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Comparison of Expression in Hemopoietic Cells by Retroviral Vectors Carrying Two Genes

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In order to identify factors that influence expression by retroviral vectors in hemopoietic cells, we have compared viral RNA levels in cells infected with several different recombinant viruses. All of the vectors tested carry the neomycin resistance gene and provide for the insertion of a second gene which, in these studies, comprised sequences from the *myc* or *myb* oncogenes or the gene encoding granulocyte-macrophage colony-stimulating factor. The vectors utilize two different strategies for the coexpression of the two genes: alternate splicing and the use of a separate internal promoter. We found that expression in hemopoietic cells could be increased by substituting sequences from the myeloproliferative sarcoma virus long terminal repeat for those of the Moloney murine leukemia virus long terminal repeat. However, none of the vectors examined was able to express a second gene at levels equivalent to those achieved by the parental vectors carrying only the neomycin resistance gene. The reasons for this varied with the different vectors and included inefficient splicing and/or a reduction in the level of unspliced transcripts upon insertion of a second gene. Although the basis of the latter phenomenon is not clear, it is probably related to the position—near the 5′ long terminal repeat—at which the second gene was inserted, since insertion of the same genes near the 3′ end of another vector had no effect on viral RNA levels. In an attempt to circumvent some of these problems, we constructed a vector that employs an internal β-actin promoter. Although this vector could express granulocyte-macrophage colony-stimulating factor sequences in a responsive hemopoietic cell line, the level of granulocyte-macrophage colony-stimulating factor produced was disappointingly low. The results from these studies suggest approaches to the design of improved vectors for effective expression of genes in hemopoietic cells.

One approach to investigating the biological properties of genes which are thought to regulate cellular growth and differentiation is to assess the consequences of introducing them into normal cells. The murine hemopoietic system is well suited to such an approach since a range of cell types at various stages of differentiation can be readily isolated and maintained in vitro under defined growth conditions (48). Several recent reports have described the use of retroviral vectors for gene delivery into hemopoietic cells (4, 10, 17, 31, 40, 52), including stem cells apparently capable of generating all elements of the hemopoietic system (6, 15, 32, 39, 65). Most vectors have been derived from Moloney murine leukemia virus (Mo-MLV) and are designed to express two genes: a selectable marker and a second gene, the biological activity of which is to be investigated. The presence of a selectable marker is desirable since it allows the isolation of infected cell populations containing the gene of interest and facilitates titration of virus-producing cell lines.

To date, many of the genes that have featured in reports of functional expression by retroviral vectors in primary hemopoietic cells (6, 15, 17, 32, 40, 44, 52) have encoded enzymes (e.g., neomycin phosphotransferase, adenosine deaminase, hypoxanthine phosphoribosyl transferase, and dihydrofolate reductase) for which there are highly sensitive assays and which are probably required at relatively low levels to exert a phenotypic effect (11, 19). However, it seemed likely that higher levels of expression were needed for genes such as *myc*, *myb*, or the gene encoding granulocyte-macrophage colony-stimulating factor (GM-CSF), because our initial experiments with retroviruses carrying these genes produced rather weak or inconsistent phenotypic changes in primary hemopoietic cells. We therefore undertook a comparative study of some of the available vectors to see which were optimal for expression of these genes. We also attempted to increase the level of expression in hemopoietic cells by replacing the Mo-MLV enhancer with that of myeloproliferative sarcoma virus (MPSV) and by constructing a novel retroviral vector (pNAc) carrying an expression cassette directed by the promoter-enhancer sequences of the human β-actin gene.

In this report, we present an analysis of the expression of the various recombinant viruses in hemopoietic cells and cell lines in vitro; a parallel study of the efficacy of certain of these viruses in vivo was presented previously (6). We found that replacement of Mo-MLV long terminal repeat (LTR) sequences with the corresponding sequences from MPSV could improve the expression in hemopoietic cells of a vector carrying only the neomycin resistance gene (Neo^r). However, inclusion of a second gene (such as *myb*, *myc*, or the gene encoding GM-CSF) invariably resulted in reduced expression of one or both genes. The vectors which utilized an internal β-actin promoter were only partially successful: although functional in hemopoietic cells, they expressed a test gene product (GM-CSF) at low levels compared with those expressed by a previously described LTR-driven vector (37). Our results have highlighted several problems in retroviral vector design and should be useful in guiding the construction of more effective vectors.

MATERIALS AND METHODS

Retroviral vectors. Retroviral vectors pZipNeoSV(X) (8), here termed pZipNeo, and pDOL (35) here termed pDolNeo,

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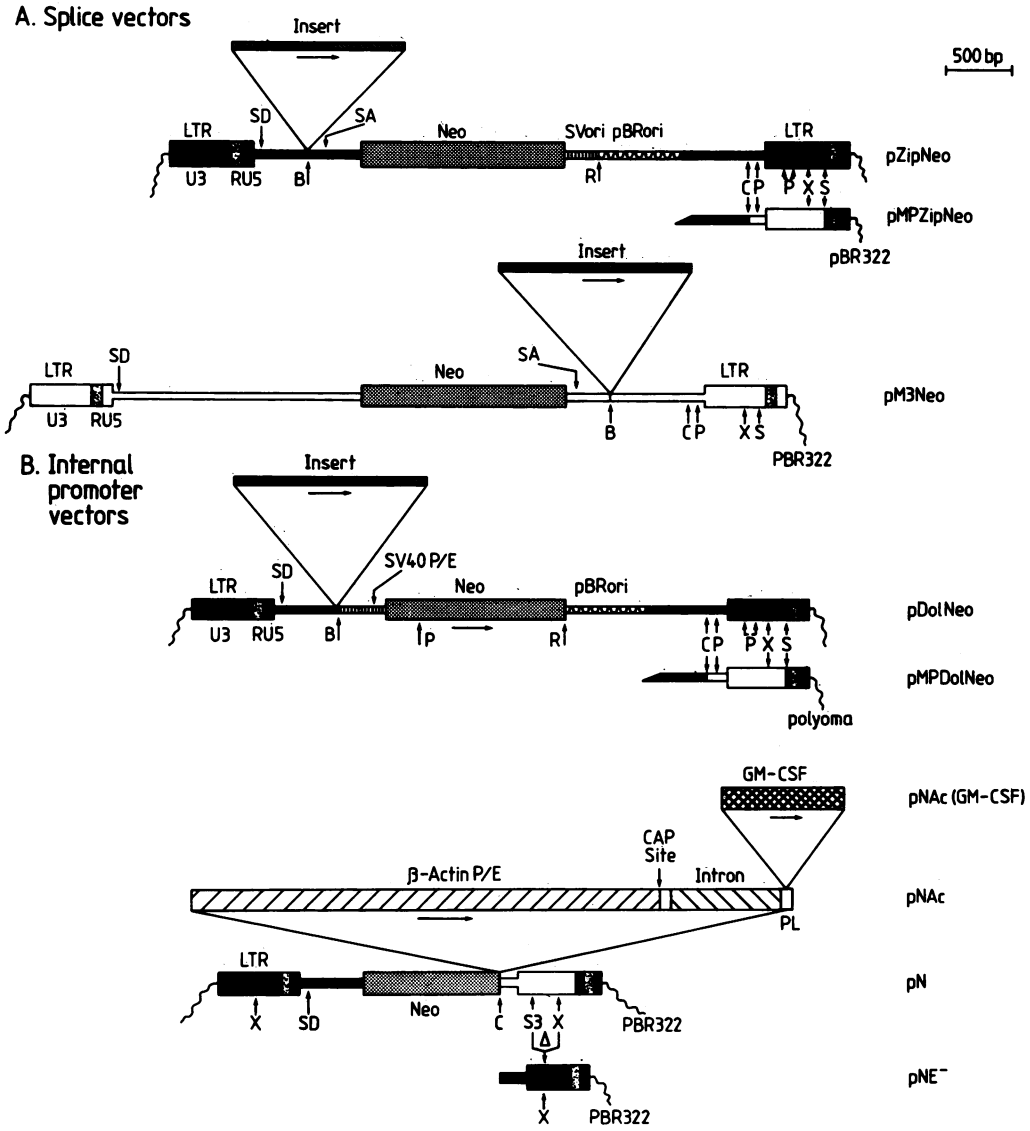

FIG. 1. Schematic representation of the proviral structures of (A) splice vectors and (B) internal promoter vectors used in this study. Symbols: (▬) sequences derived from Mo-MLV, (▭) sequences derived from MPSV;(▒) neomycin resistance gene (which confers resistance to G418). SA and SD, splice acceptor and donor sites, respectively. The positions and orientation of the various inserts placed in these vectors are shown, as are the locations of sequences derived from SV40 or PBR322: SVori, SV40 origin of replication; PBRori, PBR322 origin of replication; SV40 P/E, SV40 early promoter-enhancer. β-actin P/E, Human β-actin promoter region. The construction and origin of these vectors are described in Materials and Methods. Cleavage sites for several restriction endonucleases relevant to the construction and analysis of the vectors are also indicated as follows: B, *Bam*HI; C, *Cla*I; P, *Pvu*II (not all sites outside the LTR are shown); R, *Eco*RI; S, *Sac*I; S3, *Sau*3A (not all sites shown); X, *Xba*I. For clarity, sites within the LTRs are shown only on one LTR.

were obtained from R. Mulligan (Whitehead Institute, Boston). pM3Neo (36) was derived from MPSV and obtained from W. Ostertag (Heinrich-Pette Institute, Hamburg, Federal Republic of Germany). The basic structure of these vectors is detailed in Fig. 1. Standard methods (45) were used to construct derivatives of these vectors as follows.

(i) pMPZipNeo and pMPDolNeo, which bear a hybrid Mo-MLV–MPSV 3′ LTR, were derived from pZipNeo and pDolNeo by first isolating *Eco*RI fragments (of 2.5 and 4.0 kilobases [kb], respectively) encompassing the 3′ LTR and adjacent vector sequences. A 556-base-pair (bp) *Cla*I-*Sac*I fragment, which included all but 31 bp of the U3 region of the LTR, was removed from each *Eco*RI fragment and replaced with a corresponding *Cla*I-*Sac*I fragment derived from the 3′ LTR of pM3Neo. The modified *Eco*RI fragments were then used to reconstitute the respective vectors.

(ii) The simplified vector pN was constructed from pMPZipNeo by deleting the region between the *Bam*HI and *Cla*I sites and replacing it with a 1-kb *Bgl*II-*Sma*I fragment from pZipNeo, which carries just the neomycin resistance gene. In pNE$^-$, the enhancer was deleted from the 3′ LTR by removing the *Cla*I-*Sac*I fragment from pN and replacing it with a deleted version of the corresponding fragment from Mo-MLV. The deletion of 202 bp in this latter fragment (obtained from M. Scott, University of California, San Francisco) extended from the *Sau*3A site (Fig. 1) immediately 5′ of the 75-bp direct repeats to the *Xba*I site in the LTR.

(iii) Retroviral vectors pNAc and pNE$^-$Ac, designed to express genes under the independent control of a β-actin promoter-enhancer (Fig. 1) were constructed by inserting a human cytoskeletal β-actin expression cassette (obtained from P. Gunning, Stanford University) into the unique *Cla*I site of the vector pN or pNE$^-$. The cassette consisted of a 4.3-kb *Eco*RI-*Alu*I fragment of the human β-actin gene isolate p14TB-17 (38) encompassing the β-actin promoter and adjacent sequences, including 77 bp of the 5′ untranslated region, intron 1, and a truncated second exon in which a polylinker has been inserted 5′ of the translational start codon. The cassette was placed in the unique *Cla*I site of vector pN or pNE$^-$ (Fig. 1).

The following cDNA fragments were introduced into the *Bam*HI sites of some or all of the various vectors after the addition of *Bam*HI linkers where necessary: (i) a 1,391-bp *Xho*I fragment of murine c-*myc* cDNA (58), (ii) a 2.1-kb *Sma*I-*Bgl*II murine c-*myb* cDNA sequence constructed by joining the cDNA clones MM49 and MM46 (20) via their common *Sma*I site, (iii) a 1.1-kb fragment containing the entire v-*myb*^{AMV} coding sequence (33), and (iv) the 0.8-kb GM-CSF cDNA clone Δ7 (23).

Virus-producing cell lines. Fibroblast lines producing recombinant virus free of helper virus were generated both by stable transfection of Ψ2 cells (46) with vector DNA as described previously (11, 37), or by infection of tunicamycin-treated (0.1 μg/ml for 16 h) Ψ2 cells with supernatant from transiently transfected Ψ2 cells (10, 11, 54). (The efficiency of infection of tunicamycin-treated Ψ2 cells was approximately 60-fold less than that of untreated NIH 3T3 fibroblasts.) Virus-producing lines were maintained in Dulbecco modified Eagle medium (DME) containing 10% newborn calf serum. For each construct, 5 to 30 cloned Ψ2 lines were assayed for virus production by measuring the ability of filtered supernatants to confer G418 resistance to NIH 3T3 fibroblasts (6, 37). Briefly, supernatants from confluent monolayers were filtered and, after dilution in medium containing 4 μg of polybrene per ml, used to infect 3T3 fibroblasts; 2 or 3 days later, the cells were split 1:20 into medium containing 400 μg of G418 per ml, and colonies were counted 10 to 12 days later.

Infection of hemopoietic cells. Murine hemopoietic cell lines FDS-P1 (14, 25) and WEHI-3B(D$^+$) (51) and primary hemopoietic cells from 12- or 13-day fetal liver (29) were infected with the various recombinant retroviruses by cocultivation with virus-producing Ψ2 cells followed by selection for G418 resistance or colony-stimulating factor (CSF) independence. The various Ψ2 lines were seeded at a density of 5×10^5 per 60-mm dish in 5 ml of DME containing 10% fetal calf serum plus, in the case of FDC-P1 cells or fetal liver cells, growth factor [1% (vol/vol) WEHI 3B(D$^-$) conditioned medium (68) or 2% (vol/vol) pokeweed mitogen-stimulated spleen conditioned medium (50)]. Hemopoietic cells (1×10^5 to 2×10^5) were added and cocultivated for 2 days. Nonadherent cells were harvested, replated in fresh dishes for 4 h to allow dislodged Ψ2 cells to attach, and then harvested again. After three washes in DME, the cells were selected for resistance to 1 mg of G418 per ml or growth in the absence of added CSF as described by Lang et al. (37). Selection was maintained for at least 10 days prior to harvesting cells for further analysis, except for the fetal liver cells, which were harvested after selection for 4 days.

Assay of GM-CSF production. Medium conditioned by virus-infected FDC-P1 cells was prepared by plating cells at 5×10^5 per 5 ml in DME containing 10% fetal calf serum and allowing them to grow for 3 days, at which time medium was collected and the cells counted. CSF activity was determined by a microwell assay (49) with (uninfected) FDC-P1 indicator cells. Activity is expressed in bone marrow units (48), which were calculated from the microwell assay results after applying an empirically determined conversion factor.

Analysis of viral DNA and RNA. To minimize variation in expression arising from the site and number of proviral integrants in individual cells, DNA and RNA analyses were performed on polyclonal populations. Poly(A)$^+$ RNA was isolated and Northern (RNA) blot analysis was performed as described previously (20). Autoradiograms were quantitated with a Joyce-Loebel Chromoscan 3 densitometer and in most cases corrected for variations in loading of poly(A)$^+$ RNA as estimated by hybridization of the Northern blots with a β-2 microglobulin probe. High-molecular-weight DNA was isolated by ethanol precipitation of cell lysates dispersed in guanidine hydrochloride (5). After digestion with restriction endonucleases, Southern blot analysis was performed as described previously (12). Hybridization probes were prepared by nick translation of the appropriate DNA fragments in the presence of [α-^{32}P]dATP. The neomycin resistance gene probe was a 1.4-kb *Hin*dIII-*Sma*I fragment from pSV2Neo (56). Other probes are described in the relevant figure Legends.

RESULTS

Vectors. The basic features of the Mo-MLV-based vectors used in this study are outlined in Fig. 1. All harbor the Neo^r which, in eucaryotic cells, confers resistance to the antibiotic G418. The *Bam*HI cloning sites enable insertion of an additional gene—in this study, a cDNA encoding c-*myc*, c-*myb*, v-*myb*, or GM-CSF. The vectors fall into two categories. pZipNeo (8) and pM3Neo (36) are splice vectors in which the viral LTR promoter-enhancer controls expression of both genes; the 5′ gene is translated from the viral genomic RNA, whereas the 3′ gene is expressed via a spliced subgenomic RNA. By contrast, the pDol- (35) and pNAc-based vectors use the LTR to express only the 5′ gene, the 3′ gene being under the control of an internal promoter-enhancer.

Whereas pZipNeo and pDolNeo were both derived from Mo-MLV, pM3Neo was derived from MPSV (34). Although the Mo-MLV and MPSV LTRs are highly homologous (57), the minor differences between their sequences are apparently sufficient to account for the myeloid tropism of MPSV (60, 61). Therefore, in an attempt to increase the level of expression of the recombinant viruses in myeloid cells, we constructed derivatives of pZipNeo and pDolNeo in which most of the U3 region of the 3′ Mo-MLV LTR was exchanged for the corresponding region of the MPSV LTR (Fig. 1). (Only the 3′ LTR needs to be modified because the retroviral replication process produces a provirus which also carries the modification in the 5′ LTR [63].) The modified vectors were denoted pMPZipNeo and pMPDolNeo (Fig. 1).

Inefficient expression of the 3′ gene in splice vectors. We first investigated expression of genes inserted into the splice vectors. The M3Neo vector (Fig. 1A) was designed to express Neo^r from its genomic transcript and a second gene from the spliced subgenomic mRNA. Although genomic transcripts were readily detected by Northern blotting in cells infected with M3Neo-based viruses (see Fig. 6), no subgenomic mRNA could be detected in cells infected with either the parental virus or with derivatives carrying c-*myb*, c-*myc*, or GM-CSF (data not shown). Moreover, no GM-CSF could be detected in the supernatant of cells infected

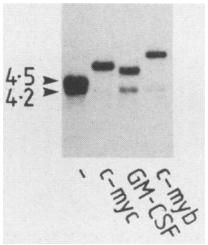

FIG. 2. Comparison of the levels of genomic and subgenomic (spliced) mRNA in NIH 3T3 cells infected with ZipNeo-based viruses bearing either no insert (−) or the gene indicated. The figure shows Northern analysis, with a Neo^r probe, of poly(A)$^+$ RNA (1 μg) isolated from polyclonal G418-resistant populations of these cells. Arrows indicate the 4.5-kb genomic and 4.2-kb subgenomic transcripts from the parental ZipNeo vector.

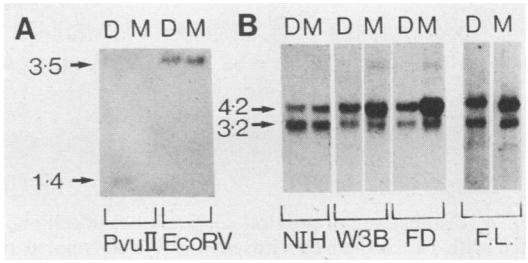

FIG. 3. Analysis of proviral structure and expression in DolNeo and MPDolNeo virus-infected cells. (A) Southern blot analysis of DNA isolated from WEHI-3B cells infected with DolNeo (D) or MPDolNeo (M) viruses and selected for G418 resistance. DNA (5 μg) was restricted with *Pvu*II or *Eco*RV and hybridized with a nick-translated DNA fragment that extended 600 bp from the unique *Bam*HI site of pDolNeo to a *Pvu*II site in Neo (Fig. 1). Sizes of the fragments are shown in kilobases in this and subsequent figures. (B) Northern blot analysis of RNA from polyclonal G418-resistant populations of NIH 3T3 fibroblasts (NIH), WEHI-3B (W3B), FDC-P1 (FD), or fetal liver (F.L.) cells infected with DolNeo (D) or MPDolNeo (M) viruses as indicated. Poly(A)$^+$ RNA (1 μg) was analyzed with a nick-translated Neo^r probe. Arrows indicate the positions of the 4.2-kb viral genomic and 3.2-kb subgenomic SV40-Neo transcripts. In this and subsequent figures, substantial gaps between lanes indicate that the photographs shown are from separate autoradiographs.

with M3Neo(GM-CSF) virus, in marked contrast to the results obtained with ZipNeo(GM-CSF) virus (37) and DolNeo(GM-CSF) (our unpublished results). Thus, an apparent splicing defect effectively silences the second gene.

The configuration of the ZipNeo vector differs from that of M3Neo, in that Neo^r is encoded by the spliced RNA and the second gene is expressed from the genomic transcript. In contrast to M3Neo, both the genomic and spliced subgenomic transcripts were produced in approximately equal amounts (Fig. 2) in NIH 3T3 cells infected with the parental virus (carrying only Neo^r). However, viruses carrying an additional gene generated considerably less of the subgenomic RNA than the full-length transcript, especially those carrying c-*myc* or c-*myb* (Fig. 2). The reduction in the level of Neo^r mRNA presumably reflects inhibition of splicing by the inserted sequences and may result in insufficient mRNA in some infected cells to confer G418 resistance, thus reducing the apparent titer of the virus. Indeed, the viral titers of Ψ2 lines transfected with pZipNeo(c-*myc*) were consistently lower than those obtained with several other ZipNeo-based vectors (Table 1 and our unpublished results), an observation which correlates with the very low level of Neo^r mRNA levels seen in NIH 3T3 cells infected with ZipNeo(c-*myc*) (Fig. 2). Interference with the expression of the Neo^r appeared to be most pronounced with v-*fos*: no G418-resistant Ψ2 lines were ever obtained, despite repeated attempts to transfect Ψ2 cells with pZipNeo(v-*fos*) DNA (unpublished data).

TABLE 1. Titers of recombinant virus-producing cell lines

Virus-producing ψ2 clone	Fibroblast titer[a] (10^4)
ZipNeo	10, 20
ZipNeo (c-*myc*)	0.2
ZipNeo (c-*myb*)	11
ZipNeo (GM-CSF)	18, 20
MPZipNeo	32, 92
MPZipNeo (GM-CSF)	7.4
DolNeo	10, 24
DolNeo (c-*myc*)	26, 26
DolNeo (c-*myb*)	26
DolNeo (v-*myb*)	70
DolNeo (GM-CSF)	1.3
MPDolNeo	32, 72
MPDolNeo (c-*myc*)	12, 38
MPDolNeo (v-*myb*)	20
M3Neo	60, 110
M3Neo (c-*myb*)	23, 100
M3Neo (GM-CSF)	2

[a] Fibroblast titers are the numbers of G418-resistant colonies produced per milliliter of filtered supernatant from the indicated ψ2 lines after infection of 3T3 fibroblasts. (Where two figures are given, these represent the results of independent assays.)

MPSV LTR sequences can enhance expression in myeloid cells. Although the titers of some of the recombinant ZipNeo viruses may have been better than the G418 resistance assay suggested, we decided to place our major emphasis on the DolNeo viruses, with which we routinely obtained recombinant viruses of adequate titer even when a second gene was inserted (Table 1). Most of the DolNeo virus-producing lines were isolated by superinfection of tunicamycin-treated Ψ2 cells (see Materials and Methods) and generally had higher titers of virus than those produced be transfection, in agreement with results reported by Hwang and Gilboa (26).

To confirm that the MPSV LTR sequences had in fact reconstituted the 5′ LTR of MPDolNeo proviruses, we compared the structure of the 5′ LTRs in cells infected with DolNeo and MPDolNeo viruses. DNA was prepared from a polyclonal population of G418-resistant WEHI-3B cells and digested with *Pvu*II, which cuts in the U3 region of the Mo-MLV LTR but not in the hybrid MPSV–Mo-MLV LTR (Fig. 1), and with *Eco*RV, which cuts within both LTRs and near the internal *Cla*I site (9). Southern blot analysis of the *Eco*RV digests with a probe from pDolNeo containing simian virus 40 (SV40) and Neo^r sequences (a 600-bp *Bam*HI-*Pvu*II fragment; Fig. 1) revealed equivalent levels of proviral DNA in both cell populations (Fig. 3A). However, only DolNeo virus-infected cells yielded a detectable *Pvu*II fragment (of 1.4 kb). This result was consistent with conversion of the 5′ Mo-MLV LTR to the predicted hybrid MPSV–Mo-MLV LTR, since only the former contains *Pvu*II sites. (The polyclonality of the infected cell population precludes detection of fragments extending from an internal *Pvu*II site [in Neo^r]of MPDolNeo to adjacent chromosomal sites.)

The effect of the LTR exchange on expression was investigated in both fibroblasts and myeloid cells. RNA was isolated from cells which had been infected with DolNeo or MPDolNeo virus, selected by growth in G418, and then

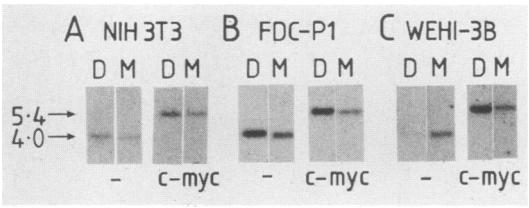

FIG. 4. Comparison of proviral copy number in cell populations infected with DolNeo-based viruses. DNA was isolated from (A) NIH 3T3, (B) FCD-P1, and (C) WEHI-3B cells infected with DolNeo (D) or MPDolNeo (M) viruses carrying no additional gene (−) or c-*myc* as indicated. After digestion with *Xba*I, the DNA was analyzed by Southern blotting and hybridization to a Neo^r probe. The amount of DNA loaded from DolNeo-infected WEHI-3B cells was only half that from the other cell populations as assessed by ethidium bromide staining.

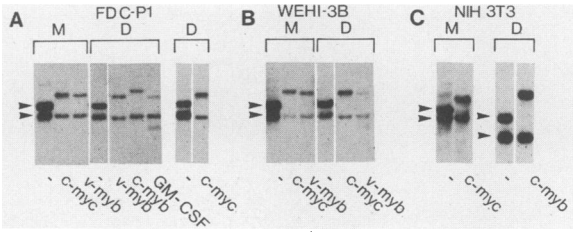

FIG. 5. Effect of insertion of *myc*, *myb*, or GM-CSF coding sequences on expression by DolNeo-based vectors in (A) FDC-P1, (B) WEHI-3B and (C) NIH 3T3 cells. Poly(A)$^+$ RNA was isolated from pooled, G418-resistant cells after infection with Dol (D) or MPDol (M) vectors bearing either no additional gene (−) or GM-CSF, c-*myb*, v-*myb*, or c-*myc* inserts as indicated. Poly(A)$^+$ RNA (1 μg) was analyzed by Northern blotting and hybridization with a nick-translated Neo^r probe. Arrows indicate the positions and sizes (in kilobases) of the genomic and subgenomic RNAs of DolNeo virus.

expanded as a polyclonal population. Northern blot analysis of poly(A)$^+$ RNA (Fig. 3B) with a Neo^r probe revealed the two expected viral RNAs: a genomic RNA of 4.2 kb and the 3.2-kb SV40-Neo^r RNA. In NIH 3T3 fibroblasts similar levels of genomic RNA were produced from the DolNeo and MPDolNeo proviruses, implying that there was no significant difference in the transcriptional activity of the two LTRs in fibroblasts.

In marked contrast, the level of viral genomic RNA was clearly greater in MPDolNeo virus-infected myeloid cells that in cells infected with DolNeo virus (Fig. 3B). This difference was seen with both the FDC-P1 cell line (2.4-fold after correction for RNA loading) and the WEHI-3B line (2.1-fold) and for the heterogeneous population of normal hemopoietic cells from fetal liver (1.9-fold). Since the two viruses were used at similar titers (Table 1), it seemed unlikely that the elevated transcription could be due to a higher average proviral copy number in the MPDolNeo virus-infected cells. This was confirmed by the Southern blot analysis shown in the first panel of each section of Fig. 4. For each cell type, the level of MPDolNeo proviral DNA was no higher than that of DolNeo virus, allowing for the approximately twofold-lower loading of DNA from DolNeo virus-infected WEHI-3B cells. We therefore concluded that, in the context of the DolNeo vector, the MPSV LTR directed higher levels of transcription in myeloid cells than did the Mo-MLV LTR. Since similar levels of SV40-Neo^r RNA were found in myeloid cells infected with either virus (Fig. 3B), the SV40 promoter is apparently indifferent to the LTR exchange.

Proviruses carrying an additional gene are poorly expressed in hemopoietic cells. To assess the utility of the different vectors bearing the Neo^r for expression of a second gene in hemopoietic cells, we first analyzed the levels of viral RNA in FDC-P1 cells infected with DolNeo and MPDolNeo viruses carrying c-*myb*, v-*myb*, c-*myc*, and the gene encoding GM-CSF. Substantially lower levels of viral genomic RNA were present in cells infected with each of these viruses than in cells harboring the corresponding parental viruses (carrying only the Neo^r) (Fig. 5A). Densitometric analysis revealed that insertion of an additional gene resulted in viral genomic RNA levels that were 2.9-fold lower with the DolNeo vectors and up to 3.2-fold lower with MPDolNeo. Moreover, the presence of a second gene effectively negated the advantage conferred by the LTR exchange (see Discussion), since the level of expression of each of the MPDolNeo viruses carrying an additional gene was similar to that of its DolNeo counterpart (Fig. 5A).

Reduced expression by the viruses carrying *myc*, *myb*, and GM-CSF was not confined to FDC-P1 cells. The presence of *myc* and *myb* sequences also reduced the level of viral transcripts in infected WEHI-3B cells (by up to 7.7-fold) (Fig. 5B); similar results were obtained with normal hemopoietic cells from 13-day fetal liver (data not shown). The effect was not due to differences in the average number of proviral inserts per cell, since this was relatively constant between the parental viruses and their derivatives, as shown in Fig. 4B and C for the *myc* viruses. Furthermore, the depressed RNA levels were not a consequence of G418 selection, since, at least for c-*myc*, the same patterns of expression were obtained in selected and unselected cells (data not shown) Whatever the basis of the phenomenon, its consequences were much more severe in myeloid cells than in fibroblasts. In NIH 3T3 fibroblasts the *myc* and *myb* viruses were expressed at levels approaching those of viruses carrying only the Neo^r (Fig. 5C). Note that the levels of the SV40-neomycin resistance gene mRNA in myeloid cells were also decreased by the presence of a second gene, although this effect was less marked and more variable than the effect on the genomic RNA (Fig. 5A and B; data not shown).

Insertion of an additional gene was as deleterious to the expression of ZipNeo-based viruses as to the DolNeo viruses. The levels of both the genomic and the spliced viral RNAs were much lower in FDC-P1 cells infected with ZipNeo(GM-CSF), MPZipNeo(GM-CSF), or ZipNeo(c-*myb*) viruses than in those infected with the parental viruses (Fig. 6); densitometric analysis revealed that the total amount of viral RNA was reduced by up to 4.4-fold.

In marked contrast to the results with the ZipNeo and DolNeo viruses, the levels of viral genomic RNA in FDC-P1 cells infected with M3Neo viruses were unaffected by the presence of *myb* or GM-CSF sequences. Thus it appears that insertion of these sequences into a viral vector does not inevitably lead to low levels of viral RNA in infected hemopoietic cells. The position of insertion and the nature of the vector are apparently critical factors (see Discussion).

Expression of an internal β-actin-driven cassette. In view of the problems described above with vectors that use the LTR to express a second gene, we constructed a new vector, in which the Neo^r is expressed via the genomic transcript and the second gene is expressed via an internal promoter. Since the SV40 promoter-enhancer is not optimal for hemopoietic cells (44; our unpublished results), we utilized regulatory sequences from a human cytoskeletal β-actin gene (38),

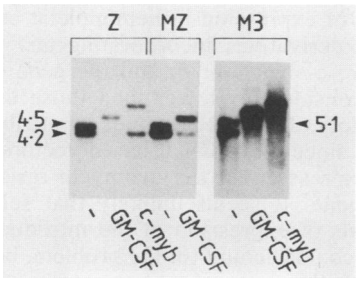

FIG. 6. Comparison of the levels of viral RNA in FDC-P1 cells infected with ZipNeo (Z), MPZipNeo (MZ), or M3Neo (M3) vectors carrying GM-CSF or c-*myb* inserts where indicated Poly(A)$^+$ RNA was isolated from virus-infected, G418-resistant cells and analyzed by Northern blotting with a Neo^r probe. Arrows at the left indicate the positions and sizes (in kilobases) of the genomic and subgenomic RNAs of the ZipNeo virus, and the arrow at the right indicates the position of the genomic RNA of M3Neo virus. Note that the smaller species seen in the M3Neo-infected cells is not the expected subgenomic RNA (because of its size and its hybridization to the Neo probe) but is probably generated by the utilization of cryptic splice signals.

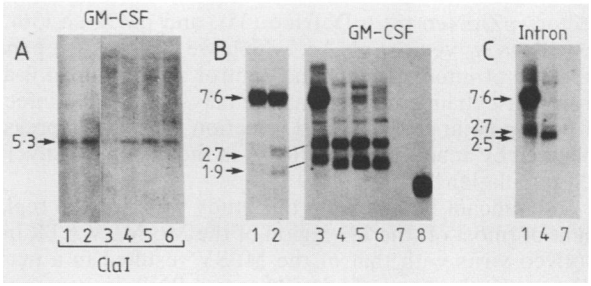

FIG. 7. Proviral structure and transcription in FDC-P1 cells infected with NAc(GM-CSF) and NE$^-$Ac(GM-CSF) viruses. (A) Southern blot analysis of integrated proviruses. DNA was isolated from polyclonal populations of FDC-P1 cells after infection with virus from individual Ψ2 lines producing either NAc(GM-CSF) (lanes 1, 2, and 3) or NE$^-$Ac(GM-CSF) (lanes 4, 5, and 6) and selected for G418 resistance (lanes 1 and 2) or CSF independence (lanes 3 and 6). DNA was cleaved with *Cla*I and hybridized to a GM-CSF probe corresponding to the cDNA clone Δ7 (23). (B) Northern blot analysis of poly(A)$^+$ RNA isolated from the same cell populations (lanes 1 to 6) as in panel A and, in addition, RNA from uninfected FDC-P1 cells (lane 7) and from concanavalin A-stimulated LB3 cells (lane 8), which produce GM-CSF and thus illustrate the normal cellular GM-CSF mRNA. Hybridization was with the GM-CSF probe; sizes of the major RNA species detected (in kilobases) are shown at the left. (C) Northern analysis with a probe corresponding to the intron (a 460-bp *Bgl*I fragment) in the β-actin cassette. Lane designations are as in panels A and B.

reasoning that these elements should allow a high level of expression in most cell types. We tested the efficacy of this vector by inserting a GM-CSF cDNA, which provides an additional selectable marker when used in CSF-dependent cells (37). This vector was designated pNAc(GM-CSF) (Fig. 1). A variant of this vector, carrying the β-actin(GM-CSF) cassette (plus an SV40 polyadenylation signal) in the reverse orientation proved to be unsatisfactory because most of the cassette was deleted in infected cells (data not shown). A derivative lacking the enhancer in the 3′ LTR, pNE$^-$Ac(GM-CSF), was also constructed (Fig. 1) (see Materials and Methods). Only the gene driven by the internal promoter should be active in cells infected with this virus, since the 5′ LTR of the resultant provirus will also lack the enhancer. Such LTR enhancerless vectors have also been described by others (10, 16, 67).

FDC-P1 cells infected with the conventional (enhancer-positive) NAc(GM-CSF) virus were selected either by the removal of CSF from the growth medium or by the addition of G418. Those carrying the enhancer-negative derivative were selected for factor independence and were, as expected, sensitive to G418. Southern blot analysis of the DNA from infected cells (Fig. 7A) showed, surprisingly, that the provirus retained the intron (Fig. 1) located in the 5′-untranslated region of the β-actin cassette; a 5.1-kb *Cla*I fragment was detected, rather than the 4.3-kb fragment expected after removal of the intron. This observation suggested that little spliced genomic RNA was present for export into virions, a view consistent with the inefficient removal of the intron from transcripts of the β-actin cassette (see below).

Analysis of the viral transcription pattern (Fig. 7B) showed that the major RNA species detected with the GM-CSF probe in FDC-P1 cells infected with the NAc(GM-CSF) virus (lanes 1, 2, and 3) was of the size (7.6 kb) expected for a complete transcript of the provirus. The level of genomic RNA was greatly reduced in cells infected with the enhancer-negative virus (lanes 4, 5 and 6), confirming that the 5′ LTR of the virus had indeed been essentially inactivated by the enhancer deletion.

Cells infected with the conventional virus and then selected by removal of growth factor (Fig. 7B, lane 3) exhibited not only the 7.6-kb RNA but also two smaller species (2.7 and 1.9 kb) corresponding in size to unspliced and spliced RNAs originating at the β-actin cap site. Significantly, these RNAs were present at only very low levels in the cells selected in G418 (lanes 1 and 2), suggesting that constitutive expression from the β-actin promoter was poor (see below). Cells infected with the enhancerless virus and selected for factor independence (lanes 4 and 6) also exhibited two transcripts of the β-actin–GM-CSF cassette, somewhat reduced in size because of the deletion within the U3 region of the LTR. The nature of the transcripts was confirmed by showing that a probe from the β-actin intron detected both the 7.6- and 2.7-kb transcripts but not the 1.9-kb RNA (Fig. 7C).

We further tested the efficacy of the NAc-based viruses by comparing the amount of GM-CSF secreted by FDC-P1 cells infected with these viruses and with the previously described ZipNeo(GM-CSF) virus (37). Polyclonal populations of NAc(GM-CSF)- and NE$^-$Ac(GM-CSF)-infected cells (selected for growth in the absence of exogenous CSF) produced only 5.6 and 2.7%, respectively, as much factor as did ZipNeo(GM-CSF)-infected cells [40 and 19 U of GM-CSF accumulated per 10^6 cells per day for NAc(GM-CSF) and NE$^-$Ac(GM-CSF), respectively, versus 704 U per 10^6 cells per day for ZipNeo(GM-CSF)]. Moreover, cells infected with the NAc-derived viruses contained at least 10-fold-lower levels of GM-CSF mRNA than those infected with ZipNeo(GM-CSF) (data not shown).

DISCUSSION

We have investigated the ability of several retroviral vectors to simultaneously express a selectable marker (Neo^r) and a second gene in hemopoietic cells. The two classes of vectors examined both utilize the viral LTR to express one gene, but whereas one class takes advantage of splicing to express the second gene, the other uses a separate internal promoter. In addition to the previously described

vectors pZipNeo (8), pDolNeo (35), and pM3Neo (36), we tested a new vector, pNAc, which we designed to express the gene of interest under the control of the human β-actin promoter-enhancer. In the main, analysis involved infection of hemopoietic cell lines and selection for Neo^r expression, followed by analysis of viral RNA in the resultant polyclonal cell populations.

A significant finding from this study was that the replacement of (most of) the U3 region of the Mo-MLV LTR in the DolNeo virus with that of the MPSV resulted in a marked increase in the level of viral genomic RNA in hemopoietic cells. Since the level of viral RNA in fibroblasts was not affected by the LTR modification, the U3 region of the MPSV LTR presumably contains a tissue-specific enhancer, as previously postulated by Ostertag and his colleagues to account for the myeloid tropism of this virus (60, 61). Surprisingly, however, insertion of either c-*myc* or v-*myb* or the GM-CSF gene into the MPDolNeo virus completely abrogated the enhancement. We do not yet know whether this stems from decreased transcription or RNA instability, although we suspect the former (see below).

The most serious problem common to all the viral vectors we examined that carried the Neo^r was their limited ability to express an additional gene. In the case of the M3Neo vector, the defect appeared to lie at the level of splicing, since no subgenomic RNA corresponding to the second gene could be detected. This may be because M3Neo contains only 85 bp upstream of the splice acceptor site, whereas optimal function of the homologous (*env*) splice site of Mo-MLV requires at least 165 bp of this region (27). Inefficient splicing also affected the ZipNeo- and NAc-based vectors, although it was generally not the major problem with either. In the case of ZipNeo, insertion of a second gene apparently interferes with the generation of the spliced subgenomic RNA which encodes the Neo^r product, an effect which substantially reduces the apparent titer of some recombinant viruses and could prevent exploitation of the selectable marker. Similar effects of inserted sequences on the generation of subgenomic viral RNAs have also been reported for other retroviral vectors (30, 53). With the NAc viruses, splicing was clearly inefficient, but the effects of this on vector function are unclear (see also below).

Although splicing inhibition reduces the versatility of splice vectors, problems have also been reported for vectors utilizing an internal promoter. Emermann and Temin (18, 19) found that selection for expression from one promoter of such vectors resulted in decreased expression from the other. Such "promoter suppression" did not seem to affect the DolNeo viruses, because the ratio of genomic to subgenomic viral RNA in infected FDC-P1 cells did not vary between selected (G418-resistant) and unselected populations (data not shown). In apparent contrast, expression from the β-actin promoter of NAc(GM-CSF) was much lower in cells selected for expression of Neo^r, which is translated from the LTR-driven transcript, than in cells selected for CSF independence. This observation cannot readily be ascribed to the presence of the LTR promoter, however, since inactivation of the LTR did not further improve expression from the internal promoter. The poor expression of the β-actin cassette within the NAc viruses thus presumably reflects either low activity of the human β-actin promoter in murine hemopoietic cells or instability of the hybrid β-actin–GM-CSF transcripts. This observation is somewhat surprising in view of the efficient transcription in fibroblasts from this cassette incorporated into another (nonviral) vector (24).

The levels of expression in hemopoietic cells of ZipNeo and DolNeo derivatives incorporating any of four genes tested (c-*myc*, c-*myb*, v-*myb*, and the gene encoding GM-CSF) were considerably lower than those directed by the parental vectors. This effect cannot be attributed to defective splicing, since the DolNeo-based vectors do not utilize splicing to express either the neomycin resistance gene or the second gene. It seems unlikely that selection against elevated levels of expression of the introduced c-*myc* and c-*myb* genes could account for the problem, because the cell lines in which the depression was most marked—FCD-P1 and WEHI-3B—are both characterized by relatively high endogenous levels of c-*myc* and c-*myb* expression. It also seems unlikely that sequences within the inserted genes decrease the stability of the viral transcripts. Although the GM-CSF cDNA insert carries a sequence within its 3′-untranslated region that has recently been shown to cause RNA instability (55), removal of this sequence did not significantly alter the level of GM-CSF viral RNA (our unpublished results). Moreover, although the cellular c-*myc* and c-*myb* mRNAs are also relatively unstable (13, 62) and carry sequences in their 3′-untranslated regions related to those in the GM-CSF mRNA, our c-*myc* and c-*myb* viruses retain very little of the 3′-noncoding region, and the v-*myb* virus retains none. Finally, expression by M3Neo-based vectors was unaffected by the presence of either c-*myb* or GM-CSF sequences (c-*myc* was not tested).

Thus, poor expression by ZipNeo- and DolNeo-based viruses containing two genes probably stems from the position in the viral genome at which the second gene is inserted, rather than from the nature of the inserts themselves. Any explanation for this effect must be able to account for its tissue specificity, since it was more pronounced in hemopoietic cells than in fibroblasts. We tend to favor the hypothesis that transcription, rather than RNA stability, is affected, since the levels of the SV40-Neo^r transcript of the DolNeo-based vectors seemed also to be depressed by the insertion of a second gene, whereas the structure (and presumably the stability) of this RNA should not be altered by the insertion. Although the cloning sites of the DolNeo- or ZipNeo-based vectors are not located within any well-defined transcriptional regulatory elements—and indeed lie between sequences not normally contiguous in a natural retrovirus—it has recently become apparent that sequences other than those in the LTR can influence viral transcription and RNA levels. For example, in avian retroviruses, regions within both *gag* (1, 7) and the 3′-untranslated region (43) have been implicated, whereas in murine retroviruses sequences in the 5′-untranslated regions can affect expression in a cell type-specific manner (2, 42, 64). Thus, if the cloning sites of the DolNeo and ZipNeo vectors are sufficiently near to or within such regulatory sequences, insertion into these sites could conceivably down-regulate transcription (or decrease RNA stability) in particular cell types. Since the levels of transcripts directed by the SV40 promoter are also decreased, its activity is presumably influenced by the presence nearby of either the LTR or the putative additional regulatory region or both.

The observations reported here on the expression of recombinant retroviruses in vitro show striking similarities to those we reported previously (6) from in vivo experiments. In the latter study, it was found that although expression of MPDolNeo (and, to a lesser extent, DolNeo) virus was readily detectable by Northern blot analysis in spleen colonies from mice repopulated with infected hemopoietic cells, no expression could be detected in spleen

colonies arising after infection with MPDolNeo(c-*myc*) virus. It was noteworthy that neither the genomic RNA nor the SV40-Neo^r mRNA was detectable, suggesting that both promoters were inactive. Although the in vitro and in vivo phenomena may be related, the magnitude of the effect in vivo was considerably greater and may involve additional factors. This latter notion is supported by our finding that, unlike the parental M3Neo virus, the M3Neo(c-*myb*) virus was not expressed in vivo (unpublished observations), even though expression in vitro of these two viruses was comparable.

Several other recombinant retroviruses carrying two genes (often under the control of viral promoters) have also been shown to be functionally silent in vivo (47, 59, 66). It has been suggested (47, 66) that retroviruses infecting hemopoietic stem cells may be repressed in a manner similar to those infecting embryonic "stem" cells (22, 28, 41) and therefore cannot be expressed in the progeny of those cells. This hypothesis certainly does not hold for viral vectors carrying only the neomycin resistance gene (6, 15, 17, 32). Why, then, should the more complex viruses be silenced? Perhaps the repression mechanism can only operate on a provirus that already shows little or no transcriptional activity, due to a phenomenon akin to that described here.

Finally, it is worth noting that, despite the problems revealed in our studies, it may be possible to improve the design of vectors which utilize LTRs to express sequences such as *myb* and *myc* in hemopoietic cells. The observations reported here suggest several approaches. Since, for example, the amount of M3Neo genomic RNA is not affected by the presence of additional sequences, it may be feasible to introduce sequences into M3Neo which allow efficient splicing (27) and thus to generate adequate levels of the subgenomic mRNA. Alternatively, given the proven efficacy of the simple (i.e., single-gene) vectors (3, 6, 15, 40), especially those containing the MPSV LTR (6; this report), it may be wise to forgo the option of drug selection and simply replace the neomycin resistance gene by the gene of interest. Current work in our laboratories is pursuing these directions.

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