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Complementation of a Primer Binding Site-Impaired Murine Leukemia Virus-Derived Retroviral Vector by a Genetically Engineered tRNA-Like Primer

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Reverse transcription of retroviral genomes is primed by a tRNA annealed to an 18-nucleotide primer binding site. Here, we present a primer complementation system to study molecular interaction of the replication machinery with the primer and primer binding site in vivo. Introduction of eight base substitutions into the primer binding site of a murine leukemia virus-based vector allowed efficient RNA encapsidation but resulted in severely reduced vector replication capacity. Replication was restored upon complementation with a synthetic gene designed to encode a complementary tRNA-like primer, but not with a noncomplementary tRNA-like molecule. The engineered primer was shown to be involved in both the initiation of first-strand synthesis and second-strand transfer. These results provide an in vivo demonstration that the retroviral replication machinery may recognize sequence complementarity rather than actual primer binding site and 3′ primer sequences. Use of mutated primer binding site vectors replicating via engineered primers may add additional control features to retroviral gene transfer technology.

Copying of retroviral RNA into proviral DNA takes place in a nucleoprotein complex that contains catalytic and structural viral proteins. The only known host-encoded component required for the process is a specific tRNA that is copackaged in the virion and serves as a primer for the viral reverse transcriptase (RT). The 3′ end of the priming tRNA molecule is annealed to the primer-binding site (PBS), a complementary 18-bp sequence near the 5′ end of the viral RNA genome. Different groups of retroviruses use different tRNA species as specific primers (19, 28, 33, 34, 37), and the tRNA primer molecule is likely to interact with both viral proteins and genomic RNA during the processes of packaging, primer annealing, and reverse transcription. Evidence for specific molecular interactions between RT and the cognate primer molecule in vitro is available for human immunodeficiency virus (HIV) (2, 3, 32) and avian leukosis virus (14, 25, 26, 29). Furthermore, the integration of the primer tRNA into a complex RNA secondary structure involving additional base pairing beyond the PBS may serve to select a single tRNA species as replication primer (1, 15, 38). Specific recognition of tRNA sequences may also be involved in subsequent steps of replication in which 18 nucleotides at the 3′ end of the tRNA primer are reverse transcribed during plus-strand synthesis into a DNA copy that, by annealing to the minus-strand DNA copy of the PBS, serves to mediate the second template shift of reverse transcription. Recent in vivo evidence for tRNA primer specificity comes from studies of HIV and avian leukosis virus demonstrating that PBS-mutated viruses designed to use tRNA species other than the cognate primer readily revert to wild type (wt) after a few passages in cell culture (10, 17, 39, 40).

For further understanding of critical macromolecular interactions required for the overall process of reverse transcription, genetic approaches may form important links between biochemical and in vivo replication studies. While genetic analysis of viral RNA and proteins has already contributed valuable information, we here present the possibility of also modifying the host-derived tRNA that plays key roles in the process. We have previously shown that PBS mutations designed not to match any naturally occurring tRNA molecule result in a strong impairment of vector transduction (20). To test the feasibility of making replication dependent on a genetically altered primer, we have used single cycle replication of murine leukemia virus (MLV)-derived vectors. Relative to other groups of retroviruses, MLV may show less stringent primer specificity. MLV RT exhibits no apparent preference for its natural $tRNA^{Pro}$ partner in various biochemical assays (14, 25). Additional evidence of less stringent tRNA primer specificity in MLV comes from in vivo use of $tRNA^{Gln}_{1,2}$ (9, 23) or $tRNA^{Lys}_{3}$ (18).

We here present results documenting a flexibility of primer and PBS sequences for MLV, which has allowed the engineering of an extensively modified complementary PBS primer set with in vivo functions in initiation of first-strand cDNA synthesis and plus-strand transfer leading to a complete provirus.

MATERIALS AND METHODS

Mutagenesis and cloning. The plasmid vector pPBS-x2 was constructed from ptvAkv-*neo* by a two step PCR-mediated site-directed mutagenesis procedure as previously described (18). The PCR primer utilized to insert the PBS-x2 mutation was 5′-CCTGGGCGGGGGTCTCCAAATCCCCTGCAACTGACCAAA TGAAAGAC-3′. Cloning into pUC19 was facilitated by the presence of unique *Eco*RI and *Xho*I sites in linker sequences inserted in the 5′ U3 region and the 3′ U5 region. The structure of pPBS-x2 was verified by sequencing of the PBS and by restriction analysis. The function of the *neo* gene was confirmed by the ability of the vector to confer kanamycin resistance in bacteria. The plasmid $pRNA^{x2}$, encoding the putative tRNA molecule, was created from synthetic oligodeoxynucleotides. Briefly, a 23-base oligodeoxynucleotide (5′-GGAAAGCTTATA AAAACTTTCAG-3′) was annealed to a 127-base oligodeoxynucleotide (5′-CC GGAATTCGAAAACGAAGAAACAAAGTTTACATCTCAGCTGCTGGTC TAGGGGTATGATTCTCGCTTAGGGTGCGAGAGGTCAGGGGTTCAA ATCCCCTGCAGCTGAAAGTTTTTAGCTTTCC-3′) containing the RNA^{x2} cassette and elongated with *Taq* polymerase. Unique *Eco*RI and *Hin*dIII sites

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facilitated cloning into pUC19. The structure of pRNA^{x2} was verified by sequencing. Likewise, for the cloning of pRNA^{x1}, a 23-base oligodeoxynucleotide (5′-GGAAAGCTTATAAAAACTTGATG-3′) was annealed to a 127-base oligodeoxynucleotide (5′-CCGGAATTCGAAAACGAAGAAACAAAGTTTAC ATCTATGAATCTGGTCTAGGGGTATGATTCTCGCTTAGGGTGCGAG AGGTCTAGGGTTCAAATCCCTAGATTCATCAAGTTTTTATAAGCTT TCC-3′), elongated, and cloned into pUC19. All oligodeoxynucleotides were purchased from DNA Technology ApS, Aarhus, Denmark.

Cell culture. BOSC 23 cells (27) were obtained from the American Type Culture Collection. To ensure efficient expression of the *env* gene, BOSC 23 packaging cells were passaged twice in HAT-DMEM selective medium (27). During the subsequent steps of transfection and virus harvesting, BOSC 23 cells were grown in Dulbecco modified Eagle medium supplemented with GlutaMAX (Life Technologies, Roskilde, Denmark), 15% fetal calf serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. Transfection of BOSC 23 cells (seeded at a density of 7×10^4 cells per cm^2 the day prior to transfection) and culturing of NIH 3T3 cells were performed as previously described (18). Briefly, BOSC 23 packaging cells were transfected with 2 μg of vector plasmid and 18 μg of supercoiled carrier DNA (pUC19) in a 2-ml transfection cocktail. For the PBS-x2 complementation analysis, either pRNA^{x1} or pRNA^{x2} was substituted for pUC19, resulting in a cotransfection with a 20-fold excess of pRNA^{x1} or pRNA^{x2} to pPBS-x2. Medium containing recombinant viral particles produced during the peak in transient expression between 48 and 72 h posttransfection was filtered through a sterile 0.45-μm filter, diluted, and transferred to recipient NIH 3T3 cells (seeded at a density of 5×10^3 cells per cm^2 the day before infection) in the presence of 6 μg of Polybrene per ml. G418 selection (0.6 mg of active G418 per ml) was initiated 48 h postinfection, and G418-resistant colonies were counted after 10 to 12 days of selection.

Analysis of transduced vectors. Individual G418-resistant colonies were isolated and expanded. A region encompassing the PBS was analyzed by PCR amplification and direct sequencing as previously described (18) by using primers specific for the U3 region and the *neo* gene.

Virion RNA analysis. For RNA dot blot analysis, total RNA was purified from 5× 1 ml of virus-containing medium by pelleting virus particles at 4°C for 1 h at 13 krpm in a benchtop centrifuge followed by guanidinium thiocyanate extraction. Crude RNA from NIH 3T3 cells was used as a negative control. Vector-specific RNA levels were determined by dot blot hybridizations (11) with ^{32}P-labelled DNA probes for *neo*. Hybridization signals were detected with a PhosphorImager SF (Molecular Dynamics). To evaluate the presence of cellular RNA the filter was reprobed with a GAPDH-specific probe as described elsewhere (11). For detection of RNA^{x2} in virus particles, total RNA was purified from 10× 1 ml of virus-containing medium by pelleting virus particles for 1 h at 13 krpm in a benchtop centrifuge followed by guanidinium thiocyanate extraction. A total of 1/10 of the purified total RNA was incubated for 10 min at 37°C in 100 μl of PCR buffer (Stratagene) containing 0.2 mM deoxynucleoside triphosphate and 3 U of *Taq* polymerase, after which 25 pmol of vector-specific (5′-TCCGAATCGTGGTCTCGCTGATCCTTGG-3′) and biotinylated RNA^{x2}-specific (5′-GGGAGCTCTAGACAGCTGCTGGTCTAGGGGTATG-3′) primer was added. PCR amplification was performed for 30 cycles at 94°C for 1.2 min and at 55°C for 1.2 min. Purification and denaturation of the PCR product were performed according to the Dynabead procedure (Dynal Inc.). Direct sequencing of the PCR product was performed with a Sequenase 2.0 sequencing kit according to the instructions of the manufacturer (U.S. Biochemicals, Inc.).

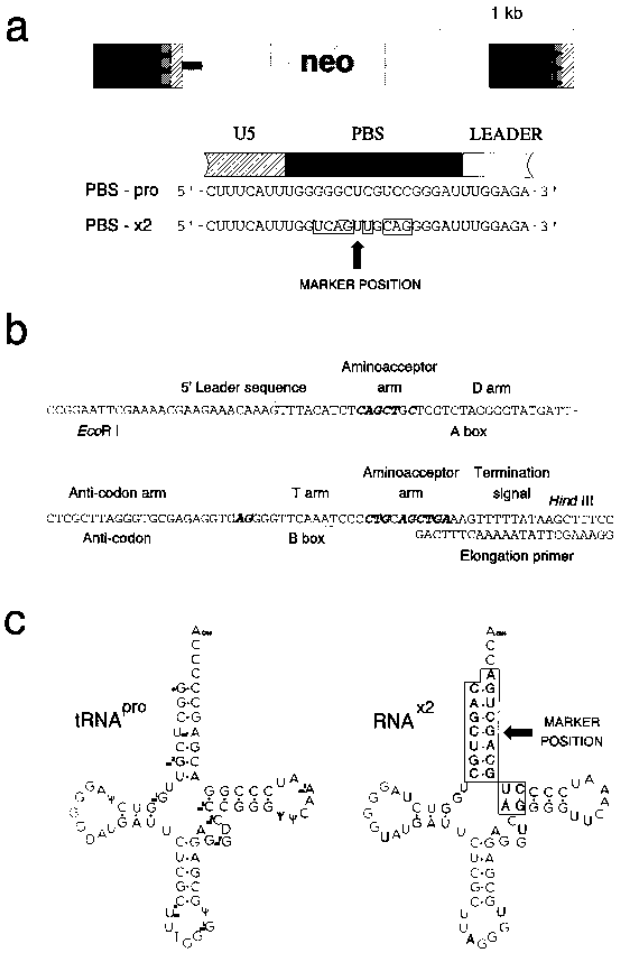

FIG. 1. Vector and engineered primer structure. (a) Structure of the 3.4-kb Akv-MLV-derived pPBS-x2 vector, with emphasis on the PBS region. Mutations relative to the wt PBS-pro are shown in boxes. Arrow, the marker position (see text for explanation). (b) Sequence of the 127-nt oligodeoxynucleotide used for cloning the RNA^{x2} gene. Genetic elements of importance for transcription and segments of the subsequent RNA^{x2} structure are shown. Mutations relative to the tRNA^{pro} gene (31) are indicated in bold italics. (c) Structure of the murine tRNA^{Pro} (after Harada et al. [13]) and putative structure of the synthetic RNA^{x2} molecule. Mutations relative to tRNA^{Pro} are shown in boxes. Arrow, the marker position (see text for explanation).

RESULTS

Construction of a PBS-mutated vector and a synthetic gene for a tRNA-like primer. To investigate the possibility of complementing the replication deficiency of a PBS-mutated retroviral vector, a new vector, PBS-x2, carrying the selectable *neo* marker, was constructed. Eight nucleotide alterations were introduced into the PBS of an Akv-MLV-derived retroviral vector by PCR-mediated site-directed mutagenesis to generate the mutant vector PBS-x2 (Fig. 1a). The mutations were chosen on the basis of an alignment of murine tRNA molecules and murine tRNA genes (36) to give the least possible match to any known murine tRNA. However, the 5′TGG motif corresponding to the CCA tail of the tRNA molecule was not modified. Likewise, the five most 3′ positions were not altered. These positions correspond to one of the intragenic promoter sequence elements of the tRNA (31) (Fig. 1b).

A genetic element encoding a putative tRNA-like primer, RNA^{x2}, matching the PBS of PBS-x2 was constructed from synthetic oligonucleotides (Fig. 1b). To distinguish sequences of RNA primer and PBS origin during replication, the RNA primer gene was designed to yield perfect Watson-Crick base pairs in only 17 of 18 positions and a G-U pair in the remaining position (Fig. 1). We have previously shown that inclusion of a single non-Watson-Crick base pair in a PBS-primer hybrid may allow efficient vector replication (18). The RNA^{x2} molecule was constructed by introducing the x2 mutations into the backbone of the gene encoding tRNA^{Pro} (31). Since efficient RNA polymerase III transcription of tRNA genes may be mediated from intragenic promoter regions (35), only 27 bp upstream and 12 bp downstream of the structural gene were included. Hence, given the small size of the tRNA genes, a plasmid, pRNA^{x2}, harboring the RNA^{x2} gene, could be made by cloning of a fragment made from chemically synthesized oligodeoxynucleotides.

Replication deficiency of PBS-mutated vector. The effect of altering the PBS of the PBS-x2 vector to a sequence not matching any known murine tRNA molecule was analyzed by retroviral vector titer assays. The BOSC 23 cell line constitutively expressing the viral *gag*, *pol*, and *env* genes (27) was used to package transiently transcribed PBS-x2 vectors, and transduc-

TABLE 1. Vector transduction efficiencies

Construct	Titer (CFU/ml)		Vector RNA in virions[a]
	Expt 1	Expt 2	
pPBS-pro + pUC19	9.6×10^5	1.2×10^5	100 ± 47
pPBS-pro + pRNA^{x2}	4.6×10^5		ND
pPBS-x2 + pUC19	6.5×10^1	$<10^1$	79 ± 17
pPBS-x2 + pRNA^{x2}	3.6×10^4	1.5×10^4	132 ± 67
pPBS-x2 + pRNA^{x1}	$<10^1$	$<10^1$	ND

[a] Averages (± standard deviations for three samples) are given in arbitrary hybridization units of *neo* hybridization. ND, not determined.

tion titers were determined by transferring virus-containing supernatant to NIH 3T3 cells followed by G418 selection. The transient titer of the Akv-neo vector carrying a wt PBS-pro ranged between 1.2×10^5 and 9.6×10^5 CFU per ml of supernatant. However, the replication capacity of the PBS-x2 vector was severely impaired with a titer reduction of about 5 orders of magnitude (Table 1).

Complementation of PBS-mutant replication by tRNA-like primer. The inability of PBS-x2 to replicate may be caused by the lack of an RNA primer with a matching 3′ end among the host cell tRNAs. To test if the PBS-x2 vector could replicate when provided with a properly modified tRNA-like primer, cotransfections of pPBS-x2 and pRNA^{x2} were performed. As shown in Table 1, cotransfecting pPBS-x2 with pRNA^{x2} resulted in reconstitution of titer levels to an order of magnitude below wt levels, thus indicating that replication of a PBS-mutated vector can be controlled by proper modification of a host cell-derived factor. Cotransfecting pPBS-pro with pRNA^{x2} did not significantly alter the replication capacity of pPBS-pro. A second genetic element, pRNA^{x1}, also encoding a putative tRNA-like RNA, RNA^{x1}, was synthesized and cloned into pUC19 to serve as a control for primer specificity. The 3′ 18 nucleotides of RNA^{x1} can potentially form only 10 Watson-Crick bp with the PBS-x2 primer binding site sequence (Fig. 2). Cotransfection of pPBS-x2 with pRNA^{x1} did not result in increased PBS-x2 transduction efficiency, hence providing evidence for the specificity of the PBS-x2–RNA^{x2} interactions.

To further study if PBS mutations or primer annealing influenced the encapsidation of vector RNA, dot blot analysis of virion RNA in the supernatants used for titration was done. We found no significant differences between samples from BOSC 23 cells carrying the vector with wt PBS, the PBS-x2

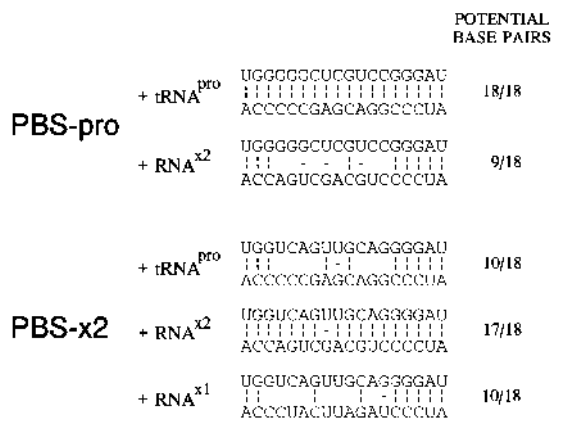

FIG. 2. Potential base pairs formed between PBS-pro and tRNA^{Pro} or RNA^{x2} and between PBS-x2 and tRNA^{Pro}, RNA^{x1}, or RNA^{x2}. |, potential base pairings; −, possible non-Watson-Crick G · U pairings.

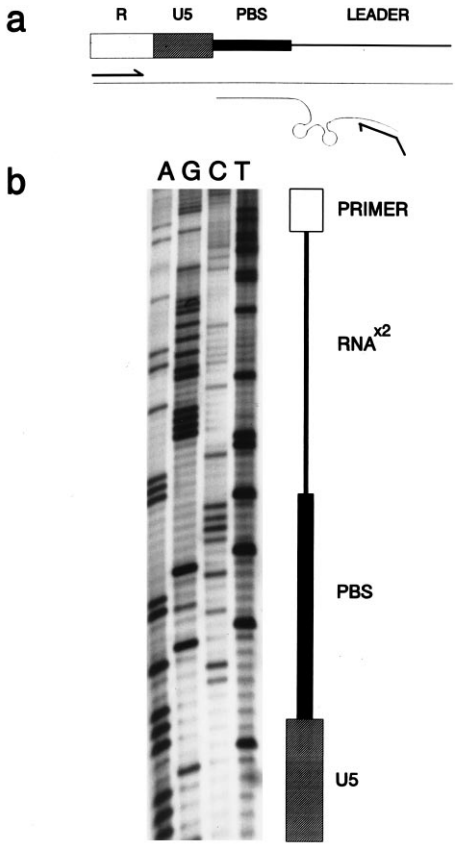

FIG. 3. Direct sequencing evidence for RNA^{x2} primer usage. Virion RNA was purified and subjected to in vitro elongation utilizing the RT activity of *Taq* polymerase, and a fragment encompassing the RNA^{x2} primer was PCR amplified and sequenced. (a) Diagram showing the positions of the utilized PCR primers. (b) Autoradiogram showing direct dideoxysequencing of RNA^{x2} annealed at the PBS.

vector alone, and the PBS-x2 vector together with the pRNA^{x2}. Specifically, the ability of the RNA^{x2} gene to increase replication by about 4 orders of magnitude was not equalled by an increase in the efficiency of vector RNA encapsidation.

Function of engineered primer in minus cDNA initiation. Most likely the RNA^{x2} gene functioned directly as an RT primer for minus-strand synthesis after annealing to the PBS. To test this assumption, total RNA was purified from PBS-x2–RNA^{x2} particles, and in vitro elongation was performed from endogenous primer molecules. PCR amplification of the cDNA-RNA^{x2} complex with primers specific for the viral genome and for the synthetic RNA^{x2} yielded the predicted 162-bp product whose structure could be confirmed by direct sequencing. Figure 3 shows the sequence extending from the viral U5 region through PBS-x2 into the synthetic RNA^{x2} primer molecule. The results show that RNA^{x2} is packaged into virions, anneals to the complementary PBS-x2, and functions correctly as a primer for first strand reverse transcription of PBS-x2. Detailed inspection of the sequencing ladders (Fig. 3) reveals a mixture of the primer and PBS complementary sequence at the marker position (Fig. 1). We assume that this mixture results from cases of RNase cleavage of the primer RNA upstream of the marker difference before priming of cDNA synthesis as previously reported (6, 12).

Function of mutant primer and PBS sequences in second-strand transfer. The mechanism of plus-strand transfer during replication of PBS-x2 in the presence of RNA^{x2} was analyzed

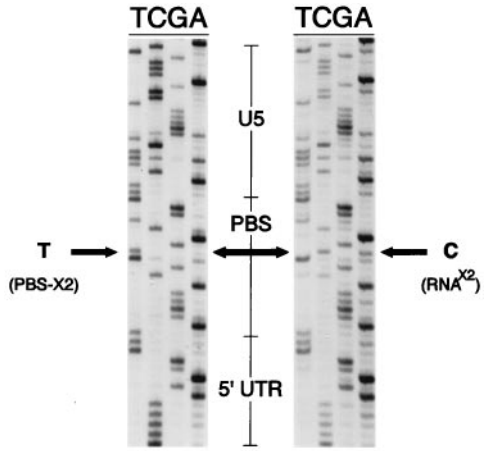

FIG. 4. Evidence for usage of the RNA^{x2} sequence in second-strand transfer. Autoradiograms showing dideoxysequencing of a region of transduced PBS-x2 vectors encompassing the PBS sequence from two representative cell clones. (Left panel) The transduced PBS originates from a copy of the original PBS-x2 sequence. (Right panel) The transduced PBS originates from copying of the RNA^{x2} primer.

by using the marker mutation at position 8 of PBS-x2 to distinguish RNA^{x2} and PBS-x2-derived sequences. During reverse transcription, the transduced PBS is generated after the second jump from a DNA copy of the vector PBS annealing to a DNA copy of 18 3′ nucleotides of the primer tRNA. Hence, the PBS sequence of a transduced provirus could originate from a copy of the genomic PBS sequence or from copying of the 3′ 18 nucleotides of the utilized primer molecule. Therefore, a PBS mutation giving a single base difference from perfect primer complementarity may revert to the primer sequence (5).

To investigate the involvement of RNA^{x2} in PBS-x2 transduction, we analyzed the fate of the introduced marker mutation. Upon analysis of PBSs transduced from the pPBS-x2–pRNA^{x2} complementation assay, correction of the introduced marker mutation to match the primer sequence was demonstrated (Fig. 4). Of nine cell clones with transduced proviruses analyzed three contained the primer sequence, four the PBS-x2 sequence and two a mixture of the two, thus providing genetic evidence for RNA^{x2} involvement in PBS-x2 transduction. We found no evidence suggesting the involvement of other primer molecules in PBS-x2 transduction in the nine cell clones analyzed. In all cases, the sequences flanking the PBS showed exact correspondence to sequences of the input vector, indicating precision in priming of first-strand synthesis and elongations after second-strand transfer. Given the 10^3- to 10^4-fold difference in transduction efficiency between pPBS-x2 cotransfected with pUC19 and pPBS-x2 cotransfected with pRNA^{x2}, detecting alternative priming events might require the analysis of a very large number of transduced clones.

DISCUSSION

During the processes of MLV reverse transcription, a tRNA derived from the former host cell constitutes the only crucial cellular factor. We have previously shown that PBS-mutated vectors derived from Akv-MLV can efficiently replicate with alternative tRNA molecules as replication primers (18); hence, base pairing between the 3′ end of the tRNA and the 18 bases of the PBS is likely to be of primary importance for MLV tRNA primer selection. However, evidence from avian viruses and HIV indicates that non-PBS interactions between the viral genomic RNA and the tRNA primer are important for efficient primer selection (1, 15, 38). Likewise, specific interactions between viral proteins of both avian viruses and HIV and their cognate tRNA molecules have been demonstrated in vitro (2, 3, 14, 25, 26, 29, 32).

In this study, we describe a complementation system for genetic manipulation of the primer used for MLV replication in vivo. Primer manipulation and subsequent in vivo testing may point to important general structures of tRNAs as well as to determinants for recognition of the cognate tRNA of a given group of retroviruses outside the annealing 3′ end. The modified tRNA^{Pro} gene used here had no registered effect on growth of the packaging cells during the period for transient production of infectious particles. A retrovirus vector, pPBS-x2, containing eight specific PBS mutations was constructed. The mutations were devised to give a low match to the 3′ end of any known murine tRNA molecule (36). In accordance with previous studies on PBS alterations (10, 17, 20, 22, 30), the PBS-x2 mutations severely diminished the replication capacity of the vector due to the pivotal functions of the PBS during retroviral replication.

To complement the replication deficiency of pPBS-x2, a gene encoding an engineered primer for reverse transcription, pRNA^{x2}, was designed by introduction of 17 bp changes into the gene for mouse tRNA^{Pro}, in a way to allow normal RNA polymerase III transcription. Cotransfections of pPBS-x2 with pRNA^{x2} resulted in partial reconstitution of the transduction efficiency to 1 order below wt levels. Hence, the introduction of eight specific mutations into the PBS strongly inhibits vector replication, but the replication capacity can be restored by the introduction of a small genetic element encoding a complementary primer. The specificity of the complementation was investigated with a second artificial tRNA-like primer molecule, pRNA^{x1}, which was designed not to match pPBS-x2 (Fig. 2). Cotransfection of pPBS-x2 with pRNA^{x1} resulted in titer below 10 CFU per ml. In addition, cotransfection of pPBS-pro with pRNA^{x2} did not significantly influence pPBS-pro transduction, indicating that the function of pRNA^{x2} reside within its capacity to base pair with pPBS-x2. Upon analysis of particles released after cotransfection of pPBS-x2 and pRNA^{x2}, the artificial tRNA-like primer, RNA^{x2}, was found annealed to the PBS and capable of initiating minus strand DNA synthesis. Although we have no direct evidence that the mutations allow the formation of a correctly transcribed and processed product, the fact that the engineered primer allows accurate initiation of minus strand cDNA indicate appropriate processing of the 3′ CCA tail. According to prevalent models for reverse transcription, RT copies only the 18 most 3′ nucleotides of the tRNA because of the 1-methyl group at the adenosine residue at position 19 from the 3′ end. The engineered primer was designed to have the tRNA^{Pro} sequence at and around this adenosine position. The precision of priming after second strand transfer was demonstrated by analyzing nine transduced PBS-x2 sequences, three of which had been corrected to perfectly match the RNA^{x2} sequence. The results presented provide in vivo evidence that the replication machinery recognizes PBS primer complementarity rather than actual PBS and 3′ primer sequences. We furthermore show that packaging of the retroviral genome is independent of proper primer annealing. The ability to engineer the primer molecule should allow tests of possible roles of the tRNA primer in genomic RNA dimerization, in RT primer-template recognition, and in facilitating the template jumps during reverse transcription. A previously described system for genetic modification of the tRNA primer for the yeast *Ty1* retrotransposon (8) relied on the use of a modified tRNA in protein synthesis as well as in reverse transcription.

The freedom to manipulate PBS and primer sequences may address two critical points in relation to the advancement of retroviral gene transfer technology and its clinical usage. Firstly, a number of current studies have shown the wt PBS of MLV proviruses to be involved in transcriptional down-regulation of vectors in important target cells (4, 7, 16, 24). Secondly, the risk for generation of replication-competent retroviruses has traditionally been reduced by manipulation of the viral packaging construct, by partition of the *gag* and *pol* genes and the *env* gene, and by the use of heterologous promoters and termination signals (21). While existing vector-containing packaging cells by definition harbor all *cis* elements and *trans* factors required for retrovirus propagation, transfer systems based on PBS-modified vectors may be completely devoid of elements with PBSs that function efficiently in normal cells. Under these conditions, regeneration of replication-competent viruses would require aberrant reverse transcription in addition to recombination.

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