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High-Efficiency Transduction of Human Lymphoid Progenitor Cells and Expression in Differentiated T Cells

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Gene therapy strategies for humans have been limited by low transduction efficiencies and poor expression of retroviral vectors in differentiated progeny cells carrying the transduced vector. Here we describe a strategy utilizing a cell surface reporter gene, murine *thy-1.2*, selectable by fluorescence-activated cell sorting (FACS), to achieve higher gene marking efficiencies. Human CD34-positive cells were transduced by a murine retroviral vector bearing the *thy-1.2* marker and pseudotyped with vesicular stomatitis virus G protein, followed by FACS to enrich for CD34-positive cells that express Thy-1.2 on the cell surface. Gene marking and expression after differentiation into thymocytes were assessed in a SCID-hu Thy/Liv mouse model for human lymphoid progenitor cell gene therapy. We found that virtually all of the differentiated T-cell progeny were marked with vector sequences. It is of particular importance that reconstitution with the selected cells resulted in expression of Thy-1.2 in up to 71% of donor-derived thymocytes. It is of note that the donor-derived thymocytes that did not express Thy-1.2 still harbored vector *thy-1.2* sequences, suggesting repression of transgene expression in some cells during progenitor cell differentiation into thymocytes. These studies provide a proof of concept for efficient expression of transgenes through T-lymphoid differentiation and a potential basis for utilizing similar strategies in human gene therapy clinical trials.

One of the key elements of effective stem or progenitor cell gene therapy is development of vectors for efficient transduction and expression of transduced genes in the differentiated progeny of progenitor cells (10, 13, 27, 35). Testing of vector efficiency is complicated by relatively poor transduction efficiencies of $CD34^+$ progenitor cells and relatively poor expression in the mature cells derived from the transduced progenitor cells (4, 5, 7, 12, 16, 17). We have utilized the severe combined immunodeficient (SCID)-hu Thy/Liv mouse model (23), a SCID mouse implanted with human fetal thymus and liver tissues, to develop an effective system for rapid experimental testing of gene therapy vectors and approaches in a setting in which hematopoietic progenitor cells can be transduced and used to reconstitute a human hematolymphoid organ (Thy/Liv). We have previously shown that an amphotropic murine leukemia virus (MuLV)-based retrovirus vector, LNL6 (25), can transduce human $CD34^+$ cells and that the genetically marked cells can reconstitute T-cell development in the SCID-hu Thy/Liv mouse (1).

In this study, we utilized this model system to demonstrate a proof of principle for a novel vector-reporter gene system. We demonstrated that immunoselection for transduced human progenitor cells, using a cell surface reporter gene, results in efficient reconstitution of the T-cell compartment of the thymus, where a significant proportion of the cells express the vector.

MATERIALS AND METHODS

Construction of retroviral vectors. The MuLV-based vectors SRαLluc and SRαLthy were derived from LXSN (25). The simian virus 40 (SV40) promoter and the neomycin resistance gene were removed from LXSN and replaced with the firefly luciferase or the murine *thy-1.2* cDNA (11, 14, 32). The SRα sequence was inserted before the 5′ Moloney murine sarcoma virus (MSV) long terminal repeat (LTR) for vector amplification in COS cells (26, 34). LlucSN was constructed from LXSN and IISVluc. LXSN was digested with *Bam*HI, filled in with Klenow fragments, and then excised by *Xho*I. The resulting 7-kb fragment was ligated to a 1.7-kb fragment of IISVluc, which contains the luciferase gene, digested with *Mlu*I, filled in with a DNA polymerase Klenow fragment, and digested with *Xho*I. SRαLlucSN was constructed from LlucSN and pcDL-SRα-296 (34). LlucSN was digested with *Sac*II and *Acc*I and filled in with T4 DNA polymerase. The resulting 5.3-kb fragment was ligated to a 3.2-kb fragment of pcDL-SRα-296, which contains the SRα sequence digested with *Xho*I and *Bam*HI, and filled in with T4 DNA polymerase. Transcription of the 5′ MSV LTR is in the same orientation as the promoter in SRα sequences. SRαLluc was constructed from SRαLlucSN. SRαLlucSN was digested with *Bam*HI and *Rsr*II and filled in with T4 DNA polymerase, and the resulting 7.5-kb fragment was religated to remove the SV40 promoter and the neomycin resistance gene from the vector. SRαLthy was constructed from SRαLlucSN and LthySN (32). SRαLlucSN was digested with *Rsr*II, filled with T4 DNA polymerase, and excised with *Eco*RI. The resulting 5.8-kb fragment was ligated to a 0.5-kb fragment of LthySN, which contains the murine *thy-1.2* cDNA, digested with *Bam*HI, filled in with T4 polymerase, and digested with *Eco*RI.

Retroviral vector production. Vesicular stomatitis virus G protein (VSV-G)-pseudotyped retroviral vectors were recovered following electroporation of three plasmids: retroviral vectors SRαLluc or SRαLthy, $SV\Psi^-env^-$MLV (19), and pHCMV-G (6). Twenty micrograms of each plasmid was added to 10^7 COS cells in 500 μl of RPMI with 20% fetal calf serum. Electroporation was achieved by a single pulse from a Bio-Rad Gene Pulser apparatus equipped with a capacitance extender unit (960 μF, 230 V). Transfected COS cells were cultured in 10 ml of Dulbecco's modified Eagle medium with 10% calf serum, 100 U of penicillin per ml; and 100 μg of streptomycin per ml, and the medium was changed on day 1 postelectroporation. Supernatants (240 ml) were collected on days 2 and 3 postelectroporation, filtered with a 0.45-μm-pore-size filter, treated with RNase-free DNase (2 μg/ml; Worthington) for 30 min at room temperature (20 to 25°C) in the presence of 0.01 M $MgCl_2$, and subjected to ultracentrifugation in a Beckman L3-50 centrifuge with an SW28 rotor at 50,000 × *g* (25,000 rpm) and 4°C for 90 min. The pellet was resuspended in 200 μl of 0.1% Hanks' balanced salt solution for 24 h at 4°C. Concentrated supernatants were stored at −70°C until use.

Infection and immunoselection of gene-transduced CD34-positive cells by fluorescence-activated cell sorting (FACS). Human CD34-positive cells were purified from a male fetal liver as previously described (1). Cells were cultured in Iscove's modified Dulbecco's medium with 100 ng each of interleukin 3 (IL-3), IL-6, and stem cell factor per ml, 20% fetal calf serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml.

CD34-positive cells (5×10^5) were infected with SRαLluc VSV-G-pseudo-

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typed virus by incubation with concentrated virus in the presence of Polybrene (8 μg/ml) at 37°C for 2 to 3 h on days 1, 2, and 3 after CD34-positive cell purification from fetal liver. SRαLluc-infected CD34-positive cells (2×10^5) were injected into each female Thy/Liv implant of irradiated animals on day 4 after CD34-positive cell purification from fetal liver.

CD34-positive cells (2×10^6) were infected with SRαLthy VSV-G-pseudotyped virus by incubating concentrated virus in the presence of Polybrene (8 μg/ml) at 37°C for 2 to 3 h on days 1 and 2 after CD34-positive cell purification from fetal liver. On day 3 after CD34-positive cell purification from fetal liver, *thy-1.2*-transduced CD34-positive cells were stained with monoclonal antibodies (MAbs) to murine Thy-1.2 (Caltag) and human CD34 (Becton-Dickinson) conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE), respectively. CD34 and Thy-1.2 double-positive cells were sorted on a FACStar^{plus} (Becton-Dickinson). Female Thy/Liv implants of irradiated animals (400 rads) were directly injected with 2×10^5 Thy-1.2 and CD34 double-positive cells on day 4 after CD34-positive cell purification from fetal liver.

Luciferase assay. To analyze luciferase expression, 10^6 cells were washed twice with phosphate-buffered saline and lysed with 100 μl of 1× luciferase lysis buffer (Promega Corp.). Ten microliters of each lysate was subjected to the luciferase assay (Promega Corp.) with a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, Calif.).

Quantitative PCR assay. Each PCR amplification was performed as previously described (1, 3, 15, 18, 21, 29, 30, 38). In brief, to detect *luc* or *thy-1.2* sequences, one of the oligonucleotide primers for each pair used was end labeled with ^{32}P, and 25 ng was included in the reaction mixture (usually 5×10^6 to 1×10^7 cpm). The second oligonucleotide primer was not labeled, and 50 ng was incorporated into each reaction mixture. Each reaction mixture contained a 0.25 mM concentration of each of the four deoxynucleoside triphosphates, 50 mM NaCl, 25 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 100 μg of bovine serum albumin per ml, and 1.25 U of *Taq* DNA polymerase (Promega). The reaction mixture was overlaid with 25 μl of mineral oil and then subjected to 25 cycles of denaturation for 1 min at 94°C and polymerization for 2 min at 65°C. The reaction was performed on a Perkin-Elmer thermocycler. Amplified products resulting from the PCR were analyzed by electrophoresis on 6% nondenaturing polyacrylamide gels and visualized by direct autoradiography of the dried gels. Quantitative analysis of the amplified products was performed with a radioanalytic imager (Ambis, San Diego, Calif.). The nucleotide sequences of the oligonucleotide primers used for *luc* DNA detection were derived from the nucleotide sequence of the *luc* cDNA (9) and are as follows: Luc1, 5′-CTCTAGAGGATGGAACCGCTG-3′; Luc2, 5′-CAGCCCATATCGTTTCATAGC-3′. The nucleotide sequences of the oligonucleotide primers used for murine *thy-1.2* cDNA detection were derived from the nucleotide sequence of the murine *thy-1.2* sequence (12) and are as follows: *thy*2, 5′-CTAGCCAACTTCACCACCAAGGA-3′; *thy*3, 5′-CTTATGCCGCCA CACTTGACCAG-3′. A pair of oligonucleotide primers complementary to the first exon of the human β-globin gene (28) was used in each reaction mixture in PCR analyses to normalize the total amount of human cellular DNA present. A pair of oligonucleotide primers complementary to the sequence of the human Y chromosome (38) was used in each reaction mixture in PCR analyses to determine the amount of donor cell-derived cellular DNA present. During PCR amplification, either labeled β-globin- or Y chromosome-specific oligonucleotides were incorporated into the reaction at 5×10^6 to 1×10^7 cpm per reaction.

Quantitation of *luc* or murine *thy-1.2* DNA during PCR amplifications was performed by analyzing a standard curve of dilution of linearized SRαLluc or SRαLthy plasmid DNA digested with *Hin*dIII, which does not cleave vector sequences. These DNAs were diluted in 0.1 μg of human peripheral blood mononuclear cell DNA per μl. The copy number of *luc* or murine *thy-1.2* included in the standard curve ranged from 54 to 14,000 or 200 to 48,000 copies, respectively. Standard curves for human β-globin and Y chromosome DNAs were obtained by amplification of 0.001 to 0.1 μg of human cellular DNA (100 to 10,000 cell equivalents) from human male CD34-positive cells.

Flow cytometric analysis. Thymocytes from Thy/Liv implants were stained with MAbs to human CD3, CD4, CD5, CD8, CD45 (Becton-Dickinson), or CD1 (Coulter) or murine Thy-1.2 (Caltag) directly conjugated with FITC, PE, or peridinin chlorophyll protein. Samples were run on a FACScan flow cytometer, and data were analyzed with the Cell Quest program (Becton-Dickinson). Ten thousand events were acquired for Fig. 2A, a and b; Fig. 3A, a and b; and Fig. 3B, a to f.

Thymocyte culture for in vitro activation studies. Thymocytes were cultured at a concentration of 10^6/ml in flat-bottom culture plates. Culture plates were coated with goat anti-mouse immunoglobulin G (IgG) (GAM; Tago, Burlingame, Calif.). GAM (10 μg/ml) in phosphate-buffered saline (PBS; pH 7.4) was added to wells and incubated for 2 h at 37°C. Plates were then washed three times with PBS. Anti-human CD3 MAbs (T3; Coulter) were added at 4 μg/ml in PBS, and the mixture was incubated at 37°C for 1 h. The immobilized GAM provides a solid phase for binding of anti-human CD3 MAbs, as previously described (20, 36). After washing, thymocytes obtained from Thy/Liv implants of SCID-hu mice were cultured in the presence of IL-2 (1 μg/ml), an anti-human CD28 MAb (1 μg/ml), and an immobilized anti-human CD3 MAb in RPMI–20% fetal calf serum supplemented with 100 U of penicillin per ml and 100 μg of streptomycin per ml. Control thymocytes were cultured in parallel in the absence of IL-2, anti-CD28 MAbs, and immobilized anti-CD3 MAbs. After 2 days of stimulation in vitro, thymocytes were analyzed by flow cytometry for murine Thy-1.2 and human CD25 cell surface markers as already described. Dead cells were excluded by seven-amino-acid–actinomycin D staining as previously described (33). [^{3}H]thymidine incorporation was measured by pulse-labeling cells for 6 h on day 2 as previously described (38).

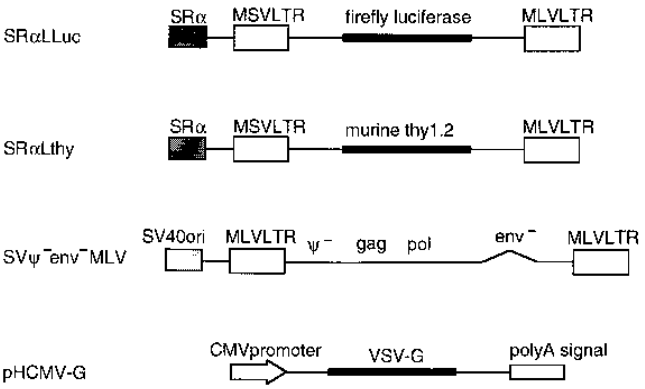


FIG. 1. Maps of luciferase and Thy-1.2 vectors. MuLV-based vectors SRαLluc and SRαLthy were developed from LXSN (25). The SV40 promoter and the neomycin resistance gene were removed from LXSN and replaced with firefly *luc* or murine *thy-1.2* cDNA (11, 14, 32). The SRα sequence was inserted before the 5′ MSV LTR for vector amplification in COS cells (25, 34). SVΨ$^-$env$^-$MLV is an MuLV *gag* and *pol* expression construct kindly provided by Dan R. Littman (19), and pHCMV-G is a VSV-G expression construct kindly provided by Jane C. Burns (6, 37). SV40 ori is an SV40 origin sequence. Ψ$^-$ indicates deletion of the retroviral packaging signal, env$^-$ means deletion of the envelope gene, and CMV is cytomegalovirus.

RESULTS

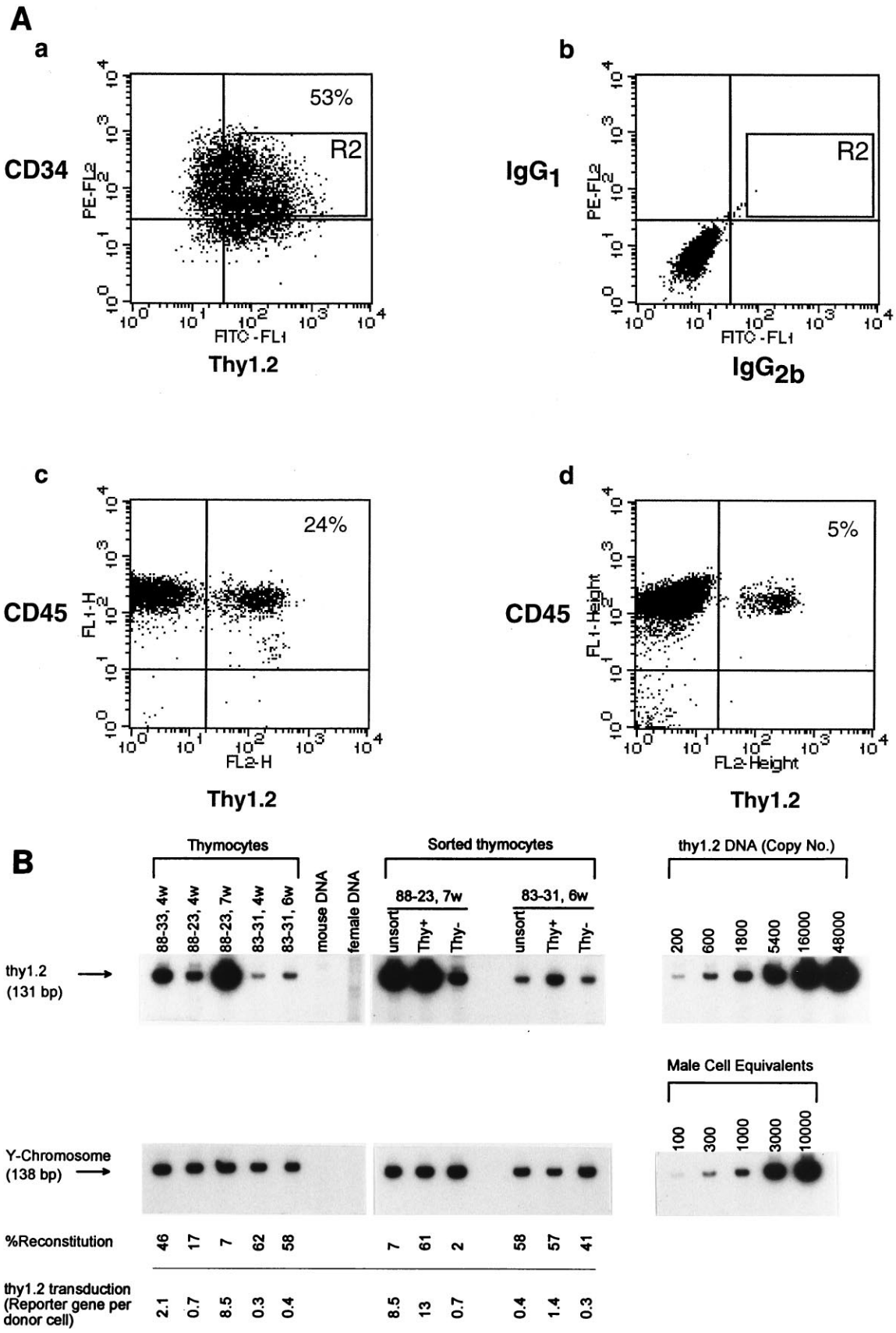
VSV-G-pseudotyped MuLV-based vectors bearing reporter genes. To facilitate the testing of different vector strategies, we utilized a transient transfection system to provide helper virus functions for the vector. We introduced the reporter gene, either firefly *luc* or murine *thy-1.2*, into MuLV-based vectors to allow sensitive detection of expression in cells (Fig. 1). Quantitative PCR was utilized to assess infection and transduction efficiencies (1, 3, 15, 18, 21, 29, 30, 38). Each of these vectors was pseudotyped with VSV-G and MuLV *gag* and *pol*, provided in trans through cotransfection. The VSV-G pseudotypes are highly efficient for transduction of a variety of human cell types, including transformed T-cell lines, peripheral blood lymphocytes, and CD34-positive hematopoietic cells derived from fetal liver. Since VSV-G pseudotypes can be concentrated by ultracentrifugation without loss of infectivity (2, 6, 37), we could achieve approximately 10- to 100-fold higher titers in human cells than with the same vectors pseudotyped with the amphotropic MuLV envelope, as measured by luciferase activity on human HeLa, Rd, MT-2, SupT1, and primary CD34$^+$ cells. Furthermore, as previously reported, VSV-G-pseudotyped virions could be concentrated and stored as frozen stocks, facilitating standardization between different experiments (6, 37).

Transduction of CD34-positive cells and reconstitution of irradiated Thy/Liv implants of SCID-hu mice. In initial studies, we tested the ability of CD34-positive cells transduced with murine retroviral vectors bearing a *luc* reporter gene and pseudotyped with VSV-G to reconstitute T-cell compartments in SCID-hu mice (Table 1). Transduced CD34-positive cells from male donors were injected into female-derived Thy/Liv implants of irradiated SCID-hu mice. Reconstitution efficiencies varied from 18 to 75%, as assessed by PCR for Y chromosome sequences 4 to 7 weeks postreconstitution. All of the

TABLE 1. Luciferase and Thy-1.2 gene transduction and expression efficiency in $CD34^+$ cells and thymocytes in SCID-hu mouse Thy/Liv implants[a]

Gene and expt no.	Source of progenitors	Mouse ID no.	Cell type	Wk post-reconstitution	% Recon-stitution	No. of reporter genes/donor cell	Expression	Normalized expression
luc								
1	$CD34^+$		$CD34^+$	0		33	1,915,625	0.58
1	$CD34^+$	76-36	Thymocyte	4	50	1.1	3,574	0.03
1	$CD34^+$	76-36	Thymocyte	7	18	2.1	999	0.004
1	$CD34^+$	79-07	Thymocyte	4	51	1.6	1,941	0.01
1	$CD34^+$	79-07	Thymocyte	7	56	1.3	485	0.003
1	$CD34^+$	76-23	Thymocyte	4	52	<0.002	<300	—
2	$CD34^+$	81-07	Thymocyte	4	75	0.09	3,506	0.39
2	$CD34^+$	81-08	Thymocyte	4	50	0.06	1,412	0.24
2	$CD34^+$	81-09	Thymocyte	4	50	0.08	2,241	0.28
2	$CD34^+$	81-10	Thymocyte	4	65	0.1	2,690	0.27
2	$CD34^+$	81-24	Thymocyte	4	42	0.06	2,578	0.43
2	$CD34^+$	83-29	Thymocyte	4	48	0.08	2,581	0.32
2	$CD34^+$	81-27	Thymocyte	4	42	0.06	1,020	0.17
2	$CD34^+$	81-11	Thymocyte	4	35	<0.002	<300	—
2	$CD34^+$	81-02	Thymocyte	4	49	3.1	5,806	0.02
2	$CD34^+$	81-01	Thymocyte	4	75	0.2	3,039	0.15
thy-1.2								
2	$CD34^+$ Thy^+	83-31	Thymocyte	4	62	0.3	2.9	4.6
2	$CD34^+$ Thy^+	83-31	Thymocyte	6	58	0.4	7.9	14
2	$CD34^+$ Thy^+	83-31	Sorted Thy-1.2^+	6	57	1.4	>92	>92
2	$CD34^+$ Thy^+	83-31	Sorted Thy-1.2^-	6	41	0.3	<0.1	<0.1
2	$CD34^+$ Thy^+	83-32	Thymocyte	4	47	0.1	2.1	4.4
2	$CD34^+$ Thy^+	83-32	Thymocyte	6	63	0.1	3.5	5.5
2	$CD34^+$ Thy^+	83-32	Sorted Thy-1.2^+	6	72	2.9	>80	>80
2	$CD34^+$ Thy^+	83-32	Sorted Thy-1.2^-	6	43	0.1	<0.1	<0.1
3	$CD34^+$ Thy^+	88-33	Thymocyte	4	46	2.1	24	52
3	$CD34^+$ Thy^+	88-23	Thymocyte	4	17	0.7	2.9	17
3	$CD34^+$ Thy^+	88-23	Thymocyte	7	7	8.5	5	71
3	$CD34^+$ Thy^+	88-23	Sorted Thy-1.2^+	7	61	13	>98	>98
3	$CD34^+$ Thy^+	88-23	Sorted Thy-1.2^-	7	2	0.7	<0.2	<0.2
3	$CD34^+$ Thy^+	88-14	Thymocyte	4	2	1.4	0.6	30
3	$CD34^+$ Thy^+	88-14	Thymocyte	7	3	1.3	1.6	53
3	$CD34^+$ Thy^+	88-14	Sorted Thy-1.2^+	7	31	2.7	ND	ND
3	$CD34^+$ Thy^+	88-14	Sorted Thy-1.2^-	7	2	1.9	<1	<1
4	$CD34^+$ Thy^+	121-27	Thymocyte	4	60	1.1	24	40
4	$CD34^+$ Thy^+	107-49	Thymocyte	4	46	0.7	14	30
4	$CD34^+$ Thy^+	121-10	Thymocyte	4	17	1.0	10	59
4	$CD34^+$	121-7	Thymocyte	4	73	0.32	2.9	3.9
4	$CD34^+$	107-46	Thymocyte	4	53	0.67	2.3	4.3
4	$CD34^+$	121-6	Thymocyte	4	23	0.27	0.82	3.5
4	$CD34^+$	121-30	Thymocyte	4	6	0.5	0.74	12.3
4	$CD34^+$	121-5	Thymocyte	4	75	<0.002	<0.1	<0.1
4	$CD34^+$ Thy^-	121-1	Thymocyte	4	103	0.07	0.48	0.46
4	$CD34^+$ Thy^-	107-2	Thymocyte	4	23	0.06	0.2	0.86
4	$CD34^+$ Thy^-	121-4	Thymocyte	4	48	0.02	0.67	1.39
4	$CD34^+$ Thy^-	107-47	Thymocyte	4	100	<0.002	<0.1	<0.1

[a] Experiments 1 to 4 were performed at different times with different CD34-positive cells from male fetal livers (gestation ages, 16 to 24 weeks). The source of progenitor cells is the cell type injected into irradiated Thy/Liv implants of SCID-hu mice. For a given experiment, the retrovirus vector stocks were the same. Cell type is the type of cells analyzed. $CD34^+$ is CD34-positive cells infected with SRαLluc. Thymocytes are cells biopsied from reconstituted Thy/Liv implants of SCID-hu mice. Sorted Thy-1.2^+ and sorted Thy-1.2^- mean murine Thy-1.2-positive and -negative thymocytes sorted by the FACStar^{plus} (Becton-Dickinson), respectively. The mouse identification (ID) number preceding the hyphen refers to the Thy/Liv implant before reconstitution. Cells were analyzed at 0, 4, 6, and 7 weeks postreconstitution (0 refers to the time prior to CD34-positive cell injection into irradiated Thy/Liv implants). Percent reconstitution was calculated by dividing the values determined by Y chromosome PCR by the values determined by β-globin PCR. The number of reporter genes per donor cell was calculated by dividing the values determined by *luc* or *thy-1.2* PCR by the values determined by Y chromosome PCR. For *luc* gene experiments, expression is reported in relative light units per 10^5 CD34-positive cells or total thymocytes, and for *thy-1.2* gene experiments, expression is reported as percent Thy-1.2 expression in total thymocytes from Thy/Liv implants. Normalized expression (relative light units per unit of *luc* DNA for *luc* gene experiments and percent Thy-1.2 in donor thymocytes for *thy-1.2* gene experiments) was calculated by the following formulas: RLU/U of *luc* DNA = (RLU/10^5 total cells)/no. of *luc* DNA copies in 10^5 total cells and % Thy-1.2 in donor thymocytes = (% Thy-1.2 in total thymocytes/% reconstitution) × 10^2). ND, not done. —, no significant level of luciferase activity. Most PCR experiments were done twice. The coefficient of variation for the values of PCR quantification was generally less than 30% (18). The percentages of Thy-1.2 and CD34 double-positive cells of each SRαLthy-infected CD34-positive cell were 10% in experiment 2, 53% in experiment 3, and 24% in experiment 4. The levels of sorting purity of Thy-1.2 and CD34 double-positive cells were 75% in experiment 2, 97% in experiment 3, and 98% in experiment 4. Sorting purity was determined by flow cytometric analysis of sorted cells. The lower levels of Thy-1.2 expression and the lower sorting purity in experiment 2 may have resulted in the lower levels of *thy-1.2* reporter gene per donor cell following reconstitution in thymocytes.


A
a
CD34
53%
R2
PE-FL2
FITC-FL1
Thy1.2
b
IgG1
R2
PE-FL2
FITC-FL1
IgG2b
c
CD45
24%
FL1-H
FL2-H
Thy1.2
d
CD45
5%
FL1-Height
FL2-Height
Thy1.2
B
Thymocytes
88-33, 4w
88-23, 4w
88-23, 7w
83-31, 4w
83-31, 6w
mouse DNA
female DNA
Sorted thymocytes
88-23, 7w
83-31, 6w
unsort
Thy+
Thy-
thy1.2 DNA (Copy No.)
200
600
1800
5400
16000
48000
thy1.2
(131 bp)
Male Cell Equivalents
100
300
1000
3000
10000
Y-Chromosome
(138 bp)
%Reconstitution
46 17 7 62 58 7 61 2 58 57 41
thy1.2 transduction
(Reporter gene per
donor cell)
2.1 0.7 8.5 0.3 0.4 8.5 13 0.7 0.4 1.4 0.3

reconstituted animals showed typical CD4-positive, CD8-positive, and CD4-CD8 double-positive thymocyte profiles (data not shown). We achieved multiplicities of infection in CD34-positive cells of greater than 1 (Table 1), most likely as a result of using highly concentrated VSV-G-pseudotyped viruses. In the thymocytes derived from the transduced CD34-positive cells, we found that the transgene level was approximately one per cell, consistent with the majority of thymocytes harboring the vector. Detectable *luc* expression was present in thymocytes of 11 of 13 reconstituted Thy/Liv implants of SCID-hu mice, ranging from 485 to 5,806 relative light units (RLU)/10^5 total thymocytes (Table 1). The calculated levels of *luc* expression (RLU per *luc* DNA copy were much lower than those of either the input CD34-positive cells (Table 1) or those of in vitro infections (typically, 1 to 10 RLU per *luc* DNA copy in human transformed cell lines; data not shown). These low levels of transgene expression are consistent with the low levels of *neo*^{r} expression that we previously reported (1) and with the general experience of others engaged in stem cell gene therapy experimentation (4, 5, 7, 12).

Immunoselection of CD34-positive cells tranduced with a *thy-1.2*-bearing vector and reconstitution of irradiated Thy/Liv implants of SCID-hu mice. We undertook a different strategy that enabled selection by FACS to enrich transduced and transgene-expressing cell populations. The *luc* gene was replaced with the murine *thy-1.2* gene, which we previously showed to be effective for monitoring of gene expression in individual cells by flow cytometry (32). One additional step was introduced into the reconstitution protocol whereby, following transduction of CD34-positive cells, the cells were purified by FACS on the basis of Thy-1.2 positivity and CD34 surface expression (Fig. 2A). In this manner, Thy/Liv implants of SCID-hu mice should be reconstituted with CD34-positive cells that are highly enriched for the transgene. At 4 to 6 weeks after introduction of CD34-positive cells into Thy/Liv implants, the levels of vector DNA ranged from 0.1 to 8.5 copies of *thy-1.2* per donor-derived thymocyte (Table 1 and Fig. 2B). We confirmed Thy-1.2 expression in human cells by demonstrating a distinct population of cells positive for both Thy-1.2 and CD45, a marker for human lymphocytes (Fig. 2A). When normalized to the percentage of donor cells, calculated by PCR for human Y chromosome versus β-globin sequences, the proportion of cells expressing Thy-1.2 ranged from 4.4 to 71% (Table 1). In other experiments, we compared reconstitution and expression of an unfractionated CD34-positive cell population with the same cells sorted into Thy-1.2-positive and Thy-1.2-negative subpopulations. As expected, we detected little or no *thy-1.2* expression in thymocytes derived from reconstitution of CD34-positive, Thy-1.2-negative cells, low levels (<3%) in thymocytes derived from unfractionated CD34-positive cells, and much greater levels (<25%) in thymocytes derived from CD34-positive, Thy-1.2-positive cells (Table 1, experiment 4).

The Thy-1.2-positive, CD34-positive cells selected by FACS should have been highly enriched for Thy-1.2-containing cells, yet there appeared to be a proportion of donor-derived thymocytes in all Thy/Liv implants which harbored *thy-1.2* DNA but did not express the transgene at a level detectable by flow cytometry. We confirmed this by sorting the thymocytes into Thy-1.2-positive and Thy-1.2-negative subpopulations and determining the levels of vector DNA in the two populations. Our results show that vector DNA was maintained in both Thy-1.2-positive and Thy-1.2-negative donor cell populations, although the Thy-1.2-positive population generally harbored higher levels, and apparently multiple copies, of *thy-1.2* DNA per cell than the Thy-1.2-negative population (Table 1 and Fig. 2B).

Three-color flow cytometric analysis determined that when thymocytes were first gated on Thy-1.2-positive cells and subsequently analyzed for expression of CD4 and CD8, the distribution of CD4- and CD8-positive cells was similar to that of the ungated population, indicating that Thy-1.2 is expressed at similar proportions on the different CD4- and CD8-positive cell subpopulations Fig. 3A, compare c with d; also see Fig. 3B). In two Thy/Liv implants, we also analyzed the CD1-positive immature and CD3-positive and CD5-positive mature thymocyte subpopulations (Fig. 3B). In general, Thy-1.2 was expressed equally in all subpopulations, indicating vector expression throughout thymopoiesis. In one Thy/Liv implant, Thy-1.2 was preferentially expressed in the CD1-expressing immature thymocyte subpopulation (Fig. 3B), but no difference was observed in the second Thy/Liv implant (data not shown).

Activation of thymocytes does not induce increased Thy-1.2 expression. Since a proportion of the cells harboring *thy-1.2* DNA were negative for Thy-1.2 expression, we tested whether we could induce Thy-1.2 expression following activation of the cells. Thymocytes from 2 Thy/Liv implants harboring luciferase vectors and 14 Thy/Liv implants harboring *thy-1.2* vectors were stimulated in vitro with IL-2 or anti-CD3 or anti-CD28 MAbs for 2 or 3 days. The rate of thymidine incorporation of the stimulated cells increased approximately 10-fold over that of nonstimulated cells, and CD25 expression increased from undetectable levels to 6 to 18% of the total thymocyte population. Thy-1.2 expression remained unchanged in the stimulated thymocytes (Table 2). Therefore, at least by this means of stimu-

FIG. 2. (A) (a and b) Selection by FACS of cells positive for human CD34 and murine Thy-1.2. (a) SRαLthy-infected, CD34-positive cells were stained for murine Thy-1.2 (Thy1.2) and human CD34 (CD34) markers, and Thy-1.2 and CD34 double-positive cells were selected by FACS. The representative data shown are from experiment 3 of Table 1, in which 53% of the cells were Thy-1.2 and CD34 double positive, relative to the gates set by using the isotype control. The R_2 gate used for sorting was set tightly and represented 39% of Thy-1.2 and CD34 double-positive cells. Sorting purity was 97%. (b) Isotype control. The same cells as those used for panel a were also stained with MAbs for mouse IgG2b (IgG_{2b}) and mouse IgG1 (IgG_1), conjugated with FITC or PE, respectively, to quantify nonspecific staining. (c and d) Flow cytometric analysis of murine Thy-1.2 and human CD45 of thymocytes from reconstituted SCID-hu Thy/Liv implants. The representative data shown are from experiment 3 of Table 1. Biopsies were obtained at 4 and 7 weeks postinjection of CD34-positive, Thy-1.2-positive cells (shown in panel a) into irradiated SCID-hu Thy/Liv implants. Thymocytes were stained for murine Thy-1.2 (Thy 1.2) and human CD45 (CD45). (c) Thymocytes taken from the reconstituted Thy/Liv implant of mouse 88-33 (see Table 1) at 4 weeks postinjection. Twenty-four percent of CD45-positive cells expressed Thy-1.2. (d) Thymocytes taken from the reconstituted Thy/Liv implant of mouse 88-23 at 7 weeks postinjection. Five percent of CD45-positive cells expressed Thy-1.2. (B) Representative murine *thy-1.2* (thy1.2) and human Y chromosome (Y-Chromosome) quantitative PCRs. The data shown are from experiments 3 (mice 88-33 and 88-23) and 2 (mouse 83-31) of Table 1. DNA from thymocytes obtained by biopsy at 6 weeks (6W) and 7 weeks (7W) postreconstitution was analyzed for *thy-1.2* gene transduction by PCR (left panels). Thymocytes were stained and sorted based on the Thy-1.2 surface marker and analyzed for *thy-1.2* gene transduction in the Thy-1.2-positive (Thy+) and -negative (Thy−) thymocytes and unsorted (unsort) populations (middle panels). The sorting purities of Thy-1.2-positive and -negative thymocytes were 80 to 98% and 99%, respectively, as determined by flow cytometric analysis of the sorted cells (Table 1). Quantitative *thy-1.2* DNA and male cell DNA standards (Male Cell Equivalents) were assayed in parallel (right panels). Mouse cell DNA (mouse DNA) and human female cell DNA (female DNA) were analyzed in parallel as negative controls. The *thy-1.2*-specific signal was compared with that of amplified Y chromosome sequences to determine the number of *thy-1.2* gene copies per donor cell (Reporter gene per donor cell; see Table 1). In other PCR analyses (data not shown), the Y chromosome-specific signal was compared with that of amplified human β-globin sequences to determine the number of donor cells per total thymocyte population (%Reconstitution; see Table 1). FL, fluorescence.

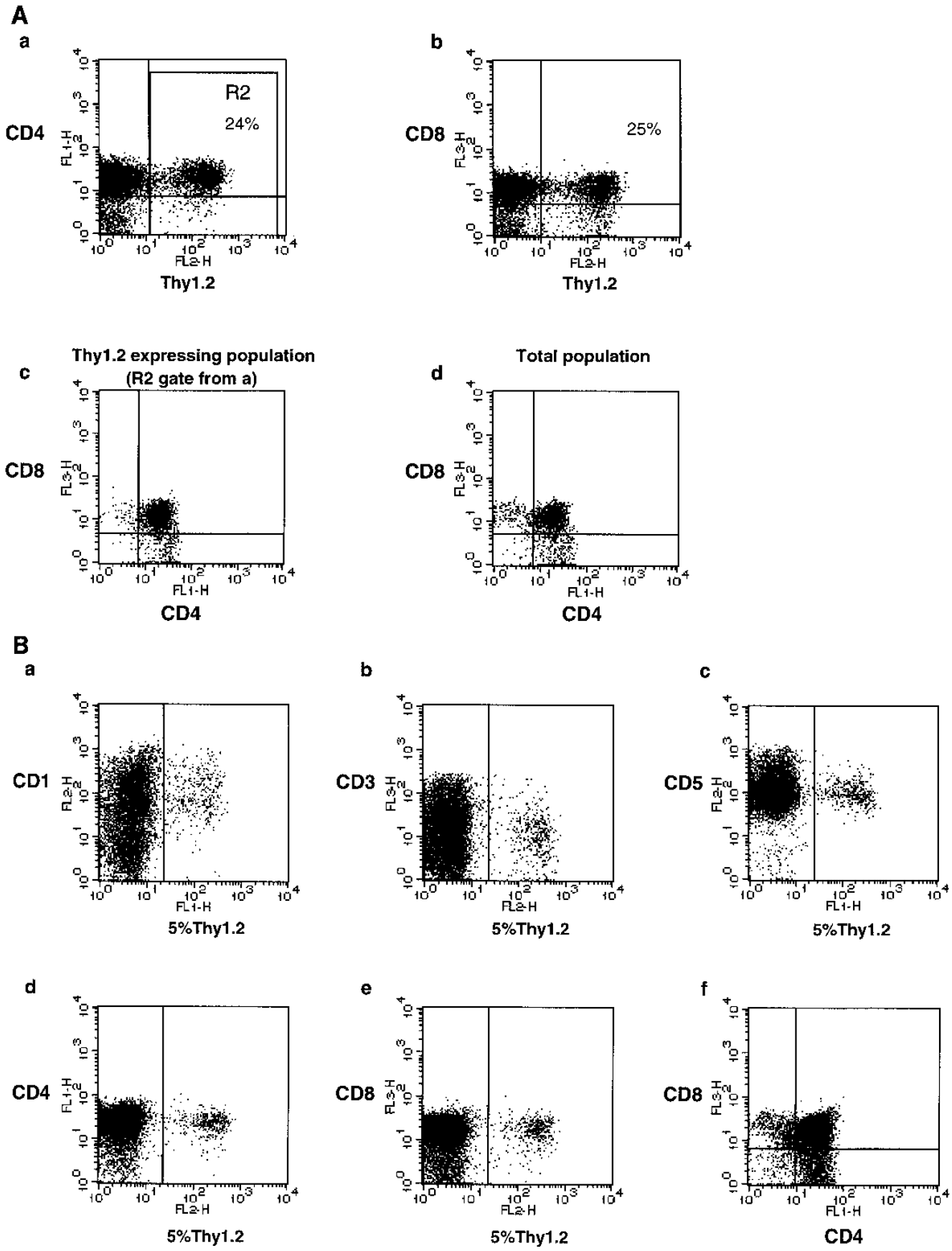

A
a
CD4
R2
24%
FL1-H
FL2-H
Thy1.2
b
CD8
25%
FL3-H
FL2-H
Thy1.2
c
Thy1.2 expressing population
(R2 gate from a)
CD8
FL3-H
FL1-H
CD4
d
Total population
CD8
FL3-H
FL1-H
CD4
B
a
CD1
FL2-H
FL1-H
5%Thy1.2
b
CD3
FL3-H
FL2-H
5%Thy1.2
c
CD5
FL2-H
FL1-H
5%Thy1.2
d
CD4
FL1-H
FL2-H
5%Thy1.2
e
CD8
FL3-H
FL2-H
5%Thy1.2
f
CD8
FL3-H
FL1-H
CD4

FIG. 3. Flow cytometric analysis of *thy-1.2*-transduced thymocytes from reconstituted Thy/Liv implants of SCID-hu mice. (A) The representative data shown are from experiment 3 of Table 1. The reconstituted Thy/Liv implant of mouse 88-33 was biopsied at 4 weeks postinjection of Thy-1.2 and CD34 double-positive cells. Thymocytes were analyzed by three-color flow cytometry for murine Thy-1.2 (Thy1.2) and human CD4 (CD4) and CD8 (CD8) markers. (a) Data for CD4 and Thy-1.2. Twenty-four percent of CD4-positive cells expressed Thy-1.2. The Thy-1.2-positive population was gated by the region shown as R2 for analysis as shown in panel c. (b) Data for CD8 and Thy-1.2. Twenty-five percent of CD8-positive cells expressed Thy-1.2. (c) R2-gated Thy-1.2-positive population analyzed for CD4 and CD8. Two thousand events were analyzed for direct comparison with panel d. (d) CD4 and CD8 staining of total thymocytes. Two thousand events were analyzed for direct comparison with panel c. (B) The representative data shown are from experiment 3 of Table 1. The reconstituted Thy/Liv implant of mouse 88-23 was biopsied at 7 weeks postinjection of Thy-1.2 and CD34 double-positive cells. Thymocytes from this implant preferentially expressed Thy-1.2 in the subpopulation of cells with higher CD1 levels. FL, fluorescence.

lation, it appears that activation of the thymocytes is insufficient to induce greater levels of Thy-1.2 expression.

DISCUSSION

In human progenitor cell transductions with amphotropic retrovirus vectors reported to date, transduction efficiencies in CD34-positive cells have been estimated to be 10 to 40%, and progeny cells harbor transduced genes at 0.01 to 5% (4, 5, 7, 12, 16, 17). We previously showed, by using the SCID-hu Thy/Liv mouse model (23), that an amphotropic retrovirus vector commonly utilized for gene transfer purposes, LNL6 (25), gave results consistent with these low efficiencies (1). Here we demonstrate that the majority of thymocytes derived from reconstitution with CD34-positive cells previously transduced with VSV-G-pseudotyped retroviral vectors can harbor vector DNA sequences. This is presumably because of the greater titers achievable through concentration of VSV-G-pseudotyped viruses, although we cannot exclude potential differences in cell tropism between VSV-G and the MuLV amphotropic envelope. Thus, one approach to the introduction of a transgene efficiently into at least the T-cell compartment is through use of high-titer VSV-G pseudotypes.

We also demonstrate that a complementary approach, perhaps more suitable in situations in which high titers are not achievable or not desired, would be to utilize vectors bearing a reporter gene encoding an immunoselectable cell surface marker (22, 31). In this manner, transduced cells can be physically separated from nontransduced cells by virtue of expression of a cell surface marker. Use of this approach may be most effective when utilizing amphotropic and/or gibbon ape leukemia virus pseudotypes (24, 25), where the efficiencies of CD34-positive cell infection are relatively low. Although the approach described here could have application for human gene therapy, a number of potential obstacles need to be addressed. For future clinical applications, it would be advantageous to develop immunoselection methods that do not rely upon FACS, such that larger numbers and more rapid purifications of transduced progenitor cells would be possible. In addition, a cell surface reporter gene may be immunogenic and lead to rejection of reconstituted cells expressing the transgene. Clinical application of such an approach would likely require use of small cell surface proteins that consist of nonimmunogenic epitopes or, alternatively, vectors that do not express the selectable transgene after reconstitution.

A similar approach was described in a murine gene therapy system (31). Another recent report described the use of a vector bearing murine CD2 and transduction of CD34-positive cord blood cells injected directly into a human fetal thymus fragment in SCID mice (SCID-hu thymus) (9). The approach differs from ours in that our SCID-hu Thy/Liv mice contain a relatively long-lived, self-renewing organ that is irradiated prior to reconstitution. Thus, our system may be more closely representative of a human stem cell gene therapy protocol in which reconstitution of an established site of hematopoiesis is undertaken after irradiation to deplete endogenous stem cells. Furthermore, our experiments involved selection by FACS of vector-expressing progenitor cells, which was not performed in the previous study.

Either one or a combination of these two approaches should allow reconstitution with 100% transduced CD34-positive cells, thus ensuring that all of the differentiated progeny would harbor the vector. In some situations, it may be desirable that only a proportion of the cells be transduced. Use of *thy-1.2* or a comparable reporter gene would allow accurate control of the proportion of transduced cells relative to nontransduced cells. Perfection of either of these two approaches would solve one of the major technical hurdles in human stem cell gene therapy.

The other major hurdle in gene therapy applications is the maintenance of efficient expression in differentiated cells derived from progenitor cells (8, 13; reviewed in references 10, 27, and 35). Consistent with the experience of other in vivo gene therapy applications, our results indicate that the MuLV-based vector expresses genes poorly in the majority of thymocytes following differentiation, in contrast to the same vectors in cultured T cells. It is of note that selection by FACS of the transduced CD34-positive population not only ensured efficient transduction but also resulted in a significant proportion of cells expressing Thy-1.2. Nevertheless, a proportion of donor-derived thymocytes harboring the transgene are unable to express *thy-1.2* at a level detectable by flow cytometry, apparently independent of activation state or the stage of thymopoiesis. Use of this model to understand the factors responsible for the higher levels of gene expression in some cells will have important implications for the effective use of gene transfer as a therapeutic modality. The SCID-hu mouse model, in combi-

TABLE 2. Activation of thymocytes with anti-CD3 and anti-CD28 mAbs and IL-2 does not induce increased Thy-1.2 expression[a]

Mouse ID no.	Stimulated thymocytes			Unstimulated thymocytes		
	% Thy-1.2 expression	% CD25 expression	[^{3}H]thymidine incorporation (cpm)	% Thy-1.2 expression	% CD25 expression	[^{3}H]thymidine incorporation (cpm)
121-27	20.6	18.3	3,258	24.6	1.1	304
107-49	13.0	17.7	1,622	13.4	0.7	170
121-10	10.9	11.8	1,115	10.1	0.38	128
107-46	3.9	16.2	2,234	5.6	1.0	222
121-30	1.2	6.4	636	0.8	0.08	173
121-1	0.7	9.2	4,962	0.6	0.9	776

[a] Thymocytes (10^6) obtained from reconstituted Thy/Liv implants were cultured for 2 days in the presence of IL-2, anti-human CD28 MAbs, and immobilized anti-human CD3 MAbs. Control thymocytes were cultured in parallel in the absence of growth-stimulating agents. After 2 days of stimulation in vitro, thymocytes were analyzed by flow cytometry for murine Thy-1.2 and human CD25 cell surface markers. Dead cells were excluded by 7-amino-acid–actinomycin D staining. [^{3}H]thymidine incorporation was measured by pulse-labeling of cells for 6 h on day 2. This experiment was repeated twice in independent mouse experiments, and results of a representative experiment are shown. Mouse identification ID numbers correspond to those in Table 1.

nation with the reporter genes we described here, will provide a rapid means to assess different vector strategies.

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