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Quantitative Analysis of Gene Suppression in Integrated Retrovirus Vectors

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Previously, we described a retrovirus vector that contained two genes: a 5′ gene under transcriptional control of the viral long terminal repeat and a 3′ gene under transcriptional control of the herpes simplex virus thymidine kinase promoter. By using a biological assay, we found that expression of the 5′ gene is suppressed when there was selection for the 3′ gene and expression of the 3′ gene is suppressed when there is selection for the 5′ gene (M. Emerman and H. M. Temin, Cell 39:459–467, 1984). In the present study, we replaced the thymidine kinase promoter with stronger promoters, and we measured expression of the genes in the retrovirus vectors by enzyme activity and RNA analysis. We found that all of the vectors displayed gene suppression when analyzed biochemically, although not when analyzed biologically. The suppressed genes produced about 10 to 50% as much product as when they were selected. The amount of suppression depended on whether the suppressed gene was 5′ or 3′ to the selected gene and from which promoter the suppressed gene was transcribed. The amount of suppression correlated with a decrease in the amount of steady-state RNA transcribed from the suppressed gene's promoter.

Retroviruses efficiently integrate a DNA copy of their genome into the chromosomes of a wide variety of vertebrate cells. The integrated form of the retroviral genome, the provirus, is maintained in the chromosome as a stable genetic element. Because a provirus is subject to cellular processes that affect the expression of chromosomal genes, retrovirus vectors can be used in the analysis of these cellular processes. We are interested in using retroviruses to determine the effects of adjacent cistrons on each other.

Previously, we constructed infectious retrovirus vectors based on spleen necrosis virus with a transcriptional promoter internal to the long terminal repeats (LTRs) of the provirus (8). We showed that a gene transcribed from the LTR is suppressed when infected cells are selected for expression of a gene transcribed from an internal promoter. Likewise, a gene transcribed from an internal promoter is suppressed when infected cells are selected for expression from the LTR. This suppression is *cis*-acting, epigenetic, and reversible (8).

In our previous study (8), suppression was measured by the ability of cells to survive in selective medium when the suppressed gene was either the herpes simplex virus thymidine kinase (*tk*) gene or the bacterial transposon Tn*5* neomycin resistance (*neo*) gene. We used the herpes simplex virus *tk* gene promoter as the internal promoter in the vector. In the present study, viruses that contain stronger internal promoters (the mouse metallothionein I (MTI) and simian virus 40 (SV40) early promoters) were used, and we measured suppression biochemically. We found in pools of cell clones that the product of the suppressed gene was about 10 to 50% as abundant as when only that gene was selected. The amount of suppression depended on whether the suppressed gene was 5′ or 3′ to the selected gene and on which promoter was directly 5′ to that gene. We found that although suppression in cells infected with these viruses was not observed with biological assays, suppression still occurred as measured biochemically by the lower amounts of the gene products in cell extracts. Furthermore, we found that suppression acted by altering the steady-state level of RNA transcribed from each promoter.

MATERIALS AND METHODS

Nomenclature. Plasmids have the letter "p" before their designations (e.g., pME111), whereas infectious retroviruses made from those plasmids do not (e.g., ME111).

Cells. Chicken embryo fibroblasts and Buffalo rat liver TK^- cells (BRL TK^- cells) were grown as previously described (7). Selection for TK^+ cells was done in 10^{-4} M hypoxanthine–5×10^{-5} M thymidine–5×10^{-7} M methotrexate (HAT medium). Selection for NEO-resistant cells was done in 400 μg of G418 (GIBCO Laboratories, Grand Island, N.Y.) per ml. Relative plating efficiencies in HAT medium of cells that were infected with a virus that contained both *tk* and *neo* genes were determined as previously described (8). On average, TK^+ colonies infected with different viruses grew at the same rate in HAT medium (data not shown).

Virus. Virus was recovered from plasmids by cotransfection of chicken embryo fibroblasts with reticuloendotheliosis virus strain A DNA as helper as previously described (8). Virus titers were determined and standardized for variations in the transfection process and in virus production as previously described (7, 8, 28). The structures of the genomes of all virus stocks were verified by analyzing the linear unintegrated DNA made after infection of chicken embryo fibroblasts as previously described (7, 11).

Plasmids. Recombinant DNA techniques were carried out as described by Maniatis et al. (15) except as noted. Plasmids pME111 and pME123 were previously described (8). They contain the *neo* gene from the Tn*5* transposon and the *tk* promoter and the *tk* gene from the herpes simplex type I virus (see Fig. 1). In these and all other constructions with the *tk* gene, the 3′ RNA processing sequences have been removed (28).

Plasmid pME131 contains the mouse MTI promoter from

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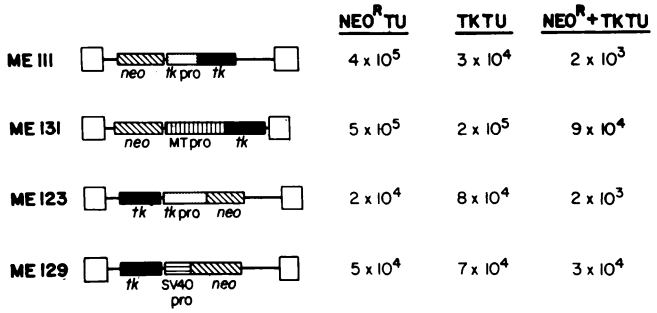

FIG. 1. Structure and transforming titers of retrovirus vectors with an internal promoter and two selectable genes. □, spleen necrosis virus LTRs; ——, spleen necrosis virus DNA; pro, internal promoter; MT, mouse MTI promoter; SV40, SV40 early promoter. NEO resistance transforming units (NEO^RTU), number of colonies per ml of virus transformed from a NEO-sensitive phenotype to be NEO-resistant phenotype; TK TU, number of cells per ml of virus transformed from a TK^- to a TK^+ phenotype; NEO^R + TK TU, number of cells per ml of virus transformed to both phenotypes simultaneously. Virus stocks were made by cotransfection of chicken cells with the indicated plasmids and reticuloendotheliosis virus strain A DNA, titers were determined on BRL TK^- cells, and then the virus titers were standardized for virus production relative to ME123 as described previously (8) and in the Materials and Methods.

the *Hin*dIII site to the *Bgl*II site (3; kindly provided by R. Palmiter). The *Bgl*II to *Bam*HI fragment of pTKterR (28; the *tk* gene without its 3′ RNA processing site) was substituted for the *tk* gene in the MTI promoter-*tk* gene plasmid pMT (3). The resultant plasmid, called pJD-MT, was constructed by J. Dougherty. The MTI promoter-*tk* gene fragment was then isolated from pJD-MT, the ends of the fragment were filled in with Klenow enzyme, and the fragment was ligated into a blunt-ended *Cla*I site at the 3′ end of a construction that contains the *neo* gene in a deleted spleen necrosis virus genome. Plasmid pME129 was constructed by deleting the *tk* promoter-*neo* fragment from pME123 and by replacing it with a fragment from pSV2*neo* (29) from the *Nde*I site in pBR322 to the *Sma*I site at the end of the *neo* gene. The SV40 early promoter included in this plasmid is from the *Hin*dIII site at position 5171 to position 270 on the SV40 map.

In vitro TK assay. The in vitro TK assays were done essentially as previously described (12, 36). Pools of clones were transferred to 35-mm-diameter dishes and were grown for 2 to 4 days. Cells were washed twice with phosphate-buffered saline, suspended in 300 μl of 50 mM Tris hydrochloride (pH 7.4)–5 mM β-mercaptoethanol–5 μM dThd, and disrupted in a sonifier (Branson Sonic Power Co., Danbury, Conn.) at microtip level 6 for 8 s. The extracts were spun for 10 min in a Microfuge (Beckman Instruments, Inc., Fullerton, Calif.), and the cell debris was discarded. Extracts were stored at −70°C for up to 1 week. The relative amount of protein in each sample was determined with the Bradford assay (2). Dilutions of a concentrated sample of uninfected BRL TK^- cell extract and bovine serum albumin served as standards. The amount of protein per TK^+ cell or per NEO-resistant cell was approximately the same (data not shown).

Equal amounts of protein from each sample (typically about 50 μg) were incubated at 37°C with (final concentrations) 50 mM Tris hydrochloride (pH 7.4)–10 mM $MgCl_2$–10 mM ATP–4.4 μM dThd–0.2 mM TTP–5 μCi [^{3}H]dThd (17 or 12 Ci/mmol; Amersham Corp., Arlington Heights, Ill.). Portions were removed at 1, 2, and 4 h to determine the conversion of dThd to TMP by spotting one-third of the sample on a disk of DE81 paper (Whatman, Inc., Clifton, N.J.). Disks were dried, washed in two changes of 4 mM ammonium formate–5 μM dThd and one change of ethanol, and counted in a scintillation counter. The amount of radioactivity bound to DE81 paper when no protein was added to the reaction was subtracted from each data point. The amount of TK activity in each extract was determined by calculating the slope of the line of disintegrations per minute bound to DE81 paper versus hours of incubation. Experiments were done in duplicate.

S1 nuclease analysis. S1 nuclease analysis was done essentially as previously described (9). Pools of transformed resistant colonies were grown until several 100-mm-diameter plates were obtained, and total RNA was extracted by the guanidium isothiocyanate method as previously described (19). To prepare a probe for ME111-infected cells (Fig. 1), the *Sac*I to *Sma*I fragment of the *tk* gene (33) was subcloned into pUC18. The probe was labeled at the *Bgl*II site with polynucleotide kinase and was recut at the *Nde*I site in pUC18. The 720-base-pair fragment was isolated from a polyacrylamide gel. The hybridization was done at 44°C for 12 h, and S1 digestion was done at 37°C as previously described (9). To prepare a probe for ME129-infected cells (Fig. 1), the SV40 promoter and the *neo* gene from pSV2*neo* (29) were subcloned into pBR322. The plasmid was then labeled at the *Bgl*II site with polynucleotide kinase and was recut at the *Pst*I site in pBR322. The 1.8-kilobase-pair fragment was isolated from an agarose gel. The hybridization was done at 56°C for 6 h, and the S1 digestion was done at 37°C. Quantitation was determined by densitometry with several different exposures of each film.

RESULTS

Construction of retrovirus vectors with two selectable genes and a heterologous internal promoter. To determine the effect of different internal promoters on suppression in integrated retrovirus vectors, we constructed several spleen necrosis virus-based vectors that contained the herpes simplex virus *tk* gene (33), the bacterial transposon Tn*5 neo* gene (13, 29), and an internal promoter (Fig. 1). Plasmids pME111 and pME123 have been previously described (8). They contain the *tk* promoter internal to the LTRs and 5′ to either the *tk* or the *neo* gene. Plasmid pME131 is analogous to pME111 except that the mouse MTI promoter replaces the *tk* promoter. Plasmid pME129 is analogous to pME123 except that the SV40 early promoter replaces the *tk* promoter.

Such constructions with two selectable genes and two promoters allowed us to select for expression of one gene and then to measure the expression of the second gene. We also measured the expression of the second gene in separate cells selected for its expression. In this way, the activity of a gene in cells selected for its expression could be compared with the activity of that gene in cells selected for expression of a closely linked gene.

Viruses with the SV40 early promoter or the mouse metallothionein promoter as the internal promoter efficiently transformed cells to a TK^+ and NEO-resistant phenotype, whereas viruses with the *tk* promoter did not. Infectious virus was made from the plasmids in Fig. 1 by cotransfection of chicken embryo fibroblasts with the plasmid and helper virus (reticuloendotheliosis virus strain A) DNA. The resultant virus stocks were used to infect BRL TK^- cells to determine

their TK-transforming titer, their NEO-resistance transforming titer, and their TK and NEO-resistance transforming titers (Fig. 1). Two major points can be found in Fig. 1. First, both the mouse MTI promoter and the SV40 early promoter are stronger promoters in BRL TK$^-$ cells in these retrovirus vectors than the *tk* promoter. The number of TK$^+$ colonies obtained when the *tk* gene is 3′ to the metallothionein promoter (ME131) is sixfold higher than when the *tk* gene is 3′ to the *tk* promoter (ME111). In addition, the number of NEO-resistant colonies is threefold higher when the *neo* gene is 3′ to the SV40 early promoter (ME129) than when the *neo* gene is 3′ to the *tk* promoter (ME123).

Second, suppression is not significantly observed in a double-selection assay with either the virus containing the mouse metallothionein promoter (ME131) or the virus with the SV40 early promoter (ME129), although it is observed with the viruses with the *tk* promoter (ME111 and ME123). That is, the number of colonies transformed to both TK$^+$ and NEO-resistance by infection with either ME111 or ME123 virus is less than 10% of the number that are transformed to a TK$^+$ or NEO-resistant phenotype alone (Fig. 1). However, when these assays were done with viruses with the stronger internal promoters (ME129 and ME131), the number of cells transformed to both TK$^+$ and NEO resistance is approximately twofold the number of cells transformed to a NEO-resistant phenotype alone (for ME129) or a TK$^+$ phenotype alone (for ME131). Previously, we showed that the inability of ME111 and ME123 viruses to transform efficiently cells to both phenotypes simultaneously is also observed when the cells are grown in one selective medium and then switched to the other selective medium (8).

All the viruses showed suppression when gene products were analyzed biochemically. i. Effect of selection for the 5′ gene on expression of the 3′ gene. To test the results in Fig. 1 biochemically, we determined the TK activity in cell extracts. In cells infected with ME111 or ME131, this assay measures the activity of the 3′ gene. BRL TK$^-$ cells were infected with ME111 or ME131 virus at limiting dilutions and then were selected for the 5′ gene (*neo*), the 3′ gene (*tk*), or both. Transformed resistant colonies on plates infected with dilutions of virus that had been shown previously to give only one provirus per cell (8) were pooled (between 15 and 50 colonies in each pool), and cell extracts were made. The TK activity in these cell extracts was determined for cells that had been selected for expression of one or both of the genes in the proviruses (Fig. 2).

The TK activity in extracts from ME111-infected cells selected for expression of the 5′ gene (*neo*) was about 10% of the TK activity in extracts from ME111-infected cells selected for expression of the 3′ gene (*tk*). That is, the 3′ gene (*tk*) was only 10% as active when we selected for the 5′ gene (*neo*) as when we selected for the 3′ gene (*tk*). Likewise, we found that the TK activity in extracts from ME131-infected cells selected for expression of the 5′ gene (*neo*) contained only about 15% as much TK activity as ME131-infected cells that were selected for expression of the 3′ (*tk*) gene. Therefore, even in cells infected with a virus in which suppression was not observed phenotypically, we found biochemically that the 3′ gene was suppressed when there was selection for expression of the 5′ gene.

The effect of selecting for expression of both genes in the provirus on the expression of the 3′ gene is also shown (Fig. 2). For both ME131- and ME111-infected cells, the amount of TK activity (the 3′ gene) in extracts of cells selected for activity of both the 5′ and the 3′ genes is about 60% of the amount of TK activity in extracts of colonies selected for only the 3′ gene (*tk*). Therefore, the 3′ gene is still suppressed in cells in the subpopulation that survives selection for both genes simultaneously, but to a lesser extent than when there is just selection for the 5′ gene. The reason that doubly resistant ME111-infected cells have the same TK activity as doubly resistant ME131-infected cells is that the doubly resistant ME111-infected cells represent a small subpopulation of the total selected cells in which there is a reduced amount of suppression (see Discussion).

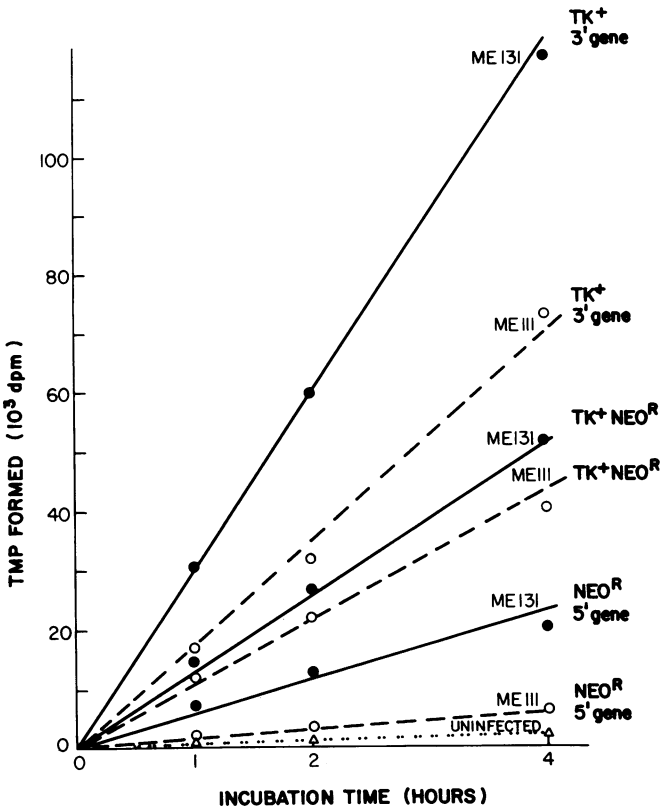

FIG. 2. The effect of selection for the 5′ gene on expression of the 3′ gene. Assay for TK activity in extracts from cells that were uninfected (△) or infected with either ME131 (●) or ME111 (○) virus and were selected for TK$^+$, for NEO resistance, or for both TK$^+$ and NEO resistance. In these vectors, the *neo* gene is 5′ and the *tk* gene is 3′. The internal promoter for ME131 is the mouse MTI promoter, and the internal promoter for ME111 is the *tk* promoter. The amount of tritiated thymidine monophosphate (TMP) in disintegrations per minute (dpm) bound to DE81 paper is plotted as a function of time of incubation. Equal amounts of protein in each extract were used for each assay. Duplicates of each extract were made from independently derived pools of clones. The absolute amount of TK activity in each extract varied from experiment to experiment, but the relative amounts of TK activity were within 10% of each other. The slopes of the lines represent the amount of TK activity in each sample.

We hypothesized that the discrepancy between the biological (Fig. 1) and the biochemical assays (Fig. 2) is the result of small changes in the amount of TK activity in cells which have a large effect on their ability to grow in medium that selects for TK$^+$ cells. To test this hypothesis, we picked several NEO-resistant ME131-infected cell clones, grew them to 10^6 cells, and measured their plating efficiency in HAT medium (to test for the TK$^+$ phenotype) relative to their plating efficiency in G418 medium (to test for the NEO-resistant phenotype). At the same time, we made cell

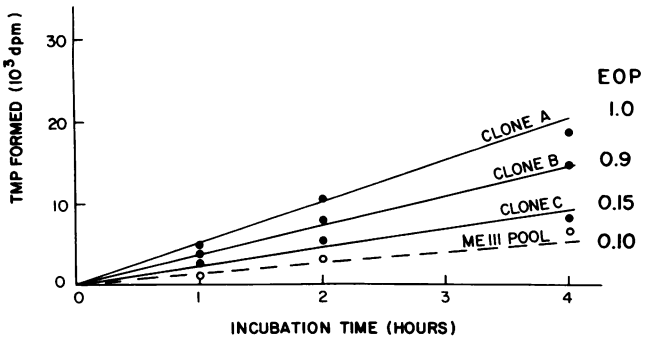

FIG. 3. Threshold amount of TK activity needed to form BRL TK$^+$ colonies in HAT medium. Assay for TK activity is described in the legend to Fig. 2 and in Materials and Methods. A, B, and C (●), three NEO-resistant cell clones that had been infected with ME131; (○), an extract from the same pool of ME111-infected NEO-resistant cells used in the experiment described in the legend to Fig. 2. EOP, Efficiency of plating, which is the number of cells from each clone or population of clones that replates in HAT medium (to select for TK$^+$) compared with the number of cells from each clone or population of clones that replates in G418 (to select for NEO resistance). Of eight NEO-resistant ME131-infected cell clones, five had the same cloning efficiency in HAT medium as that in G418. The other three had lower cloning efficiencies in HAT than in G418 media (data not shown).

extracts to determine the amount of TK activity in these clones. The TK activity in extracts from three cell clones is shown (Fig. 3), along with their relative plating efficiencies in HAT medium. Also shown is the TK activity in cell extracts from a pool of cells infected with ME111 and selected for NEO resistance and the relative plating efficiency of the pool in HAT medium. The suppressed metallothionein promoter expresses the *tk* gene at a level above the threshold of TK activity needed to form BRL TK$^+$ colonies in clones A and B. On the other hand, clone C and the pool of cells infected with ME111 virus express the *tk* gene below this threshold. Thus, a small difference in the level of TK activity (the difference between the TK activities of clones B and C) has a large effect on the phenotype of the cell clones.

ii. Effect of selection for the 3′ gene on expression of the 5′ gene. Experiments were then performed to analyze the effect of selection for the 3′ gene on expression of the 5′ gene. This experiment was done with the ME123 and ME129 viruses (Fig. 1), because with these viruses we could select for either the 5′ gene (*tk*), the 3′ gene (*neo*), or both genes and we could measure the expression of the 5′ gene (*tk*) by measuring the TK activity in cell extracts. The experiments described in the legend to Fig. 2 were repeated with ME123- and ME129-infected cells (Fig. 4). After ME123 virus infection, the TK activity in cells selected for expression of the 3′ gene (*neo*) was only 20% of the level of TK activity in cells selected for expression of the 5′ gene (*tk*). Extracts from ME123-infected cells that were selected for expression of both 5′ and 3′ genes had 70% of the level of TK activity as that in extracts of ME123-infected cells that were selected for the 5′ gene (*tk*) only.

In extracts of ME129-infected cells selected for expression of the 3′ gene (*neo*), the amount of TK activity was 40% of the activity found in extracts of cells selected for expression of the 5′ gene (*tk*). The TK activity in extracts from cells infected with ME129 and selected for both 5′ and 3′ genes was only slightly higher than the TK activity in extracts of ME129-infected cells selected for the 3′ gene alone (Fig. 4). Thus, the 5′ gene (*tk*) was suppressed in cells that were selected for expression of the 3′ gene (*neo*), even in virus-infected cells that did not show the suppression in biological assays.

We also analyzed the effect of selection for the 3′ gene (*tk*) on the expression of the 5′ gene (*neo*) by assaying in ME111-infected cells the expression of G418-aminoglycoside phosphotransferase activity by the method of Reiss et al. (25). This assay gave qualitatively the same results as the TK assay in ME123-infected cells. That is, the amount of G418-aminoglycoside phosphotransferase in cells selected for expression of the 3′ gene was less than 20% of the amount of G418-aminoglycoside phosphotransferase in cells selected for expression of the 5′ gene (data not shown). However, it was difficult to demonstrate that the assay was linear in the range of expression of G418-aminoglycoside phosphotransferase activity present in our cell extracts.

Suppression as reflected in the level of RNA from each promoter. We measured the steady-state level of RNA initiated at each promoter in ME111- and ME129-infected cells by the S1 nuclease technique. BRL TK$^-$ cells were infected with dilutions of virus expected to give only one provirus per cell and were selected for either TK$^+$ or NEO resistance phenotypes. Pools of resistant colonies (about 100 colonies in each pool) were grown to several large plates in selective medium, and total RNA was extracted. The results

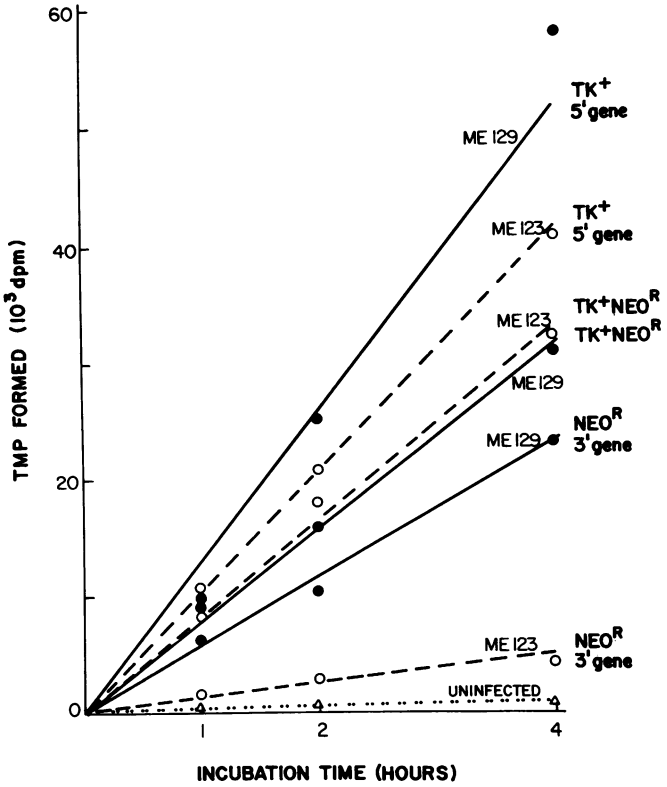

FIG. 4. Effect of selection for the 3′ gene on expression of the 5′ gene. Assay for TK activity is described in the legend to Fig. 2 and in Materials and Methods. Extracts are from uninfected cells (△) or from cells infected with either ME129 (●) or ME123 (○) and selected for either TK$^+$, NEO resistance, or both. In this case the *tk* gene is 5′ and the *neo* gene is 3′. The internal promoter for ME129 is the SV40 early promoter, and the internal promoter for ME123 is the *tk* promoter.

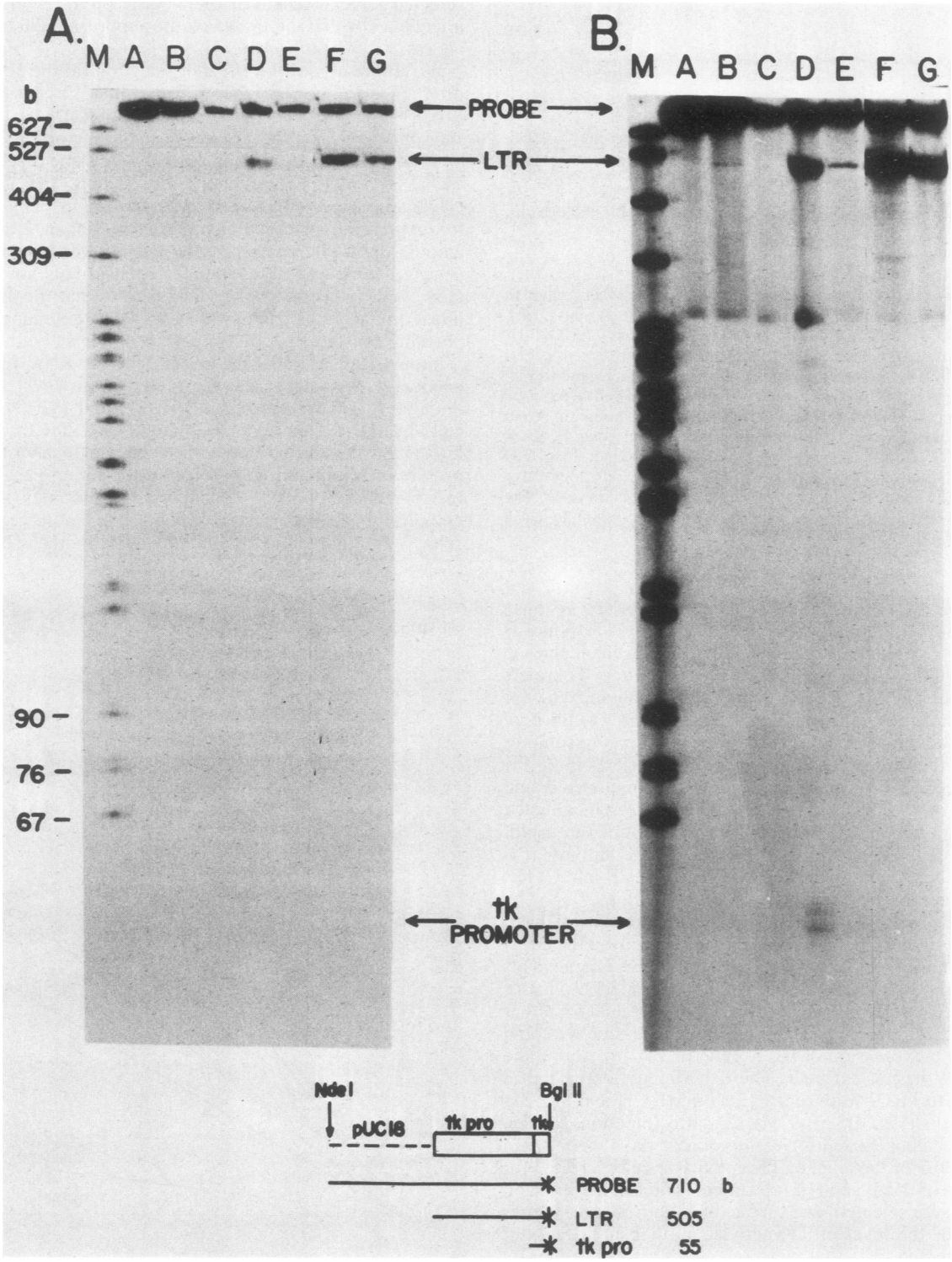

FIG. 5. S1 nuclease mapping of proviral transcripts in cells infected with ME111 and selected for expression of either the 5′ or 3′ gene. (A) Transcripts from ME111-infected cells were hybridized to a subclone of the *tk* gene. Probe and predicted protected fragments are shown. Lanes: M, pBR322 cut with *Hpa*II and end labeled; A, one-tenth the amount of probe used for hybridization; B, probe hybridized to tRNA; C, uninfected BRL RNA; D, 50 μg of RNA from a pool of TK^+ cell cones; E, 10 μg of the same RNA used in lane D; F, 50 μg of RNA from a pool of NEO-resistant clones; G, 10 μg of the same RNA used in lane G. Molecular size markers are given in bases (b). The autoradiogram was exposed to film for 10 h. (B) The same autoradiogram as in panel A was exposed to film for 72 h.

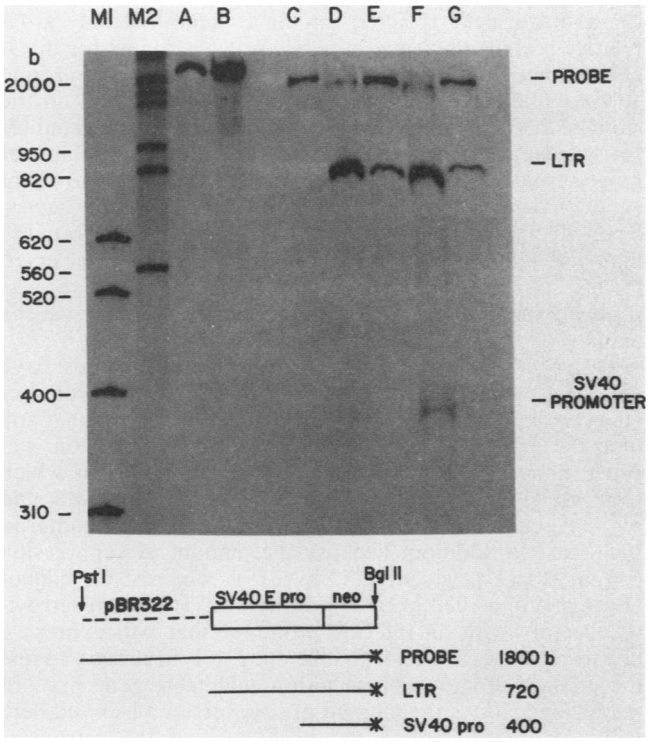

FIG. 6. S1 nuclease mapping of proviral transcripts in cells infected with ME129 and selected for expression of either the 5′ or 3′ gene. Transcripts from ME129-infected cells were hybridized to a subclone of the SV40 early promoter-*neo* gene. Probe and predicted protected fragments are shown. Lanes: M1, pBR322 cut with *Hpa*II and end labeled; M2, lambda DNA cut with *Hin*dIII and *Eco*RI and end labeled; A, probe hybridized with tRNA; B, one-tenth the probe used in the other lanes; C, BRL uninfected RNA; D, 40 μg of RNA from a pool of TK$^+$ cell clones; E, 8 μg of the same RNA as used in lane D; F, 40 μg of RNA from a pool of clones selected for NEO resistance; G, 8 μg of the same RNA as used in lane F. Molecular size markers are given in bases (b). The autoradiogram was exposed to film for 16 h.

of S1 mapping with RNA from ME111-infected cells selected for either the 3′ gene (*tk*) or the 5′ gene (*neo*) are shown (Fig. 5). The probe for this experiment was a subclone of the *tk* gene in pUC18 (see Materials and Methods) that was 5′-end labeled with polynucleotide kinase at the *Bgl*II site which is about 55 base pairs 3′ to the normal cap site for the *tk* gene (32), recut in pUC18 sequences, and gel purified. The top band in all of the lanes in Fig. 5A is the full-length probe, whereas the 505-base band is probe protected by RNA that initiated in the LTR and transcribed through the *tk* promoter. The RNA that initiates at the *tk* promoter protects 55 bases of probe. This band is not visible on the exposure shown in Fig. 5A but can be seen in the longer exposure shown in panel B. The autoradiogram in panel A was scanned by densitometry to determine the relative amounts of probe protected by each RNA sample. The steady-state level of RNA transcribed from the LTR in ME111-infected cells selected for the 3′ gene (*tk*; lane D) was only 15% of the steady-state level of RNA transcribed from the LTR in ME111-infected cells selected for the 5′ gene (*neo*; lane F). Thus, the suppression of the 5′ gene (*neo*) when the 3′ gene (*tk*) was selected in cells infected with virus that contained the *tk* promoter as the internal promoter (i.e., Fig. 4, ME123-infected cells) is reflected by a decrease in the relative amount of steady-state RNA transcribed from the LTR.

The longer exposure of Fig. 5A shown in panel B demonstrates that RNA transcribed from the *tk* promoter was present in cells selected for the 3′ gene (*tk*; lane D) but was not detectable in the cells selected for the 5′ gene (*neo*; lane F). Therefore, the suppression of the 3′ gene when the 5′ gene is selected in cells infected with virus that contains the *tk* promoter as the internal promoter can also be correlated with a decrease in the amount of RNA transcribed from the *tk* promoter.

S1 nuclease mapping was also performed with RNA from pools of ME129-infected clones that had been selected for either the 5′ gene (*tk*) or the 3′ gene (*neo*; Fig. 6). The probe for this experiment was a subclone of the SV40 early promoter-*neo* gene in pBR322 (see Materials and Methods) that was 5′-end labeled with polynucleotide kinase at the *Bgl*II site in the *neo* gene, recut in the pBR322 sequences, and gel purified. The top band in each lane of Fig. 6 is the undigested probe, the 720-base band is the correct size for a transcript that initiates in the LTR and transcribes through the SV40 promoter, whereas the 400-base bands are the correct sizes for transcripts that start at the late-early cap sites in the SV40 early promoter (34). The steady-state level of RNA that initiates in the LTR and transcribes through the SV40 promoter in cells selected for expression of the 3′ gene (*neo*) was 60% of the amount of RNA that initiates in the LTR and transcribes through the SV40 promoter in cells selected for expression of the 5′ gene (*tk*; compare Fig. 6, lanes D and E with lanes F and G; several exposures of the autoradiograph were scanned by densitometry). Thus, the suppression of the 5′ gene observed with the TK assay in Fig. 3 can be correlated with the suppression at the RNA level in Fig. 6. Both of the S1 nuclease analyses (Fig. 5 and 6) and the TK assay (Fig. 4) indicate that the amount of suppression of the 5′ LTR is less when the internal promoter is the SV40 early promoter than when the internal promoter is the *tk* promoter.

The intensities of the 400-base bands show that the suppression of the 3′ gene when there was selection for the 5′ gene reflected the amount of steady-state RNA transcribed from the internal promoter. In this case, the amount of RNA transcribed from the SV40 promoter in cells selected for expression of the 5′ gene was about 25% of the amount of RNA that initiated at the SV40 promoter in cells selected for expression of the 3′ gene (Fig. 6).

DISCUSSION

At the biochemical level, all of the retrovirus vectors that we constructed with an internal promoter displayed suppression of the activity of a gene that was either 5′ or 3′ to the gene for which we selected. As internal promoters, we used the *tk* promoter, the SV40 early promoter, and the mouse MTI promoter. By measuring specific protein activity in cells infected with viruses with an internal promoter and by selecting for either the 5′ gene or the 3′ gene, we showed that the suppressed gene produced from about 10 to 50% as much product as when it was selected. The degree of suppression depended on whether the gene was in the 5′ or 3′ position and from which promoter it was transcribed. We found that the level of steady-state RNA transcribed from each promoter was roughly proportional to the level of suppression measured by protein activity. This correlation suggests that gene suppression in retrovirus vectors is mediated by the suppression of a promoter when a nearby promoter is

selected. By using biological assays, we did not detect suppression in the viruses with internal promoters stronger than the *tk* promoter. However, we showed that this failure resulted from the fact that when the stronger promoter was suppressed, the level of expression from that promoter was still above the threshold level needed to form colonies in our biological assays.

In our previous study (8), we determined that promoter suppression is epigenetic because it is *cis*-acting and reversible. It is reversible at a high frequency when virus is recovered and at a low frequency when the cells are passaged and selected for variant cells that have regained the ability to express the suppressed gene at a high level. In another study (7), we found that all recovered viruses that are selected for expression of a 3′ gene (*tk*) in a retrovirus vector that contains three upstream promoters undergo deletion of at least one of the promoters. We propose that all of the recovered viruses in those experiments underwent deletions because suppression of a downstream promoter by two upstream promoters is too profound to be tolerated. Therefore, only variants that delete one or more of the internal promoters can express the *tk* gene at a level above that needed to give TK^+ colonies. In viruses in which there is only one internal promoter, the internal gene can be expressed efficiently, but only when the other promoter is suppressed by an epigenetic mechanism.

Clone-to-clone variability in the amount of suppression. The amount of suppression of a nonselected promoter varies from clone to clone (8; Fig. 3). S1 nuclease analysis of RNA from individual ME111-infected clones showed that the amount of RNA correlated with the phenotype of the clone (efficiency of plating in selective media) but was variable from clone to clone (data not shown). Therefore, it is important to remember that the pools of cells used in the experiments reported in this paper contained cells from several different clones that differed in the amount of transcription from each promoter. Thus, the amount of suppression determined in Fig. 2, 4, 5, and 6, is actually an average of the suppression in different clones.

Because of clone-to-clone variability in the expression of the suppressed gene, subpopulations that express the suppressed gene at a higher level than the bulk of the other cells in a population of infected cells under selection will exist. Thus, in ME111-infected cells selected for NEO resistance, there exist subpopulations of cells that are also TK^+ (Fig. 1). The TK activity in extracts from ME111-infected cells that had been selected for both 5′ and 3′ genes represents the activity of a subpopulation that expresses the *tk* gene at a level above the threshold needed for cells to survive in HAT medium (Fig. 2).

There also exist subpopulations of cells infected with ME131 or ME129 viruses and selected for NEO resistance that are not also TK^+, although this subpopulation is much smaller in ME131- or ME129-infected cells than it is in ME111- or ME123-infected cells (Fig. 1; data not shown). The subpopulation of cells infected with ME131 or ME129 that are NEO resistant but not TK^+ will have lower TK activity than cells selected for both TK^+ and NEO resistance. Therefore, the average amount of TK activity in ME131- and ME129-infected cells selected for NEO resistance only will be lower than the TK activity of ME131- and ME129-infected cells selected for both TK^+ and NEO resistance (Fig. 2 and 4).

In a previous paper (8), we selected ME111- or ME123-infected cells for one gene, and then we tested their plating efficiency in medium that selected for the second gene. On the average, cells selected for the 5′ gene had only a 4% relative plating efficiency in medium that selected for the 3′ gene, although in individual clones this number ranged from 10% to <0.01%. Cells selected for the 3′ gene had on the average a 30% plating efficiency in medium that selected for the 5′ gene, although in individual cell clones this number ranged from 100 to 0.1%. In the present study we show that small changes in the TK activity of cell clones can have a large effect on their ability to be TK^+ (Fig. 3). Therefore, the high variability in the survival of cells when counterselected for the suppressed gene seen in our previous study (8) was probably because of small clone-to-clone differences in the amount of enzyme produced in each clone.

Design of retrovirus vectors. Several other groups have also designed retrovirus vectors with an internal promoter (1, 14, 16, 17, 26, 30, 32, 35). It is difficult to determine if any of these groups also observed promoter suppression, because none of these groups constructed viruses in which they could measure the expression of a gene with and without selection for expression from the other promoter in the virus. In addition, because the amount of suppression can be slight (e.g., Fig. 4, ME129), suppression cannot always be observed in biological assays. To design retrovirus vectors with an internal promoter that will express a nonselectable gene at a high efficiency, it is necessary to test the amount of suppression with a selectable gene first. In many applications the amount of suppression observed here will not greatly reduce the usefulness of the retrovirus vector. We are currently determining if suppression occurs in retrovirus vectors with an internal promoter constructed from genomes other than spleen necrosis virus.

Alternatively, it is possible to construct retrovirus vectors in which both nonselectable and selectable genes are transcribed from the LTR and one of the genes is translated from a spliced message (4, 10). The disadvantge of such vectors is that some sequences in the intron can inhibit splicing (5; C. Miller and H. M. Temin, J. Virol., in press). Therefore, selection for one of the genes in the retrovirus vector can lead to deletions and rearrangements.

Is suppression of a promoter when a nearby promoter is fully active observed in other systems? In *Drosophila melanogaster*, the gypsy element transposes into the hairy wing and yellow loci and inactivates them (18). The unlinked recessive mutation Su(Hw) will reactivate these loci without excision of the gypsy element. Parkhurst and Corces (23, 24) have determined that the hairy wing and yellow loci are transcriptionally inactive when the gypsy element transposes into these loci, but they regain transcriptional activity in Su(Hw) flies. The mutation Su(Hw) appears to inhibit transcription from the gypsy element in *trans* (24). Therefore, transcription from gypsy could inhibit transcription of hairy wing and yellow loci by the same mechanism that occurs in our system. A similiar phenomenon is observed in yeast after integration of the Ty element near the *His4* locus (37, 38). After determining the distance between clustered genes in eucaryotes, Naora and Deacon (20) suggested that an extragenic territorial region is needed between genes for their efficient expression.

Furthermore, integration of a retrovirus into a noncoding region of a gene has, in several instances, led to inactivation of that gene (27, 31). This inactivation could be caused by the effect of the retrovirus promoter. Cullen et al. (6) determined in a transient expression assay that the 3′ LTR of an avian retrovirus was prevented from transcribing a downstream gene when the 5′ LTR was transcriptionally active, although the mechanism involved, promoter interference, may be

different from the mechanism of suppression seen in our system. On the other hand, the integration of a retrovirus either 5′ or 3′ to a gene can, in many cases, activate that gene (21, 22).

In conclusion, by measuring enzyme activity and RNA levels and by using viruses with different internal promoters, we confirmed our hypothesis that expression from one promoter is decreased when there is selection for expression from a closely linked promoter. Our data do not rule out the possibility that suppression results in the use of different, less efficient start sites than are normally used. Experiments are in progress to determine whether transcription from a promoter will directly decrease transcription from a nearby promoter or whether the suppression of expression is the result of selection for particular phenotypes.

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