KOR. JOUR. MICROBIOL. June, 1988 p. 101-105 Vol. 26, No. 2

3′ end of putative sequences of the packaging signal in Moloney-murine leukemia virus

Jong-Sang Park
Department of Chemistry, College of Natural Sciences, Seoul National University

Moloney Murine Leukemia Virus에서 포장신호의 가능한 3′ 끝의 염기서열

박 종 상
서울대학교 자연과학대학 화학과

ABSTRACT: 6 M-MuLV mutants containing deletions around the putative packaging signal were constructed by using recombinant DNA technique and transfected into NIH/3T3 cell. 2 of 6 mutants can not be packaged into virions even in the presence of the wild type helper virus. The boundary between the packagible and the non-packagible genome is located around Pvu I site, 421 nucleotide downstream from the 5′ end of M-MuLV genome. 10 base pair inverted repeat sequence (GAGUCCAAAA) which can make stem structure around Pvu I site could be the putative packaging signal.
KEY WORDS□deletion mutants around packaging signal, transfection, resistant cells, 10 base pair stem structure.

A murine retrovirus, Moloney murine leukemia virus(M-MuLV) generates two RNAs (genomic size 35S and spliced 21S)(Rothenberg *et al.*, 1978, Van Zaane *et al.*, 1977). Only the genomic size 35S RNA can be packaged into reverse-transcriptase containing particles, which can differentiate these two RNAs even though they have the same 5′ and 3′ termini. Linial *et al.*(1978) reported a deletion mutant of Rous Sarcoma Virus that was deficient in the packaging process and produced only ghost virions without RNAs. The mutant has a 300 nucleotide deletion between about 300 and 600 nucleotide downstream from the 5′ end of genomic RNA sequences. Man *et al.*(1983) described a deletion mutant of M-MuLV that was constructed by the deletion of 350 nucleotides from an infectious proviral DNA clone between the putative 5′ splice donor site(BaI I site, 214 nucleotides downstream from 5′ end of M-MuLV RNA) and the AUG of gag proteins (Pst I site, 565 nucleotides downstream from the 5′ end of M-MuLV RNA). Cell lines transfected with this mutant provirus produce reverse transcriptase-containing particles (i.e., ghost virions) that lack detectable M-MuLV RNAs. However, the cells efficiently complement the proliferation of replication-defective and packagable RNAs of M-MuLV. The deletion in the M-MuLV genome appears to define a site required in cis for packaging of M-MuLV RNAs into virions. Watanabe and Temin (1982) reported the evidence for a similar site in an avian retrovirus, spleen necrosis virus (SNV), where crucial sequences for packaging are located within the 5′ half of the nontranslated region between the right end of the 5′ LTR and gag gene.

In this report, we constructed 6 deletion mutants around the putative packaging signal and in-

vestigated whether RNAs of those mutants could be packaged and transfer their drug resistance genes thru the retrovirus-replication by the complemetation of wild-type helper M-MuLV or not. The result shows RNA sequence around Pvu I site (421 nucleotide downstream from the 5′ end of the M-MuLV genomic RNA) is critical for the RNA packaging into virions. From computer search of viral sequences we propose 10 ucleotide inverted repeat sequence GAGUCCAAAA around Pvu I site, which could make the stem structure, could be the packaging signal.

MATERIALS AND METHODS

Deletion recombinant DNA construction. From the M-MuLV provirus Bgl II-Xho I DNA fragment (Bgl II site: 2.5 k bp upstream from LTR and Xho I site: 1.5 k bp downstream from LTR) was cut out, treated with exonuclease Bal 31 and S1 nuclease, linked to Eco RI linker and inserted before the promoterless thymidine kinase (TK) gene from herpes simplex virus in Eco RI site of pBR 322 which was a gift of Dr. E. Gilboa (Gilboa *et al.*, 1982). Recombinant DNAs were transformed into C600 and grown in Ampiciline. Suriving colony were taken and their plasmid were identified. The orientation and size of M-MuLV sequences containing LTR with regard to tk gene were determined by restriction enzyme digestion (Sma I, Bal I, Rvu I, Pst I and Cla I) and agarose gel electrophoresis with proper size maker.

DNA transfection. A TK^- derivative of the NIH 3T3 cell line used in this study was kindly provided by E. Scolnick (Wei *et al.*, 1981). Cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Calcium phosphate-mediated DNA transfection (Graham *et al.*, 1973) was performed as described by Wigler *et al.* (1979). A total of 0.1 to 0.3 ug of plasmid DNA was used per 5×10^5 cells in the absence of carrier. Two days later transformed cells were maintained in growth medium(HAT) containing hypoxanthine at 15 ug/m*l*, aminopterin at 0.2 ug/m*l*, and thymidine at 5.0 ug/m*l*.

Viral Infection. Polybrene was added to the virus stock to a final concentration of 8 ug/m*l*. The medium was removed from cell culture (1×10^5 cells/6 cm plate) and 0.5 m*l* of the prepared virus stock was added per plate. The cells were incubated at 37°C for 1 hr with the plates being shaken every 10-15 minute. The cell growth medium was added back to each plate. Cells infected with recombinant virus were selected with HAT or 1.0 mg/m*l* G418.

RESULTS AND DISCUSSION

In order to map the packaging signal, several deletion recombinant DNA between Bal I site and Pst I site were constructed. Figure 1 shows the 6 identified recombinant DNA collections which contain the same LTR-TK structure cloned in the EcoR I site of pBR 322.

The difference among these recombinants exists in the size of M-MuLV sequences between the 3′ end of LTR and TK gene. The putative packaging signal of M-MuLV has been reported to be located between the 3′ end of LTR and the initiation codon of gag protein (Mann *et al.* 1983, Watanabe *et al.* 1982).

In order to introduce foreign genes through the

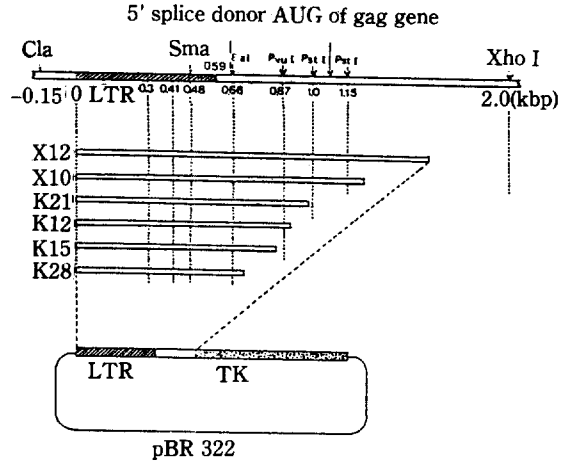

Fig. 1. *6 recombinant DNA have the same structure (LTR-TK) cloned in Eco RI site of pBR 322 and different M-MuLV sequences between Bal I site and Xho I site.* 1 unit length is 1 killo base pair (Kbp).

retrovirus replication cycle, two sequences are required. One is the 3′ viral LTR downstream of the env gene which is involved in the synthesis of the intermediate linear DNA from the genomic size 35S RNAs (Varmus H.E. 1982). The other sequence is the packaging signal which is required for the genomic sized 35S RNA to be packaged into virions (Mann *et al.* 1983, Watanabe *et al.* 1982). The second LTR was added to these 6 recombinant DNAs. Fig. 2 shows how to insert the second LTR into them in detail.

The structure B in Fig. 2 contains the whole LTR in the EcoR I site of pBR 322. The whole LTR fragment between the Cla I site in M-MuLV genome and the other Cla I site in pBR 322 was cut out from the structure B in Fig. 2 and inserted into the Cla I site of the structure A which contains the Pst I-EcoR I fragment from the M-MuLV genomic sequence and the bacterial Neo^r gene. The orientation of 3′ LTR in the structure C was determined with proper restriction enzymes. From the structure C in Figure 2, the DNA fragment between two Hind III sites (one is upstream 3′ splicing acceptor in M-MuLV genome and the other one in pBR 322) was cut out and inserted into the Hind III site of the structure D. The structure D in Figure 2 illustrates 6 different LTR-TK recombinant DNAs (X12, X10, K21, K12, K15, and K28) as mentioned above. The resulting structure E contains sequences in the following order; 5′ LTR-5′ splicing donor (Bal I site)-TK-3′ splicing acceptor-Neo^r-3′LTR, where the lengths of M-MuLV sequences between the 5′ LTR and TK are different among 6 recombinants.

NIN/3T3 TK$^-$ cells were transfected with these 6 recombinant DNAs one by one by the standard calcium phosphate method and grown in HAT medium with G418. After drug-resistant colonies appear in HAT medium, wild type M-MuLV helper retroviruses were added to medium for the viral infection. In one week supernatant was collected and added to fresh and non-infected NIH/3T3 cells. This supernatant would contain wild type helper M-MuLV RNA virion and recombinant RNA virion with TK and Neo^r gene if they are packagible. Infected cells were grown in HAT

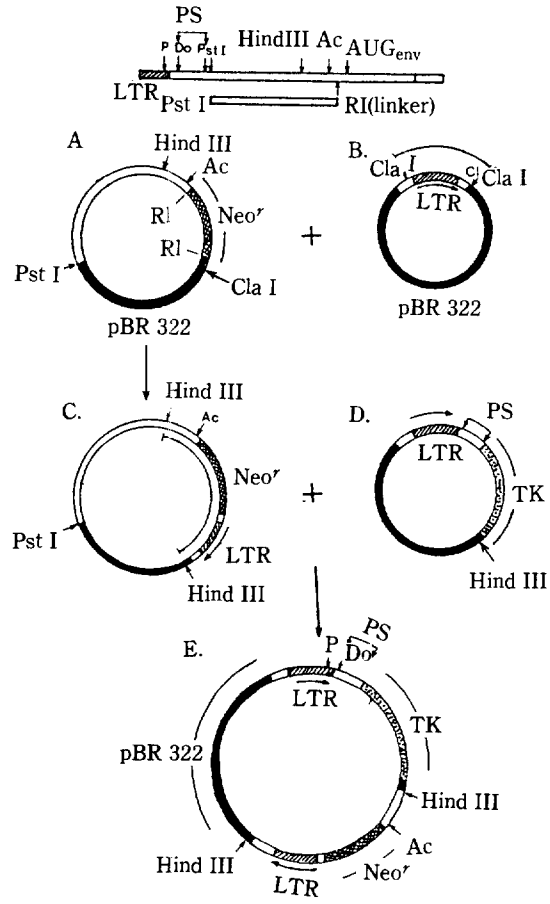

Fig. 2. *Construction of 6 recombinant DNAs whose Structure are 5′ LTR-5′ splicing Donor-3′ splicing Acceptor-Neo^r-3′ LTR.*
PS: packaging signal, Do: 5′ splicing donor, Ac: 3′ splicing acceptor.

medium and drug-resistant colonies were selected. This resistancy against HAT results from the integration of TK gene into host chromosome via infection of packaged virion. To make sure that the agents transferring TK and Neo^r gene into HAT-sensitive NIH/3T3 cells were recombinant viruses, the medium from cloned drug-resistant colonies was passed through this procedure twice. Figure 3 shows the result of this experiment + means transmissible via retrovirus life cycle. 4 of 6 recombinants can transmit TK and Neo^r gene through virus-replication. But 2 of them can not. The boundary between the transmissible and the nontransmissible locates near Pvu I site, 451 nu-

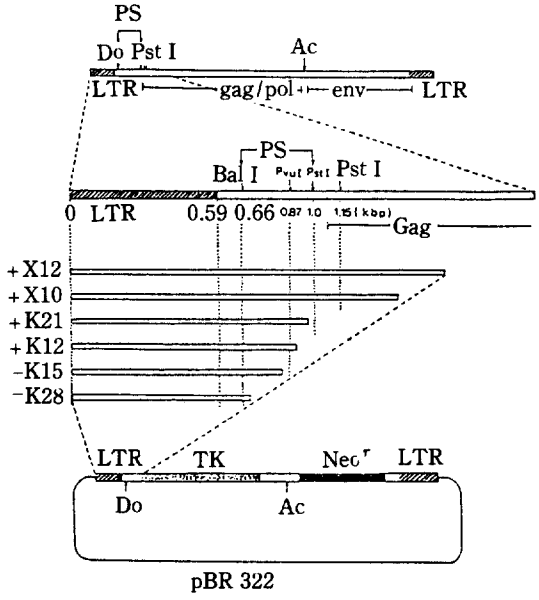

Fig. 3. *Transfection of 6 recombinant DNAs into NIH 3T3 cells and packaging experiment with wild type helper virus.*
+ means transmissible via retrovirus life cycle.
- nontransmissible.

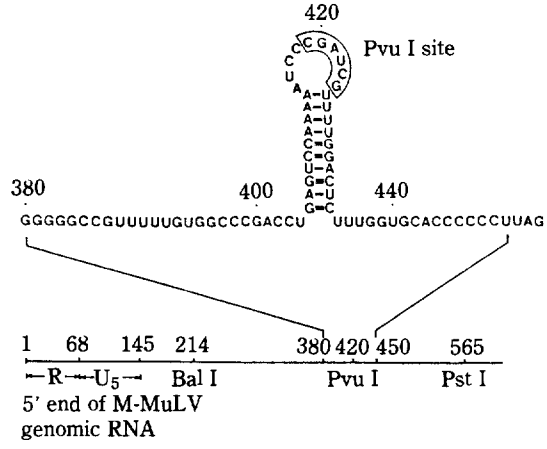

Fig. 4. *10 base pair inverted repeat (GAGUCCAAA) around the Pvul site makes the stem structure which could be the possible packaging signal sequences.*

cleotide downstream from the 5′ end of M-MuLV RNA sequence. Rather, the result says that the 3′ end of the packaging signal is near Pvu I site.

The whole M-MuLV 8332 nucleotides was sequenced and published (Shinnick *et al.*, 1981). Generally speaking, DNA binding proteins recognize some specific sequences, which have unique characteristics such as short repeat or inverted repeats. Lac operator binding site, transcriptional termination site and SV40 enhancer are good examples. On the basis of this fact, it is possible that the putative packaging signal sequences could have short repeats or inverted repeats.

By using computer programing, we looked at M-MuLV sequences near Pvu I site very carefully and found 10 base pair(bp) inverted repeat (GAGUCCAAAA), which could make 10 bp stem structure (Fig. 4). Therefore we propose this stem structure around Pvu I site in the M-MuLV genome would be the putative packaging signal. In order to prove this, more experiment should be done. For example, insert of 10 bp stem structure into the non-packagible 21S RNA and ask if RNA with this artifical packaging signal can be packaged. This kind of experiment will give the ultimate answer whether 10 bp stem structure in really involved in RNA packaging or not.

적 요

잠정적인 포장신호(packaging signal) 근처에 6개의 deletion 돌연변이를 유전자 재조합기술로 조립하여 NIH/3T3 세포에 넣었다. 6개중 4개가 야행성 레트로바이러스(M-MuLV)의 도움에 의해서 바이러스 입자에 포장되었다. 포장된 것과 포장 안된 것의 경계는 PvuI자리 근처인데 이것은 레트로바이러스 RNA의 5′말단에서부터 451 염기 하류에 존재한다. 이 PvuI자리 좌우에 10개의 염기(GAGUCCAAAA)가 반복되는데 기둥구조를 이루며 이것이 잠정적인 포장신호일지 모른다.

ACKNOWLEDGEMENT

The author thanks Dr. C.K. Park in KAIST for giving the computer programing and Mr. W.S. Hong in S.N.U. for computer searching viral sequences.

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(Received Apr. 9, 1988)