Virology 278, 368–379 (2000)
doi:10.1006/viro.2000.0683, available online at http://www.idealibrary.com on IDEAL®

Alleviation of Murine Leukemia Virus Repression in Embryonic Carcinoma Cells by Genetically Engineered Primer Binding Sites and Artificial tRNA Primers

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Received July 7, 2000; returned to author for revision September 27, 2000; accepted October 2, 2000; published online November 22, 2000

The primer binding site (PBS) plays pivotal roles during reverse transcription of retroviruses and also is the target of a cellular host defense impeding the transcription of murine leukemia virus (MLV) harboring a proline (pro) PBS in embryonic cells. Both the PBS and the tRNA primer are copied during reverse transcription and anneal as complementary DNA sequences creating the PBS of the integrated provirus. The pro PBS of MLV can be exchanged by PBS sequences matching endogenous or engineered tRNAs to allow replication of Akv MLV-derived vectors in fibroblasts. Here we use the PBS escape mutant B2 to demonstrate the capacity of the synthetic $tRNA^{B2}$ to function in reverse transcription in competition with endogenous tRNAs in fibroblasts and embryonic carcinoma (EC) cells. We further show symmetry between PBS and the primer by the ability of the synthetic $tRNA^{B2}$ to confer escape from EC repression of a PBS-Pro vector. Of a panel of vectors with the repressed pro PBS substituted for other natural or artificial PBS sequences, all except one efficiently expressed the *neo* marker gene when transferred to NIH/3T3 and EC cells, hence avoiding PBS-mediated silencing in EC cells. A non-natural PBS matching an artificially designed tRNA molecule conferred no further relief from repression than that attained with the B2 escape mutant or the natural alternative PBSs. Interestingly, a vector harboring a PBS matching $tRNA^{Lys1,2}$ suffered repression similar to the wild-type PBS-Pro but was partially rescued by a single point mutation of the PBS.

Key Words: murine leukemia virus; primer binding site; tRNA; embryonal carcinoma; silencing.

INTRODUCTION

To copy the single-stranded RNA genome of retroviruses into a double-stranded DNA provirus, the cellular tRNA copackaged in the virion serves as primer for reverse transcriptase by annealing of its 3′ 18 nucleotides to the complementary PBS near the 5′ end of the viral genome (Gilboa *et al.,* 1979). During minus-strand synthesis, the PBS present in the RNA genome is copied into DNA, whereas the corresponding sequence of the tRNA is copied during synthesis of the plus strand. Annealing of the complementary sequences then mediates the second template switch of reverse transcription and generates the PBS of the transduced provirus (Gilboa *et al.,* 1979). Different tRNAs are used by different viruses. Murine leukemia virus (MLV) (Peters *et al.,* 1977) and human T-cell leukemia virus (Seiki *et al.,* 1983) replicate via $tRNA^{Pro}$, mouse mammary tumor virus, and human immunodeficiency virus via $tRNA^{Lys3}$ (Majors and Varmus, 1983; Wain-Hobson *et al.,* 1985); visna, spuma, and Mason-Pfizer monkey viruses use $tRNA^{Lys1,2}$ (Sonigo *et al.,* 1985, 1986; Maurer *et al.,* 1988); and avian retroviruses use $tRNA^{Trp}$ (Sawyer and Dahlberg, 1973). The PBS of some endogenous murine proviruses match $tRNA^{Gln}$ (Ou *et al.,* 1983; Nikbakht *et al.,* 1985), and a PBS complementary to $tRNA^{Phe}$ is found in mouse and hamster intracisternal A particles (Cohen *et al.,* 1983; Ono and Ohishi, 1983). For a given virus, the PBS element, and thus the interacting tRNA, is highly conserved, yet replication of MLV and MLV-derived vectors is compatible with several PBS-tRNA combinations in cell cultures (Colicelli and Goff, 1987; Lund *et al.,* 1993, 1997, 2000a) and in mice (Lund *et al.,* 1999). $tRNA^{Gln}$ was found to support transduction of Moloney MLV (MoMLV) revertant *dl587*rev (Colicelli and Goff, 1987), and we have previously shown PBS-mutated retroviral vectors derived from Akv-MLV to replicate efficiently using $tRNA^{Gln1,2}$, $tRNA^{Lys3}$ (Lund *et al.,* 1993), $tRNA^{Ser}$, $tRNA^{Arg}$, $tRNA^{Phe}$ (Lund *et al.,* 2000a), and a genetically engineered tRNA molecule (Lund *et al.,* 1997).

The developmental restraints on such a pivotal element make it a candidate target for cellular mechanisms balancing virus–host interactions. Hence the PBS of MLV that matches $tRNA^{Pro}$ has been found to coincide with a negatively acting *cis*-element mediating transcriptional silencing of MoMLV and MoMLV-based vector proviruses in embryonic carcinoma (EC) cells (Teich *et al.,*

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0042-6822/00 $35.00

1977; D'Auriol *et al.,* 1981; Stewart *et al.,* 1982; Weiher *et al.,* 1987) and embryonic stem (ES) cells (Grez *et al.,* 1990; Kempler *et al.,* 1993) and in cells of the early embryo (Jähner *et al.,* 1982; Vernet and Cebrian, 1996). A single base pair change from G to A at position 15 of MoMLV PBS was identified in a search of escape mutants in EC cells and found to be sufficient for relief of a vector from repression (Barklis *et al.,* 1986). In accordance with this, the PBS of the MoMLV revertant *dl587*rev with multiple base pair differences changing it to match a $tRNA^{Gln}$ (Colicelli and Goff, 1987) is compatible with expression in ES cells (Grez *et al.,* 1990). The silencer activity of the PBS region has by several criteria been separated from its roles during reverse transcription; hence repression is seen both in transfection assays independent of the presence of retroviral proteins (Barklis *et al.,* 1986; Loh *et al.,* 1987, 1988, 1990; Feuer *et al.,* 1989), with the element in either orientation, and when placed in an intron (Petersen *et al.,* 1991) or outside of the transcriptional unit (Loh *et al.,* 1990). Detailed mapping studies have delimited the negative element to a region overlapping the PBS, and only very few alterations of the sequence retain silencer functions (Loh *et al.,* 1990; Petersen *et al.,* 1991; Kempler *et al.,* 1993). Graduated but variable levels of repression were attributed to various PBS sequences in a study of MoMLV-derived vectors with test sequences inserted downstream of a gln PBS (Yamauchi *et al.,* 1995), suggesting that some PBS sequences might escape repression only partially. Whether the singly mutated B2 PBS or the other tRNA-matching PBSs of similar activity are completely devoid of repressive effects or alternative non-tRNA-matching sequences might exist that would confer further escape from repression by the PBS silencer is not known. From *in vitro* studies of factor binding to PBS sequences, repression has been suggested to be mediated by a cellular protein, factor A (Yamauchi *et al.,* 1995). Because factor A was also detected in permissive fibroblasts (Petersen *et al.,* 1991), however, the identity of host cellular factors involved and their mode of operation in PBS silencer-mediated repression remain unclear.

In this study, we investigated repression in F9 cells using the ability of MLV to replicate via various PBS-tRNA sets. Several natural and engineered PBS-tRNA complements are tested for repression in the context of their reverse transcription function by replacing pro wild-type (wt) PBS at the homologous position in Akv MLV-derived vectors. An engineered $tRNA^{B2}$ primer encoding the B2 PBS mutation is used to discern participation of the engineered molecule in competition with endogenous tRNAs and to show symmetry during reverse transcription between the primer and the PBS in providing relief from repression in F9 cells. Alleviation of repression is conferred by all of the variant PBS sequences tested except lys1.2 PBS. A partial escape mutant containing a single base substitution compared with lys1.2 PBS is inferred from primer extension analysis of expressed proviruses.

RESULTS

Replication with a synthetic tRNA primer on a background of matching endogenous tRNAs

Replication of Akv MLV-derived vectors with a mutationally impaired PBS can be restored by an engineered complementary tRNA primer, functional in both the initiation of first-strand synthesis and second-strand transfer (Lund *et al.,* 1997). We were interested in the ability of synthetic tRNA primers to complement vectors harboring a functional PBS on a background of competing endogenous tRNAs. The Akv MLV-derived vectors PBS-Pro (Lund *et al.,* 1993) and PBS-B2 (Fig. 1A) harboring the LTR-promoted G418-selectable *neo* gene differ in the 15th position of the PBS sequence by a G-to-A mutation present in the B2 PBS (Barklis *et al.,* 1986). This mutation gives a single base difference from perfect complementarity to a $tRNA^{Pro}$ primer and can be used to determine use of a synthetic tRNA molecule in reverse transcription. We incorporated the B2 mutation into either the PBS of the vector or an engineered tRNA molecule, $tRNA^{B2}$ (Figs. 1A–1C), constructed from oligodeoxynucleotides to resemble the backbone of the gene for $tRNA^{Pro}$. The 109-bp synthetic minigene encoding the tRNA includes 27 bp upstream and 10 bp downstream of the mature tRNA gene and holds intragenic promoter regions, the A and B box, for RNA polymerase III transcription (Sprague, 1995). We have previously shown a similarly sized synthetic $tRNA^{x2}$ gene (Fig. 1B) to be proficiently expressed to complement the replication deficiency of a PBS mutated vector in transduced fibroblasts (Lund *et al.,* 1997). To control for possible adverse effects of adding synthetic tRNAs, a plasmid encoding a putative full-length $tRNA^{Pro}$, including larger regions upstream and downstream of the mature tRNA molecule that might contribute to expression (Pavesi *et al.,* 1994), was constructed by PCR amplification of 446 bp from the murine Pro-tRNA gene (Russo *et al.,* 1986). Vector constructs and artificial tRNAs were cotransfected into the BOSC 23 packaging cell line (Pear *et al.,* 1993), and transduction titers were determined by transfer of transiently produced virus particles to NIH/3T3 cells followed by G418 selection (Fig. 2).

The PBS of a transduced provirus derives from a DNA copy of the PBS sequence in the original vector annealing to a DNA copy of the 18 3′ nucleotides in the tRNA primer during reverse transcription. A marker mutation hence results in a mismatched PBS after second-strand transfer, which may be corrected by cellular repair systems according to either template or, if cell division occurs before repair, lead to two different proviruses integrated at identical locations in the cellular genome (Berwin and Barklis, 1993). In any case, a mutant might revert at high frequency (Barklis

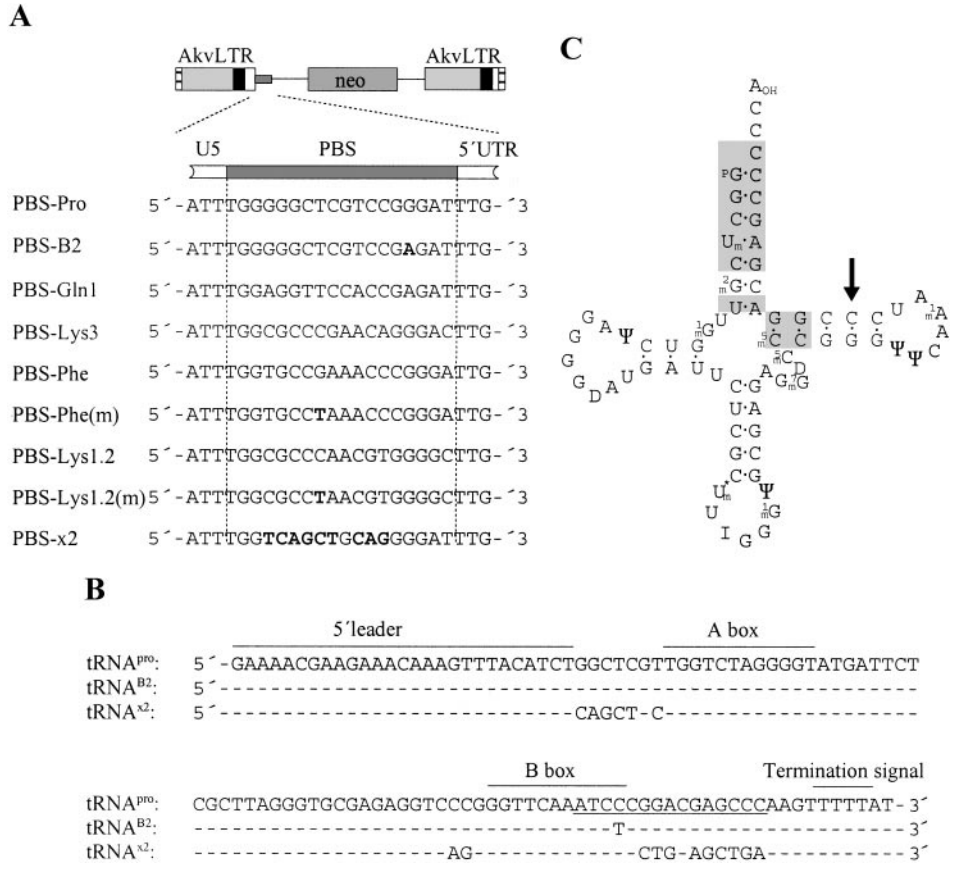

FIG. 1. Structure of vectors and engineered tRNA primers. (A) Vectors based on Akv MLV with the PBS sequence highlighted. The wt pro PBS of the 3.4-kb vector PBS-Pro was replaced with modified PBS sequences with perfect or near-perfect match to tRNA acceptors for pro (PBS-B2), gln (PBS-Gln), lys3 (PBS-Lys3), phe [PBS-Phe, PBS-Phe(m)], or lys1.2 [PBS-Lys1.2, PBS-Lys1.2(m)] or to a synthetic $tRNA^{x2}$ (PBS-x2). Nucleotide mutations differentiating PBS-B2 and PBS-x2 from PBS-Pro, PBS-Phe(m) from PBS-Phe, and PBS-Lys1.2(m) from PBS-Lys1.2 are shown in bold. The G418-selectable *neo* gene is expressed from the LTR. Striped boxes indicate linker regions in the LTRs, which are reconstituted to wt sequence after reverse transcription of the vector. (B) Sequences of engineered tRNA molecules. Primary sequence of a 109-bp subfragment of the endogenous murine Pro-tRNA gene (Russo *et al.,* 1986) encompassed in the synthetic minigenes. Nucleotides mutated in the engineered $tRNA^{B2}$ and $tRNA^{x2}$ (Lund *et al.,* 1997) relative to the Pro-tRNA gene are indicated. Elements important for transcription are shown above the sequence. Nucleotides annealing to the PBS are underlined. Linkers for cloning of the minigenes are not included in the figure. (C) Clover-leaf structure of the murine $tRNA^{Pro}$ (after Harada *et al.,* 1979). Nucleotides mutated in $tRNA^{x2}$ (shaded) and $tRNA^{B2}$ (arrow) are indicated.

et al., 1986; Rhim *et al.,* 1991), providing genetic evidence for use of a specific tRNA primer in reverse transcription (Berwin and Barklis, 1993; Lund *et al.,* 1993 and 1997). The transduced PBS sequences were analyzed by primer extension dideoxy termination on genomic DNA from G418-selected cell populations. In this analysis, the presence of pro and B2 PBS proviruses can be distinguished in terms of a 24-mer pro and a 22-mer B2 band arising from termination at the first encountered T in the template by incorporation of a dideoxy nucleotide analog (Fig. 2). Regardless of cotransfected synthetic tRNAs, PBS-Pro and PBS-B2 transduce NIH/3T3 fibroblasts with similar efficiencies in parallel experiments, ranging from 1.3×10^4 to 6.3×10^5 cfu/ml (Table 1). PBS-B2, regardless of cotransfection of $tRNA^{Pro}$, gives rise to a population of cells containing equal amounts of B2 and pro PBS proviruses as predicted from reversion to wt during reverse transcription (Barklis *et al.,* 1986) (Fig. 2, left, lanes 2 and 3). The engineered tRNA molecule per se hence does not appear to adversely affect the transduction, yet no evidence concerning participation of the molecule in transfer is obtained. However, when providing the B2 mutation in the artificial tRNA, specific use of the engineered molecule in reverse transcription is demonstrated. Accordingly, the distribution is shifted toward B2 PBS proviruses when $tRNA^{B2}$ is combined with a PBS-B2 vector (Fig. 2, lane 4), or $tRNA^{B2}$ gives rise to detectable levels of B2 PBS proviruses when in conjunction with PBS-Pro (Fig. 2, lane 5) as opposed to the pure population obtained with PBS-Pro alone (Fig. 2, lane 1). Thus the amount of artificial tRNA molecules supplied by transfecting 9 μg of supercoiled tRNA-plasmid into the packaging cells is at a level capable of competing with the endogenous tRNAs in complementation of the vectors.

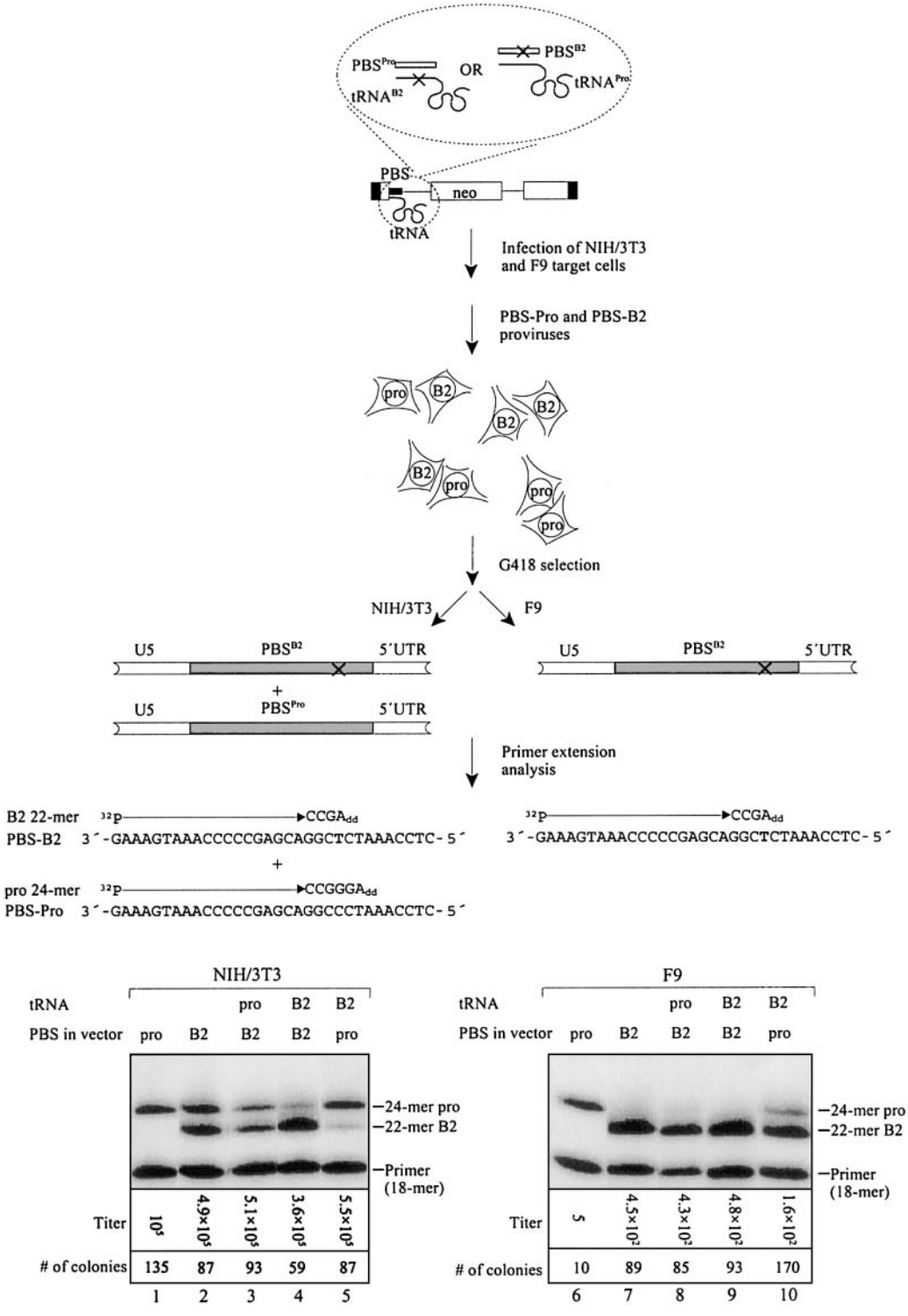

FIG. 2. Replication of vectors with a mismatched PBS-tRNA complement in fibroblasts and EC cells. Akv MLV-derived vectors containing wt pro PBS (PBS-Pro) or the single nucleotide-mutated B2 PBS (PBS-B2) were transfected into BOSC 23 transient packaging cells alone or in combination with synthetic minigenes encoding $tRNA^{Pro}$ or $tRNA^{B2}$, and viral supernatant was transferred in parallel to NIH/3T3 (left) and F9 EC cells (right). Virus particles containing a B2-pro mismatch at the PBS region in the RNA genome result in both PBS-Pro and PBS-B2 proviruses in the target cells. After G418 selection, *neo*-expressing proviruses in pools of resistant clones were analyzed by extension of an end-labeled primer (horizontal arrow) in the presence of ddATP. Twenty-two-mer B2- and 24-mer pro-indicative bands, deriving from termination at the first T in a PBS-B2 and a PBS-Pro template, respectively, were resolved by polyacrylamide gel electrophoresis, and the gel was exposed in a Personal Molecular Imager Fx and visualized by using Quantity One. In permissive fibroblasts (left), both PBS-Pro and PBS-B2 are expressed, and $tRNA^{B2}$ is used to assess participation of the non-natural tRNA in reverse transcription. In F9 EC cells (right), the PBS-Pro vector is repressed and only PBS-B2 is present after selection for *neo* expression. Transduction titers of the particular PBS-tRNA combination and number of colonies used for primer extension are indicated below each lane. Data are from experiments 5 and 7 (for PBS-Pro) of Table 1.

TABLE 1

Vector Transduction Efficiencies[a]

Vector + tRNA	Experiment	Titer (CFU/ml) F9[b]	Titer (CFU/ml) NIH/3T3[c]	NIH/3T3 F9	% PBS-Pro repression[d]
PBS-Pro	1	1.6×10^1	5.0×10^5	31,250	100
	4	2.6×10^1	4.6×10^5	17,690	100
	6	5.0×10^0	1.8×10^5	36,000	100
	7	5.0×10^0	1.0×10^5	20,000	100
	8	1.0×10^0	5.5×10^3	5500	100
	9	1.0×10^0	1.3×10^4	13,000	100
PBS-B2	1	1.8×10^2	9.7×10^4	539	2
	2	1.0×10^3	1.8×10^4	18	NA
	4	4.7×10^2	3.7×10^5	787	4
	5	4.5×10^2	4.9×10^5	1089	NA
	7	2.0×10^2	4.0×10^4	200	1
	8	1.1×10^2	1.7×10^4	154	3
	9	2.5×10^2	2.2×10^4	88	1
PBS-B2 + $tRNA^{Pro}$	3	3.1×10^2	3.3×10^5	1064	NA
	4	1.2×10^3	5.6×10^5	467	3
	5	4.3×10^2	5.1×10^5	1186	NA
	9	2.0×10^2	1.9×10^5	950	8
PBS-B2 + $tRNA^{B2}$	1	3.0×10^2	1.1×10^5	367	1
	2	3.8×10^3	1.7×10^4	4	NA
	4	3.6×10^3	5.5×10^5	153	1
	5	4.8×10^2	3.6×10^5	750	NA
	9	2.9×10^2	5.9×10^4	203	2
PBS-Pro + $tRNA^{Pro}$	3	1.3×10^1	5.0×10^5	38,461	NA
	4	8.6×10^1	6.3×10^5	7325	41
	9	1.0×10^0	9.5×10^3	9500	73
PBS-Pro + $tRNA^{B2}$	3	1.3×10^2	1.0×10^5	769	NA
	4	3.8×10^2	1.6×10^5	421	2
	5	1.6×10^2	5.5×10^5	3437	NA
	9	4.4×10^1	1.3×10^4	295	2
PBS-Gln	1	1.2×10^3	3.9×10^5	325	1
	2	4.6×10^3	1.5×10^5	33	NA
	4	6.7×10^3	1.1×10^6	164	1

MLV expression in EC cells by use of artificial $tRNA^{B2}$

In accordance with a silencer coinciding with the wt pro PBS region, the vector PBS-Pro transduces F9 cells poorly, with titers ranging from 1 to 2.6×10^1, whereas PBS-B2 containing the EC escape mutation (Barklis *et al.,* 1986) evades silencing obtaining titers from 1.1×10^2 to 10^3, although F9 cells are still less transducable than NIH/3T3 fibroblasts (Table 1). We sought to avoid the silencing of PBS-Pro by complementation with a synthetic tRNA molecule providing the B2 escape mutant. Because the absolute titer of different virus stocks is subject to experimental variation, the NIH/3T3-to-F9 ratio, the restriction index (Kempler *et al.,* 1993), is used to compare repression in EC cells of various constructs within the same experiment. Based on this ratio, PBS-B2 is repressed at a level of only 1–4% compared with PBS-Pro in repeated experiments. Transduction efficiencies obtained by PBS-B2 cotransfected with $tRNA^{Pro}$ or $tRNA^{B2}$ do not differ significantly from the results with PBS-B2 alone, indicating no adverse effect of the synthetic tRNA (Table 1). Similarly, compared with PBS-Pro alone, PBS-Pro + $tRNA^{Pro}$ is repressed at 41%. However, silencing can be evaded by complementing PBS-Pro with synthetic $tRNA^{B2}$, giving titers on F9 cells from 4.4×10^1 to 3.8×10^2 and restriction indices matching those for PBS-B2 (Table 1). A restriction index as high as 3437 obtained with the PBS-Pro + $tRNA^{B2}$ combination in one experiment might indicate that alleviation of repression is not in all cases as efficient as with the PBS-B2 vector, a finding that can be explained by the simultaneous presence of endogenous $tRNA^{Pro}$ primers, which have to be outcompeted.

The results from primer extension analysis on pools of G418-resistant F9 clones confirm $tRNA^{B2}$-mediated escape of the PBS-Pro vector as demonstrated in titering assays. PBS-B2 alone or together with $tRNA^{Pro}$ or $tRNA^{B2}$ gives rise only to detectable levels of the 22-mer B2 band (Fig. 2, right, lanes 7–9), indicating efficient silencing of revertant pro PBS proviruses; the 24-mer pro band in lane 6 derives from the few resistant colonies obtained

TABLE 1—*Continued*

Vector + tRNA	Experiment	Titer (CFU/ml) F9[b]	Titer (CFU/ml) NIH/3T3[c]	NIH/3T3 F9	% PBS-Pro repression[d]
PBS-Lys3	1	2.0×10^2	7.8×10^4	390	1
	2	5.3×10^2	1.5×10^4	28	NA
	4	4.1×10^2	4.4×10^5	1073	6
PBS-Phe	8	7.0×10^1	2.3×10^3	33	1
	8	7.0×10^1	8.6×10^3	123	2
PBS-Phe(m)	6	9.0×10^1	1.5×10^4	167	1
	6	9.0×10^1	1.5×10^4	167	1
	6	7.0×10^1	1.8×10^4	257	1
	7	6.0×10^1	9.0×10^3	150	1
	7	5.0×10^1	9.0×10^3	180	1
	7	6.0×10^1	1.0×10^4	167	1
PBS-Lys1.2	8	1.0×10^0	1.4×10^3	1400	25
	8	3.0×10^0	5.9×10^3	1967	36
	9	3.0×10^0	6.4×10^3	2133	16
	9	2.0×10^0	9.0×10^3	4500	35
PBS-Lys1.2(m)	6	6.0×10^0	1.0×10^4	1667	5
	6	8.0×10^0	1.0×10^4	1250	4
	6	5.0×10^0	8.5×10^3	1700	5
	7	1.0×10^1	1.0×10^4	1000	5
	7	7.0×10^0	5.0×10^3	714	4
	7	0	5.0×10^3	NA	NA
	9	1.2×10^1	6.3×10^3	525	4
PBS-x2	1	0	1.3×10^1	NA	NA
	2	0	3.0×10^0	NA	NA
	4	0	1.4×10^1	NA	NA
PBS-x2 + $tRNA^{x2}$	1	3.0×10^2	9.9×10^4	330	1
	2	1.0×10^3	1.0×10^4	10	NA
	3	1.3×10^3	1.4×10^5	108	NA
	4	2.5×10^3	2.8×10^5	112	1

[a] NIH/3T3 and F9 cells were transduced in parallel with dilutions of virus particles from BOSC 23 cells transiently transfected with 1 μg of vector + 9 μg of tRNA plasmid or 1 μg of vector + 9 μg pUC19 where no tRNA is indicated.

[b] F9 cells were seeded at 3×10^3 cells/cm^2 in 6-wells dishes.

[c] NIH/3T3 cells were seeded in P10 dishes or in 6-well dishes (experiments 8 and 9) at 10^4 or 5×10^3 (experiment 9) cells/cm^2.

[d] The NIH/3T3-to-F9 ratio was compared with the corresponding ratio for PBS-Pro within the same experiment. NA, not applicable.

by PBS-Pro transduction, which were expanded for primer extension analysis. However, PBS-Pro + $tRNA^{B2}$ transduction of F9 cells results in the majority of proviruses harboring a B2 PBS (Fig. 2, lane 10), demonstrating utilization of the synthetic tRNA in competition with the endogenous $tRNA^{Pro}$ and establishing symmetry between a mutation in the PBS and in the reverse transcription primer in conferring escape to EC repression. We do not know the reason for the presence of the weaker 24-mer pro band seen with PBS-Pro + $tRNA^{B2}$ (Fig. 2, lane 10) and in some cases also with PBS-B2 + $tRNA^{Pro}$ (data not shown). It is known that some sites of integration may confer expression of pro PBS-containing vectors in EC cells (Barklis *et al.,* 1986). Hence it is possible that stochastic variations in permissive integration sites, within the limited number of clones present in the pools analyzed, may affect the intensity of the pro PBS band. Compared with the distribution in unrestricted NIH/3T3 cells, the order of appearance of increasing pro PBS band intensities are essentially the same, i.e. PBS-B2 + $tRNA^{B2}$ < PBS-B2 + $tRNA^{Pro}$ < PBS-Pro + $tRNA^{B2}$, which is why the distribution in F9 might simply reflect the amount of $tRNA^{B2}$ participating in reverse transcription, shifting the transduced PBS toward the B2.

PBS determinants of MLV repression

To further characterize PBS silencer-mediated repression, we studied the negative effect of different PBS sequences when at their natural position, mediating their functions in reverse transcription. A panel of Akv MLV-derived vectors harboring a variety of PBS sequences in place of the pro PBS with perfect or near-perfect match to naturally occurring tRNAs (Fig. 1) were tested for transduction efficiencies. The introduced PBS sequences complement tRNA acceptors for glutamine (PBS-gln), lysine3 (PBS-Lys3), lysine1.2 (PBS-Lys1.2), and phenylalanine (PBS-Phe). PBS-Lys1.2(m) and PBS-Phe(m) each harbor a single base substitution, C → T and G → T, respectively (Fig. 1),

giving a C-A and a G-A mismatch in the PBS after second-strand transfer. Virus titration assays were done on NIH/3T3 and F9 cells in parallel to allow a comparison of different constructs within an experiment.

In NIH/3T3 fibroblasts, all PBS mutations sustained efficient vector replication in the presence of endogenous or cotransfected synthetic tRNA primers, confirming the ability of Akv MLV-derived vectors to replicate by use of various tRNA molecules (Lund *et al.,* 1993, 1997, 2000a). In general, for each construct, restriction indices varied from one experiment to another, but they were relatively consistent when comparing vectors within an experiment. Hence all vectors with functional PBS sequences, except PBS-Lys1.2, displayed restriction indices paralleling those for PBS-B2 and were repressed at 1–6% the level of PBS-Pro in F9 cells, indicative of relief from repression (Table 1). In particular, in parallel transductions, both PBS-Lys3 and PBS-Phe vectors behaved similarly to PBS-B2 in terms of restriction indices, as did the single mismatch vector PBS-Phe(m) (Table 1). Consistent with this, no repression of PBS-Phe(m) in F9 was observed in primer extension analysis performed on pools of G418-selected clones. Both NIH/3T3 and F9 PBS-Phe(m)-transduced cells showed equal distribution of 22-mer phe(m) and 33-mer phe PBS bands, indicative of balanced reversion to wt phe PBS in permissive cells of a PBS-tRNA complement with a single mismatch (Fig. 3, lane 4 and lanes 8–9).

A PBS point mutation escaping EC repression of lys1.2 PBS

Of the transduced vectors with alternative PBS sequences, only PBS-Lys1.2 showed substantial repression in F9 cells, at approximately one third the level of PBS-Pro (Table 1). Interestingly, when we analyze PBS sequences in pools of selected clones from cells transduced with the mutant PBS-Lys1.2(m) by primer extension, PBS-Lys1.2(m) is demonstrated to escape repression in F9 cells compared with the revertant PBS-Lys1.2 (Fig. 3). In NIH/3T3 cells, corresponding levels of lys1.2(m) 22-mer and lys1.2 27-mer bands are seen, indicating no restriction (Fig. 3, lane 3). In F9 cells, however, in accordance with the restriction seen in titer assays, the 27-mer lys1.2 provirus-derived band is hardly visible, whereas PBS-Lys1.2(m) appears to escape repression (Fig. 3, lane 7). A closer inspection of transduction efficiencies, albeit generally low on both F9 and NIH/3T3 cells in experiments 6–9, reveals a tendency for PBS-Lys1.2(m) repression to be intermediate to repression of PBS-Lys1.2 on the one hand and PBS-B2, PBS-Phe, and PBS-Phe(m) on the other, confirming escape of the lys1.2(m) PBS proviruses in the population.

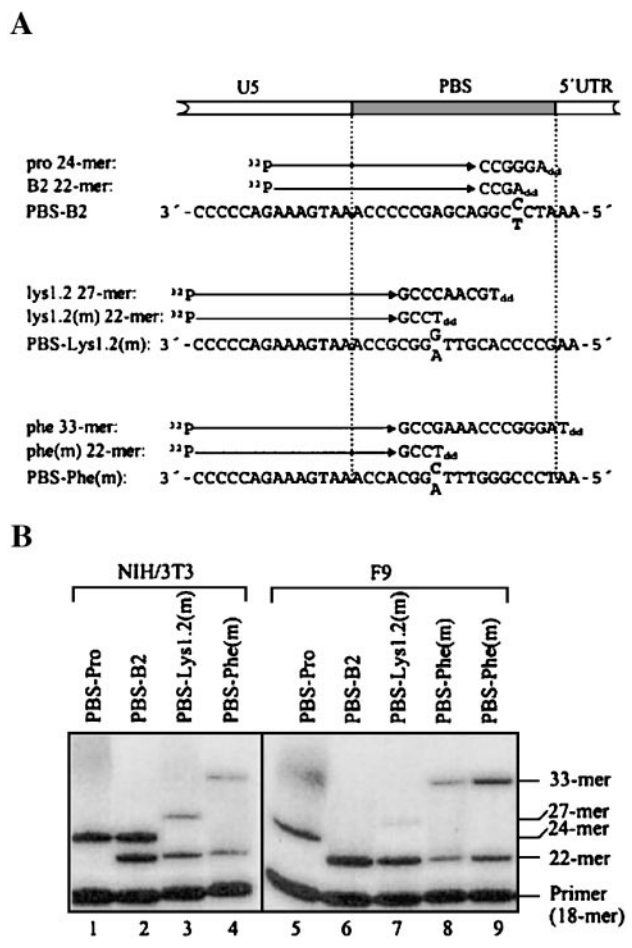

FIG. 3. Primer extension analysis of PBS-tRNA mismatched vectors. (A) Schematic representation of termination products resulting from primer extension performed with the single nucleotide-mutated PBS-B2, PBS-Lys1.2(m), and PBS-Phe(m) vectors and their respective PBS revertant proviruses, PBS-Pro, PBS-Lys1.2, and PBS-Phe, as templates. Extension of an end-labeled primer (horizontal arrow) was performed in the presence of ddATP for PBS-B2-transduced cells or ddTTP for cells transduced with PBS-Lys1.2(m) and PBS-Phe(m) vectors. (B) Primer extension analysis of PBS-Pro-, PBS-B2-, PBS-Lys1.2(m)-, and PBS-Phe(m)-expressing G418-resistant populations of NIH/3T3 (left) and F9 cells (right). Termination products were resolved by polyacrylamide gel electrophoresis, and the gel was exposed in a Personal Molecular Imager Fx and visualized by using Quantity One. The samples are from experiment 7 of Table 1.

Escape from EC repression by engineered PBS-tRNA complement

The PBS-impaired vector PBS-x2 (Lund *et al.,* 1997) harbors nine nucleotide alterations in the PBS compared with the vector PBS-Pro (Fig. 1A). The mutations were chosen to give the least possible match to any known murine tRNAs, yet conserved the 5′ TGG matching the CCA tail of a tRNA molecule and the five most 3′ nucleotides constituting a part of the intragenic B box promoter element (Lund *et al.,* 1997) (Fig. 1B). The replication capacity of PBS-x2 is severely reduced due to lack of a complementary tRNA primer but is restored to near-wt

levels by cotransfection of a matching synthetic molecule $tRNA^{x2}$ (Lund *et al.,* 1997). To assess the possibility of attaining further escape from PBS silencing than that provided by B2 or alternative tRNA-matching PBSs, PBS-x2 with the lowest homology to natural sequences was tested in our transduction system.

A very low but reproducible number of G418-resistant clones was obtained after transfer of PBS-x2 to fibroblasts (Table 1). Expressed proviruses from three individual clones were PCR amplified using a primer set that allowed specific amplification of proviruses derived from replication of the input PBS-mutated vectors (Lund *et al.,* 1993). By both the size of the amplified fragment and sequence analysis of transduced PBS and flanking sequences, exact identity to the input PBS-x2 vector was demonstrated (data not shown), indicating that low incidence reverse transcription of the vector might have occurred by priming not involving tRNA-PBS-x2 perfect complementarity (Mikkelsen *et al.,* 1996, 1998). Cotransfection of a matching synthetic $tRNA^{x2}$ (Figs. 1B and 1C) reestablished replication of PBS-x2 in NIH/3T3 cells to wt levels as shown previously (Lund *et al.,* 1997). In F9 cells, PBS-x2 is efficiently impaired displaying no transduction even after transfer of milliliter levels of viral supernatant (Table 1). However, the ability of $tRNA^{x2}$ to restore replication also applies to these cells, which are efficiently transduced by the PBS-x2-$tRNA^{x2}$ complement. Hence relief from PBS silencer-mediated repression in F9 is demonstrated both by inclusion, at the homologous position in the vectors, of alternative PBS sequences functioning in reverse transcription via endogenous tRNA primers and by use of a synthetic PBS-tRNA complement. Notably, the synthetic PBS-tRNA complement provides no further relief from repression than that conferred by the B2 mutation or by the PBS matching alternative tRNAs.

DISCUSSION

In this study, we analyzed PBS-tRNA interactions during a single round of replication of MLV by replacement with alternative sequences in terms of (1) utilization of an engineered primer in competition with endogenous tRNAs and (2) repression effects of different PBS-tRNA sets in EC cells.

The ability of MLV to replicate efficiently using various naturally occurring PBS-tRNA primer complements has been previously demonstrated (Lund *et al.,* 1993) and used in the engineering of an artificial primer complementation system (Lund *et al.,* 1997). In that system, a synthetic gene encoding a tRNA-like molecule is capable of restoring replication of a complementary vector with a mutationally impaired PBS to near-wt levels when present in the packaging cells during transient production of virus particles (Lund *et al.,* 1997). In the work presented here, we show that an engineered primer, without perfect match to the PBS, functions in reverse transcription of an Akv MLV-derived vector PBS-Pro in competition with endogenous tRNAs. As a genetic marker for primer utilization, the single base pair PBS mutant B2 was incorporated into the engineered tRNA. Because both the primer and the PBS sequence are copied during reverse transcription to generate the transduced PBS (Gilboa *et al.,* 1979), a mutation in either of them is predicted to appear in approximately half of the integrated proviruses (Barklis *et al.,* 1986; Berwin and Barklis, 1993). When we analyze proviruses of the NIH/3T3 target population by primer extension dideoxy termination (Modin *et al.,* 2000a,b), a PBS-Pro vector transfers only proviruses with pro PBS, whereas PBS-B2 vectors replicating via $tRNA^{Pro}$ alone shows equal distribution of pro and B2 PBS proviruses as predicted from reversion to wt. Cotransfecting each vector with $tRNA^{B2}$, however, shifts these distributions toward a higher frequency of B2 PBS proviruses, indicating that to some extent the engineered $tRNA^{B2}$ is used as primer during the transfer of both PBS-Pro and PBS-B2 vectors (Fig. 2, left). Previously, *in vitro* transcription and some degree of posttranscriptional modification of the engineered tRNA have been demonstrated (Lund *et al.,* 2000b). The results presented here indicate that the *cis*-elements contained in the construct are sufficient to allow function of the engineered primer even on a background of competing endogenous tRNAs. Notably, the C-to-T substitution corresponding to the B2 PBS mutation localizes to one of the intragenic RNA polymerase III promoters of the engineered tRNA gene (Fig. 1B), giving a single nucleotide deviation from the published consensus sequence of the B box (Pavesi *et al.,* 1994), presumably without detrimental effects on gene expression.

The efficient function of an engineered primer in reverse transcription was exploited to assess PBS primer contributions to transcriptional silencing of MLV in undifferentiated embryonic cells. By providing the PBS escape mutant B2 (Barklis *et al.,* 1986) in the tRNA primer, we demonstrate symmetry of the PBS and the primer during reverse transcription. A PBS-Pro vector that is efficiently silenced in the EC cell line F9 is thus relieved from repression by the engineered $tRNA^{B2}$ cotransfected into the packaging cells as measured by transduction efficiencies and confirmed in primer extension analysis showing the appearance of B2 PBS proviruses. From the results for both fibroblasts and EC cells, we conclude that cellular repair mechanisms presumably correcting mismatches in the PBS of the provirus do not distinguish between sequences derived from the PBS or the primer. For each PBS-primer combination, restriction indices varied from one experiment to another, but fluctuations were relatively consistent when comparing constructs within an experiment.

The results obtained from transduction of Akv MLV-derived vectors with substitution of the natural pro PBS

show that all except one of the alternative PBS sequences escape PBS silencer-mediated repression in F9 cells. Primer binding sites matching tRNAs gln, lys3, and phe and the engineered $tRNA^{x2}$ are all compatible with expression in F9 cells at levels comparable to what is accomplished by PBS-B2 containing the original PBS escape mutant B2 identified by Barklis *et al.* (1986). Previously, PBS sequences complementary to $tRNA^{Gln}$ (Grez *et al.,* 1990; Petersen *et al.,* 1991) and $tRNA^{Lys3}$ (Yamauchi *et al.,* 1995) have been reported to mediate replication of MoMLV-derived vectors in undifferentiated cells, whereas contributions from other alternative PBS sequences to repression were assessed from a position downstream of a gln PBS responsible for replication of the vectors (Yamauchi *et al.,* 1995). This design separated the repressor function of the test fragments from their PBS functions, yet concomitantly introduced two PBS sequences into the same vector, as opposed to one PBS present in the natural context. Under those conditions, PBS sequences for isoleucine, methionine, and tryptophan exhibited no silencing activity, and those for phe and lys3 exerted low but variable repression, reducing viral infectivities 2- to 10-fold compared with a B2 PBS, whereas a PBS matching $tRNA^{Lys1.2}$ exerted repression at 29–120% the level of a wt pro sequence (Yamauchi *et al.,* 1995). These results thus pointed to the existence of gradual levels of repression mediated by different PBS sequences. In the context of the Akv MLV-based vectors used here, we see no evidence of negative effects exerted by PBS-Phe or PBS-Lys3 in particular. The transduction data rather reflect a drawback inherent to titering assays, namely, variations in transfer efficiencies derived from variable transfection of producer cells, cell density, time of virus harvest, growth conditions, and so on (Weiher *et al.,* 1987). As demonstrated by our repeated experiments, these differences are only to a certain extent evened out by comparing restriction indices rather than absolute titers. Thus subtle differences between vectors based on titering assays alone may be interpreted with caution. For PBS-Lys3, PBS-Phe, and the single mismatch vector PBS-Phe(m), although restriction indices vary and reach 10^3 for PBS-Lys3 in one case, they are in general comparable to those for PBS-B2 within the same experiment. In support of this, we analyzed proviruses of PBS-Phe(m)-transduced cells by primer extension dideoxy termination and found balanced reversion to phe PBS in both NIH/3T3 and F9, indicating no discrimination between these two phe PBS sequences. We cannot exclude, however, that derepression could have been obtained by the choice of another point mutation in the phe PBS. Hence from the data in Table 1, it appears that all vectors with substitutions of the wt PBS except PBS-Lys1.2 escape repression to similar extents. In a study fine-mapping the inhibitory activity of the PBS region via DNA transfections, expression from a recombinant harboring the B2 PBS was identical to that from an unrestricted control lacking the entire PBS (Loh *et al.,* 1990). Our results, directly comparing transfer efficiencies of various primer binding sites, determine that the single G-to-A mutation of B2 PBS is a complete escape from PBS silencer-mediated repression. PBS-Gln harbors the identical mutation in addition to four others distinguishing it from PBS-Pro yet do not differ from PBS-B2 in terms of relief from repression. Importantly, the synthetic PBS designed to have the least possible match to any known tRNA does not, when complemented by an engineered $tRNA^{x2}$ during replication, give further alleviation than that provided by the B2 and natural tRNA-matching PBSs. The data hence do not support a model of general repression by tRNA-matching sequences.

Among the alternative PBS sequences tested here, only lys1.2 inhibits transcription substantially in EC cells, in accordance with earlier observations by Yamauchi *et al.* (1995), who found the titer of a gln PBS vector to be reduced more than 30-fold by a downstream positioned lys1.2 PBS relative to a B2 PBS. Our vector PBS-Lys1.2 displays transduction titers of $\leq$3 on F9, giving restriction indices at least 10-fold above those for PBS-B2 in parallel experiments. A variant vector PBS-Lys1.2(m) containing a single nucleotide mutation displayed corresponding transduction efficiencies. However, a closer comparison of repression levels indicates that although PBS-Lys1.2 is inhibited approximately 3 times less than PBS-Pro, PBS-Lys1.2(m) repression appears to be intermediate to that exerted by PBS-Lys1.2 on the one hand and the vectors PBS-B2, PBS-Phe, and PBS-Phe(m) on the other in parallel transductions. This was substantiated by primer extension analysis performed on PBS-Lys1.2(m)-transduced populations, where in permissive fibroblasts, the mutated vector showed balanced reversion to wt lys1.2 PBS, whereas in F9, only low levels of lys1.2 PBS revertants could be detected. Transcriptional inhibition mediated by the PBS region has been correlated to *in vitro* binding of a putative EC repressor, factor A, to a wt pro but not to a mutated B2 probe spanning the PBS region (Loh *et al.,* 1990; Petersen *et al.,* 1991; Yamauchi *et al.,* 1995). In addition, Yamauchi *et al.* (1995) tested binding to alternative PBS sequences and observed a complex with a mobility similar to that of factor A with a lys1.2 PBS probe, but because the two factors did not cross-compete for binding, they were suggested to be distinct factors (Yamauchi *et al.,* 1995). Our results lend further support to the existence of a negative element coinciding with the lys1.2 sequence and show that a single C-to-T mutation at the eighth position of the PBS partially relieves inhibition, a finding comparable to the G-to-A escape mutation in B2 PBS. However, PBS-B2 is still superior to PBS-Lys1.2(m) in conferring escape from repression, probably reflecting that it was selected because of its expression capacity in EC cells as opposed to the arbitrarily designed mutation in PBS-Lys1.2(m).

In conclusion, we find alleviation of pro PBS silencer-

mediated repression by the B2 PBS escape mutation provided in an engineered tRNA molecule or by exchange of the wt PBS with natural or synthetic tRNA-PBS sets. No further relief than that obtained with a B2 PBS or alternative tRNA-matching PBSs is achieved by the synthetic tRNA-PBS complement. In addition, we confirm that the lys1.2 PBS of visna, spuma, and Mason-Pfizer monkey viruses is also subject to transcriptional constraints in the context of a MLV vector and find a single point mutation in lys1.2 PBS to partially relieve the repression.

MATERIALS AND METHODS

PCR mutagenesis and cloning procedures

All vectors, inclusive PBS sequence, and tRNA-like primers are shown in Fig. 1. The vectors are contained in a pUC19 plasmid backbone and consist of truncated LTRs of Akv MLV (Van Beveren *et al.,* 1985) flanking 258 bp of the 5′ UTR with PBS sequence, a Tn5 fragment encompassing the neomycin phosphotransferase encoding gene (*neo*) (Beck *et al.,* 1982), and 407 nt including part of Akv MLV *env* and 3′ UTR. Construction of the plasmid vectors pPBS-Pro, pPBS-Gln1, pPBS-Lys3 (Lund *et al.,* 1993), pPBS-x2 (Lund *et al.,* 1997), and pPBS-Phe(m) (pPBS-BRN11ma in Lund *et al.,* 2000a) by PCR-mediated site-directed mutagenesis has been described previously. Briefly, the 5′ part of the vectors was amplified from the transmission vector ptvAkv*neo* (Paludan *et al.,* 1989), generating a 618-bp fragment carrying the modified PBS sequence, which was connected by PCR-aided overlap extension to the likewise PCR-amplified 3′ 2734-bp portion of the vectors. During amplification, the LTRs were truncated by the introduction of specific restriction sites into the 5′ U3- and the 3′ U5 region for subsequent cloning into pUC19 (Lund *et al.,* 1993). pPBS-B2 was generated by a similar procedure using primer 2 (5′-CCTGGGCGGGGGTCTCCAAATC**T**CGGACGAGCCCCCAAAT-3′, single nucleotide C-to-T mutation in bold) introducing the B2 PBS (Barklis *et al.,* 1986). For the construction of pPBS-Phe, pPBS-Lys1.2, and pPBS-Lys1.2(m), the PBS of pPBS-Pro was replaced by cloning of a 735-bp *Eco*RI–*Spe*I fragment amplified from ptvAkv*neo* using the previously described *Eco*RI-linker LTR primer 1 (5′-GGGAATTCTACCTTACGTTTCCCCGACCAGAGCTGATGTTCTCAG-3′) (Lund *et al.,* 1993) and a 180-nt-long oligodeoxynucleotide 3 (PBS sequence underlined) (5′-GGACTAGTACAGAATCAGACGCAGGCGCAAAAAGTAGATGCCGGCACACACACACACACGCACAAAGACAGAGACGGAGACAAAACGATCGCTGGCCAGCTTACCTCCCGACGGTGGGTCGGTGGTCCCTGGGCGGGGGTCTCCAA<u>TCCCGGGTTTCGGCACCA</u>AATGAAAGACCCCCAG-3′) introducing Phe PBS, 4 (5′-GGACTAGTACAGAATCAGACGCAGGCGCAAAAAGTAGATGCCGGCACACACACACACACGCACAAAGACAGAGACGGAGACAAAACGATCGCTGGCCAGCTTACCTCCCGACGGTGGGTCGGTGGTCCCTGGGCGGGGGTCTCCAAGCCCCACGTTGGGCGCCAAATGAAAGACCCCCAG-3′, single nucleotide C-to-T mutation in bold) containing the RNA cassette and recognition sites for *Eco*RI and *Hin*dIII (underlined), extended with Sequenase version 2.0 (Amersham Pharmacia Biotech) (Modin *et al.,* 2000), and cloned into pPUR (Clontech, Inc.) via *Eco*RI–*Hin*dIII digestion. ptRNA^{Pro} encodes 446 bp of the putative tRNA^{Pro} molecule obtained by PCR amplification of the murine Pro-tRNA gene (Russo *et al.,* 1986) using primers 8 (5′-CCG<u>CTGCAG</u>GTACCGTTGTCAATCATTAAATTTTGAACCT-3′) and 9 (5′-CCG<u>GAATTC</u>TCGAGAATTGGCCTTTTGGCTTGCG-3′) and cloned into pBluescript (Stratagene) via *Pst*I and *Eco*RI (underlined) digestion. All oligodeoxynucleotides were purchased from DNA Technology A/S (Aarhus, Denmark).

Cell cultures, transfection, and transduction

The human kidney-derived BOSC 23 packaging cell line (Pear *et al.,* 1993) was passaged three or four times in HAT-Dulbecco's modified Eagle's medium (DMEM) selective medium (Pear *et al.,* 1993) to ensure expression of the *env* gene. Subsequent maintenance was in DMEM supplemented with 10% fetal calf serum (FCS) (Gibco BRL; Life Technologies). The mouse embryonic carcinoma (EC) cell line F9 (Strickland, 1981; Bernstine *et al.,* 1983) was grown on 0.1% gelatin-coated culture flasks in DMEM supplemented with 10% FCS. NIH/3T3 mouse fibroblasts were grown in DMEM with 10% NCS. All cell culture media contained 100 U/ml penicillin and 100 μg/ml streptomycin. For transduction of target cells, BOSC 23, seeded at 7×10^4 cells/cm^2 on the day before transfection, were transfected by the calcium phosphate

method (Graham and van der Eb, 1973) without a glycerol shock with 1 μg of plasmid vector and either 9 μg of plasmid encoding synthetic tRNA or 9 μg of carrier pUC19. Medium was renewed after 12–16 h, and virus supernatant was harvested 48–72 h posttransfection, filtered through a sterile 0.45-μm filter, and transferred serially diluted to F9 and NIH/3T3 target cells [seeded at (F9) 3×10^3 and (NIH/3T3) 10^4 cells/cm^2 on the day before transduction] in the presence of 5 and 6 μg/ml Polybrene (Aldrich Chemical Co. Inc.), respectively. G418-containing (Sigma, St. Louis, MO) selective media were added 24 h posttransduction at (active compound) 600 μg/ml for fibroblasts and at 400 μg/ml for F9 cells, and resistant colonies were counted after 10–14 days of selection.

DNA preparation and primer extension analysis

For primer extension analysis of transduced vector proviruses in NIH/3T3 and F9 cells, 59–170 G418-resistant colonies were lysed with DNAzol in the culture dishes, and genomic DNA was isolated according to instructions provided by the manufacturer (Molecular Research Center, Inc., Cincinnati, OH); for PBS-Pro and PBS-Lys1.2(m), 10 and 33 G418-resistant colonies, respectively, were pooled and expanded before DNA isolation. Specific PCR amplification of integrated vector proviruses was achieved using primer 10 (5′-TTCATAAGGCTTAGCCAGCTAACTGCAG-3′) matching Akv MLV position 7838–7865, which is reconstituted in the 5′ LTR after transduction of the vectors, and a *neo*-specific primer 11 (5′-XGGCGCCCCTGCGCTGACAGCCGGAACAC-3′, with X denoting biotin). PCR amplification, performed on 1 μg of DNA template in a standard reaction buffer with 2.5 U of AmpliTaq Gold (Perkin–Elmer Cetus), was for 10 min at 95°C to activate the enzyme, followed by 1 min at 94°C, 1 min at 60°C, and 3 min at 73°C in 40 cycles. Then 70 μl of PCR amplified product was purified with Dynabeads (Dynal M-280) and denatured in NaOH, and the biotinylated strand was used in primer extension analysis with modified T7 DNA polymerase (Sequenase version 2.0, Amersham Pharmacia Biotech) essentially as described (Modin *et al.,* 2000a). Briefly, an 18-mer extension primer 12 (5′-TTTCATTTGGGGGCTCGT-3′) for PBS-Pro and PBS-B2, 13-mer (5′-GGGGGTCTTTCATTTGGT-3′) for PBS-Phe, and 14-mer (5′-GGGGGTCTTTCATTTGGC-3′) for PBS-Lys1.2 was 5′ labeled using [γ-^{32}P]dATP and T4 polynucleotide kinase and annealed to the respective template in 10 μl of the reaction buffer supplied with the Sequenase enzyme by heating to 65°C for 2 min and slowly cooling to room temperature. Extension was carried out for 10 min at 37°C after the addition of 5 μl of extension mix containing 3.25 U of modified T7 DNA polymerase and for PBS-Pro and PBS-B2, 1 mM ddATP and 0.1 mM concentration each of dCTP, dGTP, and dTTP, and for PBS-Phe and PBS-Lys1.2(m), 1 mM ddTTP and 0.1 mM concentration each of dCTP, dGTP, and dATP. Reactions were terminated by the addition of 10 μl of formamide loading buffer (80% formamide, 1 mM EDTA, 0.1% xylene cyanol FF, 0.1% bromphenol blue in 1× TBE buffer) and heated to 95°C for 2 min, and the samples were analyzed on 20% polyacrylamide gels and exposed in a Personal Molecular Imager Fx.

ACKNOWLEDGMENTS

The technical assistance of Lene Svinth is gratefully acknowledged. A.S. is supported by a grant from the Deutscher Akademischer Austauschdienst. This work was supported by contracts CT 95-0100 (Biotechnology) and CT 95-0675 (Biomed 2) of the European Commission, the Karen Elise Jensen Foundation, the Danish Cancer Society, the Danish Biotechnology Programme, the Danish Natural Sciences, and Medical Research Councils.

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