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Development of Retrovirus Vectors Useful for Expressing Genes in Cultured Murine Embryonal Cells and Hematopoietic Cells In Vivo

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A series of retrovirus vectors were constructed in which cellular promoter elements derived from the chicken β-actin and human histone H4 genes were introduced within the proviral transcriptional unit of Moloney murine leukemia virus in order to promote expression of inserted sequences. Each of these vectors gave rise to high titer of virus capable of transferring the expected proviral structure to cells. Inclusion of normal 5′ splice sequences or a portion of viral *gag* sequences in these constructions resulted in significant increases in virus titer. Each construction was transcriptionally active in NIH 3T3 cells and in undifferentiated F9 cells. One of the vectors, HSG-neo, which contained the human histone H4 promoter, was shown to be transcriptionally active in hematopoietic cells derived from long-term reconstituted bone marrow transplant recipients engrafted with transduced stem cells. These vectors should be of general use for obtaining efficient gene expression in embryonal and hematopoietic cells.

While recombinant retrovirus vectors have proved to be very useful for introducing and expressing genes in cultured cells (30), a number of investigators have experienced difficulty in obtaining high levels of gene expression after introduction of the vectors into either embryonal carcinoma (EC) cells or hematopoietic cells in vivo (2, 14, 23, 32). In the case of EC cells, the poor expression associated with the use of vectors that include the viral long terminal repeat (LTR) to promote the expression of inserted sequences appears to result from the inherent transcriptional inactivity of the viral LTR in these cells (10, 19). Recently, vectors have been developed which rely on transcriptional elements located within the proviral transcriptional unit to promote the expression of inserted sequences (13, 16, 20, 25, 31, 33). These vectors with internal promoters have been shown to function better in F9 cells than LTR-based vectors, yet in most cases have not resulted in high levels of gene expression. The reasons for the poor performance of retrovirus vectors in hematopoietic cells in vivo are not well defined. Many of the LTR-based and internal promoter vectors are clearly transcriptionally active after their introduction into mature hematopoietic cells in vitro. However, the same vectors do not function in mature hematopoietic cells in vivo when those mature cells have been derived from infected stem cells (23, 32). It appears, therefore, that the developmental history of an integrated recombinant provirus can affect its ultimate expression. The molecular basis for this apparent irreversible shutoff of vector transcription remains a mystery.

To develop vectors that would be useful for gene expression studies in a variety of developmental contexts, we have constructed and tested a series of vectors that include cellular transcriptional signals known to be functional in both early embryonal cells and cells representing a variety of stages of hematopoietic cell differentiation. In this report, we describe the transmission properties of these vectors, their transcriptional activity in both fibroblasts and EC cells, and the activity of one particular construction, HSG-neo, in hematopoietic cells derived from mice reconstituted for long periods (greater than 2 months) with transduced hematopoietic stem cells.

MATERIALS AND METHODS

Construction of recombinant MoMLV expression vectors. Moloney murine leukemia virus (MoMLV) sequences used in the construction of the pBA-neo (β-actin) and pH-neo (histone H4) vectors included the following. (i) 5′ LTR to the *Pst*I site at nucleotide 563 taken from pDOL$^-$ (16). (ii) The individual tissue-specific transcriptional signals. In pBA-neo, a 277-base-pair (bp) *Xho*I-*Hin*fI fragment of the chicken β-actin gene was used. The 3′ end was adapted with *Bam*HI linkers. In pH-neo, a 723-bp *Eco*RI-*Hin*dIII fragment of the human histone H4 promoter which contained at the 3′ end a 21-bp polylinker containing *Sal*I, *Acc*I, *Hin*cII, *Pst*I, and *Hin*dIII in that order (taken from clone δ1705 of pHu4A insert in vector M13mp8/pUC8; gift of N. Heintz, Rockefeller) was used. The 5′ end of this DNA fragment was adapted with *Xho*I linkers, and the 3′ end was adapted with *Bam*HI linkers. (iii) The bacterial neomycin phosphotransferase gene. (iv) MoMLV sequences from the *Cla*I site at nucleotide 7672 through the 3′ LTR. The 3′ LTR contains a deletion of viral transcriptional enhancer elements extending from *Pvu*II at nucleotide 7933 to *Xba*I at nucleotide 8111 (6). The chimeric proviral transcriptional units are contained within mouse genomic DNA flanking sequences of pZIPNeo (4). The plasmid backbone of pBR322 extends from *Hin*dIII at nucleotide 29 to *Eco*RI at nucleotide 4361.

Vector pHS-neo differs from pH-neo in that the mutated 5′ splice site (16) has been replaced with the normal 5′ splice site found in MoMLV (taken from plasmid pA5, gift of S. Goff). This was accomplished by switching the fragment extending from *Xba*I at nucleotide 8111 to *Pvu*I at nucleotide 419. Vector pHSG-neo contains both the wild-type 5′ splice site and a portion of *gag* (taken from plasmid pA5) extending to the *Xho*I site at nucleotide 1558. In this latter segment of DNA, a *Sac*II linker has been inserted at the *Hae*III site at nucleotide 624, resulting in the premature termination of *gag* translation.

Mammalian cell culture, DNA transfection, and viral infection. NIH 3T3 and ψ2 cells were grown in Dulbecco modified

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Eagle medium with 10% calf serum. F9 EC cells were grown in the same medium with 10% fetal bovine serum.

Transfections were performed by the method of Graham and Van der Eb as modified by Parker and Stark (26).

Stable ψ2 producer cell lines transfected with recombinant expression plasmids were made by selection of ψ2 cells in medium containing G418 (Gibco Laboratories) at a concentration of 0.36 mg of the active component of the drug per ml. Individuals G418^r colonies were picked and expanded. Virus titers of individual producers (maintained in culture) remained constant after repeated virus harvests.

Viral supernatants from stably transformed cloned ψ2 producer cells were prepared by harvesting 10 ml of normal medium, applied 18 h previously, from a confluent monolayer (10 cm diameter) and then filtering the medium (0.45-μm pore size filter; Millipore Corp.). Harvests of retroviruses taken from ψ2 cells 18 h after transfection (transient transfections) were filtered as described above. Stable and transiently rescued viral harvests were used either immediately for infection or stored frozen at −70°C.

NIH 3T3 and F9 cells were infected by incubating 5×10^5 cells with 2 ml of viral supernatant or a dilution thereof in the presence of 8 μg of polybrene (Aldrich) per ml for 2.5 h. Medium (8 ml) was then added, and cells were grown to confluence (2 days), at which time they were split into selective medium. Fresh selective medium was added at 3-day intervals. After 10 days in selection medium, plates containing populations of G418^r colonies were either stained with crystal violet or pooled and expanded for preparation of DNA and total cellular RNA.

Southern hybridization analysis. Genomic DNA was prepared from confluent 10-cm-diameter plates of cells, digested to completion with either *Kpn*I, *Xba*I, or *Nhe*I, and subjected to electrophoresis in 1% agarose gels transferred to nylon hybridization membranes (Zetabind; Cuno) as described before (21). Southern blots were probed with ^{32}P-labeled *neo* DNA prepared by oligonucleotide labeling (9).

Northern (RNA blot) hybridization analysis. Total cellular RNA was prepared from plates of confluent cells (5×10^6) as described before (5) and suspended in 20 μl of H_2O. RNA was subjected to electrophoresis in 1% agarose gels containing 20 mM MOPS (morpholinepropanesulfonic acid, pH 7.0), 5 mM sodium acetate, 0.1 mM EDTA, and 7% formaldehyde for 24 h. RNA was transferred to nitrocellulose filters and probed with ^{32}P-labeled *neo* DNA.

SP6 protection analysis of total cellular RNA. An mRNA protection assay was used to quantitate transcription with uniformly labeled antisense RNA probes. Plasmid SP6neo was constructed by cloning the *Eco*RI-*Hin*dIII fragment of pZIPneoSVX into the polylinker of SP65 (24). In vitro runoff transcription of DNA linearized at the *Sma*I site with SP6 RNA polymerase was carried out in the presence of 100 μCi of [α-^{32}P]UTP, as described before (24). In a typical reaction, 50 to 70% of the labeled triphosphate was incorporated. The 246-bp RNA probe was purified by electrophoresis on a 6% polyacrylamide–8.3 M urea gel, followed by elution by the method of Maxam and Gilbert (22). Labeled RNA was suspended in 0.002 M EDTA, pH 7.5, at 10^6 cpm/3 μl. The hybridization of labeled probe to RNA was carried out based on a modification of the protocol of Zinn et al. (34). One microliter of RNA to be assayed was added to 3 μl of probe. Each sample was adjusted to contain 80% formamide, 0.4 M NaCl, 0.04 M PIPES [piperazine-*N*, *N*′-bis (2 ethanesulfonic acid), pH 6.7], and 0.001 M EDTA and incubated for 10 min at 85°C and then for 12 to 18 h at 55°C. Following hybridization, the samples were diluted 10-fold and adjusted to 0.3 M NaCl, 0.005 M EDTA, and 0.01 M Tris (pH 7.5). RNase A and RNase T_1 (PL Biochemicals) were added to concentrations of 250 and 1 μg/ml, respectively, and incubated for 30 min at 30°C. The samples were adjusted to 0.5% sodium dodecyl sulfate and 250 μg of proteinase K (Merck) per ml and digested for 15 min at 37°C. After digestion, the samples were extracted with phenol, ethanol precipitated, suspended in sequencing loading buffer, and run on 6% polyacrylamide–8.3 M urea DNA sequencing gels (22).

Infection of bone marrow cultures and transplantation. Four days prior to extraction of bone marrow, female C3H/HeJ mice aged 7 to 15 weeks were injected via tail veins with 5 mg of 5-fluorouracil (140 to 150 mg/kg of body weight) (Hoffman LaRoche). At day 5, the mice were killed by cervical dislocation, the bones of the hind limbs were removed, and marrow was harvested in αMEM medium (Gibco) supplemented with 10% calf serum. The marrow collected from one donor mouse was evenly divided between two 10-cm-diameter plates of producer cell line ψ2HSGneo9 containing 5×10^5 cells. Cells were cocultivated for 24 h in the presence of 20% (vol/vol) conditioned medium from WEHI-3B cells and 8 μg of Polybrene. After 24 h, nonadherent bone marrow cells were harvested by gently washing the plates with their own fluid layer. Cells were spun for 5 min at 1,500 rpm, the pellet was suspended in Hanks balanced salt solution (pH 7.1 to 7.3). Viable cells were counted in a solution of 2% acetic acid. Recipient male C3H/HeJ mice, aged 7 to 15 weeks, were exposed to a total of 1,100 rads of radiation from a cesium source, which was delivered in two doses over a period of 3 h. Lethally irradiated recipients received transduced bone marrow via tail vein injection of 2.0×10^4 cells contained in 0.5 ml of Hanks balanced salt solution.

Analysis of recipient animals. At 12 days and 2.5 months posttransplantation, animals were sacrificed to obtain total bone marrow and spleen cells. Cells were prepared for total cellular RNA and DNA as described before (18).

RESULTS

Construction of internal promoter vectors. The general design of the internal promoter vectors pBA-neo and pH-neo is outlined in Fig. 1A. In each case, the vector backbone contained the minimal proviral sequences necessary for the generation of genomic RNA transcripts suitable for encapsidation into viral particles and efficient reverse transcription and integration of the recombinant proviral genome. To reduce the possibility of aberrant splicing events that might interfere with the expression of inserts, the vector backbone also contained a mutation at the normal viral 5′ splice site (5′ss) (16). In addition, the 3′ LTR in each construction was deleted of the viral enhancer sequences (6) so as to prevent the possibility of viral enhancer-dependent repression of transcription in embryonal cells, as has been reported previously by others (10). Two cellular promoter-containing sequences were chosen for incorporation into these vectors. These included (i) a 277-bp segment of the chicken β-actin gene (17, 27) extending from −276 to +1 and (ii) a 702-bp segment of the human histone H4 gene (12) extending from −694 to +8 (Fig. 1A). The actin and histone promoter sequences were chosen because both of the corresponding genes are known to be expressed in virtually all somatic and germ cells. While the transcription of the human H4 histone gene has been shown to be cell cycle regulated, disruption of the murine histone H4 gene either by introduction of an intron in the coding region or by displacing the hairpin loop

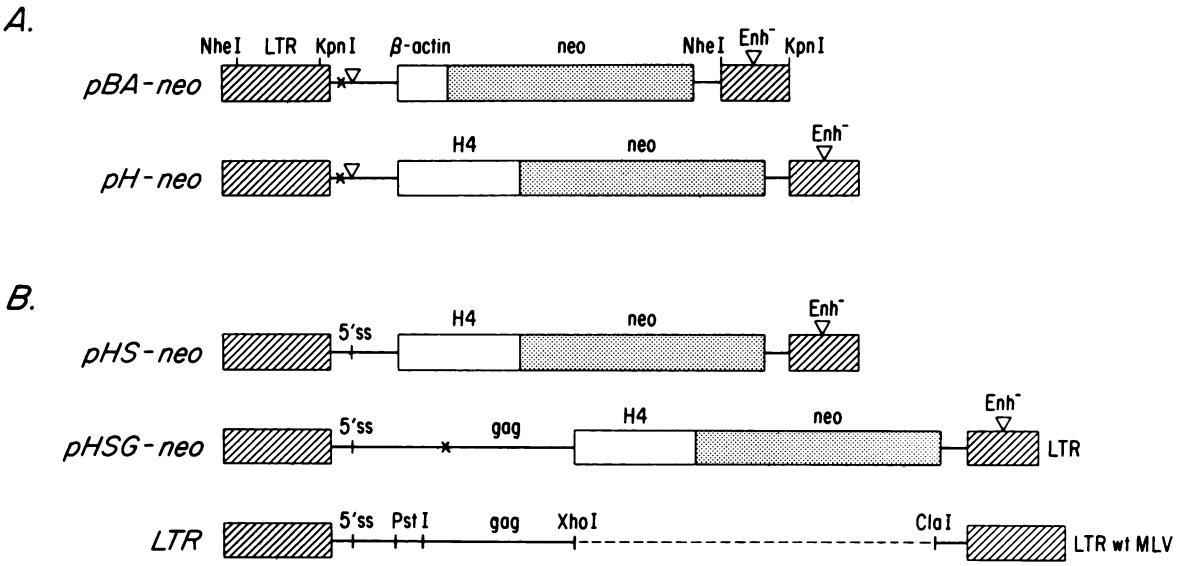

FIG. 1. Structures and derivation of recombinant MoMLV genomes containing tissue-specific transcriptional elements (▭). (A) General design of internal promoter vectors carrying either the chicken β-actin promoter (pBA-neo) or the human histone H4 promoter (pH-neo). Each construction incorporates a mutation of the 5'ss (denoted as **X** with a triangle) and deletion of viral enhancer sequences (denoted as Enh⁻ over a triangle) in the 3' LTR. (B) Two derivative vectors of pH-neo which incorporate additional viral sequences. Construction pHS-neo contains a normal 5'ss, while pHSG-neo incorporates the 5'ss along with an additional 995 nucleotides of viral *gag* sequences from *Pst*I at 563 to *Xho*I at 1558 (the x in the *gag* sequences of pHSG-neo denotes insertion of a *Sac*II linker at the *Hae*III site at nucleotide 624 that results in premature termination of *gag* translation). Symbols: ▨, LTRs; ▩, bacterial neomycin phosphotransferase gene; ——, viral DNA sequences; – – – –, wild-type MoMLV sequence. Not drawn to scale.

structure in the 3' direction results in cell cycle-independent expression of the gene (28).

Two derivatives of the histone H4 promoter-containing vector were constructed to determine whether additional viral sequences would alter the titer of virus obtained from the vectors (Fig. 1B). pHS-neo contained the normal viral 5'ss in place of the mutated 5'ss. The vector pHSG-neo contains the sequences in pHS-neo as well as additional viral sequences extending from *Pst*I at nucleotide 563 to *Xho*I at nucleotide 1558.

Effect of viral sequences on virus titer. Stably transformed clonal ψ2 packaging cell lines were generated by transfection of ψ2 cells with each vector and selecting transfected ψ2 populations directly in G418. Virus from cloned ψ2 producers of pBA-neo and pH-neo was harvested, filtered, and used to infect NIH 3T3 cells. Following selection of infected NIH 3T3 cells in G418, drug-resistant cell populations were stained and counted. A mean virus titer of 7.8×10^3 infectious particles per ml was obtained for seven different ψ2 producers of pBA-neo (data not shown), while the mean value for pH-neo titers was 8.4×10^4 (Table 1), or roughly 10-fold greater. The internal promoter elements accounted for the only difference between these constructions. To permit a careful comparison of the efficiency of the modification of the vector backbone in the constructions carrying the human histone promoter, 12 clonal ψ2 producer cell lines were generated for pH-neo, pHS-neo, and pHSG-neo, and titers of all were determined on NIH 3T3 cells. As shown in Table 1, each construction yielded a range of titers. As stated above, pH-neo yielded producer cell lines with a mean titer of 8.4×10^4/ml. Incorporation of the normal splice site into the vector pHS-neo increased the titer roughly 10-fold to 7.8×10^5, while the addition of the extra viral *gag* sequences in pHSG-neo further increased the titer about threefold to 2.1×10^6. In the case of pHSG-neo, over half of the cell lines produced high titers ($>2 \times 10^6$/ml).

Integration of the vectors in NIH 3T3 and F9 cell populations. To demonstrate that pBA-neo and pH-neo were transmitted faithfully to recipient cells, ψ2 cells were transfected by each construction (transfections of ψ2 cells with two previously characterized vectors, pZIPNEO [4] and pDOL⁻ [16], as well as pHS-neo and pHSG-neo, were carried out in parallel), and virus corresponding to each construction was then harvested 1 day after the transfection as described previously (4) and used to infect NIH 3T3 or F9 cells. Infected cell populations were selected in G418 for 10 days

TABLE 1. Virus titers of transformed producer cell lines[a]

Expt. no.	Virus titer		
	pH-neo	pHS-neo	pHSG-neo
1	1.8×10^5	1.5×10^6	1×10^5
2	1.4×10^5	1.7×10^6	7×10^4
3	3×10^4	4×10^4	2×10^6
4	2.5×10^4	7.5×10^4	2×10^6
5	3.75×10^3	1.4×10^6	2.5×10^5
6	5×10^3	3×10^4	3×10^6
7	1.5×10^5	1×10^5	2×10^6
8	1.1×10^5	2×10^4	2.5×10^5
9	2×10^4	9×10^5	4.6×10^6
10	3.1×10^3	9×10^5	2.7×10^6
11	2.25×10^4	1.5×10^6	4.5×10^6
12	3.2×10^5	1.25×10^6	3.5×10^6
Mean	8.4×10^4	7.8×10^5	2.1×10^6

[a] Virus titers of stably transformed cloned ψ2 producer cell lines containing the pH, pHS, and pHSG-neo genomes are presented as number of infectious virus particles per milliliter of producer supernatant. To determine the significance of the difference in titers between producers of pH-neo and pHS-neo and between producers of pHS-neo and pHSG-neo, titers for each group were compared by using paired *t* statistics. Comparison of the titers of pH-neo with those of pHS-neo yielded $P < 0.001$, while comparison of pHS-neo with pHSG-neo yielded $P < 0.01$. A threshold of $P = 0.02$ was considered significant.

TABLE 2. Transient titers of internal promoter vectors[a]

Vector	No. of G418^r colonies/plate	
	NIH 3T3	F9
pDOL$^-$	500	17
pZIPNEO	180	0
pBA-neo	270	30
pH-neo	380	60
pHS-neo	550	105
pHSG-neo	600	325

[a] Virus titers for each construction were determined by infecting NIH 3T3 or F9 cells with supernatants harvested from transiently transfected ψ2 cells. Presented are the results from a typical experiment.

and either stained with crystal violet to determine transient viral titers (Table 2) or expanded for genomic DNA isolation. On NIH 3T3 cells, all constructions yielded titers comparable to those obtained with the previously described vectors pDOL$^-$ (16) and pZIPNEO (4). In keeping with previous experience, the pZIPNEO construction yielded no colonies on F9 cells, and the pDOL$^-$ construction yielded very few, reflecting the low level of transcription of the constructions in F9 cells (2). In contrast, each of the internal promoter constructs yielded G418^r cell titers on F9 cells, with the histone H4 constructions yielding the greatest number.

Genomic DNA made from infected populations was cleaved with either *Kpn*I or *Nhe*I and subjected to Southern blot analysis (Fig. 2A). Both *Kpn*I and *Nhe*I cleave in the viral LTR. *Kpn*I cleaves 3′ of the enhancer deletion, and *Nhe*I cleaves 5′ of the deletion. If the correct proviral structure has been transmitted, *neo* hybridization to a Southern blot of *Kpn*I digests of both infected-cell DNA and marker plasmid DNA should show fragments of identical size. In addition, if the deleted enhancer region of the 3′ LTR is properly duplicated to the 5′ LTR following integration of the provirus, the sizes of the proviral bands should be identical after either *Nhe*I or *Kpn*I digestion of DNA. Analysis of genomic DNA taken from infected NIH 3T3 cell populations revealed comigration of bands in both infected-cell DNAs and standards digested with *Kpn*I. In the case of pBA-neo, proper replication of the enhancer deletion was established by the comigration of hybridizing bands in the *Kpn*I and *Nhe*I digestions. The presence of an *Nhe*I restriction site in the histone promoter of pH-neo precluded the above analysis. Analysis of infected F9 cell populations yielded identical results (data not shown).

Transcription of the vectors in G418-resistant NIH 3T3 and F9 cell populations. To evaluate transcription of the different constructions, total cellular RNA was isolated from infected and G418-selected NIH 3T3 and F9 cell populations (made as described above) and run on Northern gels (Fig. 2B). A ^{32}P-labeled *neo* probe was used in hybridization analysis of Northern blots. In NIH 3T3 cells, expression of the expected RNA transcript was observed for each of the internal promoter constructions, although more RNA was seen in pZIPNEO-infected NIH 3T3 cells. The minor bands which migrated more slowly than the major RNA species probably represent small amounts of transcript initiated in the LTR as a consequence of enhancement by the inserted promoter sequences. Each of the vector constructions described in Fig. 1 was transcriptionally active in F9 cells at levels comparable to those found in infected NIH 3T3 cells.

Transcription of pHSG-neo in infected unselected populations of NIH 3T3 and F9 cells. Since we have previously shown that chromosomal position can dramatically influence transcription of integrated proviruses in F9 cells (2), an

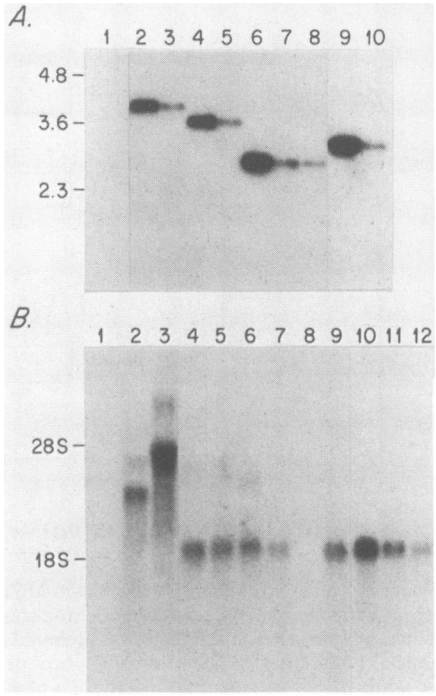

FIG. 2. (A) Southern blot hybridization analysis of vector transmission in NIH 3T3 cells. Genomic DNA (5 μg) prepared from populations of confluent cells was digested to completion with either *Kpn*I (lanes 1–7, 9, and 10) or *Nhe*I (lane 8). The blot was probed with 10^7 cpm of ^{32}P-labeled *neo* fragment. Lanes: 1, uninfected NIH 3T3 cells; 2, pDOL$^-$ marker DNA; 3, pDOL$^-$-infected cells; 4, pZIPNEO marker DNA; 5, pZIPNEO-infected cells; 6, pBA-neo marker DNA; 7 and 8, pBA-neo-infected cells; 9, pH-neo marker DNA; 10, pH-neo-infected cells. Sizes are shown in kilobases. (B) Expression of *neo* transcripts in NIH 3T3 cells (lanes 1–7) or F9 cells (lanes 8–12) infected with internal promoter vectors. Northern blot hybridization analysis of total cellular RNA (5 μg) prepared from populations of confluent cells transiently infected and selected in G418 (as above). Lanes: 1 and 8, uninfected cells; 2, pDOL$^-$; 3, pZIPNEO; 4 and 9, pBA-neo; 5 and 10, pH-neo; 6 and 11, pHS-neo; 7 and 12, pHSG-neo.

analysis of G418-selected F9 cell populations may not accurately reflect the inherent transcriptional activity of the integrated retrovirus constructions in F9 cells. To examine the expression of the vectors in a more direct way, virus harvested from a stable ψ2 cell line producing pHSG-neo was used to infect F9 cells, and 3 days later (without any G418 selection), DNA and RNA were isolated from the population of infected cells and analyzed (Fig. 3). Infections of NIH 3T3 cells were run in parallel. Comparison of the band intensities from the Southern blot analysis (Fig. 3A, lanes 4 and 8) indicated that F9 cells were slightly less well infected than were NIH 3T3 cells. Analysis of genomic DNA taken from cell populations containing a single proviral copy (lanes 3 and 7) indicated that multiple provirus copies were introduced into each cell of both the NIH 3T3 and F9 populations (lanes 4 and 8). The pHSG-neo provirus copy number in NIH 3T3 cells was 1.5-fold greater than in F9 cells, while the level of transcription of pHSG-neo in F9 cells was 6-fold below the level of pHSG-neo transcription in NIH 3T3 cells.

Transcription of the pHSG-neo genome in unselected murine hematopoietic cells in vivo. Based on the encouraging expression results obtained with pHSG-neo in F9 cells, we asked whether the pHSG-neo proviral genome would be

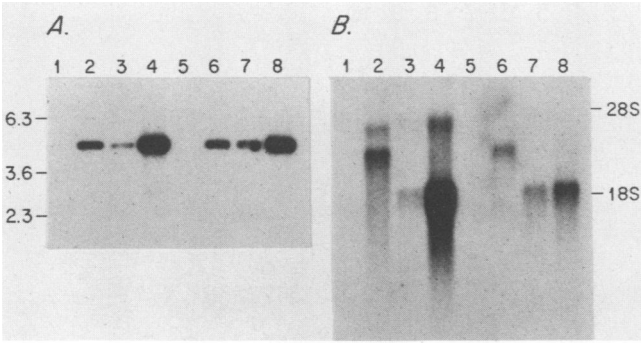

FIG. 3. (A) Transmission of HSG-neo to unselected NIH 3T3 and F9 cells infected with virus from a stable high-titer ψ2 producing cell line. Genomic DNA was isolated on day 3 postinfection, and cells were not subjected to G418 selection. Genomic DNA (5 μg) was digested with *Kpn*I and probed with ^{32}P-labeled *neo* fragment. Lanes: 1–4, NIH 3T3 cells; 5–8, F9 cells; 1 and 5, uninfected cell controls; 2 and 6, pHSG-neo marker DNA; 3 and 7, standard of single-copy pHSG-neo DNA from transiently infected G418-selected cells; 4 and 8, cells infected with virus (2 ml) from a stable high-titer (5×10^6 virus particles per ml) producer of HSG-neo. Sizes are shown in kilobases. (B) Transcriptional activity of the HSG-neo vector in unselected cells infected with virus as described for panel A. Northern blot of total cellular RNA (5 μg) probed with ^{32}P-labeled *neo* fragment. Lanes: same as in panel A, except lanes 2 and 6, which are NIH 3T3 and F9 cell populations, respectively, transiently infected with pDOL$^-$ and selected in G418.

transcriptionally active in hematopoietic cells in vivo. Murine bone marrow cells of C3H/HeJ female mice were cocultivated with ψ2HSG-neo9, a cloned producer cell line of pHSG-neo (5×10^6 G418-resistant CFU/ml) and subsequently introduced into lethally irradiated C3H/HeJ male mice via bone marrow transplantation as described before (18). Approximately 2×10^4 bone marrow cells were used for each transplantation. The efficiency of infection of bone marrow cells with the HSG-neo virus was evaluated by sacrificing two mice 12 days posttransplantation. Total cellular RNA and DNA were prepared from total spleen cells for Southern and Northern blot analysis. Digestion of total spleen cell DNA with *Kpn*I revealed that each recipient contained the expected proviral sequences at approximately 1 copy per cell, indicating a very efficient infection of CFU-S progenitor cells (Fig. 4A, compare lane 2 with lanes 5 and 7). Although poor transfer of high-molecular-weight DNA to the nylon filter made it difficult to determine accurately the number of independent integrations in each spleen DNA sample digested with *Xba*I, it appeared that 1 to 3 clones of transduced stem cells repopulated each recipient (lanes 6 and 8). Expression of *neo* RNA transcripts by the integrated HSG-neo genomes is shown in Fig. 4B (lanes 5 and 6). The expression of *neo* RNA in total spleen was approximately 1/4 the level of *neo* RNA found in a population of infected 3T3 cells harboring a single-copy provirus per cell (lanes 2 and 3). This indicated expression of proviral transcripts in the heterogeneous collection of hematopoietic cell types found in the spleen of transplant recipients 12 days posttransplantation.

Since the analysis of hematopoietic cells shortly after bone marrow transplantation does not necessarily reflect analysis of the progeny of transduced stem cells capable of permanent and complete reconstitution, transplant recipients were also examined 2.5 months posttransplantation. For this analysis, four mice were examined by an SP6 RNA protection analysis (24). Southern blot analysis of DNA from the spleen and bone marrow of each recipient indicated that the mice were reconstituted primarily with donor-derived cells (data not shown), with an efficiency of gene transfer ranging from 10 to 40% (Fig. 4C). The level of *neo* transcription in spleen and bone marrow RNA samples from the different recipients is shown in Fig. 4D. To compare the level of *neo* RNA expression in the spleen and bone marrow samples with the level of *neo* RNA in NIH 3T3 cells infected with HSG-neo to a single copy, the amount of *neo* RNA that would be detected if the spleen and bone marrow cells contained a single-copy provirus per cell was calculated. The levels of *neo* mRNA expression based on this normalization are shown in Table 3. While the levels of RNA detected were variable, they approximated the levels observed in spleen cells isolated from 12-day bone marrow transplant recipients.

DISCUSSION

The results presented here suggest that at least one construction, HSG-neo, and perhaps all of the vectors tested may be of general use for expression studies in both embryonic and hematopoietic cells. The HSG-neo vector yields high-titer-virus-producing cell lines and is transcriptionally active in both F9 cells and hematopoietic cells in vivo. The level of transcription found in both kinds of cells is high and comparable to the level found in infected NIH 3T3 fibroblasts. A major factor that appeared to affect the transmissibility of the vectors was the presence of mutations at the normal viral 5′ss. Replacement of the mutated portion of the viral genome with the wild-type splice donor sequence increased the average titer of virus-producing cell lines over 10-fold. A smaller increase (threefold) was achieved by including in the vectors additional viral sequences encoding a portion of the *gag* coding region. The data presented in this report indicate a small effect of these additional sequences on transmissibility of virus, while others have reported greater effects (1, 3). Since the titers obtained with the internal promoter vectors described in this report are comparable to those obtained by other groups, it may be that different features of their vectors account for the profound effect of the additional *gag* sequences that they report.

The studies reported here were motivated by a number of previous studies which indicated that vectors containing internal promoters appeared to function better in undifferentiated cells than vectors which used the viral LTR for the transcription of inserted sequences. Specifically, a number of groups have reported the construction and preparation of vectors containing either the simian virus 40 early promoter (13, 16, 25, 33) or the herpesvirus thymidine kinase (TK) promoter (20, 31). Both types of vectors were shown to be transcriptionally active in F9 cells, although in a number of cases the virus titers were low and the level of expression was poor. In the case of the TK promoter constructions, reasonable levels of expression have been obtained, and in one recipient, the vectors were shown to function after introduction into the mouse germ line via the infection of preimplantation embryos (29). Internal promoter vectors have also been used for the transfer of genes into hematopoietic stem cells and their progeny. While a number of groups have reported the expression of vector sequences in progenitor cells in vitro (7, 8, 13, 15) and CFU-S-derived cells in vivo (13, 18), few data on the expression of each construction at long periods after reconstitution have been reported.

In studies to be reported elsewhere, we compared the performance of the HSG vector with that of an analogous vector which contains the herpesvirus TK promoter. In that

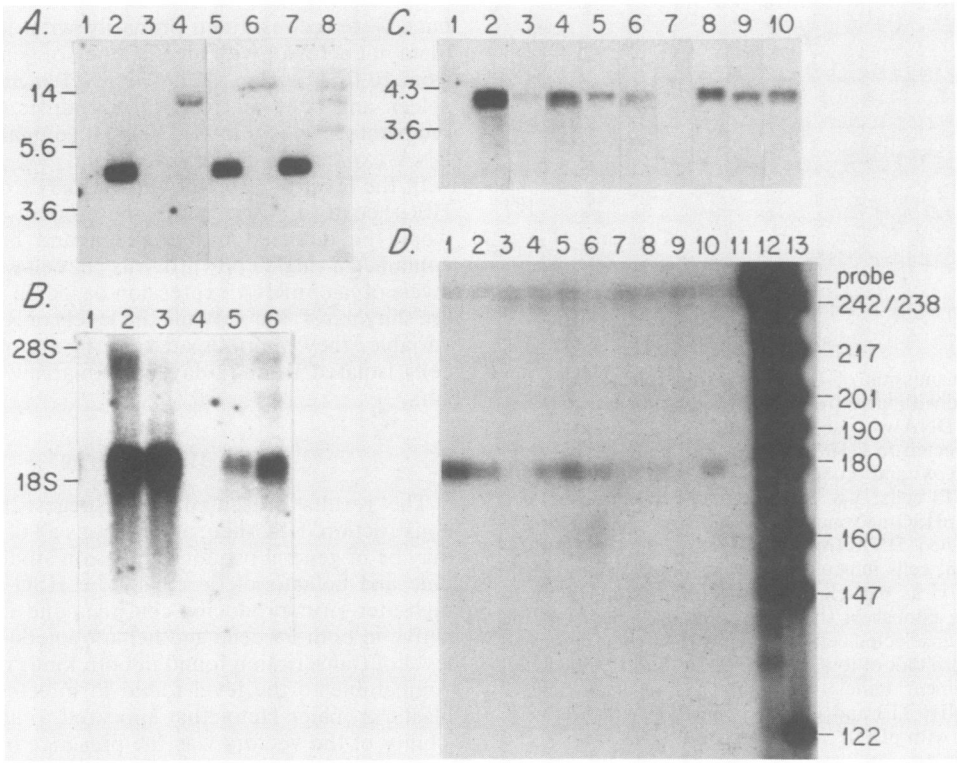

FIG. 4. Transfer and transcription of the HSG-neo genome in murine spleen and bone marrow cells from 12-day and 2.5-month transplant recipients. (A) Southern blot hybridization analysis of genomic DNA (5 μg) isolated from the spleens of male C3H/HeJ mice at day 12 after bone marrow reconstitution with 2×10^4 female C3H/HeJ donor cells infected with HSG-neo from producer cell line ψ2HSGneo9. DNA was digested to completion with either *Kpn*I (lanes 1, 2, 5, and 7) or *Xba*I (lanes 3, 4, 6, and 8), subjected to electrophoresis, and probed with ^{32}P-labeled *neo*. Lanes: 1 and 3, uninfected normal spleen DNA; 2 and 4, pHSG-neo marker DNA; 5 and 6, total spleen cells from transplant recipient mouse 1; 7 and 8, spleen cells from mouse 2. (B) Northern blot hybridization analysis of in vivo expression from the HSG-neo genome in total spleen cells at day 12 after transplantation. Total cellular RNA (5 μg) probed with ^{32}P-labeled *neo*. Lanes: 1, uninfected NIH 3T3 cells; 2 and 3, single-copy expression from the HSG-neo genome in transiently infected and G418-selected NIH 3T3 cells and F9 cells, respectively; 4, uninfected normal spleen cells; 5 and 6, HSG-neo-infected spleen cells from mice 1 and 2, respectively. (C) Southern blot hybridization analysis of 2.5-month HSG-neo transplant recipient mice. Four transplant recipients engrafted at the same time and in the same fashion as those noted in panels A and B were sacrificed at 2.5 months posttransplantation. DNA (5 μg) was prepared from total bone marrow (lanes 3, 5, 7, and 9) or spleen cells (lanes 1, 2, 4, 6, 8, and 10) and digested to completion with *Kpn*I, subjected to electrophoresis, blotted, and probed with radioactive *neo*. Lanes: 1, uninfected normal spleen; 2, same as lane 1 plus single-gene-copy pHSG-neo marker DNA; 3 and 4, HSG-neo transplant recipient mouse 3; 5 and 6, mouse 4; 7 and 8, mouse 5; 9 and 10, mouse 6. (D) SP6 protection analysis of total cellular RNA isolated from the bone marrow (lanes 3, 5, 7, and 9) or spleen cells (lanes 4, 6, 8, and 10) of 2.5-month posttransplant recipient mice described in panel C. Control lanes: 1, 0.25 μg of total cellular RNA from NIH 3T3 infectant containing single-copy pHSG-neo; 2, same but 0.125 μg; 3 and 4, mouse 3; 5 and 6, mouse 4; 7 and 8, mouse 5; 9 and 10, mouse 6; 11, tRNA; 12, undigested ^{32}P-labeled *neo* probe; 13, ^{32}P-marker DNA. Spleen RNAs from recipients transplanted with uninfected cells yielded no signal in the SP6 analysis (data not shown). Size markers in panels A and C are in kilobases, those in panel D are in base pairs.

study, expression of a human low-density lipoprotein receptor cDNA was examined in infected NIH 3T3 cells, human hepatoma (HEpG2) cells, and primary cultures of rat or rabbit hepatocytes. Analysis of RNA showed that the HSG vector yielded two- to fourfold more RNA than the TK promoter constructions (J. Wilson and R. Mulligan, unpublished). Those studies suggest that the HSG vector may have advantages over existing TK-based vectors in at least some cell types. It remains to be determined whether vectors such as pHSG will be useful for expressing all inserted sequences in all cell types. However, an encouraging fact is that all of the vectors described here yield high-titer-virus-producing cell lines with a variety of inserts. Moreover, we have demonstrated reasonable levels of expression of a variety of inserts with these vectors (11, 32a). Because of the high transmissibility of the vectors, the presence of a selectable biochemical marker in the vector appears to be unnecessary. Expression studies with a large number of different constructions are now in progress.

TABLE 3. Expression of HSG-neo[a]

Mouse no.	HSG-neo expression (% of control)	
	Bone marrow	Spleen
3	42.0	10.0
4	65.0	12.0
5	ND[b]	2.9
6	3.3	15.0

[a] Expression of HSG-neo in 2.5-month bone marrow transplant recipients. The level of *neo* mRNA is expressed as the percentage of expression of *neo* in G418-selected NIH 3T3 cells infected with a single copy of the vector HSG-neo.

[b] ND, Not determined.

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