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Stably Transmitted Triple-Promoter Retroviral Vectors and Their Use in Transformation of Primary Mammalian Cells

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Retroviral vectors were constructed which coexpressed three inserted genes from independent transcriptional promoters in singly infected cells. Several such triple-promoter vectors were constructed with various combinations of oncogenes and selectable drug resistance genes. All expressed three mRNAs of the expected size in infected cells. One vector expressing the v-Ha-*ras*, v-*myc*, and *neo* genes was characterized in detail. This retrovirus did not undergo rearrangement during the process of infection, as judged by Southern analysis, and infection of primary rat embryo fibroblasts demonstrated that *ras-myc*-cotransformed cells could be selected in G418. This demonstration that retroviral vectors can be used to express three cistrons independently increases their value as gene transfer vehicles, particularly for studies involving oncogene cooperation in primary cells.

Retroviral vectors provide an efficient means of gene transfer for cells which are relatively refractory to transfection by other methods, or which are represented at a low frequency in mixed primary populations (3, 4, 13, 32, 40). Many genes have been expressed in a variety of retroviral constructs, mostly by utilizing the transcriptional enhancer and promoter elements in the retroviral long terminal repeat (LTR) (3, 4, 16, 22). In addition, a number of vectors have been constructed in which inserted genes are expressed from heterologous internal promoters. The promoters that have been used to date include those from the simian virus 40 (SV40) early region and the herpes simplex virus thymidine kinase (HSV *tk*), mouse metallothionein, and rat growth hormone genes (10, 20, 23, 24, 35). Internal promoters represent one way in which the gene of interest can be expressed together with a selectable marker in infected cells. This strategy is useful in establishing that infection has occurred, quantifying infection frequencies, and purifying infected cells in selective medium (23, 24, 26, 36). Internal promoters are also important for expression in cells in which the retroviral LTR is not an efficient promoter (29, 38). However, experiments in the spleen necrosis virus (SNV) system suggested that retroviruses containing two internal transcriptional promoters were unstable and therefore not effective expression vectors (9).

Our goal is to determine which oncogenes are capable of transforming subsets of primary lymphoid and hematopoietic cells. Since the expression of a single oncogene is generally insufficient for the full transformation of primary mammalian cells (14, 15, 17, 25, 30, 31), we constructed a number of retroviral vectors expressing two oncogenes. However, to make the biology of cotransformation more amenable to analysis, we wanted to include a drug resistance marker in these vectors so that infected cells expressing two oncogenes could be purified in selective medium. While a coinfection strategy (with separate retroviruses) can be used (31), this is not practical with mixed primary populations if the observed infection frequency of the target cell is low.

Here we report the construction and characterization of retroviral vectors designed to express three inserted genes (two oncogenes and a selectable marker) from independently transcribed mRNAs, one transcribed from the vector LTR and two from internal transcriptional promoters.

Vector construction. Initially, two triple-promoter retroviral vectors were constructed based on the dual-promoter retroviral vector pΔRM, which encodes the v-Ha-*ras* and v-*myc*^{MC29} oncogenes (Fig. 1). pΔRM was constructed from pΔSP (Fig. 1) by (i) inserting a *Sac*I-*Pst*I fragment containing the v-Ha-*ras* gene from pHB11 (8) at the *Bam*HI site (with *Bam*HI linkers) and (ii) modifying the *Hin*dIII site of pΔSP with *Bgl*II linkers and inserting a *Bgl*II-linkered *Bst*EII-*Sph*I fragment of the v-*myc* gene encoding p110$^{gag\text{-}myc}$ (37) at this position. The *neo* gene, under the control of the HSV *tk* or adenovirus type 2 major late promoter (mlp), was inserted at the *Bsm*I site of pΔRM to form the triple-promoter vectors pΔRMtk*neo* and pΔRMmlp*neo*, respectively (Fig. 1). These plasmids were transfected into ψ2 cells (19), and virus-producing clones were isolated after selection in G418. The ΔRMtk*neo* and ΔRMmlp*neo* retroviruses conferred both focus formation and G418 resistance on BALB/3T3 cells with similar titers, while the parental ΔRM retrovirus conferred only focus formation (Table 1).

Analysis of vector mRNA expression. Northern (RNA) blot analysis of total cell RNA from transfected ψ2 and infected BALB/3T3 cells showed that the three expected vector-specific transcripts were expressed from the ΔRMtk*neo* and ΔRMmlp*neo* vectors (Fig. 2, lanes 7 to 10). While the *neo* probe hybridized to all three transcripts, a probe specific for the v-*myc* gene hybridized to the larger two transcripts only, as expected from the structure of these vectors (Fig. 1; data not shown). The relative positions of these mRNAs on the blot were consistent with their predicted sizes (Fig. 1 and 2). Encouragingly, the sizes of the transcripts in the ψ2 and 3T3 lanes were indistinguishable (Fig. 2, lanes 7 to 10; data not shown), suggesting that these three promoter vectors had been transmitted by retroviral infection without undergoing gross rearrangement. To assay for vector rearrangements at the clonal level, BALB/3T3 cells were infected with the ΔRMtk*neo* virus (multiplicity of infection, 10^{-4}) and cloned in the presence of G418. Northern analysis showed that all six clones isolated expressed vector-specific transcripts of the same size as those previously observed in bulk populations of ΔRMtk*neo*-infected 3T3 cells (Fig. 2, lanes 1 to 7).

The possibility existed that the two smaller vector-specific mRNAs detected by the above Northern analysis did not

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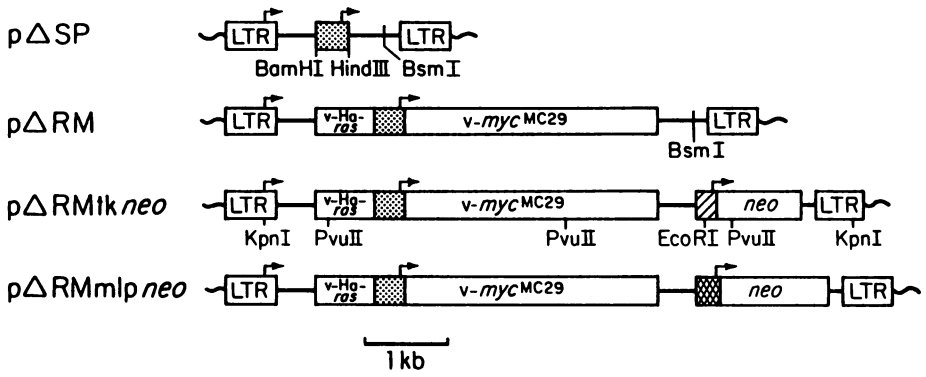


FIG. 1. Structure of retroviral vectors pΔSP, pΔRM, pΔRMtk*neo*, and pΔRMmlp*neo*. Stippled boxes represent the SV40 early region promoter, the hatched box shows the HSV *tk* promoter and the cross-hatched box shows the adenovirus type 2 major late promoter (mlp). Arrows represent transcription initiation (CAP) sites for each of the three promoters. Straight lines represent retroviral sequences derived from Moloney murine sarcoma virus-murine leukemia virus (see reference 23); wavy lines represent flanking plasmid sequences. pΔSP was derived from pLSDL (23) by (i) removing the *dhfr* sequences between the *Hin*dIII and *Nco*I sites and inserting a *Hin*dIII linker at this position and (ii) using site-directed mutagenesis to delete the Moloney murine sarcoma virus-derived splice donor (AGGT→AGGC) at nucleotide 862 of pLSDL (23). To construct pΔRMtk*neo*, pΔRM was digested with *Bsm*I and blunt ended with T4 DNA polymerase, and a fragment containing the *neo* gene under the control of the HSV *tk* promoter was inserted at this position. This *tk-neo* cassette consisted of a *Pvu*II-*Bgl*II fragment spanning the HSV *tk* promoter (21) ligated to a *Bgl*II-*Sma*I fragment of the *neo* gene (2, 33). pΔRMmlp*neo* was constructed from pΔRM by a similar strategy but with a blunt-ended fragment containing the *neo* gene under the control of the adenovirus type 2 major late promoter. The mlp-*neo* cassette consisted of a *Xho*I-*Pvu*II fragment of the adenovirus type 2 major late promoter (nucleotides 5,778 to 6,072 of the published sequence [11]) ligated to a *Hin*dIII-*Sma*I fragment of the *neo* gene.

represent transcription from the internal promoters, but rather transcripts with a spliced-on U5 segment which had originated in the 5′ vector LTR. To address this issue, we hybridized Northern blots of poly(A)$^+$ RNA from control and ΔRMtk*neo*-infected 3T3 cells with an antisense oligonucleotide probe specific for U5 sequences. This probe hybridized with the larger vector-specific transcript only (Fig. 3A). The smaller vector transcripts were readily detected with oligonucleotide probes specific for the predicted 5′ ends of these mRNAs in the SV40 (Fig. 3B) and HSV *tk* (Fig. 3C) sequences. These oligonucleotide probes were of identical length and similar T_m. Thus, the smaller transcripts detected in infected cells did not contain sequences homologous to the U5 probe and therefore in all probability represented authentic transcription from the internal vector promoters. Interestingly, the use of poly(A)$^+$ mRNA allowed the relative abundance of the SV40-driven message to be seen more clearly with oligonucleotide probes (Fig. 3B and 3C, lanes 2) or with an antisense v-*myc* probe (data not shown). Thus, the abundance of this transcript was not accurately represented in Northern blots of total RNA (Fig. 2, lane 1) owing to its proximity to the 28S rRNA.

TABLE 1. Titers of retroviruses produced by ψ2 cells

Virus[a]	G418^r CFU/ml[b]	Hygromycin^r CFU/ml[b]	Focus-forming units/ml[b]
ΔRM	0	ND[c]	1.3×10^5
ΔRMtk*neo*	4.6×10^4	ND	4.0×10^4
ΔRMmlp*neo*	3.2×10^4	ND	3.4×10^4
Δ*Hrafmyc*	ND	1.7×10^5	3.1×10^5
Δ*Hsrcmyc*	ND	3.3×10^4	2.3×10^4

[a] Each virus was harvested from the supernatant of a clone of transfected ψ2 cells (19). ψ2 cells were transfected (5) and clones were isolated as previously described (26). A single ψ2 clone was used for each construct in all experiments.

[b] Titers of virus stocks were determined on BALB/3T3 cells (1) which were then selected for growth in G418 (500 μg/ml) or hygromycin B (300 μg/ml) or for focus formation as previously described (26).

[c] ND, Not done.

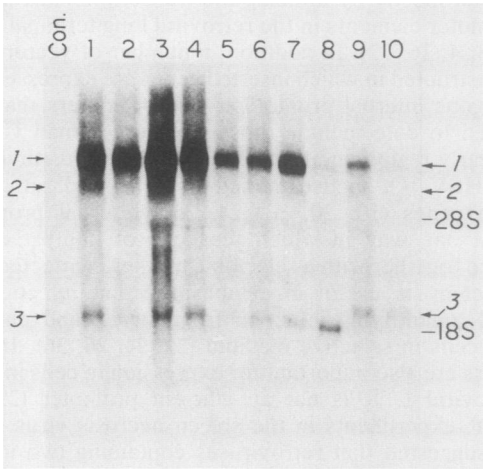


FIG. 2. Northern analysis of total RNA from infected and transfected cells. Lanes: 1 to 6, clones 1 to 6, respectively, of ΔRMtk*neo*-infected, G418-resistant BALB/3T3 cells; 7, ΔRMtk*neo*-infected BALB/3T3 cells selected in bulk for G418 resistance; 8, the clone of ψ2 cells producing the ΔRMtk*neo* virus; 9, ΔRMmlp*neo*-infected BALB/3T3 cells selected in bulk for G418 resistance; and 10, the clone of ψ2 cells producing the ΔRMmlp*neo* virus. The predicted transcript sizes to the nearest 50 nucleotides, not including poly(A) tails, for ΔRMtk*neo* (labeled on left side of figure) are: 1, 7000 nucleotides, transcribed from the vector 5′ LTR; 2, 5,400 nucleotides, transcribed from the SV40 promoter; and 3, 1,700 nucleotides, transcribed from the HSV *tk* promoter (Fig. 1). For ΔRMmlp*neo* the predicted transcript sizes (labeled on right side of figure) are: 1, 7,350 nucleotides; 2, 5,750 nucleotides; and 3, 2,000 nucleotides (Fig. 1; transcript 3 is transcribed from the adenovirus major late promoter in this vector). Electrophoresis was on a 1% agarose-formaldehyde gel (18) with 5 μg of total RNA in each lane. Blotting and hybridization conditions were as previously described (26, 39). The blot was hybridized with a ^{32}P-labeled antisense RNA transcript of the *neo* gene generated from an SP6 transcription system (Promega Biotec, Madison, Wis.). Positions of the 28S and 18S rRNAs are indicated. Exposure was for 4.5 h at −70°C with intensifying screens. Con., Control.

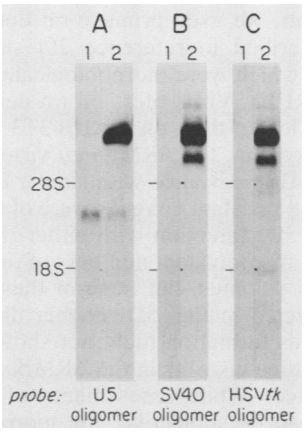


FIG. 3. Northern blots of poly(A)$^+$ RNA from ΔRMtk*neo*-infected cells probed with the U5 and control oligomers. Lanes 1 and 2 in each panel correspond to poly(A)$^+$ RNA from BALB/3T3 cells and clone 1 of ΔRMtk*neo*-infected, G418-resistant BALB/3T3 cells (Fig. 2), respectively. The antisense oligomer probes were, for U5, 3′-AGGCTTAGCACCAGAGCGACAAGGA-5′; for SV40, 3′-GA TAAGGTCTTCATCACTCCTCCGA-5′; and for HSV *tk*, 3′-TCGCTGGGACGTCGCTGGGCGAATT-5′. The 3′ ends of the oligomers are 68, ~8, and 7 nucleotides from the predicted CAP sites, respectively. A 5-μg sample of poly(A)$^+$ RNA per lane was electrophoresed in an agarose-formaldehyde gel and blotted as detailed in the legend to Fig. 2. Oligomer probes were kinased with [γ-^{32}P]ATP (New England Nuclear Corp., Boston, Mass.) and T4 polynucleotide kinase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Hybridization was at 55°C in a 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) buffer (18). Blots were washed in 6× SSC at 55°C.

Southern analysis. Genomic DNA was isolated from the infected 3T3 clones and subjected to Southern analysis. Each clone contained a single proviral insertion, since a probe specific for the *neo* gene hybridized to a single *Eco*RI restriction fragment in each of the DNA samples (Fig. 4A). The different migration rates of these restriction fragments confirmed that the clones were independently infected isolates, since their size was determined by the single *Eco*RI site in the ΔRMtk*neo* vector (Fig. 1) and by the proximity of the *Eco*RI site in the genomic sequences 3′ to the integrated provirus.

Two Southern hybridization strategies were used to assay for deletions or rearrangements of the ΔRMtk*neo* provirus in the infected 3T3 clones. To release the intact provirus, the DNA samples were digested with *Kpn*I (which cuts once in each LTR) and Southern blots were hybridized with a *neo*-specific probe (Fig. 4B), and to assay for small deletions in the vicinity of the internal transcriptional promoters (Fig. 1), the DNAs were digested with *Pvu*II and the blots were hybridized with a v-*myc*-specific probe (Fig. 4C). In both cases, the restriction fragments hybridizing with the probes were of the predicted sizes, were the same for each of the clones, and comigrated with digests of pΔRMtk*neo* DNA electrophoresed on the same gels (Fig. 1 and 4B and C; data not shown). Therefore, the triple-promoter ΔRMtk*neo* retrovirus was transmitted without undergoing detectable rearrangements or deletions in six of six clones of infected 3T3 cells which had been selected for G418 resistance.

Transformation of primary cells. As an assay for the expression of all three cistrons in the ΔRMtk*neo* vector, primary rat embryo fibroblasts (REFs) were infected with this and control retroviral constructs and selected for drug resistance. Infections with retroviruses expressing a drug resistance marker and either v-Ha-*ras* or v-*myc*^{MC29} alone (LSNL*ras* and ΔH*myc*, respectively) gave rise to drug-resistant colonies of REFs with morphologies which were distinct from those of control colonies (Fig. 5A to C). Only the ΔRMtk*neo* virus induced the formation of drug-resistant REF colonies with a grossly transformed morphology (Fig. 5D) typical of that induced by ΔRM in focus assays (Fig. 5E) and that seen after cotransfection of REFs with activated *ras* and v-*myc* oncogenes (data not shown). To obtain a more

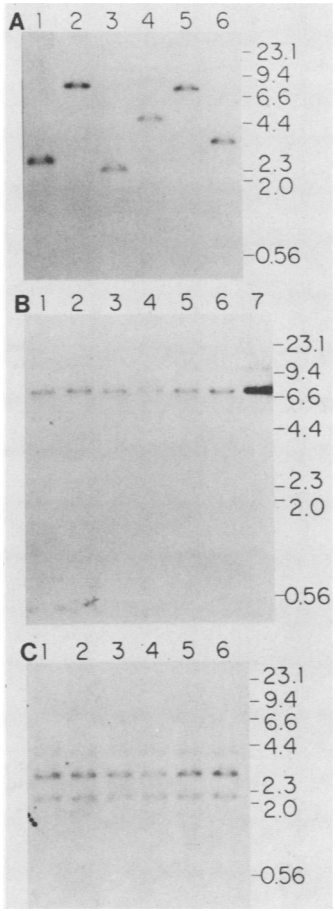
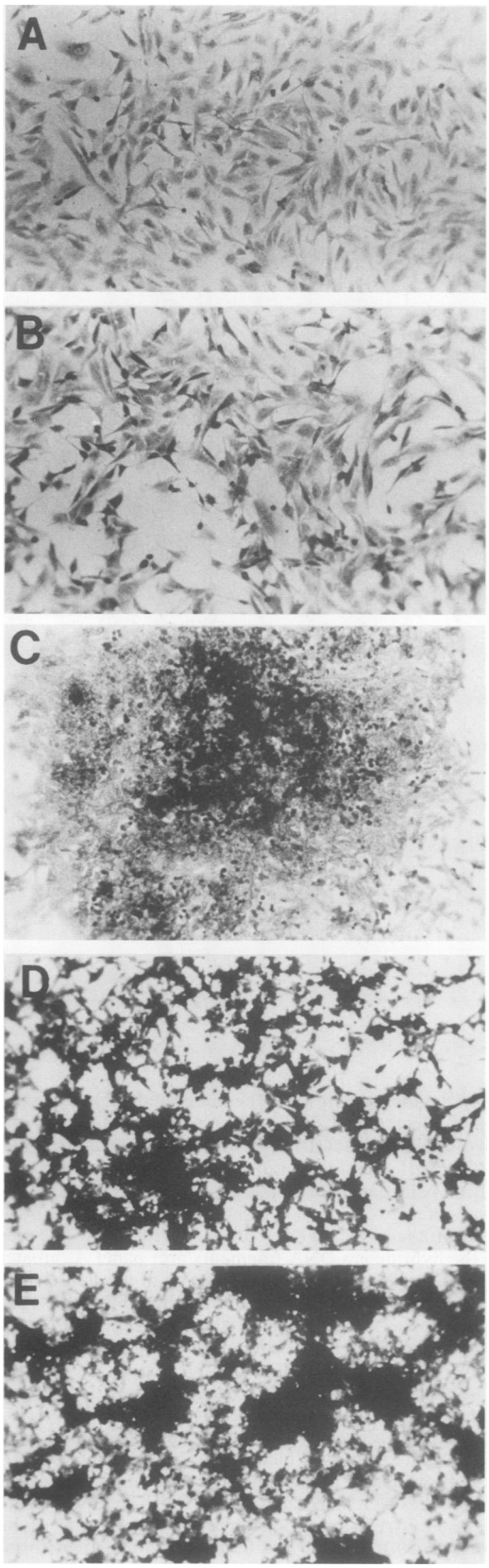


FIG. 4. Southern analysis of the integrated ΔRMtk*neo* provirus in clones of infected BALB/3T3 cells. (A) *Eco*RI digests hybridized with a *neo*-specific probe; (B) *Kpn*I digests hybridized with the same probe; (C) *Pvu*II digests hybridized with a v-*myc*-specific probe. Lanes 1 to 6 in each panel correspond to DNA samples from ΔRMtk*neo*-infected 3T3 clones 1 to 6, respectively. Lane 7 in panel B is the ΔRMtk*neo* plasmid DNA digested with *Kpn*I. The predicted sizes of the hybridizing DNA fragments are: panel A, greater than 1,800 bp, the size dependent on the location of the *Eco*RI site in the flanking chromosomal DNA 3′ to the integrated provirus; panel B, 7,000 bp; and panel C, 2,900 and 2,100 bp (see Fig. 1 for the location of relevant restriction enzyme sites in the ΔRMtk*neo* vector). The weak signal in panel C migrating at ~4,000 bp is also seen in *Pvu*II digests of uninfected 3T3 DNA (data not shown) and probably represents hybridization to murine c-*myc* sequences. Figures at the right of each panel represent molecular weight markers in kilobases. Digested DNA (10 μg) was electrophoresed in each lane of 0.6% agarose gels which were then blotted onto nitrocellulose. Nick-translated probes were generated from a *Bgl*II-*Sma*I fragment of the *neo* gene and a *Bst*EII-*Sph*I fragment of the v-*myc* gene. Hybridizations were for 20 h at 42°C in a buffer containing 50% formamide (39). Blots were washed in 1× SSC at 68°C and exposed at −70°C with intensifying screens.



quantitative result, we used primary rat dermal fibroblasts (isolated as described in reference 25) since these cells formed colonies which were morphologically less heterogeneous than the REFs. Virus titers on rat dermal fibroblasts were ~100-fold lower than on BALB/3T3 cells (data not shown). In this system, the ΔRMtk*neo* virus induced focus formation and G418 resistance with similar frequencies and >85% of the G418-resistant colonies were of the morphology depicted in Fig. 5D. Infection with either the LSNL*ras* or ΔH*myc* virus led to equivalent numbers of drug-resistant rat dermal fibroblast colonies, but none of these exhibited the morphology depicted in Fig. 5D; neither did these viruses induce overt focus formation (data not shown). These data showed that all three cistrons in the ΔRMtk*neo* vector were functionally active. While assessments of morphological transformation can be subjective, the morphological alterations observed provided a clear-cut assay for *ras-myc* cooperation in this particular system. We did not examine the phenotype of the infected primary cells with respect to their life span or anchorage dependence (14, 15, 25).

Other triple-promoter vectors. Two other triple-promoter vectors, ΔH*rafmyc* and ΔH*srcmyc*, were constructed with the same promoter organization as the ΔRMtk*neo* vector described above. However, these vectors expressed the *hph* gene, which confers resistance to hygromycin B (12, 34), instead of the *neo* gene since we have found the former to be a more effective selectable marker for lymphoid cells. Also, the v-*myc* gene was expressed from the HSV *tk* promoter in the ΔH*rafmyc* and ΔH*srcmyc* vectors (Fig. 6). Clones of hygromycin-resistant ψ2 cells isolated after transfection with these vectors yielded viruses which conferred hygromycin resistance and focus formation on BALB/3T3 cells with similar titers (Table 1). Northern analysis of total cell RNA from infected 3T3 cells selected in bulk for hygromycin resistance showed that three vector-specific transcripts hybridized to the v-*myc* probe (Fig. 7). The migration rates of these three transcripts on the gel were consistent with their predicted sizes (Fig. 6 and 7). In addition, Northern blots of poly(A)$^+$ RNA from infected cells were hybridized with the oligomer probes specific for the predicted 5′ end of each vector mRNA as described above. The results obtained were similar to those shown in Fig. 3 for the ΔRMtk*neo* vector: the U5 probe did not hybridize to the two smaller vector transcripts, which were readily detectable with the SV40 or HSV *tk* oligomer probes (data not shown).

Determinants of vector stability. These data show that triple-promoter retroviral vectors can be stably transmitted by retroviral infection. No rearrangements of the integrated ΔRMtk*neo* proviruses were detectable by Southern analysis (Fig. 5A to C), and all four of the triple-promoter vectors described here expressed the three expected transcripts in

FIG. 5. Morphology of primary REFs infected with ΔH (A), LSNL*ras* (B), ΔH*myc* (C), ΔRMtk*neo* (D), or ΔRM (E). REFs (2.5×10^4) were infected with $\sim 5 \times 10^3$ CFU of each virus in 1 ml for 20 h in the presence of 4 μg of Polybrene per ml. After a further 24 h, the cells were selected for hygromycin resistance (A and C, G418 resistance (B and D), or focus formation (E). After a further 10 days of growth, the cells were fixed with methanol and stained with methylene blue. Magnification is ×46. ΔH*myc* is the ΔH retroviral vector (see Fig. 6) with the v-*myc*^{MC29} fragment inserted at the *Bam*HI site. LSNL*ras* is the LSNL retroviral vector (26), which is similar to ΔH but contains the *neo* gene in place of the *hygro* gene, with the v-Ha-*ras* gene inserted at the *Bam*HI site. The *ras* and *myc* fragments in LSNL*ras* and ΔH*myc*, respectively, were the same as those used in the ΔRMtk*neo* and ΔRMmlp*neo* vectors.

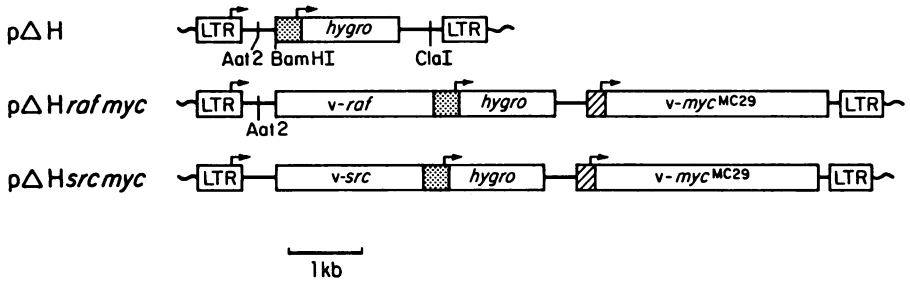


FIG. 6. Structure of the retroviral vectors pΔH, pΔH*rafmyc*, and pΔH*srcmyc*. Vector segments are labeled as described in the legend to Fig. 1. To construct pΔH, pΔSP (Fig. 1) was digested with *Hin*dIII and the *hph* gene from pLG90 (12) (a gift from L. Gritz) was inserted at this position with *Hin*dIII linkers. The *hph* gene confers resistance to hygromycin B on mammalian cells (34) and is labeled *hygro* in the figure. ΔH*rafmyc* was constructed from ΔH by (i) removing the *Aat*II-*Bam*HI fragment from ΔH and replacing it with an *Aat*II-*Bam*HI fragment containing the v-*raf* oncogene from the plasmid pF4 (28), and (ii) inserting a fragment containing the v-*myc* gene under the control of the HSV *tk* promoter into the blunted *Cla*I site of ΔH. The *tk-myc* cassette consisted of the *Pvu*II-*Bgl*II fragment of the HSV*tk* promoter ligated to a *Bgl*II v-*myc* fragment from pΔRM. ΔH*srcmyc* was constructed from ΔH by inserting a *Bam*HI-linkered *Nco*I-*Eco*RI fragment encoding v-*src* from pSRA-2 (7) at the *Bam*HI site of ΔH. The *tk-myc* cassette was inserted as in ΔH*rafmyc*.

infected cells (Fig. 2, 3, and 7). Additionally, the ΔRMtk*neo* virus was able to express the three gene products in a functional manner, as judged by the production of *ras-myc*-transformed, G418-resistant colonies of primary REFs. Thus, the insertion of multiple, independently expressed cistrons in retroviral vectors is not incompatible with the retroviral life cycle, and this strategy can be used effectively to coexpress inserted sequences in singly infected cells. While numerous factors may influence the utility of other such retroviral constructs, the deletion of the Moloney murine sarcoma virus-derived splice donor signal was probably an important factor contributing to the stability of our vectors.

In an SNV-based retroviral vector containing the α-globin and HSV *tk* promoters, infected cells which were selected for expression of the HSV *tk* cistron consistently contained proviruses with deletions of the α-globin promoter (9). It was proposed that these deletions occurred because the active α-globin promoter prevented efficient transcription of the HSV *tk* cistron in this vector, perhaps by epigenetic suppression of gene expression or transcriptional overlap interference (6, 9, 10). In our experiments, all infected cells analyzed had been selected for expression of the vector drug resistance marker, yet no rearrangements of the triple-promoter vectors were detected. The difference between these results and those obtained in the SNV system (9) remains to be established, but could be a result of the different promoter combinations used. In this respect, it is relevant that transcriptional overlap interference has been demonstrated with the human α1-globin promoter in a nonretroviral system (27).

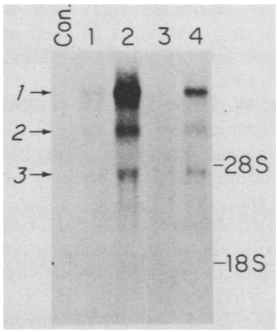


FIG. 7. Northern analysis of ΔH*rafmyc* and ΔH*srcmyc* expression in infected and transfected cells. Lanes: 1, ψ2 clone producing the ΔH*rafmyc* retrovirus; 2, BALB/3T3 cells infected with ΔH*rafmyc* and selected in bulk for hygromycin resistance; 3, ψ2 clone producing the ΔH*srcmyc* retrovirus; and 4, BALB/3T3 cells infected with ΔH*srcmyc* and selected in bulk for hygromycin resistance. Total cell RNA was subjected to Northern analysis as detailed in the legend to Fig. 2. The predicted transcript sizes (Fig. 6) are similar for both constructs and are as follows: 1, ~8,500 nucleotides; 2, 5,700 nucleotides; and 3, 3,700 nucleotides, not including poly(A) tails. The blot was hybridized with a ^{32}P-labeled antisense RNA transcript of the v-*myc* gene generated by SP6 RNA polymerase transcription. Con., Control.

In the present study, the relative abundance of the three ΔRMtk*neo* transcripts in infected 3T3 cells, as estimated by integrative scanning densitometry of the Northern blot depicted in Fig. 3C, was 42:6:1 (LTR transcript:SV40 transcript:HSV*tk* transcript). This probably reflected, at least in part, the relative transcriptional strengths of the promoters used in murine fibroblasts; however, we were unable to rule out the possibility that some transcriptional interference was occurring. Ultimately, an important determinant of the observed stability of these types of vectors may be whether or not the particular context of the promoter controlling transcription of the selectable marker allows an expression level adequate to confer the selectable phenotype on infected cells (10).

The triple-promoter vectors described here should be useful tools in determining the conditions necessary for the transformation of primary lymphoid and hematopoietic cell populations.

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