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Immunology

Long-term expression of a T-cell receptor β-chain gene in mice reconstituted with retrovirus-infected hematopoietic stem cells

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ABSTRACT To determine the feasibility of retrovirus-mediated gene transfer into stem cells for studying T-cell development, we constructed a high-titer retrovirus vector containing the neomycin phosphotransferase (*neo*) gene and a murine T-cell receptor (TCR) β-chain gene with the $V_{\beta}6$ variable segment. The TCR gene was placed under the control of the human β-actin promoter and enhancer. Bone marrow cells pretreated with 5-fluorouracil were infected by coculturing with ψ-2 virus-producing cells in the presence of recombinant interleukins 1, 2, 4, and 6 as well as interleukin 3 from WEHI-3 conditioned medium. The infected cells were transplanted into irradiated mice, and expression of the exogenous $V_{\beta}6$ gene was examined with a $V_{\beta}6$-specific monoclonal antibody, RNase protection, and polymerase chain reaction amplification. Three of seven mice expressed the retroviral TCR gene on the surface of a significant proportion of mature T cells 5–6 months after transplantation. In mice analyzed <1 month after transplantation, up to 30% of mature T cells expressed $V_{\beta}6$ TCRs, an increase of at least 20% above the level of endogenous $V_{\beta}6$ expression. DNA analysis revealed that pluripotent hematopoietic stem cells were infected by the retroviral vector in a long-term reconstituted mouse that showed increased $V_{\beta}6$ expression.

All hematopoietic cell types, including T and B lymphocytes, are derived from pluripotent stem cells of the bone marrow (BM). Committed T-cell progenitors migrate to the thymus, where T-cell precursors proliferate and are selected on the basis of the ability of the clonotypic T-cell antigen receptor (TCR) to discriminate self from nonself (1). Adoptive BM transfer into lethally irradiated hosts (2), and more recently TCR transgenic mice (3), have contributed greatly to our present understanding of the events and factors involved in T-cell differentiation. As an extension of these two animal model systems, we have examined the feasibility of introducing various genes important for T-cell development into hematopoietic stem cells by means of a retroviral vector. The infected stem cells can be transplanted into different genetic backgrounds with relative ease and the fate of the T cells that express the exogenous gene(s) can then be followed.

Retroviral vectors have been used to transfer genes into diverse cell types such as lymphocytes (4), hepatocytes (5), endothelial cells (6), and stem cells of the embryo (7) and BM (8–11). Several attempts to express exogenous genes *in vivo* using retroviral vectors were impeded by the difficulties in obtaining retroviral vectors that can infect stem cells at high efficiency on a consistent basis. This was especially true when more than one transcriptional unit was present in the vector (12). However, recent studies have suggested that consistent, long-term retroviral gene expression may be possible (13–16). Here we address the applicability of the retroviral gene-transfer technique in studying T-cell differentiation. We constructed a retroviral vector to express a well-characterized TCR β-chain gene under the control of the human β-actin promoter and enhancer. Cell lines producing high-titer recombinant virus were generated and were used to infect BM cells. BM-transplanted mice were shown to express the exogenous TCR β-chain gene in a significant proportion of thymocytes and splenic T cells 5–6 months after transplantation.

MATERIALS AND METHODS

Vector Construction and Virus-Producing Cell Lines. The basic retroviral vector containing the Moloney murine leukemia virus (Mo-MLV) long terminal repeats (LTRs) and the *neo* gene, encoding G418 resistance, was obtained from A. Bernstein (Mount Sinai Hospital, Toronto). The human β-actin promoter was excised with *Bgl* II and *Sal* I from the plasmid pβactSE (a gift from N. Yamamoto, Ontario Cancer Institute, Toronto) and cloned into the retroviral vector. The β-actin enhancer fragment, located downstream of the promoter in the first intron of the β-actin gene (17), was cloned into a *Sal* I site, downstream of the β-actin promoter in the vector. No significant difference in the promoter activity was detected when the enhancer was placed upstream of the promoter. Full-length cDNAs encoding the TCR α and β chains were isolated in our laboratory from a T-cell hybridoma, AF 3G7 (from L. Glimcher, Harvard Medical School, Boston, MA), as described (18). This cell line reacts specifically to beef insulin when presented by $I\text{-}A^{b}$-expressing cells. The AF 3G7 TCR β-chain gene is composed of variable (V), diversity (D), joining (J), and constant (C) segments as follows: $V_{\beta}6\text{-}D_{\beta}2\text{-}J_{\beta}2.3\text{-}C_{\beta}2$. The gene was cloned into the retroviral vector at the *Hin*dIII site after removal of the poly(A)-addition signal. The complete vector will be referred to as MBAE-$V_{\beta}6$ (Fig. 1*A*) and the TCR β-chain cDNA will be referred to as $rV_{\beta}6$ to distinguish it from TCRs containing an endogenous $V_{\beta}6$ gene segment. Ecotropic ψ-2 viral producer lines (19) were made, titered on RAT-II cells (12), and checked for helper virus formation by the $S^{+}L^{-}$ assay (20). The highest-titer producer lines [0.5–1 × 10^7 colony-forming units (cfu)/ml] were stored in liquid N_2 and BM cell infections were done only with freshly thawed cell lines to minimize helper cell production. Cell lines were maintained in cDMEM [Dulbecco's modified Eagle's medium with 10% (vol/vol) fetal bovine serum, 0.1 mM nonessential amino acids, and 50 μM 2-mercaptoethanol; all from GIBCO, except 2-mercaptoethanol (Kodak)].

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Abbreviations: TCR, T-cell antigen receptor; BM, bone marrow; 5-FU, 5-fluorouracil; cIFM, complete infection medium; FACS, fluorescence-activated cell sorter; Mo-MLV, Moloney murine leukemia virus; LTR, long terminal repeat; (r)IL-*n*, (recombinant) interleukin *n*; FITC, fluorescein isothiocyanate; $G418^r$, G418-resistant; cfu, colony-forming unit(s); M*n*, mouse *n*; V, variable; D, diversity; J, joining; C, constant.

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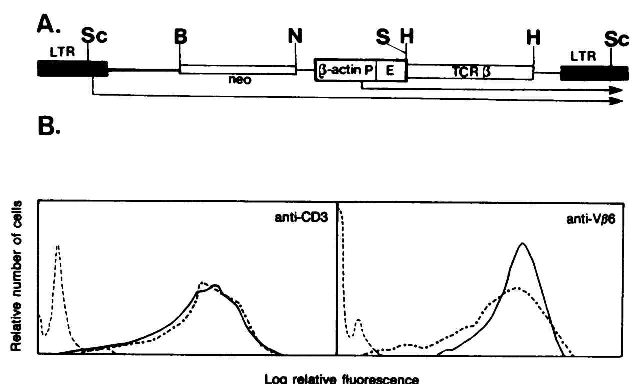

FIG. 1. (A) MBAE-$V_{\beta}6$ retrovirus vector. LTRs are from Mo-MLV; *neo*, neomycin phosphotransferase gene; P, promoter; E, enhancer; TCR-β, β-chain gene containing $V_{\beta}6$ gene segment; B, H, N, S, and Sc are: *Bgl* II, *Hin*dIII, *Nru* I, *Sal* I, and *Sac* I recognition sites, respectively. Arrows represent two mRNA transcripts expected from the vector. (B) Fluorescence-activated cell sorter (FACS) analysis of MBAE-$V_{\beta}6$-infected, G418-resistant (G418^r) BW 5147 β^- clone. Recipient cell line BW 5147 β^- (----), retrovirus-infected, G418^r BW 5147 β^- transformant (·····), and positive control AF 3G7 T-cell hybridoma (——) were stained with monoclonal antibodies specific for $V_{\beta}6$ (RR4-7) and CD3 (145.2C11).

Infection and BM Transplantation. The BW 5147 thymoma cell line and its TCR β^- mutant line (from J. Kappler, Howard Hughes Medical Institute, Denver) and a panel of beef insulin-specific T-cell hybridomas (J.W., unpublished work) were infected by coculture with high-titer MBAE-$V_{\beta}6$ virus-producing lines as described (12).

BM cells were isolated from the femurs of 10- to 14-week-old female C57BL/6 mice. Some of these mice had been injected with 5-fluorouracil (5-FU; Sigma) at 150 mg per kg of body weight 2 days prior to BM cell isolation (9). All mice were purchased from The Jackson Laboratory and maintained in the Mount Sinai Hospital Animal Colony. Infection of the BM cells with the high-titer virus-producing ψ-2 ecotropic packaging line was carried out as described (10). The complete infection medium (cIFM) consisted of cRPMI with recombinant interleukin 1 (rIL-1, 10 units/ml), rIL-2 (50 units/ml), rIL-4 (50 units/ml), rIL-6 (20 units/ml), 10% U-937 conditioned medium (CM), 15% WEHI-3B (from R. A. Phillips, Hospital for Sick Children, Toronto) CM, and Polybrene (4 μg/ml). (All recombinant interleukins were from Genzyme.) After coculture the BM cells were recovered, washed once with cRPMI, and, in some cases, selected in cIFM with G418 at 2 mg/ml (dry weight). Lethally irradiated C57BL/6 (11 Gy), BALB/c (9 Gy), and BALB/c × A/J (9 Gy) mice were each injected with $5–20 \times 10^5$ viable, infected BM cells. Mice were sacrificed 14–30 days or 5–6 months after BM transplantation.

Immunofluorescence Analysis. Spleen cells were separated into Thy-1$^+$ (T cells) and Thy-1$^-$ (non-T cells) populations by a "magnetic activated cell sorter" (21). The monoclonal antibodies used were specific for the CD3 ε chain (145.2C11, from J. Bluestone, University of Chicago), CD4 (Becton Dickinson), CD8 (Becton Dickinson), Thy-1.2, TCR $V_{\beta}6$, TCR $V_{\beta}11$ (RR4-7, RR3-15, respectively, from O. Kanagawa, Washington University, St. Louis), and TCR $V_{\beta}8$ (F23.1, from J. Kappler). The $V_{\beta}6$-specific antibody was semipurified from the culture supernatant by ammonium sulfate and caprylic acid precipitations and then conjugated with biotin (Sigma) (22). Streptavidin-fluorescein isothiocyanate (FITC) conjugate, streptavidin-phycoerythrin, goat anti-rat IgG-FITC, and goat anti-mouse IgG-FITC (all from Jackson ImmunoResearch) and goat anti-hamster IgG-FITC (Cappel Laboratories) were used as the secondary reagents. Stained cells were analyzed with an Epics V (Coulter) FACS.

DNA and RNA Isolation and RNase Protection Assay. DNA isolation, random-primer labeling of the probes with [α-^{32}P]-dCTP (Amersham), and blot analysis were done as described (23). Total cytoplasmic RNA was obtained using guanidinium isothiocyanate (24). RNA dot blot (23) and RNase protection (25) assays were carried out as described, except that when antisense RNA probes were hybridized with polymerase chain reaction (PCR)-amplified DNA, RNase digestion was carried out at 37°C for 30 min with RNases M1 and U2 (both at 10 units/ml; Pharmacia) in addition to RNases A and T1 (Sigma). The antisense RNA probes were complementary to the AF 3G7 TCR β-chain cDNA and were made using SK vectors (Stratagene) from the sequence spanning the last 50 base pairs (bp) of $V_{\beta}6$, all of $D_{\beta}2$ and $J_{\beta}2.3$, and the first 55 bp of $C_{\beta}2$.

PCR. One to 3 μg of DNase-treated RNA was heated to 60°C and then reverse-transcribed at 42°C for 1 hr with the PCR primers and Mo-MLV reverse transcriptase (Boehringer Mannheim). The oligodeoxynucleotide primers were GCCAGCAGTTCTGGGGGG (sense, $V_{\beta}6$ to $J_{\beta}2.3$, including junction nucleotides), CCAGAAGAACGAGATGGC (sense, $V_{\beta}6$), TGATGGCTCAAACAAGGAGAC (antisense, C_{β} internal), GCCAAGCACACGACGGTAGCC (antisense, C_{β} external), and CTAGCTTGCCAAACCTAC (antisense, LTR). After reverse transcription the samples were heated to 94°C for 10 min, cooled on ice, amplified using *Thermus aquaticus* DNA polymerase (Cetus) for 30 cycles (Ericomp; 94°C, 1 min; 56°C, 2 min; 72°C, 3 min), and then incubated for 15 min at 72°C. One-twentieth of the product was used for RNase protection. A similar protocol was used with the $V_{\beta}6$-sense and LTR-antisense primers to detect the provirus. Subsequent PCR products were used for RNase protection.

RESULTS

MBAE-$V_{\beta}6$ Activity *in Vitro*. The activity of the human β-actin promoter/enhancer in the retroviral vector was initially examined by infection of various T-cell lines. We used T-cell lymphoma lines that lacked a functional β chain as well as antigen-reactive T-cell hybridomas with fully functional TCRs as the recipients of r$V_{\beta}6$. Infection of the BW 5147 TCR β^- mutant, which lacks surface expression of the TCR–CD3 complex but has a functional α chain, with a high-titer MBAE-$V_{\beta}6$-producing line (10^7 G418^r cfu/ml) resulted in >35% of the cells becoming G418^r after one infection cycle. This frequency of transformation can be increased to ≈60% when the infection cycle is repeated two or more times (26).

In >80% of BW 5147 β^- transformants, $V_{\beta}6$ TCR mRNA could be detected by RNA dot blot assays (data not shown). These transformants expressed a relatively uniform level of r$V_{\beta}6$ on the surface as detected by staining with the $V_{\beta}6$- and CD3-specific antibodies (Fig. 1*B*). As expected, MBAE-$V_{\beta}6$ was functional in all other T-cell lines tested.

TCR Gene Transfer into Hematopoietic Stem Cells and Cell Surface Expression of r$V_{\beta}6$ *in Vivo*. Nine mice transplanted with 5-FU-treated BM cells that had been infected with MBAE-$V_{\beta}6$ in cIFM were sacrificed after 13–30 days and examined for cell surface $V_{\beta}6$ expression on thymocytes and splenic T cells. The proportions of T cells with surface $V_{\beta}6$ expression in short-term reconstituted mice (M1–M9; for simplicity, all mice are referred to as M*n*, mouse no. *n*), supported by DNA and RNA analyses (see below), are listed in Table 1. All treated control mice (see legend to Table 1 and Fig. 2) and age- and sex-matched untreated mice showed a consistent frequency of endogenous $V_{\beta}6$ expression: Six to 7% of thymocytes and 9–11% of splenic T cells expressed $V_{\beta}6$ in all three strains of mice used. As expected, cell surface expression of $V_{\beta}6$ was restricted to the T-cell lineage. The highest staining of $V_{\beta}6$ in splenic T cells was found in M8 (34% vs. 10% in control mice; Fig. 2*A*) and the largest increase in thymocytes was detected in M4 (18% vs. 6% in

control mice). Four mice (M1, M4, M7, and M8) showed increased expression of $V_\beta6$ in both spleen and thymus, two (M5 and M6) had increased expression only in spleen, and one (M2) showed a significantly higher level of $V_\beta6$ expression in thymocytes.

To correlate the increased proportion of T cells expressing $V_\beta6$ in reconstituted mice with the retrovirus infection efficiency, the proviral copy number was determined (Table 1 and Fig. 3*A*). In short-term reconstituted mice tested the range of provirus copy number per cell was <0.1 to about 0.3.

Table 1. Mice reconstituted with retrovirus-infected BM cells

Mouse*	Tissue†	DNA copy no.‡	RNA expression§	$V_\beta6$ surface expression ¶
M1	BM	+(P)	+	<1
	T	+(P)	+	*16*
	ST	+(P)	+(P)	*28*
M2	BM	+	+	<1
	T	+	+	*18*
	ST	+	−	12
M4	BM	+	NT	NT
	T	+++	+	*18*
	ST	+++	+	*20*
M5	BM	NT	NT	<1
	T	NT	NT	8
	ST	NT	NT	*20*
M6	BM	++	+(P)	NT
	T	+(P)	−	5
	ST	+	+(P)	*15*
M7	BM	NT	NT	NT
	T	NT	+(P)	*11*
	ST	NT	+(P)	*16*
M8	BM	NT	NT	NT
	T	+(P)	NT	*13*
	ST	+(P)	+(P)	*34*
M11	BM	+	+	NT
	T	−	−	7
	ST	−	−	*14*
M12	BM	+++	NT	NT
	T	+++	+(P)	*16*
	ST	++	+	*16*
M15	BM	++	NT	NT
	T	++	+(P)	*15*
	ST	+(P)	+	*15*
M17	BM	++	+	<1
	T	+++	+	*14*
	ST	+++	+	*21*
M20	BM	+(P)	+(P)	<1
	T	−	−	8
	ST	NT	NT	10

*Only the mice that exhibited positive results in at least one of the three categories of analysis are listed. All mice were C57BL/6 except M14–M17 (BALB/c × A/J) and M5, M6, and M18–M21 (BALB/c). M1–M9 were analyzed within a month after transplantation. M10–M21 were examined after 5 months.

†T, thymocytes; ST, splenic T cells.

‡Provirus copy number was determined as described in legend to Fig. 3: +, ≤0.1 copy per cell; ++, ≤0.25; +++, ≤0.5; (P) denotes provirus detection by PCR amplification only and the copy-number parameters do not apply. NT, not tested, because of insufficient amount of DNA.

§+, Presence of retrovirus-specific transcripts as detected by RNase protection; (P), retrovirus-specific transcripts detected by a combination of PCR amplification and RNase protection.

¶Determined by FACS analysis. Values represent the proportion of cells staining with the RR4-7 antibody in each mouse (see Fig. 2), and those that are significantly higher than the negative controls are italicized. The control mean values of five mice (two C57BL/6, two BALB/c, and one BALB/c × A/J) transplanted with mock-infected C57BL/6 BM cells were 6.2 ± 0.6% and 9.6 ± 0.8% for thymocytes and splenic T cells, respectively.

In general, higher proportion of T cells expressing $V_\beta6$ corresponded to higher proviral copy number in both the short- and long-term reconstituted mice. Moreover, in mice that did not show an increased proportion of $V_\beta6$-expressing T cells, the provirus was either undetectable or present in extremely low numbers (<0.05) in a tissue-restricted manner (M2 splenic T cells and M6 thymocytes). In M6, which showed increased $V_\beta6$ expression on splenic T cells only, the almost negligible amount of provirus in the thymus, combined with a significantly higher number of splenic T cells infected with the retrovirus, suggests that the infected splenic T cells were the product of (*i*) infected precursors with limited ability to self-renew, (*ii*) infected stem cells no longer actively generating immature thymocytes (11), or, less likely, (*iii*) long-lived mature T cells. M2, which showed a high level of $V_\beta6$ expression on thymocytes only, is difficult to interpret since the thymocytes derived from T-cell precursors expressing $rV_\beta6$ should also give rise to mature T cells in the periphery in the absence of specific negative regulation on $rV_\beta6$-expressing thymocytes. Perhaps the thymocytes expressing $rV_\beta6$ had not migrated (or could not migrate) out of the thymus at the time of analysis.

To determine the extent of retroviral gene expression in long-term reconstituted mice, we examined the proportion of T cells expressing $V_\beta6$ in 12 mice that were sacrificed 5–6 months after transplantation (M10–M21, Table 1). The success of the long-term reconstitution and retroviral gene expression seems to depend on the infection condition, in that only the mice transplanted with 5-FU-treated BM cells infected in cIFM (three out of seven mice: M12, M15, and M17) showed increased expression of $V_\beta6$ on thymocytes and splenic T cells. Two mice transplanted with untreated BM cells infected in cIFM and three mice transplanted with 5-FU-treated BM cells infected in the absence of recombinant interleukins failed to show long-term $rV_\beta6$ expression. This result supports the findings by others (9, 13, 15) implicating the growth factors and 5-FU treatment as the critical steps for successful long-term retroviral gene expression. In contrast, G418 selection does not appear to be an absolute requirement for successful reconstitution, since only M15 was transplanted with infected BM cells that were selected with G418.

Two typical examples of long-term reconstituted mice that showed increased $V_\beta6$ expression are shown in Fig. 2 (M15 and M17; no increase in M18). M17 expressed $V_\beta6$ in 21% of splenic T cells, and this increase was specific for $V_\beta6$ since the frequency of cells expressing $V_\beta8$ (or $V_\beta11$; data not shown) did not vary. In the thymus, the separation of $V_\beta6^+$ from $V_\beta6^-$ thymocytes is less distinct because immature thymocytes, about 85% of total thymocytes, express lower levels of TCR on the surface than mature thymocytes. However, the thymocytes of M15 clearly expressed more $V_\beta6$ than the thymocytes of the control mouse (Fig. 2).

The examination of the proviral copy number in long-term reconstituted mice revealed varying proportions of cells infected with the retrovirus, ranging from 0.1 to 0.5 proviral copy per cell (Fig. 3*A*). Overall, the increases in $V_\beta6$ expression among long-term reconstituted mice did not reach the high proportions seen in some short-term reconstituted mice. As pointed out before, this may be attributed to the contribution of more differentiated progeny of the stem cells that had been infected and expressing $rV_\beta6$ in short-term reconstituted mice, but only the contribution of infected stem cells can be detected 5 months after transplantation.

To confirm that the $rV_\beta6$-expressing cells originated from a common stem-cell ancestor, we performed Southern blot analysis with *Sal* I (which cuts only once within the vector) on DNA from various tissues of M12 (Fig. 3*B*). The presence of identically sized bands (the common retrovirus integration bands) in different cell types indicates that some of the cells in different tissues were generated from a common self-

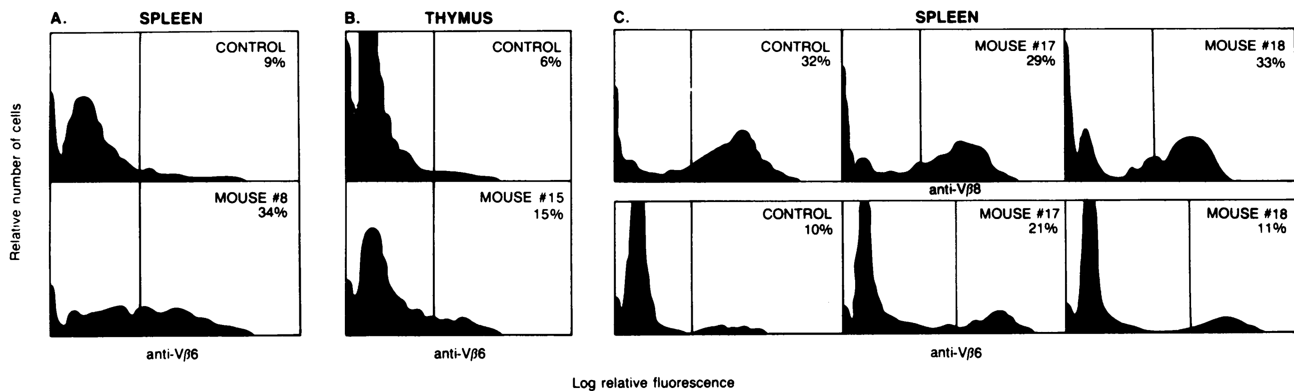

FIG. 2. FACS analysis of splenic T cells and thymocytes from BM-transplanted mice. (*A* and *B*) Proportion of splenic T cells of M8 (*A*) and thymocytes of M15 (*B*) expressing $V_{\beta}6$ as detected by monoclonal antibody RR4-7. Secondary antibody was goat anti-rat IgG-FITC in *A*. All others were stained with streptavidin-FITC. Negative control, splenic T cells from a C57BL/6 mouse transplanted with untreated syngeneic BM cells. (*C*) $V_{\beta}8$ (*Upper*) and $V_{\beta}6$ (*Lower*) expression in splenic T cells of long-term reconstituted M17 and M18 as determined by staining with monoclonal antibodies F23.1 and RR4-7, respectively. Negative control, splenic T cells isolated from one of four C57BL/6 mice transplanted with syngeneic BM cells infected with N2 retrovirus (a gift of J. Dick, Hospital for Sick Children, Toronto). This vector has the *neo* gene under the control of a Mo-MLV LTR. With splenic T-cell FACS profiles, the cursors were set in each case so as to exclude cells expressing low levels of TCR.

renewing pluripotent stem cell(s) of the BM. The number of bands suggests that three infected cells, each with one integrated retrovirus vector, originating from the BM contributed to the generation of cells in both lymphoid and myeloid lineages at the time of analysis.

RNA Analysis of the Reconstituted Mice. RNase protection assays were performed to demonstrate that the transcription of $rV_{\beta}6$ was responsible for the increased frequency of surface $V_{\beta}6$ expression in T cells. Since the probability of

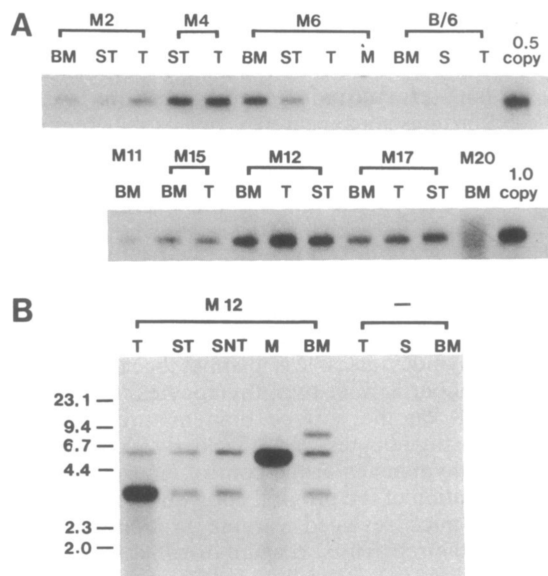

FIG. 3. (*A*) Provirus copy number. DNA (10 μg) from various cell types was digested with *Sac* I and Southern blots were hybridized with ^{32}P-labeled DNA probe complementary to the *neo* gene. Bands represent the 4.4-kbp *Sac* I fragment expected from unaltered provirus structure. ST, splenic T cells; T, thymocytes; M, mast cells obtained by culturing BM cells for >3 weeks in the presence of IL-3; B/6, C57BL/6 mouse transplanted with mock-infected syngeneic BM cells; 0.5 or 1 copy, BW 5147 thymoma line infected with the vector and containing 0.5 or 1 provirus per cell. Copy number was determined after DNA quantity normalization of the blots with the Molecular Dynamics computing densitometer. (*B*) Analysis of DNA samples from M12. Size markers (kbp) are shown at left. SNT, Thy-1^{-} cells from the spleen; –, DNA samples from a C57BL/6 mouse transplanted with mock-infected syngeneic BM cells; S, total spleen cells. All lanes were loaded with 10 μg of *Sal* I-digested DNA.

endogenous $V_{\beta}6$-containing β-chain mRNAs having exactly the same nucleotide sequence as the AF 3G7 β-chain gene is extremely small [TCR αβ junctional repertoire size is estimated to be $>10^{15}$ (27)], antisense RNA probes that were perfectly complementary to the $rV_{\beta}6$ V-D-J junctional region were made. These probes protected a 155-nucleotide fragment only in RNA samples from the cells expressing $rV_{\beta}6$ (band 1 on the autoradiograph of Fig. 4, lanes M4, M15, and M17, but not M11). The 155-nucleotide protected band seen with the thymocyte RNA from M4 is appreciatively higher in intensity than those detected in other mice and this correlates roughly with the higher frequency of $V_{\beta}6$ expression on the thymocytes of M4.

To increase the sensitivity of detection, we amplified RNA from selected samples by PCR, and a modified RNase protection assay was performed with the amplified products (data not shown). Again, the increased proportions of T cells expressing $V_{\beta}6$ correlated with the presence of $rV_{\beta}6$-specific transcripts in the cells (Table 1). The retrovirus-specific transcripts were also detected in the splenic non-T-cell population in M12 and M15 and in the erythrocytes of M12 (data not shown), indicating that MBAE-$V_{\beta}6$ is active in various lineages of infected stem cells or progenitors.

In >30 mice transplanted with treated BM cells—including negative controls—the increased frequency of $V_{\beta}6$-expressing T cells correlated with the presence of provirus and $rV_{\beta}6$ transcripts. Conversely, no provirus or $rV_{\beta}6$ transcripts were found in mice that showed a normal proportion of $V_{\beta}6$ T cells. This strict concordance was not evident in two short-term reconstituted mice (M2 and M6) in which the provirus was present in a tissue-restricted manner and at extremely low copy numbers (see above) and in one long-term reconstituted mouse (M11). FACS analysis of M11 indicated that 14% of the splenic T cells expressed $V_{\beta}6$, but the $rV_{\beta}6$-specific transcripts and provirus could not be found in either thymus or spleen. However, the $rV_{\beta}6$-specific transcripts and provirus were detected in the BM. In this particular case the proportion of the splenic T cells expressing endogenous $V_{\beta}6$ seems abnormally high, for unknown reasons, and the presence of $rV_{\beta}6$-specific transcripts in the BM cells only is another example of organ-restricted retroviral gene expression (10). Similar BM-restricted $rV_{\beta}6$ expression was detected in M20 (data not shown).

DISCUSSION

The surface expression of exogenous TCR β chain in the T-cell lineage of reconstituted mice was achieved using a high-titer

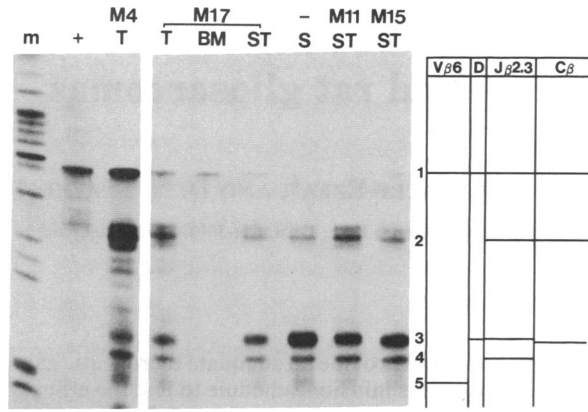

FIG. 4. RNase protection analysis. Lanes represent 20 μg of total RNA hybridized with RNA probes, except lane +, where 4 μg of positive control RNA from AF 3G7 was used, and lane M, size markers. With RNA samples from splenic T cells (ST) and thymocytes (T), the endogenous expression of one or more of the AF 3G7 β-chain gene segments ($V_{\beta}6$, $D_{\beta}2$, $J_{\beta}2.3$, or $C_{\beta}2$) in uninfected T cells results in the protection of subfragments. The presumed identity of each band is indicated at right. Band 1 represents the 155-nucleotide fragment expected of RNAs perfectly complementary to the probe. The exact identity of bands 3 and 4 could not be determined. Since BM cells normally do not transcribe fully rearranged TCR β-chain genes and since there is only one functional $V_{\beta}6$-containing β-chain gene in AF 3G7, subfragments were not detected in these samples. Lane m, ^{32}P-end-labeled *Hpa* II-digested pBR322 marker; −, negative control RNA from a C57BL/6 mouse reconstituted with mock-infected syngeneic BM cells.

virus (10^7 G418^r cfu/ml) and a number of interleukins that affect stem-cell proliferation during the infection stage. The importance of 5-FU treatment, IL-3, and IL-6 indicates that consistently effective retrovirus-mediated gene transfer into stem cells will require a detailed understanding of the factors involved in stem-cell proliferation and differentiation (13, 15, 28).

In general, when significant retroviral DNA sequence was detected, an increase in $V_{\beta}6$ surface expression followed. Although the possible downregulation of the internal β-actin promoter in the vector by the presence of nearby LTR sequence or by the retroviral integration into transcriptionally inactive chromosomal sites cannot be ruled out, the β-actin promoter in the retroviral vector was able to faithfully transcribe the TCR gene in most cell types *in vivo*. Proviral copy number-independent variation in gene expression may reflect the retrovirus integration site-controlled variation in transcription rate, specific requirements for proper TCR complex formation in T cells, and the activity of any given infected stem cells or progenitors in the generation of hematopoietic cells at a particular time (11). Since the maximum provirus copy number detected was 0.5 per cell, increased infection frequency should result in more T cells expressing the retroviral gene, even though the extent of increase in gene expression is difficult to predict and will certainly vary from one host to another regardless of infection frequency.

The level of long-term gene expression should increase with further improvements in the infection procedure (13, 15, 26) and vector construction (29). We have reported the level of retroviral TCR gene expression in terms of the proportion of T cells expressing the protein on the cell surface as detected by monoclonal antibodies. In cells that expressed $rV_{\beta}6$, the density of TCR on the cell surface was comparable to that of cells expressing endogenous $V_{\beta}6$ TCR (Fig. 2). For detection of surface expression, the β chain must be present in sufficient quantity to associate with the endogenous α chain and CD3 complex. Therefore, it is possible that we underestimated the extent of retroviral gene transcription, since some cells may have expressed the $rV_{\beta}6$ at a level insufficient for antibody detection. Regardless, the demonstration of high proportions of total T cells expressing $rV_{\beta}6$ at a comparable level to the endogenous TCR genes in a number of mice indicates that the technique can easily be used to study TCR selection in the thymus.

Clearly, retroviral vectors can be a versatile tool for transferring and expressing exogenous genes in animals to study gene function. We have indicated that more than one transcriptional unit can be inserted into the vector (*neo* and TCR genes) without major deleterious effects. Since dominant drug selection does not appear to be critical, one or more genes involved in T-cell development, such as the genes encoding specific TCRs, CD4, CD8, and interleukins, may be simultaneously introduced into different target cells, including pluripotent and lineage-restricted stem cells, T-cell precursors, and peripheral blood lymphocytes. The development of these infected cells can then be examined in genetically diverse host environments.

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