JOURNAL OF VIROLOGY, Feb. 1995, p. 748–755
0022-538X/95/$04.00+0

Vol. 69, No. 2

Multiple Modifications in *cis* Elements of the Long Terminal Repeat of Retroviral Vectors Lead to Increased Expression and Decreased DNA Methylation in Embryonic Carcinoma Cells

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Received 1 August 1994/Accepted 24 October 1994

Infection by murine retroviruses in embryonic carcinoma (EC) and embryonic stem cells is highly restricted. The transcriptional unit of the Moloney murine leukemic virus (MoMuLV) long terminal repeat (LTR) is inactive in EC and embryonic stem cells in association with increased proviral methylation. In this study, expression in F9 EC cells was achieved from novel retroviral vectors containing three modifications in the MoMuLV-based retroviral vector: presence of the myeloproliferative sarcoma virus LTR, substitution of the primer binding site, and either deletion of a negative control region at the 5′ end of the LTR or insertion of a demethylating sequence. We conclude that inhibition of expression from the MoMuLV LTR in EC cells is mediated through the additive effects of multiple *cis*-acting elements affecting the state of methylation of the provirus.

Retroviral vectors which are transcriptionally active in embryonic cells could have a wide range of applications. Because of their high efficiencies of transduction and integration, retroviral vectors are valuable tools for the generation of transgenic mice and embryonic stem (ES) cell chimeras and in cell marking studies during embryonic development (6, 20). Moloney murine leukemia virus (MoMuLV)-based retroviral vectors are among the most commonly used retroviral vectors for gene transfer purposes. However, expression from the transcriptional unit of the MoMuLV long terminal repeat (LTR) has been shown to be inhibited in ES cells and in embryonic carcinoma (EC) cells.

Several mechanisms have been implicated in the transcriptional silencing of the MoMuLV LTR. First, the enhancer repeat unit of the MoMuLV LTR was demonstrated to be nonfunctional in embryonic cells (23, 40). The inactivity of the enhancer is mediated by its interaction with negatively acting cellular factors (1, 16, 28, 41, 42). A variant LTR was isolated from the myeloproliferative sarcoma virus (MPSV) (8) and shown to express more strongly than the MoMuLV LTR in EC cells (17, 19, 23, 42) and in hematopoietic cells (3, 29, 36). The fundamental difference between the MoMuLV and the MPSV enhancer repeats is the presence in MPSV of a consensus site for binding the transcription factor Sp1 (31).

Second, negatively acting *cis* elements have been characterized in the MoMuLV sequences. One such element, located at the 5′ end of the LTR, is a conserved sequence present in over 90% of mammalian type C retroviruses and is referred to as the negative control region (NCR) (14). The NCR sequence has been shown to bind a cellular factor, thereby mediating transcriptional repression (13). Another well-defined inhibitory element is located at the primer binding site (PBS) of the MoMuLV leader region (12, 24, 39, 42). The sequence from the MoMuLV PBS acts by binding a cellular factor which inhibits RNA transcription (22, 25, 30). A specific mutation converting bp +160 in the PBS from guanine to adenine prevents binding of the inhibitory protein and abrogates the negative effects of the PBS on transcription (22, 30, 42). An endogenous murine retrovirus, *dl*587rev, was isolated from murine genomic sequences and found to contain a novel PBS sequence which includes adenine at position +160 (10). Inclusion of the *dl*587rev PBS in retroviruses allowed increased expression in EC cells compared with the wild-type MoMuLV PBS (1, 17).

Third, extensive de novo methylation of cytosine residues in the proviral MoMuLV LTR has been detected in embryonic cell lines (28, 35). Despite the fact that the causal role of methylation in mediating repression of gene expression is still ambiguous, methylation has been associated with the block in transcription of many different genes (4, 5).

Efforts have been made to produce retroviral vectors which overcome the inherent inactivity of the MoMuLV transcriptional unit in embryonic cells. One approach has been to produce vectors with an internal promoter to mediate gene transcription in ES cells, while using the retroviral LTR only for producing full-length viral genome in the packaging cell (2, 18, 34). However, there is evidence that the MoMuLV enhancer and PBS may have negative effects even on heterologous promoters placed internally (2, 16). Another approach involves incorporation of various sequence elements which are active in ES cells, to replace the MoMuLV sequences, such as the enhancer from the MPSV variant (19) or from a mutant polyomavirus (23), or the PBS from *dl*587rev (17, 42). These attempts had limited success, perhaps because of the interaction of multiple inhibitory elements in or near the LTR. Consequently, single modifications might not be sufficient to completely overcome the barrier to transcriptional activity.

In this report, we describe the construction of a series of retroviral vectors containing modifications of the MoMuLV transcriptional unit. The modifications included (i) substitution of the MoMuLV enhancer to the one from MPSV, (ii) deletion of the NCR, (iii) substitution of the MoMuLV PBS with the

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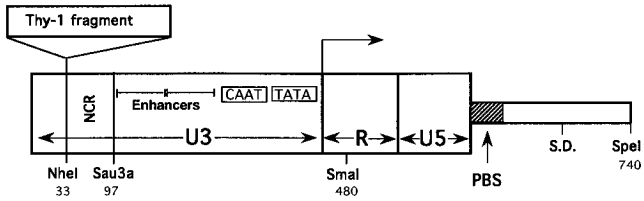

FIG. 1. Schematic diagram depicting the locations of the modifications introduced in the LTR and leader regions of the retroviral vectors. The Thy-1 fragment was inserted at the *Nhe*I site. The NCR was deleted from the *Nhe*I to *Sau*3a sites. The MPSV LTR differs from the MoMuLV LTR in the enhancer repeats. The location of the PBS is also shown. S.D., splice donor.

PBS of the *dl*587rev strain, and (iv) insertion of a demethylating fragment cloned from the 5′ upstream region of the murine Thy-1 gene. The latter sequence has been reported to inhibit de novo methylation of adjacent heterologous sequences in a plasmid transfected into ES cells (38) and in a retroviral vector transduced into ES cells (7a). To assess the specific contribution of each of the MoMuLV sequences in vector expression, the alterations were introduced into a MoMuLV-based retroviral vector, either singly or in multiple combinations. The modified retroviral vectors were analyzed for gene transfer, gene expression, and proviral methylation status in F9 EC cells.

We observed increased gene expression in EC cells in association with decreased methylation of vectors containing three modifications compared with the standard MoMuLV-based vector. These results suggest that multiple elements of the MoMuLV sequences act simultaneously to inhibit transcription in embryonic cells.

MATERIALS AND METHODS

Retroviral vectors. The MPSV 3′ LTR was provided by W. Ostertag (Heinrich-Pette Institute, Hamburg, Germany), the *dl*587rev 5′ LTR and leader region were provided by S. Goff (Columbia University, New York, N.Y.), the Thy-1 fragment in Bluescript plasmid was provided by M. Szyf (McGill University, Montreal, Quebec, Canada), and the G1Na plasmid was provided by P. Tolstechev (Genetic Therapy, Inc., Gaithersburg, Md.). The LN vector was constructed and packaged in the laboratory of A. Dusty Miller (Fred Hutchinson Cancer Center, Seattle, Wash.).

The MPSV LTR was used to replace the 3′ MoMuLV LTR of G1Na to make *MPneo*. The NCR was removed from the MPSV LTR as an *Nhe*I (at nucleotide 33 in the LTR)-to-Sau3a (at nucleotide 97 in the LTR) fragment. The cut ends of the LTR were ligated together after fill-in by Klenow DNA polymerase to make the MPncr 3′ LTR; this was then used to replace the 3′ LTR of G1Na, yielding *MPncrneo*. The Thy-1 fragment in the plasmid Bluescript was opened at the *Sma*I site immediately 3′ of the insert, and a synthetic oligonucleotide encoding an *Xba*I site (New England Biolabs, Beverly, Mass.) was ligated in place. The Thy-1 piece was isolated as an *Xba*I-*Xba*I fragment and cloned into the *Nhe*I site of the MPSV LTR in Bluescript. The Thy-1-substituted MPSV LTR was inserted in place of the 3′ LTR in G1Na to make *MPthyneo.*

The 5′ LTR and psi region from plasmid LN was subcloned as an *Eco*RI-*Eco*RI fragment. The *Kpn*I-*Spe*I fragment encompassing the PBS was removed and replaced by the *Kpn*I-*Spe*I fragment from *dl*587rev. The 5′ LTR/leader region fragment containing the *dl*587rev PBS was then returned to the MPneo, MPthyneo, and MPncrneo plasmids to produce *MPdlneo*, *MPthydlneo*, and *MPncrdlneo.*

To make *LNncrneo*, the NCR (*Nhe*I at position 33 to *Sau*3a at position 97; Fig. 1) was deleted from the 3′ LTR of G1Na. Specifically, a portion of the 3′ LTR from G1Na was subcloned into Bluescript as a *Cla*I-*Sst*I fragment. Then, PCR was performed with primers overlapping the *Sau*3a site (bp 97), 5′-GAC C**GCTAGC**AGATCTAGGTCAGG-3′ (sense), and the *Sst*1 site (bp 413), 5′-CTGGAGCTCGGGGAGCAGA-3′ (antisense). The sequence of the sense primer included a 5′ overhang which contains the recognition site for NheI (boldfaced in the primer sequence), followed by sequences overlapping the *Sau*3a site converting it to a *Bgl*II site (underlined in the primer sequence). The 320-bp PCR product, which lacks the NCR, was digested with *Nhe*I and *Sst*I and used to replace the 380-bp *Nhe*I-to-*Sst*I fragment of the 3′ LTR, effectively removing the NCR and adding a novel *Bgl*II site. The *Cla*I-*Xba*I fragment containing the NCR deletion in Bluescript was used to replace the *Cla*I-*Xba*I portion of the 3′ LTR in G1Na plasmid, producing LNncrneo.

The vector plasmids MPneo, MPdlneo, MPncrdlneo, LNncrneo, MPthyneo, and MPthydlneo were transfected into the ecotropic packaging cell line GP+E-86 by using transfection reagent (DOTAP; Boehringer Mannheim Corp., Indianapolis, Ind.) and selected in 0.5 mg of active G418 (Geneticin; GIBCO-BRL, Bethesda, Md.) per ml. Culture supernatant was collected and used to transduce the PA317 amphotropic packaging cell line. Subsequently, the PA317 cells were selected in G418, and cell clones were isolated. Supernatant from the clones was titered by serial dilution on NIH 3T3 fibroblasts. High-titer clones were derived from the PA317 pools of MPneo, MPdlneo, and MPncrdlneo, whereas high-titer pools of MPthyneo, MPthydlneo, and LNncrneo were used in subsequent analyses.

Cell lines. PA317, NIH 3T3, and F9 cell lines were obtained from the American Type Culture Collection. GP+E-86 cells were a generous gift from A. Bank (Columbia University, New York, N.Y.). The F9 cell line was grown on gelatin-coated tissue culture plates in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal calf serum (FCS). NIH 3T3 cells were grown in DMEM supplemented with 10% calf serum. The PA317 cells were grown in DMEM containing 10% FCS. GP+E-86 cells were grown under selection pressure in DMEM supplemented with 10% newborn calf serum, hypoxanthine (15 μg/ml), xanthine (250 μg/ml), and mycophenolic acid (25 μg/ml) (26). To prepare viral supernatant, the medium was changed to DMEM supplemented with 10% FCS.

Vector titering on F9 and 3T3 cells. Viral supernatants in DMEM containing 10% FCS were harvested from confluent 100-mm-diameter tissue culture plates of vector producing PA317 fibroblasts and passed through 0.45-μm-pore-size filters. They were serially diluted 10-fold in a total volume of 5 ml. The dilutions were as follows: undiluted, 1:10, 1:100, 1:1,000, 1:10,000, and no virus. Two milliliters of each dilution was overlaid on each of F9 cells and 3T3 cells plated 24 hours earlier at 2.5×10^4 cells in six-well tissue culture dishes. Transduction was performed in the presence of 8 μg of Polybrene per ml for 2 h. Then, the cells were washed in phosphate-buffered saline and cultured in their respective media. Twenty-four hours later, G418 (GIBCO-BRL) was added at 0.5 mg/ml. Selection was carried out for 12 to 14 days, until no cells were seen in the nontransduced wells and visible colonies were formed in the transduced wells. Then, the cells were washed in phosphate-buffered saline and stained with 0.5% crystal violet in methanol. G418-resistant ($G418^r$) colonies were counted and the $G418^r$ CFU per milliliter of viral suspension was calculated. The relative ability of the different vectors to express in F9 cells was quantitated by dividing the effective titer ($G418^r$ CFU per milliliter) on F9 cells by the titer ($G418^r$ CFU per milliliter) on 3T3 cells, therefore accounting for differences in the number of infectious viral particles among preparations.

Stable retroviral transductions. F9 and 3T3 cells were transduced, in parallel, with four rounds of exposure to viral supernatant for 3 h each in six-well plates. Then, the cells were cultured for 2 weeks, and cell pellets were prepared for DNA and RNA extractions.

DNA and RNA analyses. Genomic DNA and total cellular RNA were extracted from the retrovirally transduced F9 and 3T3 cell pellets for Southern and Northern (RNA) blot analyses. DNA was isolated by sodium dodecyl sulfate-proteinase K and RNase digestion at 55°C for 3 to 4 h. The digested samples were extracted with phenol-chloroform; the DNA was precipitated in ethanol and resuspended in Tris-EDTA buffer (32). A quantitative Southern blot was performed to measure proviral copy number. Control and sample DNAs (10 μg) were digested with the restriction enzyme *Sst*I (GIBCO-BRL), which cuts once in each LTR, releasing the full-length proviral sequence. To derive a standard curve, DNA from a PA317 clone containing a single copy of the *neo* gene was diluted with DNA from the parental PA317 cells; the dilutions were as follows: 100, 50, 10, 5, and 0%. The digested DNAs were electrophoresed on a 1.3% agarose gel, denatured, and blotted onto a nylon membrane. Then, the filter was probed with ^{32}P-labeled neomycin resistance (*neo*) DNA (11) and used to expose Kodak X-Omat films (Eastman Kodak, Rochester, N.Y.) at −70°C. After satisfactory exposures were obtained, the membrane was stripped and rehybridized with the 1.6-kb human glucocerebrosidase cDNA probe to permit quantitation of differences in DNA loading. Again, the filter was analyzed by autoradiography. Densitometric analysis was performed on the Southern blot, using a U.S. Biochemical (Cleveland, Ohio) SciScan 5000.

RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform method described previously (9). RNA (15 μg) was electrophoresed on a 1.2% formaldehyde gel, denatured, neutralized, and transferred to a nylon membrane by capillary blotting. The filter was hybridized with the ^{32}P-labeled *neo* DNA probe and used to expose X-ray films at −70°C. The filter was analyzed with a Betascope 603 blot analyzer (Betagen, Waltham, Mass.) to quantitate the level of RNA. Then, the filter was stripped, rehybridized with the mouse β-actin DNA probe, and again analyzed by autoradiography.

Methylation analysis. Genomic DNAs (15 μg) were digested with the restriction enzyme *Bam*HI (New England Biolabs) to reduce the size of the high-molecular-weight DNA and then digested with *Eco*RV (GIBCO-BRL). Then, half of each DNA sample was digested with the methylation-sensitive restriction enzyme *Sma*I (New England Biolabs). To monitor completeness of the enzyme digestions, a sample (20 μl) of the digestion mixture was mixed with lambda DNA (GIBCO-BRL) for *Eco*RV digestion and adenovirus type 2 DNA (GIBCO-BRL) for *Sma*I digestion. The mixtures were incubated in parallel with the main samples at 37°C and subsequently analyzed on an agarose gel. *Eco*RV- and

TABLE 1. Modified retroviral vectors

Vector name	Enhancer	PBS	NCR	Thy-1
LN	MoMuLV	MoMuLV	Present	No
MPneo	MPSV	MoMuLV	Present	No
MPdlneo	MPSV	*dl*587rev	Present	No
LNncrneo	MoMuLV	MoMuLV	Absent	No
MPncrdlneo	MPSV	*dl*587rev	Absent	No
MPthyneo	MPSV	MoMuLV	Present	Yes
MPthydlneo	MPSV	*dl*587rev	Present	Yes

*Eco*RV-*Sma*I-digested genomic DNAs were electrophoresed on a 1.5% agarose gel, denatured, and blotted to a nylon membrane. Blots were hybridized with a 285-bp probe from the 5′ end of the *neo* gene at the *Not*I site to the *Pvu*II site in the G1Na plasmid. Several exposures of the blot were obtained on X-ray films. The autoradiograms were analyzed on a U.S. Biochemical SciScan 5000, measuring the relative intensities of the *Sma*I-sensitive and *Sma*I-resistant bands.

Sequencing of the proviral 5′ LTR in F9 cells. The 5′ LTR and leader regions of the proviruses were sequenced, first in the PA317 cells and then in the F9 cells, to ensure correct duplication and maintenance of all modifications after packaging and serial transduction. The proviral sequences were amplified by PCR of genomic DNA, using primers to the 5′ end of the LTR (5′-GACCCCACCTG TACGTATGGCAA-3′, sense) and 5′ end of the *neo* gene (5′-GCTGGCCAG GTTAACTCCC-3′, antisense). The 1.7- to 1.9-kb PCR products were purified by electrophoresis on a 1.2% agarose gel and extracted by using the Qiaex gel extraction kit (Qiagen Inc., Chatsworth, Calif.). Then, sequencing was performed with the CircumVent thermal cycle sequencing kit (New England Biolabs) by internal labeling with [^{35}S]dATP and cycle sequencing. For sequencing the enhancer region, the primer at the 5′ end of the LTR (described above) was used; for the PBS region, oligonucleotides from the splice donor site (5′-GCTGGC CAGGTTAACTCCC-3′, antisense) and the R/U5 region (5′-TGCATC CGAATCGTGGTCTC-3′, sense) were used. A primer from the Thy-1 sequence (5′-TCGGGGTGGAGCAGTCTTCT-3′, sense) allowed sequencing of the enhancer region of the two vectors containing the Thy-1 fragment.

RESULTS

Construction of the vectors. To study the involvement of specific sequences in repression of MoMuLV LTR transcription in EC cells, six novel vectors were designed. The vectors contain modifications in the *cis* elements of the parental Mo MuLV-based vector. Each modification was targeted to alleviate a potential site of repression. The diagram in Fig. 1 illustrates the locations of the different elements being studied. The modified LTR in the retroviral vectors promoted expression of the bacterial *neo* gene, which was used as a reporter gene for subsequent analyses. Table 1 describes the features of each of the modified vectors. LN is a classical MoMuLV-based retroviral vector (27) which is transcriptionally inactive in EC cells and was included in the experiments as a baseline control for vector expression. All vectors used except for LNncrneo contained the MPSV LTR enhancer instead of the MoMuLV LTR enhancer. The NCR was defined as the sequence from the *Nhe*I site to the *Sau*3a site at the 5′ end of the LTR. This sequence was deleted in the LNncrneo and MPncrdlneo vectors. MPdlneo, MPncrdlneo, and MPthydlneo vectors contained substitution of the MoMuLV PBS by the *dl*587rev PBS ("dl" in vector names). The Thy-1 demethylating fragment ("thy" in vector names) was inserted at the *Nhe*I site located at the 5′ end of the LTR in the MPthyneo and MPthydlneo vectors.

The modifications of the U3 regions (substitution of the enhancer, deletion of the ncr, and addition of the Thy-1 fragment) were made in the 3′ LTR of the vector plasmids. The vector plasmids were transfected into GP+E-86 ecotropic cells and cross-transduced into PA317 amphotropic cells, and clones producing high-titer amphotropic virus were derived. Upon reverse transcription of vector genomic RNA into proviral DNA, the U3 region of the 3′ LTR is incorporated into the 5′ LTR. To verify that this process occurred correctly, the 5′ LTR and leader regions of each vector provirus in the PA317 packaging cells were amplified by PCR and subjected to sequence analysis. The intended modifications of the LTR and PBS sequences were present for each vector.

Assay for transfer of G418 resistance to F9 cells. We characterized the ability of the modified retroviral vectors to express the *neo* gene in EC cells and to confer G418 resistance to the transduced cells. Serial dilutions of the retroviral vectors

TABLE 2. Titering assay for transfer of neomycin resistance to F9 cells[a]

Vector	Cells	Neo^r CFU/ml of viral preparation						
		Expt 1	Expt 2	Expt 3	Expt 4	Expt 5	Expt 6	Avg
LN	F9	9.0×10^4	0.75×10^4	0.05×10^4	0.2×10^4	1.3×10^4	0.02×10^4	
	3T3	50.0×10^5	10.0×10^5	1.6×10^5	8.8×10^5	8.0×10^5	0.85×10^5	
	F9/3T3	0.0180	0.0075	0.0031	0.0023	0.0162	0.0023	0.0082
MPneo	F9	2.9×10^4	0.65×10^4	0.65×10^4	0.2×10^4	3.8×10^4	0.08×10^4	
	3T3	4.0×10^5	1.8×10^5	1.5×10^5	3.2×10^5	9.5×10^5	0.63×10^5	
	F9/3T3	0.0725	0.0361	0.0433	0.0069	0.0400	0.0127	0.0352
MPdlneo	F9	2.5×10^4	1.3×10^4	0.2×10^4	0.4×10^4	1.5×10^4	0.02×10^4	
	3T3	10.0×10^5	4.0×10^5	1.5×10^5	2.1×10^5	2.9×10^5	0.36×10^5	
	F9/3T3	0.0250	0.0325	0.0133	0.0191	0.0517	0.0056	0.0245
LNncrneo	F9			0.12×10^4	0.2×10^4	1.0×10^4	0.01×10^4	
	3T3			0.75×10^5	2.7×10^5	5.0×10^5	0.31×10^5	
	F9/3T3			0.0160	0.0078	0.0200	0.0032	0.0118
MPncrdlneo	F9	5.7×10^4	10.0×10^4	1.75×10^4	12.0×10^4	69.0×10^4	0.7×10^4	
	3T3	3.2×10^5	3.5×10^5	2.3×10^5	3.5×10^5	4.0×10^5	0.5×10^5	
	F9/3T3	0.1781	0.2857	0.0761	0.3429	1.7250	0.1400	0.4346
MPthyneo	F9	9.0×10^4		0.12×10^4	0.18×10^4	2.3×10^4	0.02×10^4	
	3T3	23.0×10^5		0.65×10^5	1.15×10^5	3.5×10^5	0.18×10^5	
	F9/3T3	0.0391		0.0185	0.0157	0.0657	0.0111	0.0300
MPthydlneo	F9	1.8×10^4	1.0×10^4	0.4×10^4	1.45×10^4	14.0×10^4	0.32×10^4	
	3T3	2.0×10^5	2.0×10^5	0.75×10^5	0.9×10^5	3.0×10^5	0.13×10^5	
	F9/3T3	0.090	0.050	0.0533	0.1611	0.4667	0.2462	0.1779

[a] Abilities of the modified retroviral vectors to express in the F9 EC cell line were assayed by counting the number of Neo^r CFU per milliliter formed after transduction of F9 and NIH 3T3 cells with serial dilutions of viral preparations.

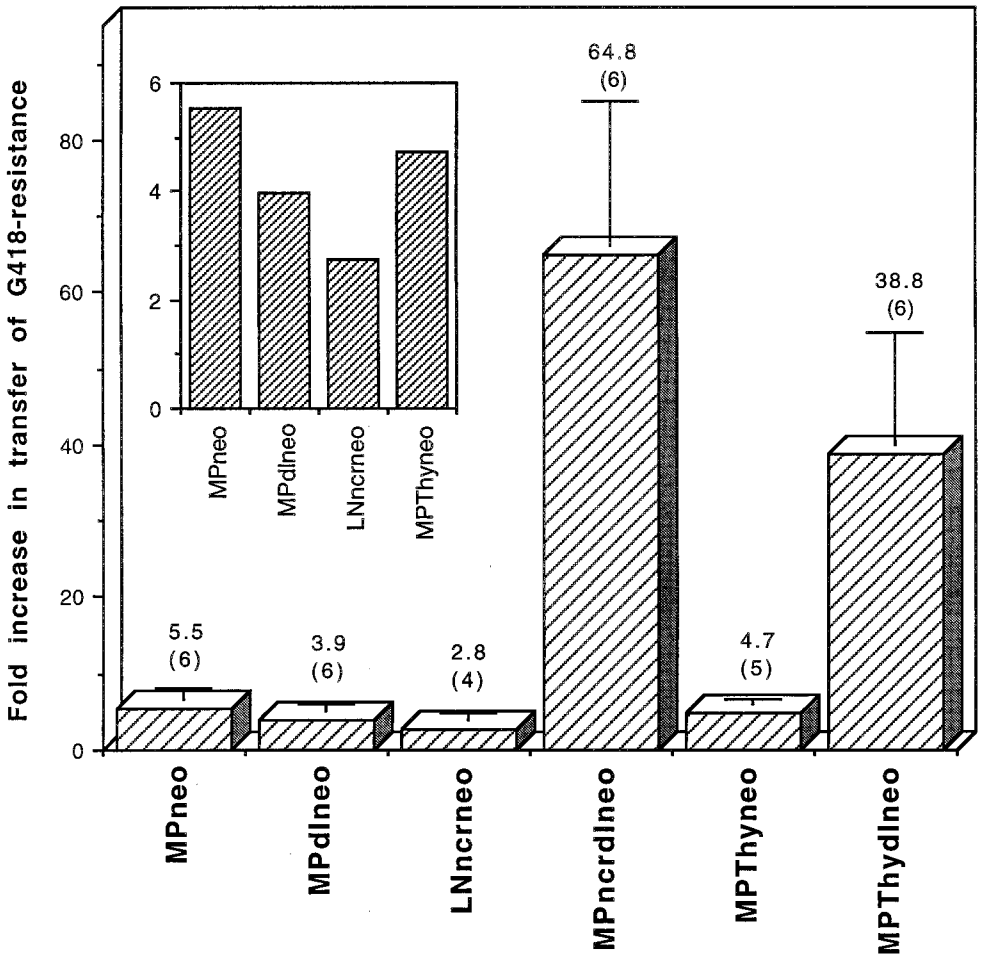

FIG. 2. Fold increases in the transfer of G418^r resistance by the modified vectors onto F9 cells assayed by titering. The ratio of F9 to NIH 3T3 G418^r CFU per milliliter of each vector from Table 1 was divided by the ratio calculated for the MoMuLV-based vector LN from the same experiment. The numbers in parentheses correspond to the numbers of experiments performed. Error bars depict the calculated standard errors of the means. The insert represents magnification of the values from the four vectors MPneo, MPdlneo, LNncrneo, and MPthyneo, which show lower fold increases in transfer of G418^r resistance.

were used to transduce, in parallel, the F9 EC cell line and the NIH 3T3 fibroblast cell line. The numbers of G418^r CFU per milliliter were scored. Table 2 summarizes the data acquired from six separate experiments. The results obtained on NIH 3T3 fibroblasts were used to normalize the results obtained with F9 cells for variations occurring in viral preparations and differences in the titer of the various packaging cell lines.

As expected, the standard MoMuLV-based vector, LN, showed restricted activity on F9 EC cells compared with NIH 3T3 fibroblasts (Table 2). LN was able to transfer G418 resistance to F9 cells only 1/120 (or 0.0082) as efficiently as to NIH 3T3 cells. The presence of the MPSV enhancer instead of the MoMuLV enhancer (MPneo) resulted in a slight increase in the relative number of G418^r CFU per milliliter formed on F9 cells (0.0352). Substitution of the MoMuLV PBS with the *dl*587rev PBS (MPdlneo) did not further increase the effective titer on F9 cells (0.0245). However, the MPSV-based vector containing deletion of the NCR in addition to the *dl*587rev PBS (MPncrdlneo) was able to transfer G418 resistance to F9 cells almost half as efficiently as to NIH 3T3 fibroblasts (0.4346). On the other hand, deletion of the NCR alone from the MoMuLV LTR (LNncrneo) did not have any significant effect on titer over the MoMuLV-based control vector LN (0.0118).

Insertion of the demethylating Thy-1 sequence at the 5′ end of the LTR (MPthyneo) had no notable effect on infectivity of F9 cells. Nevertheless, presence of the Thy-1 fragment in addition to the *dl*587rev PBS resulted in a vector (MPthydlneo) that transferred G418 resistance to F9 cells one-fifth as efficiently as to NIH 3T3 fibroblasts.

Figure 2 illustrates the results described in Table 2. The bars represent the ratio of number of G418^r CFU per milliliter on F9 cells of each modified vector normalized for titer on NIH 3T3 cells (F9/3T3 ratio of modified vector), divided by the baseline number of G418^r CFU per milliliter of the LN vector (F9/3T3 ratio of LN). Therefore, the graph depicts the fold increase in G418 resistance conferred by the six modified vectors compared with the standard MoMuLV-based vector LN. Although the MPSV enhancer alone, or along with either substitution of the PBS or insertion of the Thy-1 fragment, resulted in a slight increase in expression over LN (5.5-, 3.9-, or 4.7-fold increase, respectively), the two vectors containing three modifications, MPncrdlneo and MPthydlneo, resulted in mean increases in activity of 64.8- and 38.8-fold over LN, respectively.

Stable transduction into F9 cells and transcription analysis. The differences in the transfer of G418 resistance of each vector in F9 cells assayed by titering could be due to reasons

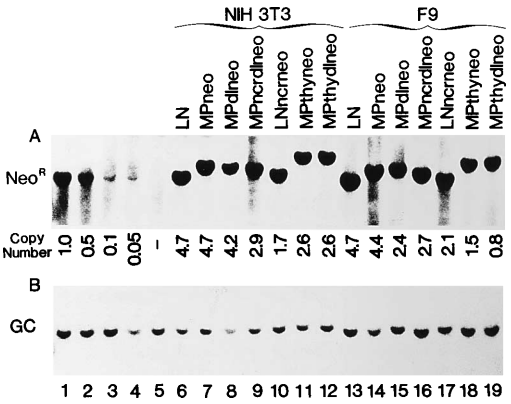

FIG. 3. Quantitative Southern blot analysis of gene transfer efficiency by the retroviral vectors into NIH 3T3 and F9 cells. Stably transduced NIH 3T3 and F9 cell lines were obtained after four rounds of transduction. Genomic DNA was isolated and digested with the restriction enzyme *Sst*I, which cuts once in each of the 5′ and 3′ LTRs. The blot was probed with the *neo* gene (A) and with the human glucocerebrosidase (GC) cDNA as an internal control for loading (B). Lanes 1 to 4 are serial dilutions (1, 0.5, 0.1, and 0.05 copy per cell) of DNA extracted from a clonal PA317 cell line containing a single copy of the *neo* gene. Differences in the sizes of the proviruses are due both to differences in size between regions upstream of the 3′ LTR of G1Na and MPSV and to the modifications incorporated in the vectors. The films were analyzed by densitometry, and the values obtained with the *neo* probe in panel A were normalized for loading differences by dividing with the values obtained from analyzing panel B. A standard curve was plotted, and the copy number values were derived by linear regression.

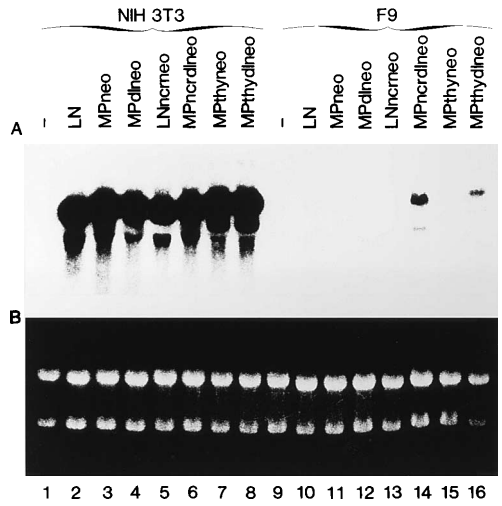

FIG. 4. Northern blot analysis of the stably transduced NIH 3T3 and F9 cells. (A) Northern blot hybridized with ^{32}P-labeled 1.3-kb *neo* DNA. (B) Ethidium bromide-stained picture of the gel before transfer and hybridization.

other than increase in RNA transcription, such as variations in the gene transfer efficiencies of the different vectors. To assess the possible association of increased G418 resistance conferred by the modified vectors with increased RNA transcription, we stably transduced F9 and NIH 3T3 cells with the various vectors and established transduced cell pools without G418 selection. DNA and RNA were extracted from the transduced cells for gene transfer and RNA transcription analyses. The 5′ LTR/U3 and leader regions of the six novel retroviral vectors in the transduced F9 pools were sequenced. Preservation of the correct modifications after transduction and expansion of the cells was confirmed.

Gene transfer by the retroviral vectors was assessed by performing a quantitative Southern blot analysis of the transduced cells (Fig. 3). Genomic DNA was digested with *Sst*I, which cuts once in each LTR. The proviral DNA was detected with ^{32}P-labeled *neo* DNA probe. Subsequently, the same blot was stripped and rehybridized with an endogenous marker, the glucocerebrosidase cDNA, to assess DNA loading. The Southern blot included a standard curve for copy number made by serially diluting genomic DNA extracted from a PA317 cell line containing one copy of the LN provirus per cell with DNA from the parental PA317 cell line (Fig. 3, lanes 1 to 5). A copy number standard curve was plotted by measuring the intensity of the provirus *neo* signal by densitometry and normalizing the results to the glucocerebrosidase endogenous marker, thereby accounting for differences in loading between DNA samples. The provirus copy number in the transduced NIH 3T3 cells (Fig. 3, lanes 6 to 12) and F9 cells (Fig. 3, lanes 13 to 19) was derived from the standard curve. Similar transduction efficiencies were achieved by the different vectors in the F9 EC cells and in the NIH 3T3 fibroblast cells, with limited experimental variations. Thus, the differences in effective titer are not due to variable gene transfer.

Northern blot analysis of proviral transcription in the transduced NIH 3T3 and F9 cells is represented in Fig. 4. All seven vectors expressed at similar levels in the NIH 3T3 fibroblasts (Fig. 4A, lanes 1 to 8). However, no RNA transcripts in the F9 cells were detected from the LN, MPneo, MPdlneo, LNncrneo, and MPthyneo vectors (Fig. 4A, lanes 10 to 13 and 15). On the other hand, the two vectors containing three modifications, MPncrdlneo and MPthydlneo, promoted detectable levels of RNA transcription in F9 cells (Fig. 4A, lanes 14 and 16).

To confirm the quality of the RNA samples, the same Northern blot was stripped and rehybridized with the mouse β-actin cDNA (data not shown). We observed that the levels of β-actin RNA transcripts were consistently lower in F9 cells than in NIH 3T3 cells and could not be used as an internal standard between the two cell lines. Therefore, the ethidium bromide staining of the gel before transfer and hybridization is shown instead (Fig. 4B). The levels of Neo^r transcripts from each of the proviral vectors in NIH 3T3 and F9 cells were quantitated by measuring ^{32}P emission, using a Betascope 603 blot analyzer. After normalization of values for the relative copy number of vector provirus per cell among all cell lines, transcription by MPncrdlneo and MPthydlneo in F9 cells was calculated to be 2 to 5% of the level seen in NIH 3T3 cells.

Methylation analysis. Since methylation is associated with the inactivity of the MoMuLV LTR in EC cells (35), we assessed the effects of the modifications introduced into the MoMuLV-based retroviral vectors on the state of methylation of the provirus in the stably transduced cells. The mouse genomic DNA contains a high copy number of endogenous retrovirus sequences; therefore, we were limited to a protocol which allowed analysis of the *Sma*I site located 30 nucleotides downstream of the transcriptional start site in the 5′ LTR (Fig. 5A). Genomic DNA, extracted from the stably transduced NIH 3T3 and F9 cells (the cell lines derived for the analysis of proviral RNA transcription), was digested with *Eco*RV, which cuts within the enhancer repeats and 280 bp downstream of the 5′ end of the *neo* gene, releasing a 2.0-kb provirus-specific fragment on Southern blots. Half of each of the *Eco*RV-treated samples was digested with the methylation-sensitive enzyme *Sma*I. A 285-bp DNA probe from the 5′ end of the *neo* gene was used to hybridize the resulting Southern blot. If the CpG sequence of the *Sma*I site is not methylated, there is reduction in size of the detected fragment from 2.0 to 1.7 kb;

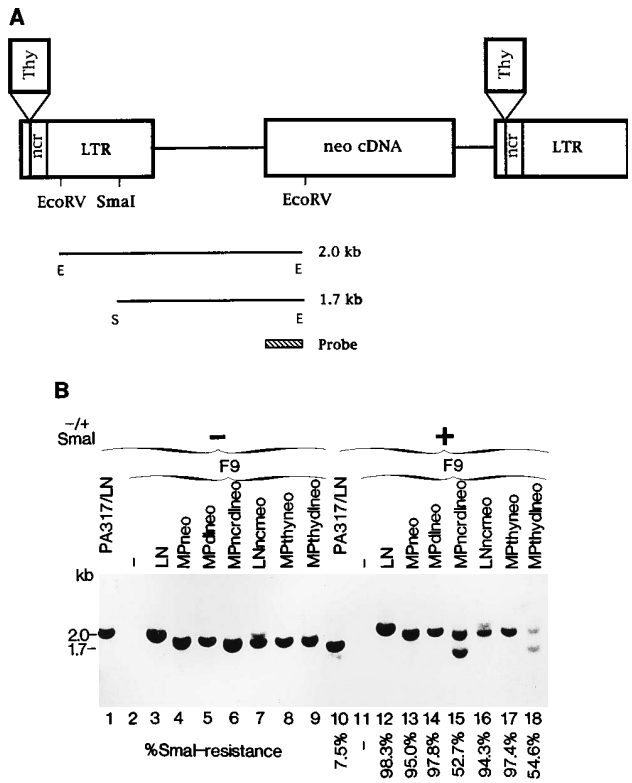

FIG. 5. Methylation analysis of the retroviral vectors in F9 cells by Southern blotting. (A) Schema of the modified retroviral vectors showing the locations of the restriction enzyme sites and the probe used for the methylation analysis. (B) Genomic DNAs from stably transduced F9 cells were digested with *Bam*HI and either *Eco*RV alone (lanes 1 to 9) or *Eco*RV and *Sma*I (lanes 10 to 18). The values for *Sma*I resistance of the proviruses were measured by densitometry as the relative intensities of the 2.0- and 1.7-kb bands generated after *Sma*I digestion. Each value represents the average obtained from analyzing two different exposures of the same blot.

however, if the site is methylated, the uncleaved 2.0-kb fragment persists after *Sma*I digestion.

The *Sma*I site in the MoMuLV LTR of the LN vector is heavily methylated in F9 cells, showing resistance to *Sma*I digestion of 98.3% (Fig. 5B, lane 12). The vectors MPneo, MPdlneo, LNncrneo, and MPthyneo did not show detectable decreases in methylation; levels of *Sma*I resistance were recorded as 95.0, 97.8, 94.3, and 97.4%, respectively (Fig. 5B, lanes 13, 14, 16, and 17). In contrast, the vectors MPncrdlneo and MPthydlneo were much less methylated than the parent vectors, with 52.7 and 54.6% *Sma*I resistance, respectively (Fig. 5B, lanes 15 and 18). We conclude that the simultaneous incorporation of three modifications in a MoMuLV-based vector caused a decrease in the state of methylation of the provirus in EC cells, accompanying an increase in RNA transcription.

DISCUSSION

In this report, we describe the design of novel retroviral vectors containing modifications of the transcriptional unit of the MoMuLV-based vector in an attempt to overcome expression inactivity in EC cells.

First, the different modified retroviral vectors, containing *neo* as a reporter gene, were assayed for the ability to transfer G418 resistance to the F9 EC cell line. The retroviral vectors incorporating the MPSV LTR enhancer repeat instead of the MoMuLV LTR enhancer, either alone or in combination with the *dl*587rev PBS, caused only a slight increase on the formation of G418^r CFU per milliliter in F9 cells (5.5- and 3.9-fold, respectively) over the LN vector. These results coincide with data previously published by Weiher et al. (42). Akgün et al. (1) and Grez et al. (17) have also studied a variant retrovirus containing both the mutant PBS and the MPSV enhancer. They detected an increase in the transfer of G418 resistance in EC cells and ES cells when the *neo* gene was used as a reporter. However, the vector that they derived, named MPSV, contains a variant of the MPSV LTR (PCMV) and was subsequently shown to include a specific rearrangement at the *gag-neo* junction that allowed expansion of the host range.

Alternatively, we designed retroviral vectors to study the effects of the NCR described by Flanagan et al. (14) and, distinctly, the insertion of the demethylating Thy-1 fragment described by Szyf et al. (38) on transcription repression. The role of these elements in transcription by retroviral vectors in embryonic cells has not been previously examined. We observed that either of these changes alone did not significantly affect expression from the MoMuLV-based vector. However, when multiple modifications were made simultaneously in a vector, the level of expression in EC cells increased to a level readily detectable by Northern blot analysis. Addition of the Thy-1 fragment or removal of the NCR sequence from MPdl neo led to vectors with 9- and 16-fold-higher titers on F9 cells; addition of the *dl*587rev PBS to MPthyneo caused an 8-fold increase in titer. Thus, no single element appears to be predominant in causing decreased expression, but rather the interaction of multiple elements appears to be responsible. We propose that EC cells contain multiple negatively acting factors which act independently but similarly to inhibit transcription from the MoMuLV LTR.

Second, to elucidate the mechanisms underlying the differences in effective titer between the modified vectors, we studied EC cells and NIH 3T3 fibroblasts which were stably transduced in parallel. The extent of gene transfer, as measured by proviral copy number, did not differ significantly among the different constructs and between cell lines. Thus, the early steps of retroviral infection, including receptor binding, entry, uncoating, reverse transcription, and integration, appear to be unaffected by the alterations introduced in the LTR and PBS. Therefore, the improved titers obtained from MPncrdlneo and MPthydlneo on F9 cells are not due to higher gene transfer efficiencies by these vectors.

Northern blot analysis showed that RNA transcripts from the standard LN vector containing the MoMuLV LTR and PBS were not detectable in F9 cells, as expected from previous studies. The vectors containing one or two alterations also failed to produce detectable levels of vector-derived transcripts. On the other hand, the levels of transcripts in the F9 cells were significantly higher from the two vectors showing increased titer. These findings suggest that the improved titers of MPncrdlneo and MPthydlneo were due to increased transcriptional activity of the modified LTR and PBS. Quantitative measurements of the levels of vector transcripts from MPn crdlneo and MPthydlneo showed expression at 2 to 5% in the F9 EC cells compared with the 3T3 fibroblasts. However, the modest level of expression in F9 cells was sufficient to increase the effective titer to almost half of that seen in NIH 3T3 cells. These observations suggest that expression of the *neo* gene above a minimal threshold is sufficient to confer the drug-resistant phenotype to F9 cells.

Third, we investigated the state of methylation of the variant proviral vectors in F9 cells. The analysis focused on the *Sma*I site located at position +30 in the proviral 5′ LTR. A strong correlation between decreased methylation of the particular

*Sma*I site and active expression was observed. In F9 cells, the parental LN vector and the derivatives with one or two modifications showed extensive methylation in parallel with inhibition of expression. On the other hand, the two vectors containing three modifications resulted in decreased methylation associated with increased expression.

Unfortunately, these findings do not resolve the long-standing debate over the causal role of methylation on gene inactivity. Inclusion of the Thy-1 fragment, as a demethylating sequence, did cause increased expression from the MPSV LTR with the *dl*587rev PBS that was associated with decreased methylation. However, deletion of a region from the LTR which inhibits transcription, the NCR or the MoMuLV PBS, had the same effect on the state of methylation and expression. Therefore, the isolated occurrence of either decreased DNA methylation or increased transcription, which would have revealed the direction of causality for expression inactivity, was not observed in this system. Previous studies, which monitored the temporal state of methylation of the wild-type MoMuLV after infection of EC cells, found that expression inactivity preceded the appearance of detectable levels of methylation (15, 22).

The observations presented in this report have important implications for the use of retroviral vectors for gene expression in stem cells and their progeny. Retroviral vectors are highly efficient at stably introducing new genes into the chromosomal DNA of target cells. Vectors incorporating the described transcription control elements to express biologically relevant genes, such as those encoding transcription factors, growth factors or their receptors, oncogenes, or other morphogenetic proteins, may produce sufficient quantities of gene products to be useful for studies in cultured ES cells or transgenic mice.

ES cells have also been used as models of hematopoietic stem cells. In permissive culture conditions with appropriate combinations of growth factors, ES cells can differentiate into mature hematopoietic cells in vitro and after introduction into mice in vivo (21, 43). We have recently reported frequent expression failure from the MoMuLV LTR in long-lived murine hematopoietic stem cells studied by serial transplantation of transduced bone marrow (7). Under these conditions, the inactive LTR also becomes increasingly methylated. Therefore, the novel vectors which we describe here may also be useful for gene transfer experiments into hematopoietic stem cells and more specifically for the purpose of long-term gene therapy via bone marrow cells.

ACKNOWLEDGMENTS

We thank Wanda Krall, Punam Malik, and Paul Robbins for carefully reading the manuscript.

This study was supported by National Institutes of Health grant DK-42694 and March of Dimes grant 1-FY92-0551. D.B.K. is a recipient of American Cancer Society Junior Faculty Research Award JFRA-320.

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