Extended Minus-Strand DNA as Template for R-U5-Mediated Second-Strand Transfer in Recombinational Rescue of Primer Binding Site-Modified Retroviral Vectors

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We have previously demonstrated recombinational rescue of primer binding site (PBS)-impaired Akv murine leukemia virus-based vectors involving initial priming on endogenous viral sequences and template switching during cDNA synthesis to obtain PBS complementarity in second-strand transfer of reverse transcription (Mikkelsen et al., J. Virol. 70:1439–1447, 1996). By use of the same forced recombination system, we have now found recombinant proviruses of different structures, suggesting that PBS knockout vectors may be rescued through initial priming on endogenous virus RNA, read-through of the mutated PBS during minus-strand synthesis, and subsequent second-strand transfer mediated by the R-U5 complementarity of the plus strand and the extended minus-strand DNA acceptor template. Mechanisms for R-U5-mediated second-strand transfer and its possible role in retrovirus replication and evolution are discussed.

Retroviruses harbor a diploid single-stranded RNA genome which constitutes the source for generation of double-stranded DNA by reverse transcription. DNA synthesis is initiated from the 3′ end of a host-derived tRNA matching the 18-nucleotide primer binding site (PBS) located downstream from the U5 region. The resulting minus-strand strong-stop DNA is in turn transferred to the 3′ end of either one of the copackaged RNAs. This first-strand transfer (or jump) is facilitated by the complementarity of the terminal R regions and furthermore by the reverse transcriptase RNase H-mediated degradation of 5′ R and U5 RNA in the RNA-DNA hybrid generated (3, 11, 29, 40, 57). Minus-strand DNA molecules shorter than strong-stop DNA (designated weak-stop DNA) are occasionally generated by premature termination and strand transfer (2, 20, 21, 26, 44, 58, 67), indicating that R-region homologies shorter than the entire length of R are sufficient for transfer to occur. After transfer of weak- or strong-stop DNA, minus-strand DNA synthesis advances toward the 5′ end of the RNA template. Plus-strand synthesis, which is primed from a purine-rich RNA fragment upstream from the U3 region (18, 28, 45, 46), is believed to proceed until the first modified tRNA nucleotide is reached, leading to regeneration of the PBS matching the primer tRNA (48, 56). The tRNA primer is subsequently removed by RNase H-mediated degradation (5, 37, 50, 52). The complementarity of the plus-strand 3′ PBS obtained by copying the tRNA primer and the minus-strand 3′ PBS mediates the second-strand transfer (1, 10), and, finally, plus- and minus-strand synthesis are completed.

Except for the apparent requirement for PBS complementarity in the second jump of reverse transcription (10, 56, 58), little is known about the transfer reaction and the acceptor template involved. Clearly, correct strand transfer cannot occur before complementary sequences have been copied during plus- and minus-strand synthesis (58). In theory, such sequences may include not only the PBS but also non-PBS sequences (22, 23, 36, 38, 41, 42) and, in particular, the R-U5 region upstream from the PBS. Copying of the R-U5 region during minus-strand synthesis may depend on whether minus-strand synthesis has been initiated from both PBSs in the genomic RNA dimer (leading to degradation of R-U5 by RNase H) and whether read-through of the PBS is influenced by potential tRNA occupancy of the PBS.

Copackaging of heterologous viral RNAs and a subsequent high rate of recombination during reverse transcription have been demonstrated in numerous studies (14–17, 39, 54, 55, 59, 66, 68). Reverse transcription-mediated recombination may involve endogenous virus-like elements, as seen in studies of various replication-defective retroviral mutants (6, 7, 9, 31, 32, 34, 51). Endogenous viral RNAs found to be encapsidated in virus particles (13, 32, 43, 49) may thus serve to provide the functional sequences required for repair of deleterious viral mutations. Such recombinational rescue mechanisms may include template shifting during minus- or plus-strand DNA synthesis and may be influenced by the character of the two strand transfers of reverse transcription (39, 59, 66).

**Forced recombination of PBS-modified vectors.** In agreement with the essential role of the PBS in initiation and completion of reverse transcription (35, 47, 62), we previously observed a strong restriction in transduction of Akv murine leukemia virus (MLV) vectors with PBSs having only partial (PBS-XXX) or no [PBS-UMU and PBS-Met(j)] homology with the 3′ end of any known murine tRNA molecule (32). In experiments based on virus production in NIH 3T3 cell-derived packaging cell lines (9′2 and OE) some of the transduced proviruses were found to harbor sequences originating from both vector and endogenous virus, suggesting that the impairment of the PBS was circumvented in some cases by reverse transcription-mediated minus-strand recombination with an endogenous virus containing a functional PBS (32). Repair of PBS mutants involved initiation of cDNA synthesis from the functional glutamine PBS of copackaged MLV-like endoge-
nous virus (MLEV) RNA, an interstrand first-strand transfer followed by minus-strand synthesis through the neo gene, and template shifting within the 5' untranslated region (5' UTR) to allow for the interaction of complementary glutamine PBS sequences during second-strand transfer. Hence, transduction of PBS knockout vectors in a single-cycle transfer protocol, as delineated in Fig. 1B, is performed under triple selection for (i) reverse transcription initiation, (ii) second-strand transfer, and (iii) expression of the neo gene (Fig. 1A). In the present report, we focus on recombination-mediated transduction of proviral sequences which have retained the original, mutationally impaired PBS (referred to as type 2 proviruses in reference 32).

Sequence analysis of transduced PBS and LTR sequences. Transduced G418-resistant NIH 3T3 target cells from five different series of virus transfer experiments (more than 80 clones altogether) were individually screened by PCR to detect proviruses resulting from recombination of the vector with MLEV. Initially, the origin of the PBS was determined by sequence analysis of a 1.37-kb PCR fragment spanning U3, R, U5, PBS, the 5' UTR, and part of the neo gene; the proviruses harboring the original, mutated PBS were subjected to further analysis. According to the model for reverse transcription (10), the minus-strand strong-stop DNA containing the R and U5 regions copied during minus-strand strong-stop synthesis is transferred to the 3' end of the genome in the first jump of reverse transcription. Therefore, we then tested by PCR amplification and subsequent sequence analysis whether the 3' long terminal repeat (LTR) of mutant PBS-harboring subclones contained non-Akv sequences. The PCR was performed with a neo primer and a primer specifically recognizing the MLEV molecular marker XIV (Fig. 2A). Indeed, in four cases (clones P3, T1.2, KL#19, and 33E) we found the specific MLEV pattern of scattered molecular differences from Akv (Fig. 2A, lower panel). In contrast to the R and U5 regions, the U3 region was found to be identical to Akv U3. These findings indicated that transduction of at least some of the type 2 proviruses involved initiation of reverse transcription on the functional glutamine PBS of MLEV.

In conclusion, the structures of the transduced recombinants harboring the marker gene suggest a transfer mechanism involving initiation of minus-strand synthesis at PBS-Gln of co-packaged MLEV RNA. Rescue by utilization of the functional glutamine PBS as a substitute for the impaired PBS, therefore, provides a tool to specifically study plus-strand transfer based on non-PBS complementary sequences. It is noteworthy that our data also show that the rescue mechanism involved leads to

**Fig. 1. Principles of forced recombination.** (A) Nonfunctional PBS sequences introduced into Akv MLV-based vectors harboring the neomycin resistance gene (neo). Selection for (i) initiation of minus-strand synthesis, (ii) successful second-strand transfer, and (iii) expression of the marker gene represents an effective selection pressure that allows for detection of recombinational vector rescue. (B) Experimental approach. PBS-modified vectors in a single-cycle vector replication protocol were investigated utilizing NIH 3T3-derived virus producer cells and NIH 3T3 target cells. Three different PBS-modified constructs were utilized, harboring PBS sequences that were designed to unlikely match the 3' end of any known murine tRNA molecule (32). The PBS sequences introduced included PBS-XXX (retaining the nucleotides complementary to the tRNA CCA tail), PBS-UMU (in which all of the wild-type PBS positions were altered), and PBS-Met(i)int (matching an internal fragment of tRNA<sub>Met</sub> suggested to serve as a primer in Drosophila copia retrotransposon replication [19]). G418-resistant colonies were cloned and subjected to sequence analysis in order to elucidate individual transduction pathways. Genomic DNAs from G418-resistant clones were prepared as previously described (27).
the generation of proviruses, which do not contain identical 5' and 3' LTR sequences.

**Model for alternative recombinational vector rescue.** In our previous work, we observed that type 1 PBS-Gln-containing proviruses are generated through 5' UTR minus-strand recombination-based patch repair of PBS-impaired vectors (Fig. 3A) (32). Based on the observations presented here, we propose that PBS-modified vectors are alternatively rescued through an initial priming on the copackaged MLEV followed by interstrand minus-strand transfer and minus-strand synthesis through the neo gene and the impaired PBS. Concomitantly, plus-strand DNA is generated and transferred to complementary minus-strand R-U5 sequences. As such, generation of a minus-strand DNA 3' R-U5 tail allows for alternative plus-strand transfer potentially leading to Akv-MLEV heteroduplex DNA formation in the 5' LTR R and U5 regions. However, none of the four clones contained mixed 5' LTR marker positions, most likely reflecting that mismatches were corrected prior to cell division by cellular DNA mismatch repair (42) or that one of the daughter cells of the infected cell had died after the first cell division (12). Alternatively, base pairing of growing minus- and plus-strand 3' ends may involve only a limited region of complementarity (see discussion below) that may not lead to heteroduplex formation at any marker position. Our data suggest that proviruses P3 and T1.2 result from second-strand transfer between markers XIII and XIV, whereas all MLEV markers have been copied prior to strand transfer in 33E. For KL#19, which harbors only Akv markers in the 5' LTR, we cannot delineate whether strand transfer occurred prior to copying of MLEV marker IX or whether the proviral structure is the result of daughter cell death or correction after the Akv strand. We designate the
alternative pathway for recombinational rescue R-U5-mediated second-strand transfer recombination (Fig. 3B).

R-U5-mediated second-strand transfer. According to the currently recognized model for reverse transcription of retroviral RNA (10), the PBS copied during generation of full-length plus-strand strong-stop DNA is unmasked by degradation of the tRNA template by reverse transcriptase RNase H activity (5, 10, 37, 52), thereby allowing for subsequent PBS-mediated second-strand transfer. In R-U5-mediated second-strand transfer transduction, in contrast, strand transfer does not involve complementary PBS sequences. Therefore, we were faced with the challenge of modeling unconventional second-strand transfer of a nascent or partly degraded plus strand being part of a DNA-DNA duplex to a nascent or possibly complete R-U5-extended minus-strand DNA.

Considering the structural features of the circular RNA-DNA intermediate in reverse transcription and the time course of minus- and plus-strand synthesis, we propose three distinct models for non-PBS-mediated plus-strand transfer (Fig. 4). The suggested models are based on the assumption that minus-strand synthesis is completed before plus-strand synthesis (model II), or that strand transfer is mediated by the interaction of nascent minus and plus strands (model III). In model I, transfer of an incomplete plus-strand DNA may be the result of a reverse transcriptase-mediated template switch in which the nascent plus strand is transferred to the completed minus strand, the template for continued plus-strand synthesis. This mechanism would require exposure of R-U5 sequences by limited unwinding of the DNA-DNA duplex mediated potentially by the reverse transcriptase (8) or by the nucleocapsid protein which recently has been shown to facilitate DNA duplex melting (60). Model II, in contrast, implies that the 3' end of the nascent minus strand invades the DNA duplex containing complete plus-strand strong-stop DNA. Hence, this transfer mechanism involves partial degradation of the plus strand, allowing for continued plus-strand synthesis through the modified PBS sequence. Alternatively, the template for the growing plus strand may be displaced by the nascent minus strand, thereby mediating premature plus-strand transfer (model III). Although minus-strand-mediated displacement may also require limited unwinding of the DNA duplex, models II and III are in accordance with the fact that the invading minus strand is unmasked by RNase H degradation of the RNA template and are moreover in accordance with in vitro studies demonstrating an extensive DNA duplex displacement capacity of the Moloney MLV reverse transcriptase apparently coupled to minus-strand DNA synthesis (64). Furthermore, in agreement with models II and III, it appears from previous studies that plus-strand synthesis is initiated prior to completion of the minus strand (4, 24, 25). However, in the present study based on molecular markers within relevant regions, we cannot distinguish among the models discussed.

Our observations support previous studies of spleen necrosis virus (38, 41, 42) and Rous sarcoma virus (36) indicating that
tRNA removal in recombinational transduction mediated by unconventional second-strand transfer. Models I and III (Fig. 4) predict that if the primer tRNA is removed (see below), it is removed late in reverse transcription after synthesis of the complete plus strand, since RNase H specifically degrades only RNA in DNA-RNA hybrids (5) and, therefore, will not remove a tRNA that has not been copied during plus-strand synthesis. To test whether tRNA sequences could be found as part of the integrated recombinant proviruses downstream from the 3′ LTR, we performed sequence analysis of the unknown DNA flanking the downstream proviral LTR. Flanking DNA was amplified by a two-step semirandomly primed PCR approach (53). Briefly, in the initial PCR step, a specific biotinylated primer matching Akv U3 and a panel of degenerate primers were utilized in a series of PCRs performed with genomic DNA which had previously been digested with PvuI (at a unique site downstream from the Akv 5′ LTR) to avoid amplification of internal proviral sequences. The PCR products were purified and utilized as templates in a second PCR in which products of the initial PCR were reamplified by using a nested U3-specific primer together with a linker-specific primer. The resulting PCR products were purified and sequenced. As shown in Fig. 2A (lower panel), we were not able to detect sequences originating from the primer tRNA in the flanking regions in any of the clones analyzed, suggesting that the tRNA had been removed correctly before viral integration. Therefore, we propose, in cases of premature plus-strand transfer (models I and III), that the tRNA primer is removed subsequent to second-strand transfer after completion of viral DNA synthesis. However, it is noteworthy that such late tRNA removal would result in the generation of a single-stranded 18-nucleotide 3′ extension that may be degraded by cellular nucleases prior to integration or by the integrase during the process of integration. Support for the latter explanation comes from in vitro studies by Vink et al. (61), who found that human immunodeficiency virus type 1 substrates with single-stranded 6-deoxynucleotidyl extensions 3′ of the CA sequence could be cleaved and integrated by human immunodeficiency virus type 1 integrase. It should also be noted that if our data reflect strand invasion by the growing minus strand (model II [Fig. 4]), the tRNA would be removed conventionally after completion of plus-strand strong-stop DNA.

R-U5 second-strand transfer in MLV replication. The question of whether transfer of an incomplete or degraded plus strand is a frequent event in MLV reverse transcription or is seen here only as a result of a marked selection pressure remains. Since specific PBS-tRNA interactions are of major importance in MLV primer selection (27), we do not expect any tRNA to bind the modified PBS in our recombination system based on PBS nonfunctionality. Consequently, cDNA synthesis is not initiated on the vector RNA and, moreover, potential tRNA binding will not interfere with PBS read-through during cDNA synthesis. The result is a minus-strand 3′ R-U5 extension generated due to the lack of RNase H-mediated RNA degradation subsequent to minus-strand strong-stop synthesis. Evidence has not been provided that both PBS sequences in a wild-type virus are bound by their matching tRNAs. Indeed, studies by Whitcomb et al. (63) have demonstrated that approximately 70% of the avian leukemia virus PBS sequences are occupied by matching tRNA primers, thus suggesting that minus-strand DNA synthesis is initiated from only part of the PBS sequences in a virus population. Therefore, we cannot exclude the possibility that interstrand minus-strand transfer in reverse transcription sometimes is followed by an intrastrand non-PBS-mediated plus-strand transfer. It was recently demonstrated that genetically distinct retroviruses hav-

![Diagram](http://jvi.asm.org/download-pdf/vol72-no25/note-2523_0001.jpg)
ing similar PBS sequences may recombine in vivo (66). Interestingly, R-U5-mediated second-strand transfer, as described in the present report, may allow for recombination of distinct retroviral species that differ within the PBS region but that have some homology within the R-U5 region. Hence, generation and transfer of incomplete plus-strand DNA, which were here selectively seen in reverse transcriptase-mediated recombination of PBS-impaired retroviruses, may play a role in retrovirus replication and evolution.

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REFERENCES


