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Consistent, persistent expression from modified retroviral vectors in murine hematopoietic stem cells

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ABSTRACT **Retroviral vectors based on the Moloney murine leukemia virus (MoMuLV) have shown inconsistent levels and duration of expression as well as a propensity for the acquisition of *de novo* methylation *in vivo*. MoMuLV-based vectors are known to contain sequences that are capable of suppressing or preventing expression from the long terminal repeat. Previously, we constructed a series of modified retroviral vectors and showed that they function significantly better than MoMuLV-based vectors *in vitro*. To test the efficacy of the modified vectors in hematopoietic stem cells *in vivo*, we examined gene expression and proviral methylation in differentiated hematopoietic colonies formed in the spleens of mice after serial transplantation with transduced bone marrow (2°CFU-S). We found a significant increase in the frequency of expression with our modified vectors (>90% expression in vector DNA containing 2°CFU-S) over the frequency observed with the standard MoMuLV-based vector (28% expression in vector containing 2°CFU-S). Expression from the modified vectors was highly consistent, with expression in >50% of the vector-containing 2°CFU-S from all 20 transplant recipients analyzed, whereas expression from the standard MoMuLV-based vector was inconsistent, with expression in 0–10% of the vector containing 2°CFU-S from 8 recipients and expression in >50% of the vector-containing 2°CFU-S from 4 other recipients. In addition, we established that the modified vectors had a lower level of DNA methylation than the control vector. These findings represent significant advances in the development and evaluation of effective retroviral vectors for application *in vivo*.**

Hematopoietic stem cell (HSC)-based gene therapy is a potentially attractive method of treatment for a wide variety of congenital and acquired diseases (1). Vectors based on well characterized murine retroviruses can be used to efficiently introduce therapeutic or marker genes into HSC. There are several prerequisites for effective HSC-based gene therapy: the efficient transduction of HSC, consistent and stable gene expression at therapeutic levels regardless of the site of proviral insertion, and gene expression that persists throughout the duration of the patient's disease.

Serial transplantation of murine bone marrow provides a powerful *in vivo* model system to study HSC-based gene therapy. It permits the evaluation of vector function in true stem cell progeny and, therefore, may provide the most accurate long term prediction of vector utility in a hematopoietic stem cell transplantation assay or protocol.

Previous studies that relied on the murine bone marrow serial transplantation system (2, 3) and other *in vivo* models have suggested that Moloney murine leukemia virus (MoMuLV)-based retroviral vectors are problematic and unreliable for gene expression *in vivo.* Still, retroviral vectors have several characteristics that make them attractive for use in somatic gene therapy: moderate transgene size carrying capacity, relatively efficient gene transfer, and permanent introduction of therapeutic sequences. In addition, their wide host cell range permits noninvasive treatment of a large variety of tissues. Therefore, it is imperative that improved retroviral vectors be developed and characterized for use *in vivo*.

In previous studies (2, 3), bone marrow transduced with standard MoMuLV-based retroviral vectors was used to reconstitute the hematopoietic systems of lethally irradiated mice after introduction into the circulation via tail vein injection (Fig. 1). Two weeks after bone marrow transplantation, some of the hematopoietic stem cells and progenitors formed macroscopic colonies or foci in the spleen, termed colony forming units–spleen (1°CFU-S). When these 1°CFU-S were excised and examined for vector presence, expression, and methylation status, the vector was found to be present in a large proportion of the colonies (approaching 100%), was expressed in nearly all of the colonies, and was unmethylated substantially (2). When bone marrow from these primary recipient mice was harvested from the hind limbs 2–6 months after 1° transplantation and was used to reconstitute serially the hematopoietic systems of irradiated secondary recipient mice, the provirus was detected in ≈83% of the colonies formed in the spleen 12–14 days after secondary transplantation (2°CFU-S). However, the MoMuLV-based vectors were found to be transcriptionally inactive in 70–80% of the 2°CFU-S and were significantly methylated (2).

It is likely that the primary cause for this discrepancy in expression activity in 1°CFU-S and expression inactivity in 2°CFU-S is the difference in the nature of the cells present in the donor marrow at the time of transduction, which then give rise to these colonies. Most of the 1°CFU-S are derived from a heterogeneous population of relatively differentiated, lineage-committed hematopoietic progenitor cells (9). In contrast, the cells that are transduced, survive 2–6 months after transplantation in the primary recipient, and then give rise to the 2°CFU-S in the secondary transplant recipients have been shown to be pluripotent HSC (9, 10). Thus, the relatively mature cells that form 1°CFU-S are less restrictive to expression from the retroviral vectors than are the pluripotent HSC that form the 2°CFU-S. Laker *et al.* (6) have demonstrated that suppression of retroviral expression is coincident with the differentiation of primitive embryonic stem cells to produce mature hematopoietic cells. By analogy, suppression of retroviral expression may occur during the course of extensive differentiation of the pluripotent HSC to form 2°CFU-S but not during the less extensive differentiation

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Abbreviations: MoMuLV, Moloney murine leukemia virus; LTR, long terminal repeat; CFU-S, colony forming units–spleen; HSC, hematopoietic stem cell; PBS, primer binding site.

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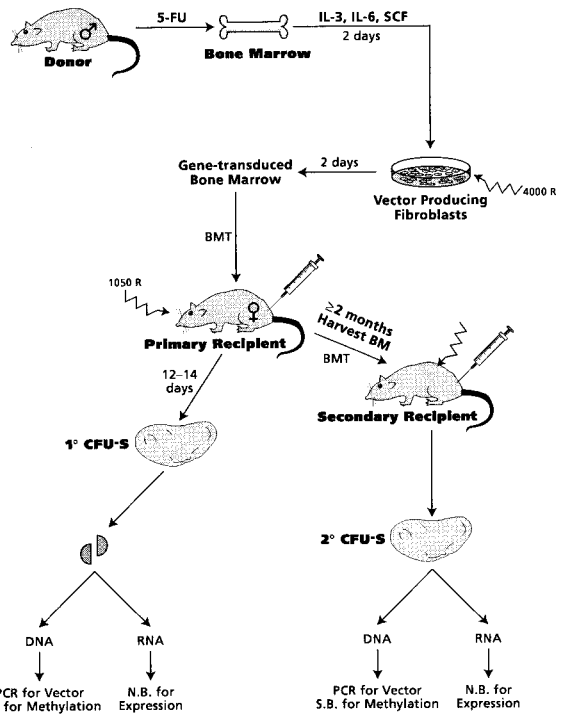

FIG. 1. Overview of the murine serial bone marrow transplant model.

that would be required for a more mature progenitor cell to form a 1°CFU-S.

Several reports indicate that achieving and maintaining detectable levels of expression from MoMuLV-based vectors *in vivo* is more difficult than anticipated and that the obstacles are multifactorial (6, 11, 12). These problems may be caused, in part, by several sequence elements that are common to MoMuLV and its close relatives and have been implicated as suppressors of long terminal repeat (LTR)-directed transcription in HSC and embryonal cell lines. These include the MoMuLV enhancer repeats (6, 13–16), the primer binding site (PBS) region (6, 17–24), the negative control region upstream of the enhancer repeats (25–27), and the embryonal long terminal repeat binding protein, *Drosophila fushi* tarazu factor 1 or embryonal carcinoma cell factor 1 binding site just downstream of the negative conserved region and immediately upstream of the enhancer repeats (6, 27–29).

Positive results *in vitro* (30, 31) using modified retroviral vectors prompted us to explore the utility of these constructs *in vivo*. The vectors were constructed with modifications to the long terminal repeats to address five potentially suppressive genetic or epigenetic entities. The first alteration was to replace the MoMuLV enhancer repeats for those from a closely related virus, the myeloproliferative sarcoma virus, which has greater transcriptional activity in HSC (13, 32, 33). The second alteration shared by all of our modified vectors is a point mutation in the consensus binding site for the embryonal long terminal repeat binding protein (27), also known as *Drosophila fushi* tarazu factor 1 (28) or embryonal carcinoma cell factor 1 (6, 28, 29). Tsukiyama *et al.* (28), demonstrated that mutations at this site (in conjunction with another site upstream) were sufficient to disrupt binding of the embryonal long terminal repeat binding protein. A different mutation in the same site is present in the MESV vector, which was shown to be suppressed after hematopoietic differentiation *in vitro* (6). The third alteration incorporated into our modified vectors is the substitution of the MoMuLV PBS region with the PBS from the dl587rev virus (23) to prevent binding of the repressor binding protein, which interferes with LTR-directed transcription (21, 22). The fourth vector alteration, found only in the MND-neo vector (Fig. 2), is the removal of the negative control region to prevent binding of the YY-1 protein, which can down-regulate expression from the LTR (26, 27, 34). The fifth variation was the insertion of a hypomethylation signal from the mouse *Thy-1* gene (35) into the U3 region of the MTD-neo vector LTR to ward off potentially suppressive *de novo* methylation of the provirus (Fig. 2; refs. 30 and 31).

We examined gene transfer, vector expression, and the extent of proviral methylation for three modified constructs and one standard MoMuLV-based control in hematopoietic colonies formed in the spleens of lethally irradiated mice after serial transplantation (2°CFU-S) with vector-transduced bone marrow. We found that the MoMuLV-based vector and one of our modified constructs were expressed poorly in the spleen colonies (10–28%) whereas two of our modified constructs were expressed with significantly higher frequency in the 2°CFU-S (90%).

MATERIALS AND METHODS

Construction and Packaging of Retroviral Vectors. The LN vector in which the MoMuLV LTR drives expression of the neo gene was constructed and packaged by using the PA317 cell line in the laboratory of A. Dusty Miller (Fred Hutchinson Cancer Research Center, Seattle). The MD-neo, MND-neo, and MTD-neo vectors, previously known as Mp-dl-neo, Mp-ncr-dl-neo, and Mp-thy-dl-neo, respectively, were constructed as described in Challita *et al.* (1995) and Robbins *et al.* (1997). MD-neo, MND-neo, and MTD-neo were packaged by transfection into the GP+E 86 cell line (36) (a generous gift from Arthur Bank, Columbia Univ., New York). The relevant differences between the vectors are highlighted in Fig. 2.

Transduction and Transplantation of Murine Bone Marrow. The murine serial transplant schema is outlined in Fig. 1. Male donor mice (strain C57/B6; Charles River Breeding Laboratories) were treated with 150 mg/kg 5-fluorouracil (SoloPak Laboratories, Franklin Park, IL) by i.v. injection 2 days before bone marrow harvest. Bone marrow was flushed from the femoral and tibial cavities with basal bone marrow medium: Iscove's modified Dulbecco's medium (GIBCO/BRL) with 30% fetal bovine serum (Irvine Scientific), 1% BSA, 10^{-6} M hydrocortisone, 100 mM 2-mercaptoethanol, 2 mM L-glutamine, and 100 units/ml penicillin–streptomycin. After washing in basal bone marrow medium, bone marrow cells were resuspended in basal bone marrow medium supplemented with 200 units/ml human interleukin 6 (Amgen Biologicals), 200 units/ml murine interleukin 3, and 2.5 ng/ml murine stem cell factor (BioSource International, Camarillo, CA). Cells were cultured for 48 hr at 2×10^6/ml before cocultivation with vector-producing fibroblasts. Vector-

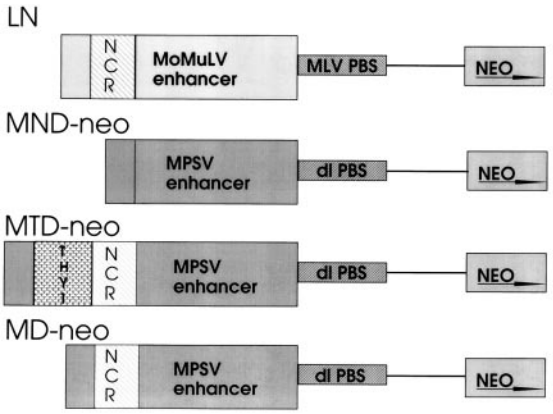

FIG. 2. Diagram of the retroviral vectors used in the serial transplants. The relevant differences are highlighted. MPSV, LTR from the myeloproliferative sarcoma virus; NCR, negative control region; dl PBS, PBS from the dl587rev virus; Thy, 214-bp hypomethylation signal from the murine Thy-1 gene promoter/enhancer.

producing fibroblasts were irradiated at 40 Gy and plated at 6×10^6 cells per 100 mm^2 plate in basal bone marrow medium supplemented with IL-3, IL-6, and murine stem cell factor 24 hr before the addition of 8×10^6 bone marrow cells. Bone marrow cells were cocultivated for 48 hr on lethally irradiated vector-producing fibroblasts with 4 μg/ml polybrene, after which the transduced bone marrow cells were recovered with forceful pipetting. Cells were washed in Hank's balanced salt solution and were resuspended in Hank's balanced salt solution with 50 units/ml heparin immediately before injection/transplantation. Female syngeneic recipient animals were prepared for transplantation with a lethal dose of irradiation (10.5 Gy from a Cesium 137 source) in split doses of 6.5 and 4 Gy separated by 24 hr. Animals were injected i.v. (tail vein) with 2–3 $\times$ 10^6 nucleated cells transduced with one of the LN, MD-neo, MTD-neo, or MND-neo retroviral vectors. Tetracycline ($\approx$100 mg/ml) was added to the drinking water of transplant recipients to control mortality from sepsis. After a period of 2–4 months, bone marrow from the primary (1°) transplant recipients was harvested and used to reconstitute a second generation (2°) of lethally irradiated female recipient mice. After (12–14 days) the secondary transplants, the colonies that form on the spleen (2°CFU-S) were analyzed for vector presence, vector expression, and methylation of the provirus.

Sample Preparation/Nucleic Acid Recovery. For analysis of vector expression in 1° and 2°CFU-S, spleens of transplanted animals were removed 12–14 days after bone marrow transplantation, and individual macroscopic colonies were excised with extreme care to prevent cross-contamination of samples. From each animal, 5 to 15 foci were isolated and minced; one half of each colony was digested in proteinase K buffer and was used for DNA extraction, and the other half was placed in 4M guanidinium isothiocyanate and used for RNA extraction.

PCR. Spleen foci were digested with proteinase K for 1 hr at 56°. Genomic DNA from individual colonies were isolated by using phenol/chloroform extraction and ethanol precipitation. Samples were screened for the presence of vector sequences by PCR. The upstream primer 5′-CTGTGCTCGACGTTGTCACT-3′ and the downstream primer 5′-GCTCTTCGTCCAGATCATCC-3′ recognize sequences within the neo gene and amplify a 200-bp region. The 30-cycle reaction was carried out in a 50-μl volume containing 100 ng of genomic DNA, 50 pmol of each primer, 10$\times$ reaction buffer II (Perkin–Elmer/Cetus), and 1.25 mmol/liter $MgCl_2$ in a Perkin–Elmer/Cetus 9600 thermocycler. Samples then were electrophoresed on a 1% agarose gel containing ethidium bromide and were photographed on a UV light box.

Southern Blotting/Methylation Analysis. DNA (10–15 μg per sample) was digested with *Bam*HI, which does not cut in the vectors, to shorten the genomic strands, and either *Nhe*I (LN, MD-neo) or *Eco*RV (MND-neo, MTD-neo), to excise an LTR-to-LTR proviral fragment. Digested samples then were precipitated in ethanol and were resuspended in Tris·EDTA buffer (10 mM Tris·Hcl, pH 7.8/1 mM EDTA). To determine the methylation status of the proviral 5′ LTR, half of each sample of DNA was subjected to further digestion with *Sma*I while half was mock digested with buffer alone. Complete digestion with *Nhe*I, *Eco*RV, and *Sma*I was ensured by monitoring parallel digestions of Adenovirus type 2 DNA or phage Lambda DNA (GIBCO/BRL) in which 10% of the genomic DNA reaction mixture was diverted to incubate with the monitor DNA. After gel electrophoresis and transfer to nylon membrane (Pall), blots were prehybridized in 0.5 M sodium phosphate (pH 7.5) 1 mM EDTA, 0.7% SDS, and 1% BSA for 1 hr. A full length bacterial neo gene probe was labeled with [α-^{32}P]dCTP (Amersham) by using a random primer kit (Prime-It, Stratagene) and was used as a hybridization probe. Hybridization was conducted overnight at 65°C in a shaking water bath. After washing two times with 1$\times$ standard saline citrate and 0.1% SDS at 65°C, nylon membranes were allowed to expose Kodak XAR film at -70°C.

Northern Blotting. Total cellular RNA was prepared by homogenization of samples in 4 M guanidinium isothiocyanate followed by phenol extraction and ethanol precipitation. RNA (10–15 μg per sample) was electrophoresed in agarose gels with Mops buffer and then was transferred to nylon membranes. Blots were hybridized with the α-^{32}P-labeled neo gene probe as described above. To normalize for RNA loading and to eliminate false negative results, blots were stripped and rehybridized with a mouse β-actin probe.

RESULTS

Transplant/Gene Transfer. The retrovirally transduced bone marrow from each primary transplant recipient was used to transplant 3–5 secondary recipient mice. After 2° transplantation, 176 mice were evaluated. Of these informative animals, 60 were repopulated with marrow transduced with the LN vector, 43 were repopulated with MTD-neo vector transduced marrow, 31 were repopulated with MD-neo vector transduced marrow, and 41 were repopulated with MND-neo vector transduced marrow. Gene transfer rates, as measured by DNA PCR for vector sequences in 2°CFU-S, were 184/227 = 81% for the LN vector, 141/164 = 86% for MTD-neo, 53/80 = 66% for MD-neo, and 120/139 = 86% for the MND-neo vector.

Gene Expression. Expression of the neo gene under control of the LTR of each vector was assessed by performing Northern blots on total RNA from 2°CFU-S that contained vector sequences as measured by DNA PCR. Samples that were negative for neo expression by Northern blot analysis were eliminated from the analysis if they did not show the presence of intact RNA on rehybridization with an isotopically labeled β-actin probe. A representative Northern blot is shown in Fig. 3. This blot indicates that, in the 2°CFU-S derived from transduced HSC from primary donor mouse A (samples A3-A5), the LN vector was expressed in sample A3 but not in A4 or A5. Similarly, the LN vector was inactive in all 2° foci derived from donor B (samples B2-B4) but was expressed in 3/3 foci derived from donor C (samples C2-C4). The MTD-neo vector was active in the foci derived from donor E (lanes E4-E5) but not F5, and the MND-neo vector was expressed in foci derived from donor H (samples H2 and H50) and J (samples J4 and J5) but not donor I (I3).

The Northern blot analysis results for all of the evaluable samples are summarized in Table 1. When the results are grouped by vector, the standard MoMuLV vector LN was expressed in 28% (n = 102) of the foci evaluated. The doubly modified MD-neo was expressed in only 10% (n = 49) whereas the triply modified MTD-neo and MND-neo vectors were expressed in 90% (n = 90 and 115, respectively) of the foci analyzed. Examination of Table 1 indicates that the probability of neo expression from the MoMuLV LTR in LN was highly variable (0–100%) and was subject to a strong founder effect. For example, most of the foci containing the LN vector did not show expression, regardless of which donor mouse gave rise to the colony. However, the LN vector did show expression in 87% and 100% of the foci derived from donors LN C and L 16, respectively (Table 1). Expression from the MD-neo vector was generally poor, ranging from 0 to 40% of the colonies from each donor. In contrast, the MTD-neo and MND-neo vectors expressed in the majority (50–100%) of the 2° foci derived from every primary donor.

Using Fisher's Exact test to compare simultaneously the proportion of LN vector containing samples in which expression was detectable to the proportion of MD-neo-, MTD-neo-, and MND-neo-containing samples in which expression was detectable revealed that the differences in the rates of expression presented in Table 1 are significantly different ($P < 0.001$). Direct statistical comparison (Student's t test) between pairs of samples, grouped by vector, revealed that the rate of expression exhibited in the foci containing the LN vector was significantly different from that observed with either the MTD-neo vector ($P = 0.0003$) or the MND-neo vector ($P = 0.0002$) but not the MD-neo vector ($P = 0.293$). Application of the same analysis to the results from the

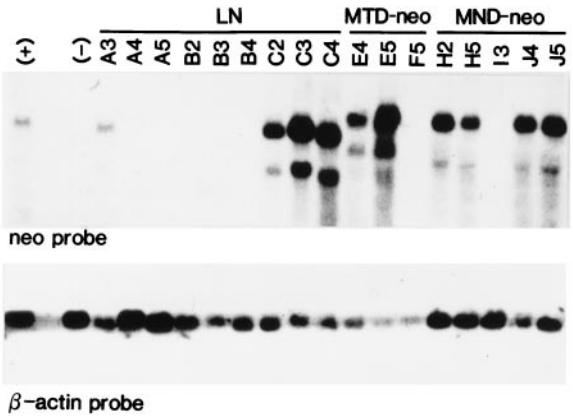

FIG. 3. Northern blot analysis of vector-derived RNA from individual 2°CFU-S. Total cellular RNA was isolated and electrophoresed on a 1.2% agarose gel and was transferred to a nylon membrane. The membrane was hybridized with a ^{32}P-labeled neo gene probe, was stripped, and was reprobed with a labeled β-actin cDNA to confirm the presence and integrity of the RNA.

other samples showed that the rate of expression observed in the foci containing the MD-neo vector was statistically different than that measured from the MTD-neo and MND-neo vectors (P = 0.000002 and 0.000004, respectively). The high frequency of expression observed in foci containing the MTD-neo and MND-neo vectors were not statistically distinguishable (P = 0.908).

Methylation. The methylation state of the 5′ LTR of the vectors was analyzed by using the methylation-sensitive restriction enzyme *Sma*I. Fig. 4*a* shows methylation-sensitive restriction enzyme analysis of proviral DNA extracted from 2° spleen foci transduced with the LN vector and derived from 1° transplant recipient LN A. The LN A5 and A6 samples (Fig. 4*a*, lanes 1 and 3, respectively) show minimal digestion with *Sma*I (Fig. 4*a*, lanes 2 and 4), indicating a high degree of methylation. Densitometric analysis indicated that the LN A5 and LN A6 samples were 90% and 91% methylated, respectively. Sample LN A7 (Fig. 4*a*, lane 5) is almost completely resistant to cutting with *Sma*I (Fig. 4*a*, lane 6; 93% methylated by densitometry). Sample LN A8 is uninterpretable (Fig. 4*a*, lanes 7 and 8). LN A9 (Fig. 4*a*, lanes 9 and 10) is 96% methylated as shown by its lack of visible digestion with *Sma*I. Sample LN A5 is included on the Northern blot shown in Fig. 3. It is worth noting that none of the samples depicted in Fig. 4*a* showed expression by Northern blot analysis.

The samples shown in Fig. 4*b* also are extracted from 2° spleen foci containing the LN vector but are derived from a different primary transplant recipient, LN C. The methylation status of these samples is significantly different from the previous set. The LN C1, -2, -3, and -4 samples (Fig. 4*b*, lanes 1–8) could be digested almost completely with *Sma*I. Densitometry revealed that these samples were only 4%, 33%, 14%, and 1% methylated, respectively. The vectors in all of these samples were expressing by Northern blot analysis, and samples LN C2, -3, and -4 are included on the blot shown in Fig. 3. In contrast, the last sample shown in Fig. 4*b*, LN C12, was more heavily methylated (44%) and was one of the two foci derived from 1° recipient LN C that did not express by Northern blot analysis.

Densitometric analysis of multiple blots revealed that the mean level of methylation measured for the LN vector in 2°CFU-S was 46% (n = 45). The MTD-neo vector had a mean methylation value of 22% (n = 37), which was found to be significantly different than the value measured for LN (P = 0.00013). Similarly, the 29% level of methylation observed in the MND-neo vector (n = 24) was also significantly different than that measured for the LN vector (P = 0.012). The MD-neo vector had a mean methylation value of 100%, but the small sample size made meaningful statistical analysis impossible. Statistical analysis of the methylation data from the LN, MND, and MTD samples by using the Bonferroni multiple comparison test indicated that both the MND-neo and MTD-neo vectors were significantly less methylated than the LN construct but were not statistically different from one another.

Table 1. Vector expression 2°CFU-S

Vector	1° recipient	2° CFU-S NB+/ 2° CFU-S tested	% 2° CFU-S NB+
LN			
	A	1/15	7%
	C	13/15	87%
	L4	7/13	54%
	L5	4/8	50%
	L10	0/9	0%
	L11	0/9	0%
	L12	0/9	0%
	L13	0/8	0%
	L14	0/4	0%
	L15	0/4	0%
	L16	4/4	100%
	L17	0/4	0%
		Total: 29/102 = 28%	
MD neo			
	D1	1/5	20%
	D2	0/5	0%
	D3	0/5	0%
	D4	0/5	0%
	D5	0/5	0%
	D6	2/5	40%
	D7	0/5	0%
	D8	0/4	0%
	D9	1/5	20%
	D10	1/5	20%
		Total: 5/49 = 10%	
MTD neo			
	E	14/14	100%
	F	6/10	60%
	T3	11/13	100%
	T4	10/10	100%
	T5	14/14	100%
	T6	10/10	100%
	T7	5/7	71%
	T9	4/4	100%
	T10	4/4	100%
	T11	3/4	75%
		Total: 81/90 = 90%	
MND neo			
	H	11/12	92%
	J	12/12	100%
	M3	4/7	57%
	M4	5/10	50%
	M5	10/10	100%
	M6	13/14	93%
	M7	16/17	94%
	M8	8/8	100%
	M10	13/13	100%
	M11	12/12	100%
		Total: 104/115 = 90%	

NB+, showing positive expression by Northern blot analysis.

Expression vs. Methylation. Combined statistical analysis of the methylation and expression data indicated that the proviruses (regardless of vector origin), contained within foci that scored positively on Northern blots, were significantly less methylated (22%) than those in which expression was undetectable (54%; P = 0.000091; Bonferroni method; Table 2). Scheffe's test showed that the extent of methylation found in LN and MND-neo samples that were expressing by Northern (28% and 12%, respectively) was significantly lower than that found in samples in which the same vectors were not expressing (62% and 45%,

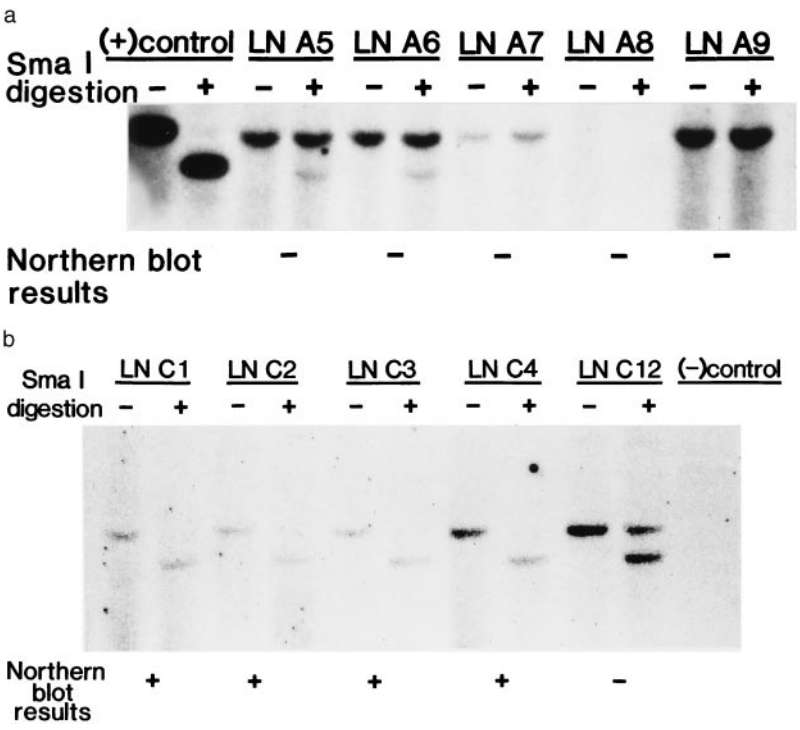

FIG. 4. Southern blots to determine the extent of proviral methylation. (*A*) The marrow used to repopulate the animals represented was derived from primary recipient LN A. Total genomic DNA was isolated from 2°CFU-S containing the LN vector and was analyzed for methylation at the *Sma*I site by Southern blot analysis as described in *Materials and Methods*. Densitometry was performed to quantitate the intensities of the *Sma*I-resistant fragments (upper bands) and *Sma*I-sensitive fragments (lower bands). The proportion of the total density measured from both bands that was contributed by the *Sma*I-resistant fragment represents the extent of proviral DNA methylation. (*B*) The marrow used to repopulate the animals represented was transduced with the LN vector and was derived from primary recipient LNC. Total genomic DNA was extracted and analyzed as above.

respectively; $P = 0.038$; Fig. 5). This observation suggested an inverse correlation between vector expression and proviral methylation for these two constructs *in vivo*. Of interest, the methylation and expression data from the MTD-neo vector did not display this inverse relationship and showed statistically indistinguishable mean levels of methylation in samples that were expressing and in those that were not (21% and 24%, respectively; Fig. 5).

Further examination of the data by using Scheffe's multiple comparison test illuminated significant differences in vector performance regarding the probability of expression as a function of proviral methylation. The LN vector is significantly less likely to express by Northern blot analysis in 2°CFU-S in which it is methylated than in those foci in which it is not methylated [LN vs. LN ($P = 0.00086$)]. The MND-neo vector was also significantly more likely to express in 2°CFU-S in which it was not methylated than in foci in which the provirus was methylated [MND vs. MND ($P = 0.015$)]. The data from foci containing the MTD-neo vector did not show a statistical relationship between methylation and expression. [MTD vs. MTD ($P = 0.77$)].

DISCUSSION

Serial transplantation of transduced murine bone marrow is a stringent model to test, *in vivo*, the long term potential of vectors intended for use in gene therapy protocols targeting hematopoietic stem cells. It permits evaluation of vector efficacy in transduced committed progenitor cells as well as in the progeny of true pluripotent stem cells. Using this system, it is possible to study gene transfer and vector expression in essentially any tissue of hematopoietic origin at many stages of differentiation. Because effective HSC-based gene therapy will require vector expression in true HSCs and their progeny, we chose to limit our observations on vector performance to cells known to be derived from true HSCs, based on the formation of colonies in the spleens of secondary transplant recipients, 2°CFU-S.

Table 2. Vector expression in 2°CFU-S vs. methylation at *Sma*I

Vector	*Sma*I Resistant,%	
	Northern blot (−)	Northern blot (+)
LN	62, $n = 27$	28, $n = 18$
MD-neo	100, $n = 4$	100, $n = 1$
MND-neo	45, $n = 8$	12, $n = 16$
MTD-neo	24, $n = 10$	21, $n = 27$
Total	54, $n = 49$	22, $n = 62$

Expression from the MoMuLV LTR can be achieved with high frequency (80–100%) in 1° colonies formed in the spleens of lethally irradiated mice after bone marrow transplantation (2). However, expression from MoMuLV in 1°CFU-S is the result of the transduction of committed progenitor cells that are less refractory to MoMuLV expression (10, 37). Expression from the MoMuLV LTR in spleen colonies formed after secondary transplantation of transduced bone marrow (2°CFU-S) is far less likely than in the 1° colonies (10–30%; ref. 2). This discrepancy is most likely caused by the fact that the 2°CFU-S are formed from true hematopoietic stem cells and their progeny (10), which are more restrictive to expression from MoMuLV (11).

Founder Effect and Integration Site Bias. Analysis of the 2°CFU-S from the mice transplanted with marrow transduced with the LN vector revealed a high degree of variability in the frequency of expression between groups of foci derived from

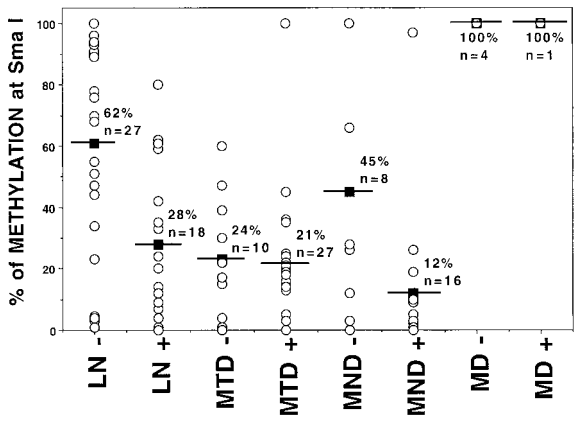

FIG. 5. Proviral methylation data grouped by vector and expression profile. For each construct, the data are segregated into two columns: nonexpressing (−) on the left and expressing (+) on the right. Within each column, the extent of methylation values measured by densitometry are plotted against the *y* axis (0–100%).

different marrow donors. Numerous investigators (2, 38–40) have demonstrated, by analyzing the patterns of proviral integration, that reconstitution of a mouse's hematopoietic system after bone marrow transplantation was accomplished by only 1–3 transduced stem cells. The implication of this finding is that this oligoclonality creates a founder effect in which the vector in each primary animal represents a unique proviral integration site. Therefore, to examine vector activity in a variety of chromosomal settings, it was necessary to generate large numbers of primary transplant recipients to be used as marrow donors in the secondary transplants.

The finding of expression from the LN vector in foci derived from only a few primary donors implies that the MoMuLV LTR is extremely susceptible to positional variegation of expression in the hematopoietic tissues. This high rate of positional variegation of expression is similar to that seen in transgenic mice in which the reporter gene is under the control of minimal promoter/enhancer elements (39). Pawliuk *et al.* (40) observed similar positional variegation of expression in 2°CFU-S (≈10% expressing) with a construct driven by the unmodified myeloproliferative sarcoma virus LTR. However, when they examined long term reporter gene expression in 2°CFU-S from the murine stem cell virus LTR, which contains some of the same modifications as our MND-neo, MTD-neo, and MD-neo vectors, they found an increase in the proportion of marker expressing cells (≈55% expressing).

Our findings that the majority (90%) of the secondary foci derived from every primary donor transduced with the MTD-neo or MND-neo vectors showed neo expression indicates that these vectors are less susceptible to positional variegation of expression. The resistance of the MND-neo and MTD-neo vectors to transcriptional inactivity *in vivo* resulted in a significant increase in the probability of expression from these constructs (from 28% to 90%). Because the sites of retroviral vector insertion are random, the ability to express irrespective of chromosomal location is vital to achieving consistent, persistent gene expression *in vivo*. The results of Pawliuk *et al.* (40), in conjunction with the observations presented here, suggest that modifications to the control elements of the vector LTRs may be necessary to achieve maximal long term expression *in vivo.*

Methylation and Vector Expression *in Vivo*. We previously have documented an inverse relationship between proviral expression and methylation of the *Sma*I site in the 5′ LTR of MoMuLV-based vectors (2, 31). To examine whether the modified vectors MND-neo, MTD-neo, and MD-neo displayed this relationship *in vivo* in 2°CFU-S, we conducted methylation analysis by using the methylation-sensitive restriction enzyme *Sma*I to perform Southern blots.

As expected, the MoMuLV-based LN vector exhibited a strong correlation between methylation of the provirus and transcriptional inactivity. Similarly, the MND-neo vector showed a significant correlation between proviral methylation and the lack of expression, although far more of MND-neo vectors were expressed and were methylated minimally.

The methylation data and expression results from the MD-neo and MTD-neo vectors suggest that methylation may play a role in suppression of LTR directed transcription. The MD-neo and MTD-neo vectors differ only by the insertion in MTD-neo of the putative hypomethylation signal from the Thy-1 gene identified by Szyf *et al.* (35). Yet, expression from the MD-neo vector was poor and statistically indistinguishable from the LN vector. Additionally, the MD-neo vector was methylated completely in all of the foci analyzed. In contrast, the MTD-neo vector expressed in nearly all of the foci tested. Of interest, the MTD-neo vector was hypomethylated in all of the foci (22% average *Sma*I resistance), even when not expressed, and thus did not display an inverse correlation between methylation and expression. In conjunction with the MD-neo data, these results suggest that the Thy-1 fragment is functioning to protect the MTD-neo vector from *de novo* methylation, permitting gene expression at high frequency. The Thy-1 fragment may be acting to dissociate methylation and transcriptional inactivity for the MTD-neo vector, which would be beneficial for the maintenance of long term expression *in vivo*.

The net result of the modifications that we have incorporated into our MND and MTD vectors is more consistent expression in hematopoietic stem cells and their differentiated progeny. The dramatic increase in the probability of expression from the modified vectors should translate to a much higher frequency of gene-expressing cells after transplantation of transduced hematopoietic stem cells. This could be particularly valuable in applications in which expression of the introduced gene is needed in a high proportion of cells (e.g., for hemoglobinopathies) or when the introduced gene provides no selective advantage and the target cells are difficult to transduce or the gene product is secreted and needed in large amounts. These findings represent a significant step toward the development of effective vectors for exogenous gene expression *in vivo* and could be useful in a variety of research or clinical applications.

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