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Genetics

Handicapped retroviral vectors efficiently transduce foreign genes into hematopoietic stem cells

(c-*myc* gene/immunoglobulin genes/*W*-mutant mice/*scid*-mutant mice/development *in vivo*)

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ABSTRACT **Retroviral vectors, designated handicapped, are described. These are genetically defective viruses that allow transfer of nonselectable genes under the transcriptional control of internal promoters. The basic handicapped vector (pHHAM) is derived from Harvey, Abelson, and Moloney murine retroviruses. It contains a 327-base-pair deletion in the 3′ long terminal repeat that spans enhancer and promoter sequences. The deletion is successfully transferred to the 5′ long terminal repeat after reverse transcription of viral RNA, yielding a provirus incapable of synthesizing viral transcripts. HHAM viruses containing the mouse c-*myc* gene under the control of immunoglobulin κ chain gene regulatory elements, along with a selectable gene (*neo*) driven by a weak promoter (*tk*), were stably transmitted to cultured mouse B cells. The donor c-*myc* gene was transcribed from the κ promoter in these cells. Helper-free virus-producing cell lines were generated at titers favorable for the efficient introduction of HHAM viruses, even without selection, into hematopoietic stem cells from mouse bone marrow. When returned to unirradiated congenic recipient mice, the cells were capable of long-term reconstitution of the myeloid and lymphoid lineages of *W*/*W*^{v} mutants and the lymphoid system of *scid* mutants.**

Defective recombinant retroviruses have been used to transfer a selectable gene into mouse hematopoietic stem cells capable of long-term reconstitution of irradiated recipients (1, 2). Many genes of interest are, however, nonselectable, and they would ideally be placed under the transcriptional control of tissue-specific regulatory elements. We have, therefore, designed a series of retroviral vectors, termed handicapped vectors, in which the viral transcriptional unit has been effectively removed, allowing transduced genes to be expressed from experimentally chosen internal promoter sequences.

In humans, mice, and rats, deregulated expression of the c-*myc* protooncogene, resulting from recombinations with immunoglobulin loci, has been implicated in the etiology of B-cell neoplasms (3). Transgenic mice containing c-*myc* genes under the control of immunoglobulin gene enhancers have in fact been shown experimentally to have a predisposition toward B-cell malignancy (4). We have here linked the mouse c-*myc* gene to immunoglobulin gene regulatory elements as a model system to introduce into hematopoietic stem cells a nonselectable gene to be expressed later in the appropriate blood-cell lineage.

MATERIALS AND METHODS

Mice. Mice of the B.C-20 strain are *Igh-1*^{a}/*Igh-1*^{a}; *Igh-6*^{a}/*Igh-6*^{a} congenic partners of C57BL/6 (*Igh-1*^{b}/*Igh-1*^{b}; *Igh-6*^{b}/*Igh-6*^{b}) mice. *Hbb*^{d}-C57BL/6 mice are homozygous for the *diffuse* electrophoretic variant of the β chain of hemoglobin; C57BL/6 standard mice have the *single* (*Hbb*^{s}/*Hbb*^{s}) variant. *W*/*W*^{v}-C57BL/6 (*W*/*W*^{v}) mice are mutants at the dominant white spotting locus causing a hematopoietic stem-cell defect and anemia. The *scid* (severe combined immune deficiency) mutation is homozygous in the C.B-17 strain (5), an *Igh-1*^{b}/*Igh-1*^{b}; *Igh-6*^{b}/*Igh-6*^{b} congenic partner of BALB/c (*Igh-1*^{a}/*Igh-1*^{a}; *Igh-6*^{a}/*Igh-6*^{a}).

Retroviral Vectors. Our pHHAM vectors (for handicapped vector derived from Harvey, Abelson, and Moloney murine retroviruses) were constructed in a series of steps from the chimeric murine retroviral vector pAFVXM (a gift from Michael Kriegler) using standard techniques (6). pAFVXM differs from conventional retroviral vectors in having nonidentical long terminal repeat (LTR) sequences. The 5′ LTR has sequences from Abelson murine leukemia virus (A-MuLV) and from Harvey murine sarcoma virus (Ha-MuSV), while the 3′ LTR has Moloney murine leukemia virus (Mo-MuLV) and A-MuLV sequences. In addition, the 5′-donor splice site, used in generating a subgenomic mRNA encoding the viral envelope glycoprotein, was removed (Fig. 1). This was done by substituting the corresponding sequences from Ha-MuSV for those in the Mo-MuLV-derived vector pEVX (8), as the 5′-splice site of Ha-MuSV was destroyed by recombination with rat sequences (7).

From this starting material, we derived the prototype handicapped-vector backbone pHHAM (9). Into the pHHAM backbone was inserted the herpes virus thymidine kinase (*tk*) gene promoter (10), to drive the bacterial neomycin resistance (*neo*) gene (11); and mouse immunoglobulin gene enhancer and promoter sequences (12, 13), to drive the c-*myc* gene (14). Details of the backbone construction and the subsequent insertions are presented under *Results*. All plasmid constructs were pUC derivatives (15).

Infection of a Mouse B-Cell Line. Infection of nonadherent WEHI-231 B cells (16) was carried out by cocultivation for several days with a monolayer of virus-producing ψ2 fibroblasts (17) in the presence of polybrene at 2 μg/ml. Infectants were propagated in the presence of G418 at 1 mg/ml after removal of residual virus-producing fibroblasts by replating on consecutive days.

Infection of Mouse Hematopoietic Stem Cells. Mice used as bone marrow (BM) donors were treated prior to BM removal with 5-fluorouracil (5-FUra) at 150 mg/kg for 2 or 4 days (18). BM was flushed from hind limbs with ice-cold Iscove's modified Dulbecco's medium (IMDM) containing 10% (vol/vol) heat-inactivated fetal bovine serum. Erythrocytes

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Abbreviations: LTR, long terminal repeat; A-MuLV, Abelson murine leukemia virus; Ha-MuSV, Harvey murine sarcoma virus; Mo-MuLV, Moloney murine leukemia virus; BM, bone marrow; 5-FUra, 5-fluorouracil; RBC, erythrocytes; THSC, totipotent hematopoietic stem cell(s).

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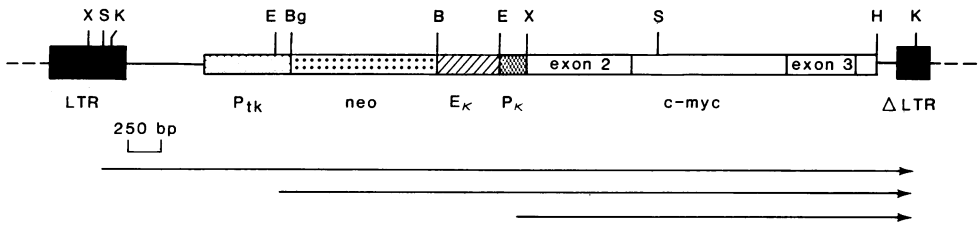

FIG. 1. Structure of the pHHAM(tk-neo)$E_\kappa P_\kappa$myc vector. The 5′ LTR up to the *Kpn* I site is from A-MuLV (nucleotide 32) (7). The remainder of the 5′ LTR and adjacent internal sequences are from Ha-MuSV (up to the Ha-MuSV *Bgl* II site at nucleotide 271) (7). To these sequences are joined Ha-MuSV sequences from an *Sst* II site at nucleotide 940 to a *Sma* I site at nucleotide 1009. Adjacent to these sequences are pEVX sequences from a *Bal* I site (Mo-MuLV nucleotide 212) (7) to a *Bgl* II site in the polylinker (8). The *tk-neo* gene unit was inserted at this site upstream of the $E_\kappa P_\kappa$-*myc* unit. The 3′ end of the vector is from pEVX from a *Hin*dIII site in the polylinker (8) to a *Pvu* II site in the 3′ LTR (Mo-MuLV nucleotide 7935/−329) (7). The remainder of the 3′ LTR is from A-MuLV (from a *Bss*HII site at nucleotide 5068/−1) and contains the deletion (denoted ΔLTR) described in the text. The three transcriptional units are indicated by horizontal arrows. B, *Bam*HI; Bg, *Bgl* II; E, *Eco*RI; H, *Hin*dIII; K, *Kpn* I; S, *Sst* I; X, *Xba* I.

(RBC) were removed by lysing in 0.17 M NH_4Cl on ice for 10 min followed by washing in IMDM. Nucleated BM cells were plated on subconfluent monolayers of virus-producing $\psi 2$ cells (17) at a density of 5×10^5 cells per ml in IMDM supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum, 10% (vol/vol) conditioned medium from WEHI-3B(D^-) cells as a source of interleukin 3 (19), 10% (vol/vol) conditioned medium from 5637 cells as a source of hemopoietin 1 (20), and polybrene at 2 μg/ml. After 48 hr, nonadherent BM cells were removed, concentrated by centrifugation, and suspended in 0.5 ml of Dulbecco's phosphate-buffered saline. Typically, 10^7 BM cells 2 days after 5-FUra treatment or 10^6 cells 4 days after 5-FUra were injected via the tail vein into *H-2*-matched congenic recipients.

Blood and Tissue Analyses. Blood was collected from the incised tail tip or the retroorbital sinus. RBC lysates were analyzed for strain-specific hemoglobin variants by electrophoresis on cellulose acetate (21). BALB/c strain immunoglobulin allotype was detected in serum by the Ouchterlony double-diffusion immunoprecipitin assay, using *Igh-1*^{a}-specific antiserum (a gift from Roy Riblet). Spleen cells for DNA analysis were obtained from partial splenectomies of mice anesthetized with sodium pentobarbital.

Molecular Analyses. Total RNA was isolated by the procedure of Chirgwin *et al.* (22) after centrifugation through a 5.7 M CsCl/0.1 M EDTA, pH 7, cushion. Poly(A)$^+$ RNA was purified by oligo(dT)-cellulose chromatography. RNA blotting was performed as described by Thomas (23), except that transfer was to nylon membranes in 10 mM sodium phosphate buffer, pH 7. DNA was purified from the same centrifuged sample from which total RNA was obtained. DNA after digestion with restriction endonucleases was transferred from agarose gels to nylon membranes in 0.4 M NaOH (24). DNA and RNA hybridizations were carried out in the presence of 1% $NaDodSO_4$, 0.5% nonfat powdered milk, and 10% (wt/vol) dextran sulfate at 42°C (24). Hybridization probes were restriction fragments radiolabeled with ^{32}P by the random primer technique (25) to specific activities of $2–5 \times 10^8$ cpm/μg.

RESULTS

Construction of the pHHAM(tk-neo)$E_\kappa P_\kappa$myc Retroviral Vector. Starting with the retroviral vector pAFVXM, sequences within the U3 region of the 3′ LTR were deleted from nucleotide −328 (A-MuLV nucleotide 4741) to nucleotide −2 (A-MuLV nucleotide 5067) by digestion with *Pvu* II and *Bss*HII, respectively (7). This deletion removes most of the enhancer 75-base-pair (bp) direct repeats and all promoter sequences (26). Because the U3 region of the 3′ LTR serves as the template for the corresponding region in the 5′ LTR (27), the provirus obtained after reverse transcription of viral RNA lacks enhancer and promoter elements in both LTRs, thereby eliminating viral transcription. The resulting vector was designated pHHAM to indicate its handicapped status as well as its chimeric origin from Harvey, Abelson, and Moloney viruses.

A series of eight pHHAM-derived viruses was then constructed, each with different internal transcriptional controls governing two structural genes. In all cases, the nonselectable mouse c-*myc* gene is the gene of interest, and the bacterial *neo* gene is present to allow selection (by resistance to the antibiotic G418) of virus-producing cell lines after transfection and of virally infected cells. The c-*myc* gene is driven by immunoglobulin μ- and κ-chain enhancer and promoter sequences either in homologous combination, as a possible optimal basis for appropriate regulation (28), or in heterologous combination, creating the possibility of a novel pattern of expression. The *neo* gene is under the control of the herpes virus *tk* promoter, either in the same or direct orientation as normal viral transcription, or in the reverse orientation. This relatively weak and widely expressed promoter (29) was chosen to minimize transcriptional interference with tissue-specific transcription of the c-*myc* gene in B cells (30). The experiments described here were all conducted with the first of these viruses to be obtained in a satisfactory titer and a stable state.

The pHHAM(tk-neo)$E_\kappa P_\kappa$myc vector in Fig. 1 contains a 0.7-kilobase (kb) *Bam*HI–*Bgl* II fragment of the *tk* promoter (10); a 1.2-kb *Bgl* II–*Bam*HI fragment from pBR-neo, containing the *neo* gene (11); a 475-bp *Alu* I fragment of the mouse immunoglobulin κ-chain gene enhancer (E_κ) (12), a 200-bp *Ava* II–*Bgl* II fragment with a mouse immunoglobulin κ-chain variable region promoter (P_κ) (13), and a 2.7-kb *Xba* I–*Hin*dIII fragment with the second and third exons of the translocated c-*myc* gene from the mouse plasmacytoma MOPC-315 (14). Thus, the E_κ and P_κ elements are coupled to the *myc*-coding region (31) and the *tk-neo* and $E_\kappa P_\kappa$-*myc* transcriptional units are oriented in the same direction.

Helper-Free Cell Lines Producing Recombinant Virus. Plasmid DNA from pHHAM(tk-neo)$E_\kappa P_\kappa$myc was introduced into the $\psi 2$ packaging cell line (17) by the calcium phosphate precipitation method (32). This cell line produces all the viral proteins required in *trans* for production of infectious virus, without producing any detectable wild-type virus. Individual $\psi 2$ colonies were isolated and expanded into mass cultures in medium containing G418 at 400 μg/ml (11). The titers of several cell lines were determined by infection of NIH 3T3 cells, followed by selection in G418. The highest titers obtained were $1–2 \times 10^5$ G418-resistant colony-forming units/ml. Pooled G418-resistant colonies from five cell lines with high titers were expanded and analyzed by Southern blots for the presence of intact viral sequences. All cell lines

were found to transfer the internal sequences of the pHHAM(tk-neo)$E_\kappa P_\kappa$myc vector without aberrant rearrangement (data not shown). One line, designated ψKK(D)-4, has not regenerated the deletion in the 3′ LTR during the transfection process and produced HHAM(tk-neo)$E_\kappa P_\kappa$myc virus at a titer of 8×10^4 G418-resistant colony-forming units/ml.

Infection of Cultured Mouse B Cells and Transcription of the Donor c-*myc* Gene. As an initial test for expression of $E_\kappa P_\kappa$-*myc* sequences in B cells, the mouse B-cell line WEHI-231 (16), which produces membrane IgM(κ), was infected by cocultivation with a monolayer of ψKK(D)-4 cells. Infectants were then expanded for DNA and RNA analyses. A Southern blot of DNAs digested with *Eco*RI and *Kpn* I, after hybridization with a probe containing the second and third exons of the mouse c-*myc* gene (Fig. 2*A*), revealed a 2.0-kb band (lane 2) from infected cells that was not present in DNA from uninfected controls (lane 1). This is the size expected for a processed HHAM(tk-neo)$E_\kappa P_\kappa$myc provirus that lacks the 1.1-kb intron in the parental construct and has preserved the original deletion in the 3′ LTR.

RNA gel blot analysis of poly(A)$^+$ RNA was performed to determine whether the *tk* and κ promoters are functional in the infected WEHI-231 cells. Elimination of LTR-driven transcription should result in only two transcripts, of 4.0 kb and 2.0 kb, from the processed HHAM(tk-neo)$E_\kappa P_\kappa$myc provirus (Fig. 1). In addition to a 2.4-kb transcript corresponding to endogenous c-*myc* mRNA (Fig. 2*B*, lane 1), RNA species of 4.0 kb and 2.0 kb were in fact detected with a c-*myc* exon 2 probe (Fig. 2*B*, lane 2). The 4.0-kb band was missing, as expected, in RNA isolated from WEHI-231 cells infected with a similar virus whose *tk-neo* component is in the reverse orientation (Fig. 2*B*, lane 3); and a 4.2-kb RNA species was found in RNA from WEHI-231 cells infected with another virus having the immunoglobulin μ enhancer in place of E_κ (Fig. 2*B*, lane 4). Thus, the 4.0-kb band corresponds to *tk-neo* "readthrough" transcripts. The 2.0-kb *myc* transcript present in all samples except the one from uninfected controls originates from P_κ. This conclusion was supported by the absence of a 2.0-kb transcript when the same blot was rehybridized with a c-*myc* exon 1 probe: in all cases, only the endogenous 2.4-kb c-*myc* mRNA was detected (Fig. 2*C*). The

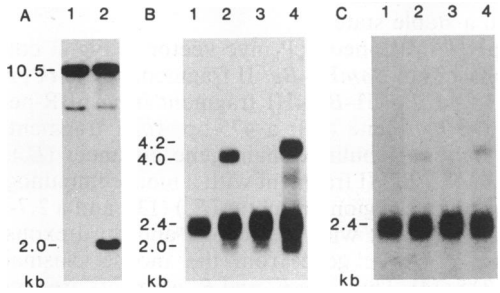

FIG. 2. DNA and RNA analyses of WEHI-231 B cells infected with HHAM viruses. (*A*) Genomic DNA (10 μg) from uninfected (lane 1) or HHAM(tk-neo)$E_\kappa P_\kappa$myc-infected WEHI-231 cells (lane 2) was digested with *Eco*RI plus *Kpn* I and analyzed by Southern blotting (24) with a c-myc probe specific for exons 2 and 3 (31). This probe detects the c-*myc* gene (10.5 kb) and a *myc*-related gene in DNA from uninfected cells. (*B*) Poly(A)$^+$ RNAs (4 μg) were analyzed by RNA gel blotting (23) with a c-myc exon-2 probe (31). Lanes: 1, uninfected WEHI-231; 2, WEHI-231 cells infected with the D-virus (direct) HHAM(tk-neo)$E_\kappa P_\kappa$myc; 3, WEHI-231 cells infected with the R-virus (reverse) HHAM(neo-tk)$E_\kappa P_\kappa$myc; 4, WEHI-231 cells infected with the HHAM(tk-neo)$E_\mu P_\kappa$myc virus, containing the immunoglobulin μ enhancer (a 700-bp *Xba* I–*Eco*RI fragment) (33) in place of E_κ. (*C*) The blot in *B* was washed and rehybridized with a probe containing the first exon of the c-*myc* gene (34). The bands at 4.0 kb (lane 2) and 4.2 kb (lane 4) are due to incomplete removal of the probe in *B*.

results demonstrate that pHHAM vectors can transduce two functional internal transcriptional units in an intact form into mouse B cells in culture.

Infection of Hematopoietic Stem Cells and Long-Term Reconstitution of Mutant Mice. Two mutant strains of mice were used as recipients for normal BM cells after infection of the cells *in vitro* with the handicapped retrovirus. Both mutants provide a selective advantage to normal syngeneic or congenic BM cells and, therefore, enable irradiation of the host to be circumvented. One series involved W/W^v recipients, with a stem cell defect and an associated macrocytic anemia. Normal BM cells are known to replace W/W^v host cells at the level of totipotent hematopoietic stem cells (THSC) that yield normal differentiated cellular progeny in myeloid and lymphoid lineages (35). In the present experiments, (B.C-20 × Hbb^d/Hbb^d-C57BL/6)F_1 females served as BM donors for W/W^v-C57BL/6 males. In the other series, homozygous *scid* mutants were the recipients. They are defective in lymphopoiesis and are devoid of mature B and T cells (5). BALB/c females were BM donors for *scid*-C.B-17 females.

BM was isolated from 2- to 3-month-old donors previously injected intravenously with 5-FUra (18). Nucleated BM cells were cocultured with ψKK(D)-4 cells for 48 hr in medium supplemented with the hematopoietic growth factors interleukin 3 and hemopoietin 1, which act synergistically on the most primitive multipotent hematopoietic colony-forming cells identified in *in vitro* assays (20). Selection with G418 (1, 2) was not included in these early experiments to gauge the baseline efficiency of BM infection and to minimize transcriptional interference between the *tk-neo* and the downstream $E_\kappa P_\kappa$-*myc* units (30).

Three months after injection by tail vein into age-matched recipients, RBC samples from peripheral blood of six W/W^v mice all had 90–100% hemoglobin of the donor-strain electrophoretic type, based on densitometric analysis of the dried gel (Fig. 3 *Upper*). When serum samples from the same animals were analyzed, two had low levels of donor-strain immunoglobulin allotype (data not shown), indicating limited presence of donor-strain B cells. However, retests of five survivors 8 months after engraftment clearly showed allotype of the donor strain in all (Fig. 3 *Lower*). In contrast, all nine *scid* hosts, even after 3 months, had substantial amounts of immunoglobulin-producing B cells of the donor strain (Fig. 3 *Lower*). Similar results were obtained in an ELISA with a rat

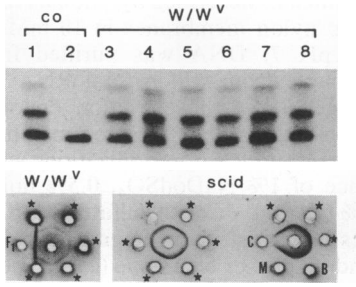

FIG. 3. Hemoglobin and immunoglobulin-allotype analyses of W/W^v and *scid* mice engrafted with HHAM(tk-neo)$E_\kappa P_\kappa$myc-infected BM. (*Upper*) Hemoglobin electrophoretic variants of donor (lane 1) and host (lane 2) types, in cellulose acetate electrophoresis of RBC lysates, indicate extensive replacement with donor-derived RBC in 6 W/W^v recipients 3 months after engraftment (lanes 3–8). (*Lower*) Five of the W/W^v recipients (starred wells) have donor-strain immunoglobulin at 8 months, as shown by the Ouchterlony immunoprecipitin reaction with an $Igh\text{-}1^a$-specific antiserum (center well). The F_1 well is an F_1 positive control. All nine *scid* recipients (starred wells) had substantial levels of donor-strain immunoglobulin in serum by 3 months. Control wells: C, C57BL/6; M, 1:1 (vol/vol) mixture of C57BL/6 and BALB/c; B, BALB/c.

monoclonal antibody specific for donor-strain IgM. After 3 months, W/W^v recipients gave only background signals, whereas *scid* recipients produced IgM at 60–100% of control levels (data not shown).

The proportion of donor-strain cells in hematopoietic tissue was determined by means of a length heterogeneity in the 5′-flanking region of the immunoglobulin μ heavy-chain constant region (C_μ) genes distinguishing BALB/c and C57BL/6 mice (36). When hybridized with an immunoglobulin μ-chain constant region probe, a 6.9-kb band was visualized in DNA, after digestion with *Eco*RI plus *Bam*HI, in BALB/c controls (Fig. 4, lane 17), whereas control C57BL/6 DNA yielded an 8.9-kb band (Fig. 4, lane 18). DNAs isolated from spleen derived from partial splenectomies of all W/W^v and *scid* recipients 3 months after BM injection exhibited both 6.9-kb and 8.9-kb bands (Fig. 4). The expected restriction pattern for the W/W^v cases is a superposition of the F_1 (donor) pattern (lane 1) and the C57BL/6 (host) pattern (lane 18). Thus, all W/W^v mice (lanes 2–7) were substantially repopulated with donor cells. From densitometric analysis of the autoradiogram, between 70% (lane 7) and 90% (lane 2) of the spleens of these animals are comprised of donor-strain cells. The expected restriction pattern for the *scid* cases is a simpler superposition of the pure-strain BALB/c (donor) and C57BL/6 (host) patterns. The results show that comparable numbers of BM cells introduced into *scid* mice resulted in lower repopulation levels, ranging from 30% (lane 13) to 50% (lane 11). Those *scid* recipients receiving substantially fewer BM cells were repopulated to relatively lesser extents (lanes 8, 15, and 16). Within each mutant host group there is no significant difference between reconstitution with 10^7 BM cells 2 days after 5-FUra or 10^6 cells 4 days after 5-FUra.

The difference in donor-derived reconstitution of the spleens of unirradiated W/W^v and *scid* recipients is striking (≈80% versus 40% average after 3 months). This probably partly reflects the presence of a THSC defect in W/W^v, leading to preferential seeding with normal cells at the THSC level (35). A further defect occurs in erythropoiesis of W/W^v and results in selective expansion of normal cells in the erythroid lineage (35), as confirmed here with the hemoglobin marker for donor-strain RBC (Fig. 3 *Upper*). (There is no RBC marker in the *scid* experiment.) While donor-derived cells are more prevalent in W/W^v spleens than in *scid* 3 months after engraftment (Fig. 4), those cells are likely to be predominantly myeloid. This is also indicated by the relatively lower levels of donor-strain serum immunoglobulin allotype in W/W^v than in *scid* recipients (Fig. 3), where defective lymphopoiesis provides an advantage for selective lymphoid expansion.

Proviral Sequences in Hematopoietic Cells of Reconstituted Mice. To learn whether donor-strain hematopoietic cells contained recombinant proviral sequences, *Eco*RI-digested spleen DNAs were hybridized with a neo probe. *Eco*RI cleaves the HHAM(tk-neo)$E_\kappa P_\kappa$myc provirus twice, excising a 1.8-kb *neo*-specific fragment (Fig. 1). A 1.8-kb band was present in all samples from the six W/W^v recipients (Fig. 5 *Upper*, lanes 2–7) and five *scid* recipients tested (lanes 9–12 and 14). Spleen DNA from one *scid* recipient also contained a 5.5-kb *neo*-reactive fragment (lane 12). This is apparently the result of a mutation that destroyed the *Eco*RI site between E_κ and P_κ, as a 3.8-kb *myc*-specific band was observed, after digestion with *Eco*RI plus *Kpn* I, in addition to the expected 2.0-kb band (data not shown).

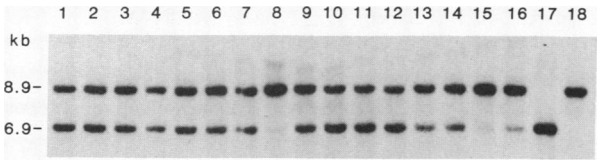

FIG. 4. Evaluation of donor-derived reconstitution in spleens of engrafted mice. A Southern blot of spleen DNAs (10 μg), after digestion with *Eco*RI plus *Bam*HI, was hybridized with a probe containing the first and second exons of the mouse immunoglobulin μ-chain constant region gene (37). Lanes: 1, F_1; 2–7, W/W^v recipients; 8–16, *scid* recipients; 17, BALB/c; 18, C57BL/6.

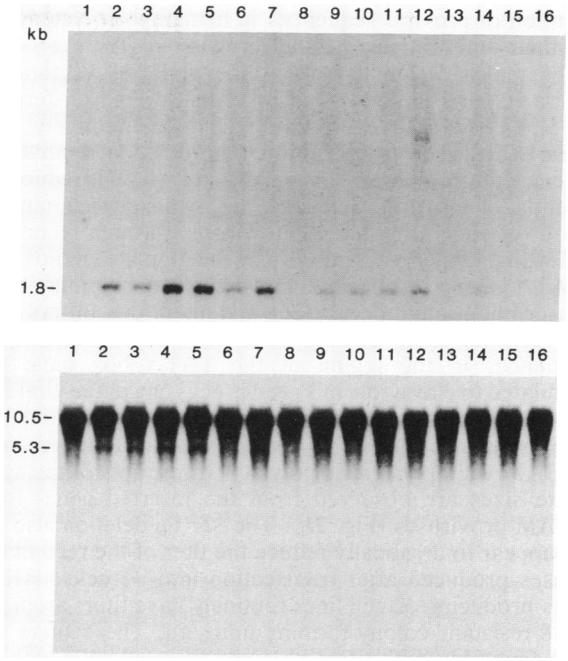

FIG. 5. HHAM(tk-neo)$E_\kappa P_\kappa$myc proviral sequences present in spleen DNAs of reconstituted mice. (*Upper*) Southern blot of *Eco*RI-digested spleen DNAs (10 μg) hybridized with a *Pst* I fragment containing sequences from the *neo* gene (38). (Sample order is the same as in Fig. 4.) (*Lower*) The same spleen DNAs digested with *Kpn* I were analyzed with the c-myc exon 2 probe.

The efficiency of infection of hematopoietic cells was estimated by digesting spleen DNAs with *Kpn* I, which cleaves within the LTRs of the HHAM(tk-neo)$E_\kappa P_\kappa$myc provirus, and hybridizing with a c-*myc* exon 2 probe. The autoradiogram (Fig. 5 *Lower*) is overexposed to intensify the 5.3-kb band corresponding to the proviral sequences. All animals identified as positive in Fig. 5 *Upper* contained the 5.3-kb provirus-specific band. These results demonstrate that the HHAM(tk-neo)$E_\kappa P_\kappa$myc provirus is stable in chromosomal DNA of hematopoietic stem cells and their cellular progeny after long-term reconstitution of the hematopoietic system of recipients. By comparison with the 10.5-kb endogenous c-*myc* band, present at two copies per diploid genome, and assuming one viral integration per cell, we estimate that 20–40% of spleen cells in W/W^v recipients have the proviral sequences. Estimates for spleen cells from comparable *scid* recipients are 5–10%. Taking into account the relative proportion of donor-strain hematopoietic cells in the spleens of the two types of recipients (Fig. 4), as many as 25–50% of the hematopoietic stem cells repopulating these animals were apparently infected with the recombinant virus, despite the absence of selection for infected cells during the culture period.

DISCUSSION

We have described the construction of genetically handicapped retroviral vectors capable of efficiently transducing intact nonselectable genes and their associated transcription-controlling sequences into target cells *in vitro*; and stable

retention of proviral sequences in hematopoietic stem cells and their differentiated cellular progeny *in vivo*.

The major design feature of the pHHAM vectors is a 327-bp deletion made in the 3′ LTR that, when transferred to the 5′ LTR during reverse transcription, effectively eliminates LTR-driven transcription. The deletion removes enhancer and promoter sequences and should reduce the potential of activating cellular genes by insertional mutagenesis or of generating infectious viruses through recombination with endogenous viruses. The deletion also removes the "TATA" box responsible for precise positioning of the transcription-initiation site (26), and, therefore, differs in this respect, and some others, from the "SIN" vectors (39). Sequences in the region of the TATA box have been postulated to play a role in 3′-end processing of the viral RNA through secondary-structural interactions (40). If so, then other sequences must be performing this function in the pHHAM vectors because poly(A)$^+$ transcripts of the appropriate sizes are observed from the inserted genes in the HHAM proviruses (Fig. 2*B*). The 327-bp deletion also does not appear to drastically reduce the titer of the recombinant viruses produced after transfection into $\psi 2$ cells: HHAM-virus-producing $\psi 2$ cell lines routinely have titers around 10^5 G418-resistant colony-forming units/ml. These titers compare favorably with titers obtained from $\psi 2$ cells with conventional retroviral vectors (17).

The pHHAM(tk-neo)$E_\kappa P_\kappa$myc vector transferred two tandem internal transcriptional units into WEHI-231 B cells in a nonrearranged form, and both transcriptional units were expressed in these cells (Fig. 2*B*, lane 2). Therefore, although the presence of two tandem internal promoters in a retroviral vector has been found to lead to structural instability (41), that does not occur with pHHAM(tk-neo)$E_\kappa P_\kappa$myc nor with most other pHHAM vectors developed in this laboratory (Fig. 2*B*; unpublished data). Stable viruses have also been obtained with pHHAM vectors in which the orientation of the selectable gene is reversed (Fig. 2*B*; unpublished data).

BM hematopoietic cells infected with the HHAM(tk-neo)$E_\kappa P_\kappa$myc virus were used to reconstitute mutant mice whose spleen cells then had intact viral sequences. Whether seeding is at the THSC level, with proviral DNA in all blood lineages, remains to be determined. THSC seeding and lineage derivations can be readily analyzed by retransplantation into irradiated recipients (42). In any case, the gene transfer is efficient, even in the absence of prior selection for virally infected BM cells, with the recombinant proviral sequences in ≈25% (and possibly up to 50%) of donor-strain hematopoietic cells. Preselection *in vitro* in G418 may increase the efficiency of hematopoietic stem cell infection with our vectors close to 100%, as preselection has been found to improve efficiency of reconstitution with other vectors (1, 2). Moreover, pHHAM vectors in which the *tk-neo* transcriptional unit opposes LTR-driven transcription (Fig. 2*B*) might be suitable for BM preselection as transcriptional overlap with the 3′-nonselectable gene would be avoided.

The handicapped vectors provide favorable possibilities for studies of tissue-specific expression of nonselectable genes, effects of genes implicated in normal and abnormal hematopoiesis, and interaction of hematopoietic cells with their environment during differentiation in animals that are otherwise free of these sequences. pHHAM vectors should also offer an opportunity to evaluate the feasibility of somatic cell gene therapy in human patients.

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