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Manipulation of Mouse Hematopoietic Progenitors by Specific Retroviral Infection*

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Previous studies have identified an enhancer 3′ of the *scl* gene that can direct transgene expression to hematopoietic progenitors and stem cells. Here we use this enhancer to restrict expression of the avian leukosis virus receptor, TVA, to hematopoietic stem cells and progenitors in bone marrow and fetal liver and demonstrate that retroviral infection can be used to specifically introduce exogenous sequences. We show that a majority of CFU-S_{12} multipotential progenitor cells can be transduced *in vitro*. Uniquely, transduction of TVA^+ progenitors with a retrovirus encoding a puromycin resistance gene allows selection and expansion of a multipotential hematopoietic progenitor population that can be superinfected with high efficiency. Using this system we show for the first time that v-Myb oncoproteins expressed from avian viruses can induce a leukemic transformation in the mouse. The phenotype of the transformed cells is similar to that which is seen in the chicken and is likewise dependent on the particular structure of v-Myb. This implies that the basic mechanisms of action of mutated transcription factors in the etiology of leukemia are conserved between birds and mammals.

Targeting hematopoietic progenitors is of great interest from the point of view of fundamental experimentation into events that regulate their commitment and differentiation and as a means to model the events leading to the initiation and progression of some types of leukemia and a variety of myeloproliferative diseases. Retroviral and lentiviral vectors are commonly used to introduce genetic material into cells of interest (1). Directing infection toward specific cells has been attempted by targeting cell surface proteins that are expressed on a restricted subset of cells, but the successes have been limited (2, 3). Attempts to target retroviral infection to hematopoietic progenitors have exploited the presence of SCF[1] receptor (c-Kit) and CD34 on their surface (4, 5) or have utilized a modified retroviral envelope; however, only very low efficiencies could be obtained compared with conventional retroviral vectors (6, 7).

Avian retroviruses, unlike their mammalian counterparts, cannot replicate in mammalian cells, because of the lack of a cell surface receptor and incomplete intracellular virus assembly (8). However, engineered expression of the subgroup A avian leukosis virus (ALV-A) receptor, TVA, confers susceptibility to infection by ALV-A viruses and by murine retroviruses pseudotyped with the ALV-A envelope protein (9, 10). By expressing TVA from tissue-specific promoters, it has been possible, for example, to restrict retroviral infection to muscle cells (11), glial cells (12), and megakaryocyte-lineage cells (13).

Recent identification of a hematopoietic progenitor-specific enhancer from the *scl* (*tal1*) gene offers the opportunity to achieve the desired pattern of expression of TVA. The *scl* gene encodes a basic helix-loop-helix transcription factor that is essential for the normal development of the blood and blood vessels (reviewed in Ref. 14). It is expressed in the hematopoietic stem cell and possibly earlier before commitment to cells with restricted hemogenic potential. An enhancer element located 3′ of the *scl* gene can direct reporter gene expression to the majority of early hematopoietic progenitors in transgenic mice (15). Long term re-population assays have shown that the *scl* enhancer can direct transgene expression to early myeloid and erythroid progenitors and to the vast majority of hematopoietic stem cells in adult bone marrow (16).

Here we describe the use of the *scl* 3′ enhancer to direct expression of a *tva* transgene to the hematopoietic stem cell and progenitor compartment in adult bone marrow and during embryonic development. TVA expression in these tissues is shown to be a specific and efficient means to permit introduction into multipotential hematopoietic progenitors of exogenous coding sequences.

Leukemic transformation is frequently associated with the expression of transcription factors that have acquired distinct regulatory properties as the result of mutation, often brought about by fusion to sequences by specific chromosomal rearrangements. The consequences of the perturbed transcription factor function leading to leukemia are thought to operate in hematopoietic progenitors and precursors. Attempts to model

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[1] The abbreviations used are: SCF, stem cell factor; ALV, avian leukosis virus; E, embryonic day; PE, phosphatidylethanolamine; FITC, fluorescein isothiocyanate; FACS, fluorescence-activated cell sorter; GFP, green fluorescent protein; EGFP, enhanced GFP; PURO, puromycin; AMV, avian myeloblastosis virus; TPO, thrombopoietin; IL, interleukin; FL, FLT-3 ligand; 5-FU, 5-fluorouracil.

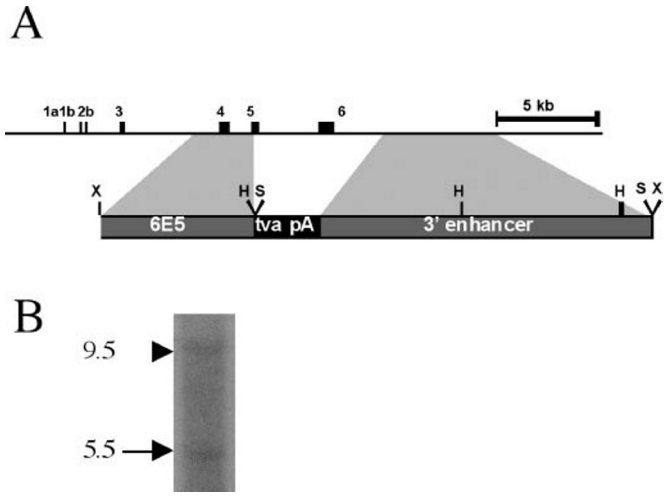

FIG. 1. **Murine scl-tva transgenic construct.** *A*, the genomic structure of the *scl* gene is depicted, with the *numbered boxes* representing exons. The schematic *below* illustrates the transgenic construct consisting of a 1.0-kbp fragment-encoding TVA flanked by a 2.8-kbp promoter *scl* fragment (6E5) encompassing exon 4 and a 5.5-kbp fragment from the 3′ end of the *scl* gene containing the enhancer element. An *Xho*I digest released a 9.0-kbp insert that was used for microinjection. Relevant restriction enzyme recognition sites are shown (*H*, *Hin*dIII; *S*, *Sal*I; *X*, *Xho*I). *B*, Southern blot analysis of *scl-tva* transgene copy number. Genomic DNA from *scl-tva* mice was digested with *Bgl*II and run on a 1% agarose/Tris-acetate EDTA buffered gel.

the initiation and progression of leukemic development have met with varied success, a major difficulty being the specificity and efficiency of introduction of the mutated transcription factors into relatively rare and inaccessible progenitors in the sites of hematopoiesis. Here we show that infection through the TVA receptor in cells from *scl-tva* mice can be used to assess the transformation potential of oncogenic transcription factors. Our results with v-Myb proteins encoded by avian acute leukemia viruses suggest that this approach is effective *ex vivo*, and the wider potential for *in vivo* application is discussed. Interestingly, the phenotype of transformation achieved using murine cells very closely resembled that seen using chicken hematopoietic cells suggesting that basic mechanisms of leukemia involving transcriptional regulators are conserved.

EXPERIMENTAL PROCEDURES

Generation of Transgenic Mice—The cDNA encoding the 950-bp TVA transcript was obtained as a 1.0-kbp *Hin*dIII-*Eco*RI fragment from pGPIbα-tva and ligated upstream of the murine *scl* 3′ enhancer element (a 5.5-kbp *Bgl*II fragment of the mouse *scl* locus (15)) and downstream of the murine *scl* promoter fragment (a 2.8-kbp of mouse *scl* exon 4) to generate p6E5/TVA/3′E (Fig. 1*A*). A 9.0-kbp *Xho*I fragment from p6E5/TVA/3′E was injected into CBA/Ca x C57B6J F2 fertilized eggs using standard procedures (17). Distal tail segments from potential founder mice were digested overnight at 55 °C in 100 mM Tris-HCl, 5 mM EDTA, 0.2% SDS, and proteinase K, and DNA was precipitated with ethanol and resuspended in 200 μl of sterile H_2O. The transgene was detected by PCR using primers specific for the tva gene (TVA1, CTGCTGCCCGGTAACCTGACCGG and TVA2, GCCCTGGGGAAGGTCCTGCCC yielding a 520-bp product). Amplification was performed in the presence of 6% Me_2SO using 30 cycles of 95 °C/30 s, 55 °C/30 s, and 72 °C/30 s. Founder mice were crossed to C57BL/6, and germ line transfer confirmed in the F1 progeny. Mice were subsequently maintained by crossing to C57BL/6. Two founder *scl-tva* mice were generated and established as lines (lines 12 and 28). Line 12 was used in all the experiments described although both lines exhibited essentially identical expression of TVA.

Transgene copy number was determined by probing a Southern blot of *Bgl*II digested tail DNA from F1 heterozygotes using a 300-bp fragment corresponding to the scl +19 enhancer. The probe detects an endogenous gene *Bgl*II fragment of 5.5 kbp whereas the transgene produces a 9.5-kbp fragment for concatemer integration and an additional band of >7.1 kbp corresponding to the end of the concatemer. Based on comparison of the intensity of signals of hybridization to the 5.5- and 9.5-kbp fragments we estimate that there are three copies of the transgene in *scl-tva* line 12 (Fig. 1*B*).

Immunofluorescent Staining and Flow Cytometry—Single cell preparations of bone marrow or E12 fetal liver were prepared by standard techniques. Red cells were depleted from the preparations by lysis in ACK buffer (18). Monoclonal antibodies were used either directly conjugated (Ter119-biotin, c-Kit-PE, CD11b-biotin, CD34-FITC, CD34-biotin, *Sca*I-biotin) or indirectly labeled (CD41) using anti-rat IgG1-FITC secondary antibody. With the exception of anti-rat IgG1-FITC (Sera Labs, Oxford, UK), all monoclonal antibodies were obtained from Pharmingen. Affinity-purified rabbit polyclonal antibody directed against TVA was obtained and used as described previously (13). For staining, 0.5–1.0 × 10^6 cells were washed in phosphate-buffered saline, resuspended in 100 μl of FACS buffer (0.3% bovine serum albumin and 0.1% NaN_3 in phosphate-buffered saline), and then incubated with 1 μl of Fc block (anti-CD16/32; Pharmingen) for 5 min followed by 45 min in primary antibody. Cells were washed twice in FACS buffer, resuspended in 100 μl of FACS buffer, and then incubated with the appropriate secondary antibody for 30 min. Cells were washed and immediately analyzed using a BD Biosciences FACSCalibur and CellQuest software.

A population of cells containing hematopoietic stem cells was purified from 3-month-old *scl-tva* bone marrow. Briefly, a single cell suspension of ACK-treated cells (4 × 10^7 cells) was bound for 15 min at 4 °C to FITC-conjugated rat anti-mouse antibodies against the lineage markers B220, CD4, CD8, CD5 Gr1, Mac1, and Ter119. After washing, antibody-bound cells were depleted by binding to magnetic beads (4 × 10^8) coated with sheep anti-rat IgG (Dynal). The resultant population of cells (Lin^-; 4 × 10^6 cells) was then stained with anti-c-Kit-PE and anti-*Sca*I-biotin followed by Cy5-conjugated streptavidin, and the Lin^-*Sca*I^+c-Kit^+ subpopulation was sorted using a Cytomation MoFlo FACS machine. Approximately 2 × 10^4 cells (0.05% of total bone marrow) were obtained.

Retroviral Vectors and Production of Infectious Retrovirus—Replication competent avian retroviruses were generated by transfecting the chicken fibroblast cell line, DF1, with RCAS vectors. The cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 100 units/ml penicillin G, and 0.1 mg/ml streptomycin sulfate. For production of virus, DF1 cells 70% confluent in 10-cm plates were transfected with 10 μg of RCAS vector DNA using the calcium phosphate co-precipitation method. Medium was changed 6–8 h after exposure to the precipitate and the day following transfection. 5 days post-transfection, greater than 95% of the DF1 cells showed expression of the exogenous gene, and viral supernatant was harvested and passed through a 0.45-μm syringe filter. The RCAS system typically produces viral titers of 5 × 10^6 or greater, especially using RCAS vectors modified by insertion of the Bryan strain *pol* region (RCASBP; see Ref. 8). Such RCASBP-based vectors were employed that expressed either an enhanced green fluorescent protein (RCAS-EGFP) or the puromycin resistance gene (RCAS-PURO; see Ref. 13). Retroviruses expressing the E26 and AMV v-Myb transforming oncoproteins were generated by transfecting the plasmids pMI3 and pMI4, respectively (19), into 293T cells, together with plasmids encoding Gag-pol and EnvA, as described previously (13).

Culture and Infection of Bone Marrow and Fetal Liver Cells—Freshly harvested femurs and tibias were flushed with Hanks'-buffered salt solution containing 1% bovine serum albumin. E12 fetal livers were dissected out, and a single cell suspension was obtained by passage through a 25-gauge needle. Mature red blood cells were depleted by incubation in ACK buffer for 5 min at room temperature. The cells were resuspended in specific growth medium to promote progenitor maintenance and expansion (20, 21). All media contained Iscove's modified Dulbecco's medium supplemented with 10% horse serum (Sigma), 100 units/ml penicillin G, and 0.1 mg/ml streptomycin sulfate. Cytokine supplements included SCF (Peprotech, Rocky Hill, NJ) at 20 ng/ml, human thrombopoietin (TPO; a kind gift from Genentech, San Francisco, CA) at 25 ng/ml, IL-3 (Peprotech, Rocky Hill, NJ) at 10 ng/ml, and IL-6 and FLT-3 ligand (FL; R & D Systems, Minneapolis, MN) at 10 ng/ml. For morphological analysis, cytocentrifuged bone marrow and fetal liver cultures were stained using May-Grünwald Giemsa.

In vitro bone marrow and fetal liver infections were performed using 500 μl of filtered virus stock with 8 μg/ml polybrene and 0.5–1.0 × 10^6 cultured cells in 500 μl of growth medium in a 6-well dish. The cells were incubated at 37 °C for 3 h with rocking every 20–30 min and then diluted with 2.5 ml of growth medium.

Colony Assay—Infected bone marrow or fetal liver cells were plated in 2.5 ml of 1% methylcellulose medium (Methocult M3434; Stem Cell

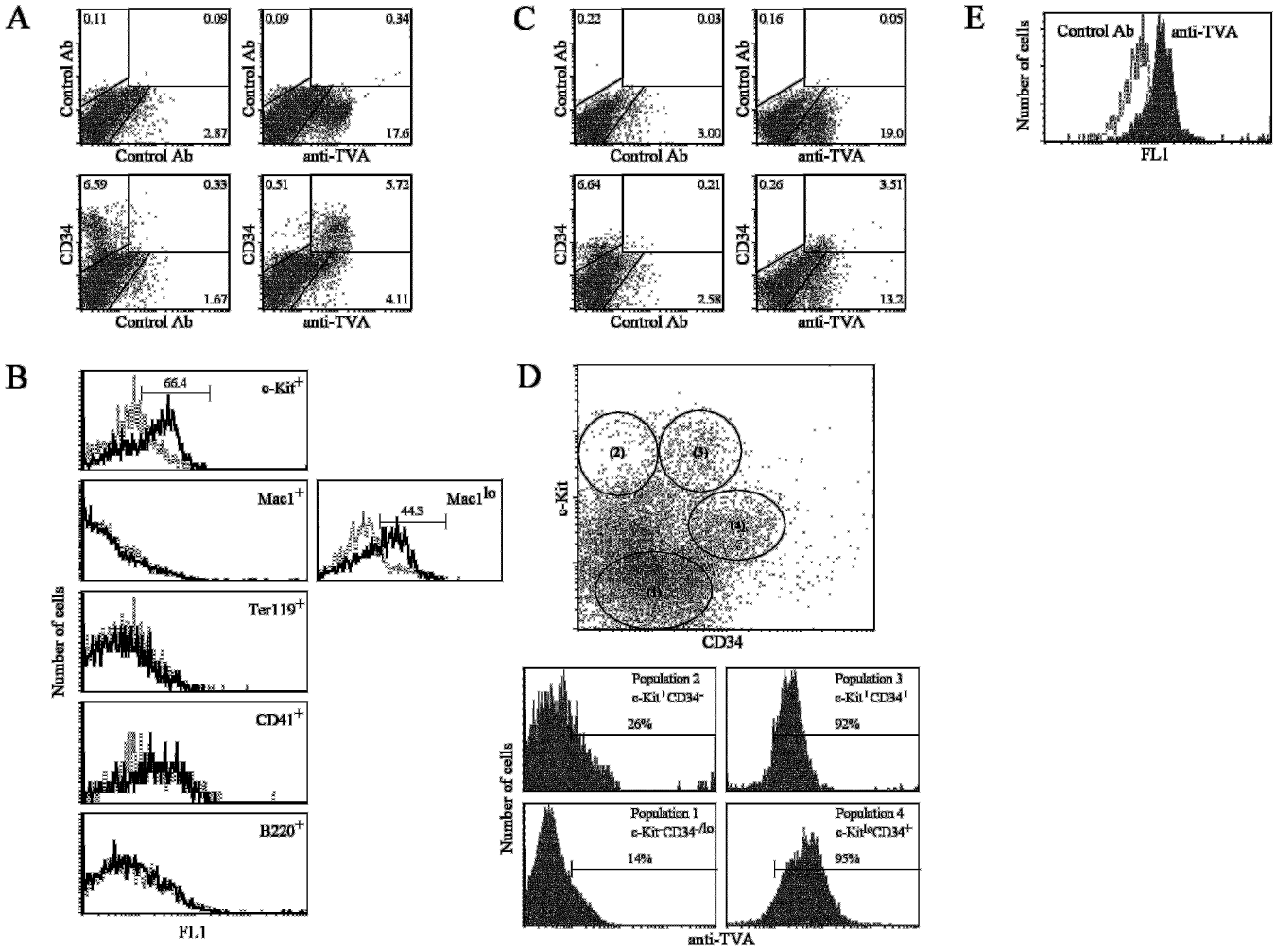

FIG. 2. **TVA expression in bone marrow and fetal liver.** *A*, bone marrow from 6–8 week old *scl-tva* mice was harvested, and red cells were lysed selectively. Samples were treated on ice with Fc receptor block followed by incubation with anti-TVA and lineage-specific antibodies or isotype controls. Cells were washed and then incubated with anti-rabbit FITC (for anti-TVA and the control rabbit antiserum) or streptavidin-PE (for the biotinylated lineage-specific antibodies). *B*, bone marrow cells were prepared and stained as described in *A*. Cells expressing the lineage-specific antigen were gated on FL2, and the FL1 fluorescence because of staining with either the control rabbit antiserum (*dotted line*) or anti-TVA antibody (*solid line*) is represented as a histogram. *C*, fetal livers were harvested from E12 *scl-tva* embryos, and dissociated cells were stained as in *A*. *D*, fetal liver cells harvested and treated as above were subjected to three-color immunofluorescence staining with anti-TVA, anti-CD34, and anti-c-Kit. Cell suspensions were stained as described in *A* except that the secondary staining was with anti-rabbit-FITC and streptavidin-PE (for the anti-CD34). The anti-c-Kit was directly conjugated with PE. The *upper panel* illustrates the staining pattern for anti-CD34 and anti-c-Kit. Cells within regions 1–4 were then gated separately, and the extent and FL1 fluorescence indicative of staining with anti-TVA is shown in histogram format in the *lower panels*. *E*, purified $Lin^{-}Sca1^{+}c\text{-}Kit^{+}$ bone marrow cells from an *scl-tva* adult were stained with anti-TVA and a control rabbit antibody. Cells were washed and then incubated with anti-rabbit-FITC. The FL1 fluorescence because of staining with either the control rabbit antiserum (*open*) or anti-TVA antibody (*solid*) is represented as a histogram.

Technologies, Vancouver, British Columbia) containing Iscove's modified Dulbecco's medium, 15% fetal calf serum, 0.1 mM 2-mercaptoethanol, 2 mM L-glutamine, 1% bovine serum albumin, insulin (10 mg/ml), iron-saturated human transferrin (200 mg/ml), IL-3 (10 ng/ml), IL-6 (10 ng/ml), SCF (50 ng/ml), Epo (3 units/ml), and TPO (25 ng/ml). Triplicate cultures were incubated for 7 days at 37 °C in a fully humidified 5% CO_2 atmosphere, and colony numbers were assessed on day 3 (CFU-E), day 6 (BFU-E), and day 10–12 (CFU-mixed and myeloid colonies) using both light and fluorescent microscopes.

CFU-S_{12} Assay—The spleen colony assay of Till and McCulloch (22) was used to assay for the stable infection of CFU-S (colony-forming unit-spleen) multipotent progenitors. E12 *scl-tva* fetal liver cells were cultured for 3 days in Iscove's modified Dulbecco's medium containing 10% horse serum, penicillin/streptomycin, FL (10 ng/ml), SCF (20 ng/ml), IL-6 (10 ng/ml), and TPO (25 ng/ml) and were infected with filtered supernatant from RCAS-EGFP-infected DF1 producer cells. C57BL/6 female recipients 8 to 12 weeks of age were exposed on the day of transplantation to a single dose of 10.0 gray of radiation from dual opposed Co^{60} sources at an exposure rate of 0.2 gray/min. 5×10^4 infected cells were injected into irradiated mice. The mice were sacrificed 12 days later, and their spleens were dissected, fixed in Bouin's solution for 5 min, then transferred to 10% neutral buffered formalin. Prior to fixation, GFP-positive colonies were visualized using an inverted fluorescence microscope at low power. Quantification of the total number of colonies was performed after fixation, which renders them more visible under normal light.

In Vivo Infections—6- to 8-week-old *scl-tva* mice and non-transgenic controls were primed by subcutaneous injection of 150 mg/kg 5-fluorouracil (5-FU) and injected intravenously via the tail vein with 1×10^6 RCAS-EGFP DF1 producer cells on day 4 post-5-FU administration. 10 weeks and 6 months after injection of the virus-producing cells, the mice were sacrificed, and bone marrow was harvested. Single cell suspensions were generated by passage through a 25-gauge needle. Cell suspensions were treated with ACK buffer to lyse red blood cells prior to immunofluorescence and flow cytometric analysis.

RESULTS

The scl 3′ Enhancer Targets TVA Expression to the Progenitor Cell Compartment—To analyze TVA expression in hematopoietic progenitors from *scl-tva* transgenic mice, we prepared bone marrow from adults at 6–8 weeks of age and from the fetal livers of E12 embryos. Cell staining using an anti-TVA rabbit antiserum demonstrated TVA expression on 14.7% of

red cell-depleted bone marrow cells (Fig. 2*A*) and 16.0% of red cell-depleted fetal liver cells (Fig. 2*C*). Simultaneous staining for markers characteristic of immature hematopoietic cells showed that virtually all of the $CD34^+$ cells in both bone marrow and fetal liver were also TVA^+ (Fig. 2, *A* and *C*). In Fig. 2*B* additional stainings of bone marrow are represented to show that TVA is expressed on cells with characteristics of progenitor cells but not on cells expressing terminal differentiation antigens. Hence, gating on cells positive for the individual antigens showed that ~66% of the c-Kit^+ population is TVA^+ whereas cells expressing low levels of the Mac1 antigen were 44% positive for TVA. Conversely, few, if any, cells expressing high levels of Mac1 or positive for the erythroid marker Ter119, the megakaryocyte marker CD41, or the B cell marker B220 were simultaneously positive for TVA.

To further define the nature of the $CD34^+TVA^+$ population and what the $CD34^-TVA^+$ cells represent we performed three-color staining of fetal liver cells for TVA, CD34, and c-Kit. This revealed that 92% of c-Kit^+CD34^+ cells were TVA^+ (Fig. 2*D*, *population 3*) whereas $CD34^+$ cells that expressed low levels of c-Kit were 95% TVA^+ (Fig. 2*D*, *population 4*). TVA^+ cells that were $CD34^-$ appeared to be mainly c-Kit^+ (Fig. 2*D*, *population 2*). The three-color staining also demonstrated that cells expressing low levels of c-Kit and low levels of CD34 were mostly TVA^- (Fig. 2*D*, *population 1*).

Next, using immunomagnetic bead depletion and high speed sorting of immunofluorescently labeled cells, we enriched a population that contained hematopoietic stem cells with the surface antigen phenotype Lin^-Sca1^+c-Kit^+ (23). These sorted cells were positive for staining using the anti-TVA antibody (Fig. 2*E*). This pattern of TVA expression was therefore consistent with that reported previously (15) for the *scl* 3′ enhancer as detected by use of a lacZ reporter and implies that TVA is present on hematopoietic stem cells, progenitors, and precursor cells.

Hematopoietic Cells from scl-tva Mice Can Be Infected with Avian Retroviruses—Next we infected fetal liver cells from E12 *scl-tva* embryos using RCAS-EGFP in the presence of FL, IL-6, SCF, and TPO to maintain the progenitor cell compartment *in vitro* (20, 21). Flow cytometric analysis of the infected population after 2 days in culture showed that ~7.0% of the cells expressed detectable levels of GFP as compared with the control cells, which were obtained by RCAS-EGFP infection of non-TVA-expressing fetal liver (Fig. 3, *A* and *B*). Simultaneous immunofluorescent staining of the infected cells indicated that the GFP-expressing cells were predominantly positive for the progenitor markers CD34, c-Kit, and, to a lesser extent, Sca-1 (Fig. 3, *C–E*) but that only a small proportion exhibited positivity for the erythroid marker Ter119 (Fig. 3*F*). These data demonstrate that this system can be used to target retroviral infection to a subset of hematopoietic cells that express the markers of hematopoietic progenitors and precursors.

Progenitor Activity of scl-tva Fetal Liver Cells Infected with RCAS-EGFP—Colony assays were performed to define functionally the nature of hematopoietic progenitor and precursor cells that could be infected through the TVA receptor. Fetal liver cells were harvested from E12 embryos, cultured in 10 ng/ml FL, 20 ng/ml SCF, 25 ng/ml TPO, and 10 ng/ml IL-6 and infected with RCAS-EGFP for two successive days cells followed by seeding in methylcellulose for determination of specific CFU content. Although three distinct types of GFP-positive colonies were noted based on their morphology (mixed, pure myeloid, and pure erythroid), the ratio of GFP^+ to GFP^- mixed colonies (2.0) was much greater than that seen for pure myeloid (0.7) or pure erythroid colonies (0.15) (Fig. 4*A*). These results indicate that the majority of the multipotential progenitors but only a minority of the unilineage progenitors had been

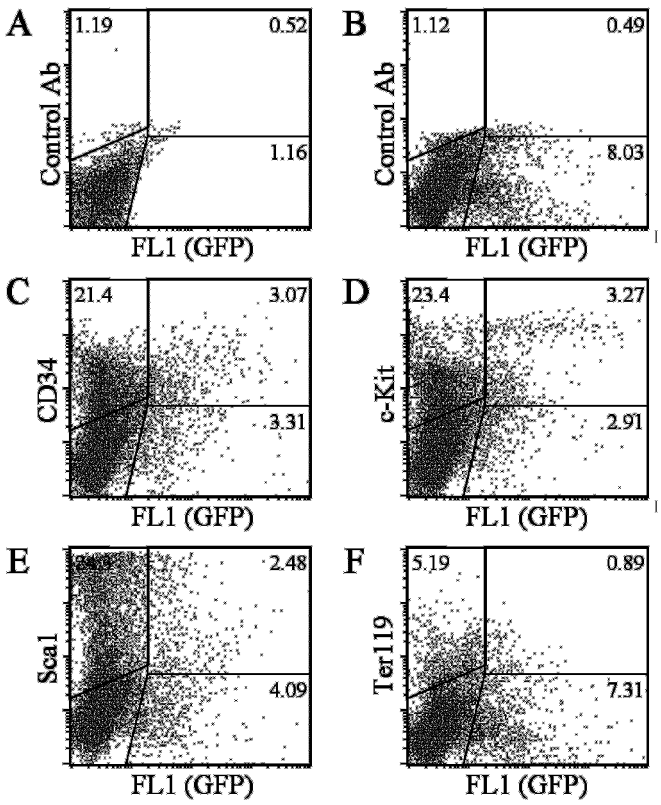

FIG. 3. **Fetal liver cells from scl-tva mice can be infected with Env-A-coated retroviruses.** Fetal livers were harvested from E12 *scl-tva* embryos. After red cell lysis, cell suspensions were cultured in FL, SCF, TPO, and IL-6 and were infected with RCAS-EGFP for two successive days. GFP expression was analyzed on day 3 by flow cytometry. Samples were treated with Fc receptor block followed by incubation with biotinylated lineage-specific antibodies followed by streptavidin-PE. *Panel A* represents RCAS-EGFP-infected fetal liver cells from a non-transgenic mouse. *Panels B–F* represent RCAS-EGFP-infected *scl-tva* fetal liver cells. The extent of green fluorescence is indicated on the *horizontal axis* whereas red fluorescence in the *vertical* direction indicates the extent of streptavidin-PE binding to control antibody (*A* and *B*) or specific antibodies against CD34 (*C*), c-Kit (*D*), Sca-1 (*E*), and Ter119 (*F*).

infected. We cannot, however, determine whether GFP expression in lineage-committed cells is the result of direct, albeit less efficient, infection of monopotent precursors or of post-infection commitment of infected multipotent progenitors before seeding into semi-solid medium.

The CFU-S assay as first described by Till and McCulloch (22) provides one means to selectively quantify multipotential progenitors present in a population of cells. Such assays were therefore performed to determine what proportion of multipotential progenitors present in an *ex vivo* culture could be infected via the TVA receptor. Lethally irradiated mice were transplanted with 5×10^4 *scl-tva* fetal liver cells that had been infected with RCAS-EGFP virus and cultured for 2 days in the growth factor mixture described above. The percentage of fetal liver cells injected into the mice that expressed GFP was similar to that described for the *in vitro* RCAS-EGFP infections in Fig. 3*B* (7.0%). Examination of the spleens of the transplanted mice after 12 days revealed an average of 6.3 colonies per spleen (Fig. 4, *B* and *C*), with 68.5% of the colonies expressing GFP (Fig. 4*C*). These data demonstrate that the majority of CFU-S_{12} progenitors which are maintained in a multipotential state during culture and infection *ex vivo* can be infected with RCAS-EGFP using this technique.

Production of an Enriched Population of Early Hematopoietic Progenitors from scl-tva Bone Marrow—Bone marrow from *scl-*

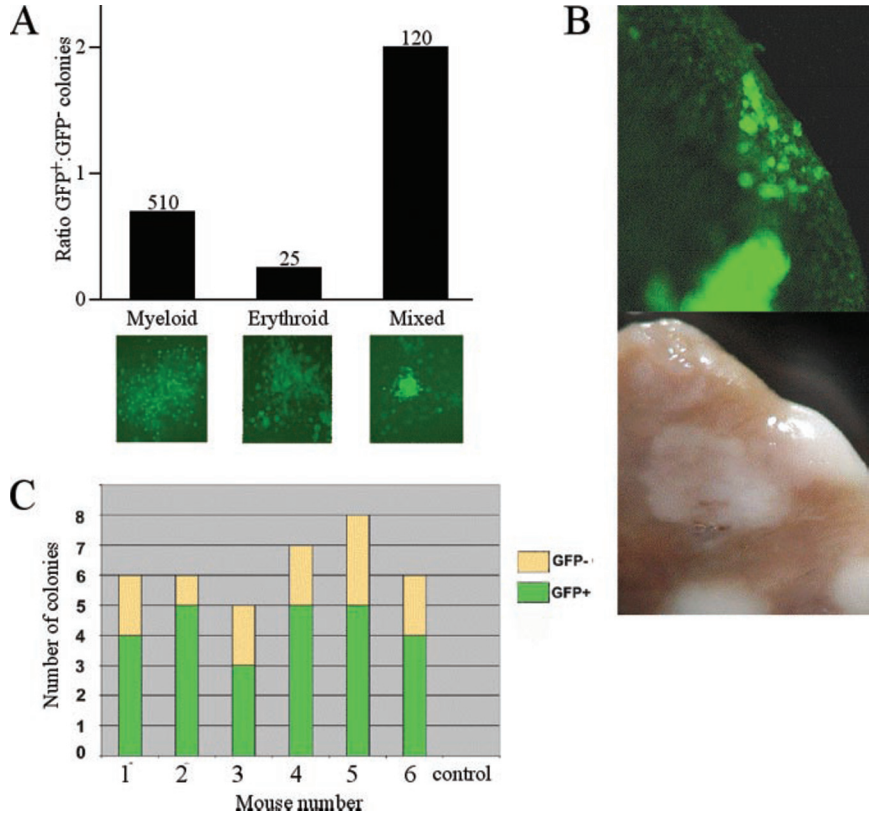

FIG. 4. **Progenitor activity of scl-tva fetal liver cells infected with RCAS-EGFP.** *A*, fetal liver cells were harvested from E12 embryos. After red cell lysis, cell suspensions were cultured and infected with RCAS-EGFP as described in the legend for Fig. 3. On day 2, 1×10^4 infected cells were plated in methylcellulose colony assays. Colonies were counted on days 3 and 10 following the initial plating. GFP-expressing colonies were visualized using an inverted immunofluorescence microscope. *B* and *C*, 5×10^4 E12 *scl-tva* fetal liver cells that had been cultured and infected with RCAS-EGFP as described for *A* were injected into irradiated 8–12-week-old C57BL6 mice. The mice were sacrificed 12 days later, and their spleens were removed, fixed in Bouin's solution for 5 min, and then transferred to 10% neutral buffered formalin. Prior to fixation, GFP-positive colonies were visualized using an inverted fluorescence microscope at low power (*B*, *upper panel*), and following fixation the colonies were counted under a dissecting microscope (*B*, *lower panel*). *C*, the histogram details the number of GFP-positive (*green*) and GFP-negative (*yellow*) colonies formed on the spleen of each recipient mouse.

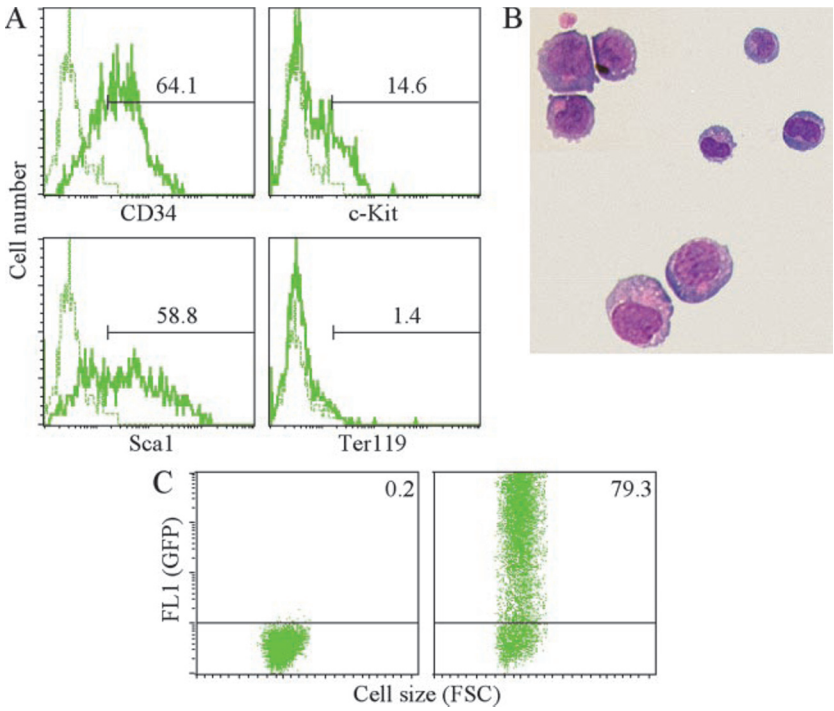

FIG. 5. ***scl-tva* puromycin-selected bone marrow.** Bone marrow was cultured in FL, SCF, TPO, and IL-6 and infected with RCAS-PURO for 2 days followed by selection in 1 μg/ml puromycin for 7 days. *A*, immunofluorescence staining of puromycin-selected cells. Samples were treated with Fc receptor block followed by incubation with biotinylated lineage-specific antibodies and streptavidin-PE. *B*, May-Grünwald staining of puromycin-selected cells after 5 days of selection. *C*, demonstration of multiple gene transduction in *scl-tva* bone marrow. Harvested bone marrow was cultured in medium containing SCF and IL-3. Cells were first infected with RCAS-PURO, selected in medium containing puromycin for 1 week, and then infected with RCAS-EGFP. Cells were exposed to viral particles in a 50/50 mix of growth medium and viral supernatant for 3 h per infection. Superinfected cells were analyzed by flow cytometry 2 days after exposure to the RCAS-EGFP supernatant. The level of fluorescence detected in the FL1 channel is plotted in relation to the forward side scatter (*FSC*) of the population. The *left-hand panel* illustrates the background green fluorescence of cells selected in puromycin whereas the *right-hand panel* shows the population of puromycin-resistant cells 2 days after RCAS-EGFP superinfection.

tva mice was cultured in FL, SCF, TPO, and IL-6 and infected with RCAS-PURO. These growth conditions maintain the early compartment of hematopoietic progenitors while still allowing distinct lineage differentiation to take place (20, 21). Following selection for 7 days in puromycin (1 μg/ml), a population of cells emerged that showed the hallmarks of immature hematopoietic progenitors. The majority of cells expressed the progenitor markers CD34 and *Sca*I (64.1 and 58.8%, respectively), whereas 14.6% expressed c-Kit (Fig. 5*A*). Conversely, almost none of the selected cells expressed Ter119, a marker of the erythroid lineage (Fig. 5*A*). May-Grünwald staining of the selected population showed a mixed population of blast-like cells (Fig. 5*B*). Methylcellulose colony assays revealed that an average of 71% of the puromycin-selected cells formed colonies (data not shown).

In mammalian cells the viral *env* gene is expressed at rela-

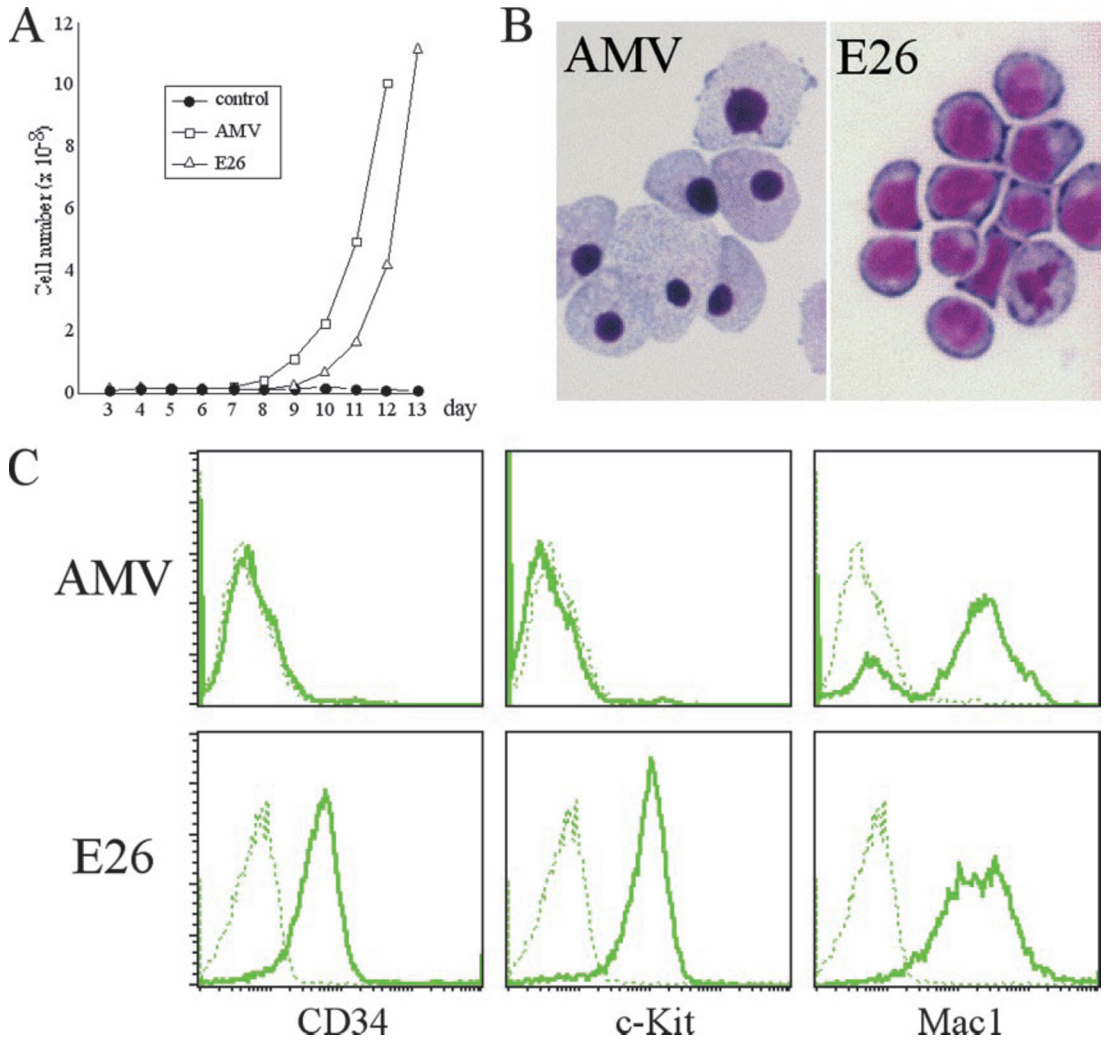

FIG. 6. **Transformation of hematopoietic progenitors following infection through TVA.** 5×10^5 *scl-tva* bone marrow cells were cultured in FL, SCF, TPO, and IL-6 and infected with E26, AMV, or a control virus for 2 days. On day 3 the cells were transferred to medium containing no added growth factors. Cultures were diluted as appropriate to maintain a cell density of $\sim 1 \times 10^6$/ml. *A*, cell numbers were determined daily and are represented as the cumulative total. *B*, May-Grünwald staining of E26- and AMV-transformed cells after 12 days of growth. *C*, immunofluorescence staining of transformed cells. Samples were treated with Fc receptor block followed by incubation with biotinylated lineage-specific antibodies and streptavidin-PE.

tively low levels so that TVA-expressing cells are susceptible to superinfection by additional avian retroviruses. To test the feasibility of multiple sequential infection of hematopoietic progenitors derived from *scl-tva* bone marrow, cells infected with RCAS-PURO were selected in puromycin for 7 days and then infected with RCAS-EGFP. During the same 7-day period, non-TVA-expressing control bone marrow cells infected with RCAS-PURO and grown in the presence of puromycin all died (data not shown). FACS analysis of the superinfected cells demonstrated that 79% of the puromycin-resistant cells express GFP at 2 days post-RCAS-EGFP infection (Fig. 5*C*). Together, these data indicate that the *scl-tva* mice can be used to produce a highly enriched population of early hematopoietic cells that can then be further genetically manipulated by retroviral superinfection.

Targeting Potential Oncoproteins to Hematopoietic Progenitors—As a further illustration of the feasibility of using the *scl-tva* transgene as a means to target hematopoietic progenitors we infected bone marrow with avian leukemia viruses that have been shown previously to transform progenitor or precursor cells in the chicken (19, 24). Specifically, we made use of the E26 virus encoding the 135-kDa Gag-myb-ets fusion oncoprotein (25, 26), which has been shown to transform both multipotential myeloid and bipotential myelomonocytic progenitors (24), and the AMV virus encoding the 45-kDa truncated c-Myb protein, which transforms monoblastic cells (19). E26 and AMV viruses produced from the DF1 packaging line were used to infect 5×10^5 *scl-tva* bone marrow cells for 2 days in the presence of FL, SCF, TPO, and IL-6. Control infections were performed using an empty retroviral vector. On day 3 the cells were placed in medium containing 15% fetal calf serum and no added growth factors. Monitoring of cell numbers showed a clear outgrowth of transformed cells from the virus-infected cultures (Fig. 6*A*). At day 12 the E26- and AMV-transformed cells had the characteristic morphology of immature blasts and monoblasts, respectively (Fig. 6*B*). These phenotypes were reflected in the expression of surface markers (Fig. 6*C*). Hence, the E26-transformed cells immature cells were positive for CD34 and c-Kit (Fig. 6*C*), whereas the AMV-transformed monoblasts expressed the marker Mac1 but were negative for CD34 and c-Kit expression. During continued culture, the progenitor phenotype of the E26-transformed cells was progressively lost as cells differentiated toward adherent, $Mac1^+$ monocyte-macrophage cells (data not shown).

Targeted Retroviral Infection of Hematopoietic Progenitor Cells in Vivo—In a preliminary attempt to demonstrate the

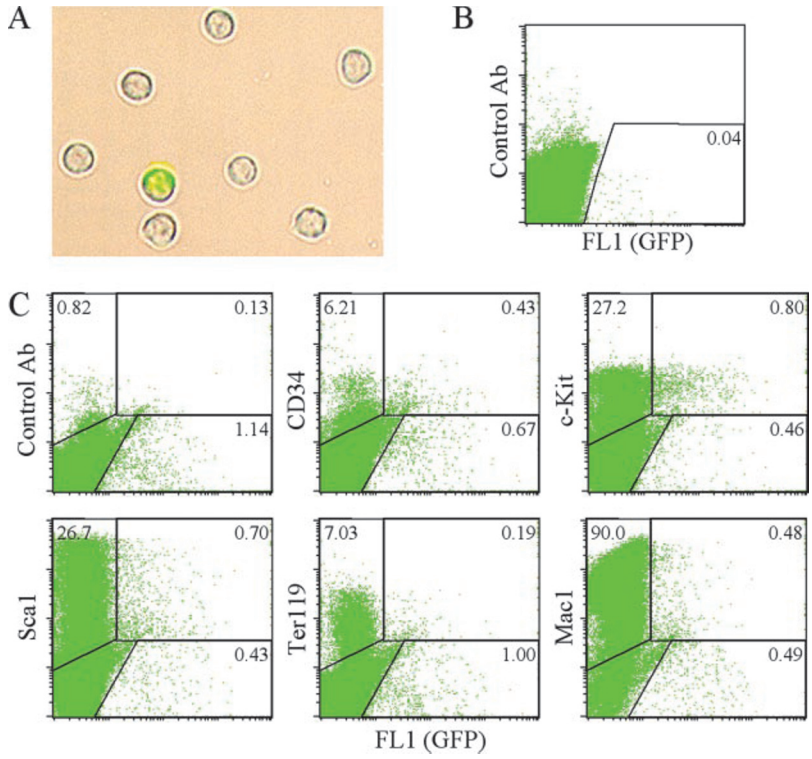

FIG. 7. ***In Vivo* infection.** *scl-tva* mice and non-transgenic controls were primed with 5-FU and injected 4 days later with 1×10^6 RCAS-EGFP DF1 producer cells. 10 weeks or 6 months later the mice were sacrificed, and bone marrow and spleens were harvested and treated to lyse red cells prior to immunofluorescence analysis. *A*, merged fluorescent and transmission images of spleen cells from an RCAS-EGFP-infected *scl-tva* mouse. *B*, flow cytometric analysis of 5×10^5 bone marrow cells from an RCAS-EGFP-infected *scl-tva* transgenic mouse 10 weeks after injection of the virus-producing cells. *C*, two-color immunofluorescence analysis of bone marrow cells from an RCAS-EGFP-infected *scl-tva* transgenic mouse six months after injection of the virus-producing cells. The extent of green fluorescence is indicated on the *horizontal axis* whereas the *vertical axis* indicates streptavidin-PE binding to control antibody or specific antibodies against CD34, c-Kit, Sca-1, Ter119, and Mac1 as indicated.

feasibility of targeted infection of progenitor cells *in vivo*, 6- to 8-week-old *scl-tva* mice and two non-transgenic controls were primed by subcutaneous injection of 150 mg/kg 5-FU. 4 days later they received 1×10^6 RCAS-EGFP DF1 producer cells via tail vein injection. Ten weeks following injection of the virus-producing cells, *scl-tva* mice and controls were sacrificed, and bone marrow and spleens were harvested. Bone marrow cells and spleen suspensions from six *scl-tva* mice were examined for the presence of infected cells. Fluorescence microscopy of the spleen suspension revealed the presence of rare GFP^+ cells (Fig. 7*A*). Flow cytometric analysis of bone marrow showed that ~0.04% were GFP^+ whereas none could be detected in the bone marrow and spleen suspensions derived from the control mice (Fig. 7*B*) (data not shown). In all six mice, the GFP^+ cells were shown to be $Mac1^{lo}$, $Sca I^+$, and c-Kit^+, consistent with them being hematopoietic progenitors. The possibility that GFP^+ DF1 virus producer cells had persisted was extremely unlikely; however, this was ruled out, because DF1 cells were shown not to react with the Mac1, *Sca*I, or anti c-Kit antibodies used (data not shown). A similar analysis of bone marrow from an animal 6 months after infection revealed that the proportion of GFP^+ cells was 1.1% (Fig. 7*C*). Two-color immunofluorescence analysis of bone marrow cells from this animal showed that the population of GFP^+ cells exhibited characteristics of both progenitor/precursors and cells with a more differentiated phenotype. Hence, a proportion of the GFP^+ population were clearly c-Kit^+ and $CD34^+$ whereas some GFP^+ cells expressed the markers Ter119 and Mac1 (Fig. 7*C*). These data illustrate that *in vivo* infection of hematopoietic progenitors is feasible and that infected cells are able to persist long term in the bone marrow.

DISCUSSION

TVA-based retroviral transduction has been developed as a means to introduce exogenous genetic material selectively and efficiently into somatic cells both *ex vivo* and *in vivo* (8). There are several advantages of infection through the TVA receptor using an ALV-based retroviral vector system in mammalian cells. Specificity of infection is determined solely by the expression domain of the TVA-expressing transgene. Moreover, highly efficient infection is facilitated, because ALV vectors can be quickly and easily grown to high titers as replication-competent viruses on avian cells. The fact that the viral *env* gene is inefficiently expressed in mammalian cells means that superinfection is not precluded as would be the case using conventional murine retroviral systems. This, therefore, allows for the expression of more than one gene in a given cell by multiple sequential infections.

Until now, the only application of TVA technology within the hematopoietic system has been for the infection of cells committed to the megakaryocyte lineage (13). Here, we have generated and characterized a new TVA transgenic mouse strain, *scl-tva*, which makes it possible to infect hematopoietic cells prior to commitment to terminal differentiation. Our results demonstrate that the *scl* 3′ enhancer can direct *tva* transgene expression to the hematopoietic progenitor compartment in a manner very similar to that seen for *lacZ* in the original characterization of this enhancer (15). The fact that most, if not all, $CD34^+$ cells also express the *tva* transgene implies that a large part of the progenitor population can potentially be targeted by retroviral infection. Moreover, the presence of TVA on $Lin^- Sca I^+$c-Kit^+ cells indicates that hematopoietic stem cells could also be infected. Using the *scl-tva* mouse we have shown that avian retroviruses carrying a gene product of choice can selectively infect hematopoietic progenitors and precursors. Among the TVA^+ population are multipotential cells, which, based on the CFU-S_{12} assay, can be infected *ex vivo* at high efficiency (~70%) and still maintain their capacity as functional progenitors when transplanted *in vivo*. Indeed, this is the best indication of the efficiency of infection of progenitors given that it was not possible to assess the absolute level of infection by RCAS-EGFP, because during culture of the infected population many of the initially TVA^+ cells would have become TVA^-, and those cells seen as being GFP^+ at the time of analysis were hence the result of a complex dynamic of self-renewal, differentiation, and cell death.

The *scl-tva* mouse will certainly be useful for studying the

influence of transcription factors or other proteins on hematopoietic lineage choice. By permitting infection and expression of a particular factor prior to lineage commitment, it should be possible to influence lineage choice and differentiation *ex vivo*. In this context, it is particularly interesting that by infection of *scl-tva* hematopoietic cells with RCAS-PURO followed by antibiotic selection we were able to produce an enriched population of primary multipotential progenitors that were highly susceptible to superinfection by a second retrovirus. We are currently investigating these cells in greater detail with regard to their phenotype and potential to undergo effective multilineage differentiation.

We have shown that infection of murine hematopoietic progenitors with the viruses E26 and AMV expressing oncogenically activated derivatives of the transcription factor c-Myb results in the outgrowth of homogenous populations of cells. Interestingly, although E26 and AMV are avian leukemogenic viruses, the phenotypes of the infected cell populations derived from mouse bone marrow resembled those obtained when the viruses transform chicken hematopoietic progenitors (19, 24). This degree of similarity between the effect of transforming Myb proteins in mouse and chicken is particularly interesting with respect to the E26 Gag-myb-ets protein. The immature progenitor phenotype of the expanded population infected with E26 appears to be analogous to the multipotent progenitor transformed from chicken hematopoietic cells (24), although our culture conditions seemed to favor eventual commitment to monocytic differentiation. In contrast, an earlier alternative approach at examining the effect of Gag-myb-ets on murine hematopoietic cells involving insertion of the oncoprotein coding sequences between murine retroviral long terminal repeats gave rise to transformed erythroid cells (27).

From the viewpoint of the manipulation of lineage choice of multipotential progenitors the *scl-tva* system also has the potential to be extended through use of viruses derived from the RCAN retroviral backbone. These viral vectors lack a splice acceptor and allow for the use of a specific internal promoter to drive exogenous gene expression (8). Other investigators (28) have shown that a retrovirus containing an erythroid-specific promoter can be used to transfer genes into the erythroid lineage with resultant high level gene expression. However, these studies have been conducted only in cells already committed to the erythroid lineage. Using an RCAN-based virus capable of expressing EGFP from an internal erythroid promoter/enhancer we have preliminary results that suggest that cells derived from the *scl-tva* mouse can be infected at an immature stage but that high level exogenous gene expression can be restricted to the red cell compartment (data not shown).

Simple retroviruses fail to integrate in non-replicating target cells because of a block that occurs before entry into the nucleus of the infected cell (29). This is a problem relevant to the targeting of hematopoietic stem cells, because they are mostly quiescent (30). In this context, we have recently been able to pseudotype a feline immunodeficiency virus-based lentiviral vector with subgroup A avian leukosis virus envelope protein.[2] This should allow the use of a lentiviral vector capable of infecting non-dividing cells while still utilizing the targeting system enabled by the specific expression of TVA.

In vivo gene transfer into hematopoietic cells is extremely enticing, but in practice this has proven to be difficult (31). Previous observations based on purification of bone marrow cells expressing the *lacZ* transgene driven by the *scl* promoter/enhancer (16) strongly suggest that it should be possible to target hematopoietic stem cells using the *scl-tva* mouse. Although the efficiency was low, our initial attempts at targeted *in vivo* infection have shown that this appears to be possible in that we obtained infected cells that persisted long term and had the characteristics of hematopoietic progenitors.

Even with a low efficiency of infection of stem cells or multilineage progenitors *in vivo* the *scl-tva* mouse has potential utility in the generation of models of hematological malignancies in which it is anticipated that the transduced gene will confer a considerable proliferative advantage. Such an approach would also make it possible to investigate the influence of genetic background on the activity of the gene of interest. We are presently investigating the potential of this approach with a number of fusion proteins associated with specific myeloid leukemias, and in this context our results with the avian v-Myb-encoding leukemic retroviruses are very encouraging.

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