors were then tested for their relative titer, which is dependent upon efficient packaging in the packaging cell line and efficient replication in the target cell line used for titer determination.

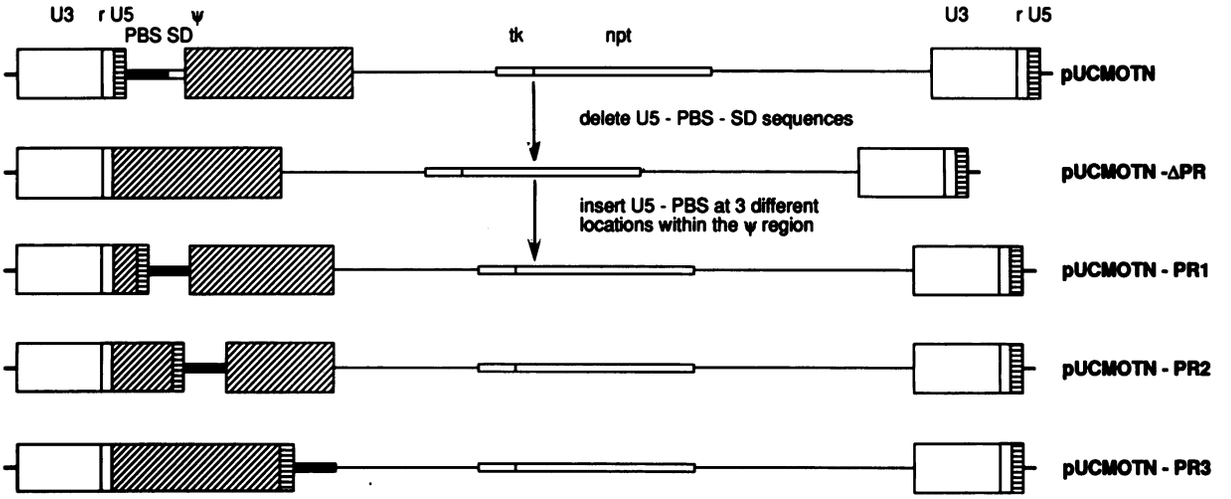

Fig. 2. Strategy used for construction of packaging retroviral vectors. The U5-PBS-SD sequences were deleted from a subclone of pUCMoTN, and the resulting plasmid (ΔPR) was *in vitro* mutagenized to reinsert U5-PBS sequences at three separate locations within the Ψ signal, at the end of nucleotides 355, 565, and 1039, to generate PR1, PR2, and PR3 vectors, respectively. The pUCMoTN vector was then modified to contain the U3-r-Ψ sequences from ΔPR to generate pUCMoTN-ΔPR or the U3-r-Ψ-U5-PBS-Ψ′ sequences from PR1, PR2, or PR3 to generate pUCMoTN-PR1, pUCMoTN-PR2, and pUCMoTN-PR3, respectively.

Location of Insertion

DNA insertions within the LTR could be made either within the U3, r, or U5 sequence or at the U3-r or r-U5 junction. For reasons described below, the r-U5 junction was selected.

The U3 region contains promoter sequences, and is transcribed only from the 3′ LTR prior to termination of transcription. These sequences would thus be present only in the viral RNA and would never be transcribed as part of the cellular RNA. Thus, it was decided not to insert the Ψ signal in this region. For DNA insertions at the U3-r junction, both the 5′ and the 3′ LTRs had to be modified, otherwise an abortive replication would have occurred in the target cell upon jumping of the Ψ-r-U5 cDNA from the 5′ end of the RNA to the U3-r region at the 3′ end of the RNA. For this reason among others, DNA was not inserted at the U3-r junction. The r region is involved in strand switching during the '−' strand cDNA synthesis. Therefore, any modification of the r region within the 5′ LTR would have to coincide with an identical modification in the 3′ LTR to permit efficient base-pairing during strand switching. Downstream of the U5 region, foreign material could be inserted without interfering with viral replication. However, after replication, DNA integration may be dependent upon the integrity of the 5′ U3 and 3′ U5 sequences (17). Hence, addition of any material 5′ to the U3 region or 3′ to the U5 region could result in abortive integration. Thus, the r-U5 junction appeared to be the most appropriate location to insert the Ψ signal. Furthermore, secondary structure prediction using the Zucker's computer program (18) revealed that the r and U5 sequences, containing 68 and 77 nucleotides respectively, can base-pair with each other and that the r-U5 junction has a high probability of forming a loop (Fig. 1).

Size of the Ψ Signal

The Ψ signal is composed of nucleotides 215−1039 of MoMuLV RNA (4−9). Nucleotides 204−210 of MoMuLV RNA correspond to the splice donor site. Thus, the Ψ signal spanning nucleotides 211−1039 of MoMuLV RNA was inserted into the r-U5 region of the 5′ LTR. However, since the additional 829 nucleotides within the LTR could have deleterious effects on viral replication, constructs with smaller Ψ signals spanning nucleotides 211−355 and 211−565, were also made.

Design and Construction of Vectors with Ψ Signal in the 5′ LTR

The following strategy (Fig. 2) was used to move the Ψ signal from its original location to within the 5′ LTR, between the r and U5 junction. The retroviral DNA sequences present in the original pUCMoTN vector are U3-r-U5-PBS-SD-Ψ--//--U3-r-U5. The U5-PBS-SD sequences were deleted by *in vitro* mutagenesis. This deleted plasmid, designated △ PR, was then further mutagenized *in vitro* to allow the insertion of U5-PBS sequences after 355, 565, and 1039 nucleotides of the MoMuLV RNA, thereby leaving varying lengths of the Ψ signal between the r and U5 sequences. This would lead to the generation of 5′ LTRs containing a 211−355, 211−565, or 211−1039 nucleotide-long Ψ signal. The remainder of the original packaging signal (Ψ′) from nucleotide 356−1039 or 566−1039 would be located downstream of the 5′ LTR.

Replication, Integration, and Packaging of Retroviral Vectors

The ability of the packaging retroviral vectors to replicate, integrate, and encapsidate vector RNA was tested by transfection of the Psi-2 packaging cell line and subsequent determination of the titer of the vector particles produced.

The Psi-2 packaging cell line, expressing MoMuLV *gag*, *pol*, and *env* genes, was transfected with pUCMoTN, pUCMoTN-ΔPR, pUCMoTN-PR1, pUCMoTN-PR2, and pUCMoTN-PR3 vectors. As expected, similar number of G418-resistant colonies were obtained from all of these vectors (Table 1). The 5′ and the 3′ LTR sequences in the vectors pUCMoTN-PR1, pUCMoTN-PR2, and pUCMoTN-PR3, as obtained from *E. coli*, are U3-r-Ψ-U5-Ψ′--//--U3-r-U5. Upon transfection of the Psi-2 cell line, these vectors will integrate within the cellular genome and give rise to vector RNA containing r-Ψ-U5-Ψ′--//--U3-r-polyA sequences, which should be packaged and subsequently released in the medium.

The vector particles released from the Psi-2 cell lines transfected with pUCMoTN, pUCMoTN-ΔPR, pUCMoTN-

Table 1. Properties of various packaging retroviral vectors.

Vector	Ψ signal inserted (nucleotides)[1]	Ψ' signal downstream of the 5'LTR (nucleotides)[1]	Transfection of Psi-2 Cells	Infection of NIH/3T3 cells	
			# $G418^R$ colonies	cfu[2]/ml	Relative titer
–			0	0	0
pUCMoTN	0	211–1039	61	1.6×10^6	1
pUCMoTN-ΔPR	0	211–1039	48	5.5×10^2	1/2900
pUCMoTN-PR1	211–355 (145 bp)	356–1039	36	1.0×10^3	1/1600
pUCMoTN-PR2	211–565 (355 bp)	566–1039	78	6.5×10^3	1/246
pUCMoTN-PR3	211–1039 (829 bp)	0	34	2.8×10^5	1/5.7

[1] Numbering of nucleotides is from the MoMuLV RNA; the 1st nucleotide of this RNA is numbered '+1'.
[2] Colony forming units.

PR1, pUCMoTN-PR2, or pUCMoTN-PR3 were purified and their titer was determined by infecting NIH/3T3 cells and then counting the number of resultant G418-resistant colonies. Upon infection, retroviral RNA is reverse transcribed into DNA and then integrates into the host chromosome where it is then forwardly transcribed to mRNA to give G418-resistance.

The titer of the MoTN-PR1 and MoTN-PR2 vectors were 1600 and 246 fold lower, respectively, than that obtained for the original MoTN vector (Table 1). The titer of the MoTN-PR3 vector, containing 211–1039 nucleotide-long Ψ signal was only 5.7 fold lower as compared to that of MoTN.

The fact that the MoTN-PR3 vector was able to infect efficiently NIH/3T3 cells indicates that this vector, containing modified LTRs, can replicate and integrate efficiently, and that the presence of an altered LTR containing 829 extra nucleotides of the Ψ signal had only a small effect on these steps. The high titer obtained for the MoTN-PR3 vector also indicates that the Ψ signal present within the 5' LTR, containing nucleotides 211–1039 of the MoMuLV RNA, is necessary and sufficient to allow RNA packaging.

The two other Ψ signals tested, in vectors MoTN-PR1 and MoTN-PR2, were only 145 and 355 nucleotides-long. Since the presence of an extra 829 nucleotides within the 5' LTR in MoTN-PR3 did not affect viral replication, it seems unlikely that the low titer obtained for MoTN-PR1 and MoTN-PR2 (Table 1) is due to inefficient replication. Rather, one would expect that this low titer likely results from an incomplete Ψ signal. Since the original Ψ signal in the MoTN-PR1 and MoTN-PR2 vectors contains a 95 bp (U5-PBS sequences) insertion, one would also expect that DNA insertions at nucleotides 355 or 565 of the Ψ signal diminishes packaging.

CONCLUSION AND SIGNIFICANCE

The design, construction, and titer of MoTN-PR3 vector containing the Ψ signal (829 nucleotides) between r and U5 sequences of the 5' LTR, are reported. The high titer obtained for these vector particles is indicative of efficient replication, integration, and packaging.

Although only the 5' LTR was modified in the pUCMoTN-PR3 vector, correct 3' LTR containing the U3-r-Ψ-U5 sequences should be generated during viral replication (19) and thus should allow packaging of downstream cellular RNAs, if any. The utility of these packaging retroviral vectors in packaging cellular RNAs, and the feasibility of cloning genes adjacent to the retroviral insertion-sites will now be evaluated.

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