Volume 14 Number 23 1986 Nucleic Acids Research

Comparison of promoter suppression in avian and murine retrovirus vectors

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Received 26 August 1986; Revised and Accepted 7 November 1986

ABSTRACT

Previously, we described "promoter suppression" in infectious retrovirus vectors with two genes and an internal promoter. Here, we examined several parameters of promoter suppression and found that the amount of suppression in an integrated retrovirus vector was dependent both on whether the vector was derived from spleen necrosis virus or murine leukemia virus and on which internal promoter was present in the vector. Murine leukemia virus vectors showed less suppression than analogous spleen necrosis virus vectors. Furthermore, the amount of suppression was not dependent on either the relative strengths of the promoters nor the distance between the promoters. Moreover, we found that in vectors in which one promoter was suppressed, there was an inverse correlation between the DNaseI sensitivity of the chromatin surrounding a promoter and the suppression of its expression.

INTRODUCTION

Retroviruses are RNA viruses that replicate through a DNA intermediate that integrates into the cell genome. The integrated form of the retroviral genome, the provirus, is maintained in the chromosome as a stable genetic element. This property of retroviruses makes them attractive vectors for the stable insertion of exogenous genes into the chromosomes of vertebrate cells. 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 have been studying the expression of genes in infectious spleen necrosis virus (SNV)-derived retrovirus vectors that contain a promoter internal to the long terminal repeats (LTR) of the provirus. We found that the expression of the gene under the control of the LTR promoter is suppressed when there is selection for the expression of the gene under the control of the internal promoter (1,2). Likewise, the expression of the gene under the control of the internal promoter is suppressed when there is selection for expression of the gene transcribed from the LTR (1,2). This suppression is cis-acting, epigenetic, and reversible (1). In addition, we

showed that suppression acts by altering the steady-state level of RNA transcribed from each promoter (2).

In this study we examined several other parameters of promoter suppression in retrovirus vectors. We examined the amount of suppression in vectors constructed from the genome of a murine leukemia virus (MLV), and we found that there was much less suppression in these vectors than in the analogous SNV-based viruses. The suppression of promoters in SNV-based viruses was mostly relieved by using an internal promoter from the MLV U3 region, but it was not relieved by increasing the distance between the promoters. We also found that within the same provirus there was a difference between the chromatin structure of a suppressed promoter relative to a promoter which was not suppressed as judged by different sensitivities to DNaseI digestion of the chromatin surrounding each promoter.

MATERIALS AND METHODS

Nomenclature.

Plasmids have the letter "p" before their names (e.g. pMEl11), whereas viruses made from those plasmids do not (e.g. MEl11). Cell clones harboring a provirus are named by the name of the provirus, followed by an arbitrary letter or number designation (e.g. MEl11.F).

Cells.

Buffalo rat liver TK^- cells (BRL TK^- cells), chicken embryo fibroblasts (CEF), and D17 cells (a dog osteosarcoma cell line) were grown as previously described (1,3). Selection for TK^+ cells was done in 10^{-4} M hypoxanthine, 5 x 10^{-5} M thymidine, and 5 x 10^{-7} M methotrexate (called HAT medium in this paper). Selection for G418-resistant cells (called NEO^R cells in this paper) was done in 400 µg/ml G418 (Gibco).

Virus.

SNV-based virus was recovered from plasmids by co-transfection of CEF with each plasmid and reticuloendotheliosis virus strain A (Rev-A) DNA as helper by the DMSO/polybrene method (4). Virus titers were determined and standardized for variations in the transfection process and in virus production as previously described (1). Helper-free virus was recovered by transfection of the D17-C3 helper-cell line as described (3). MLV-based virus was recovered by transfection of D17 cells with the appropriate plasmid, and infection of NEO^R cells with amphotrophic MLV 4070A (5). The virus was harvested five days post-infection.

Plasmids.

Recombinant DNA techniques were carried out as described by Maniatis et. al. (6) except as noted. The plasmids pME111 and pME123 were previously described (1,2). They contain the neo gene (7) from the Tn5 transposon, and the tk promoter and gene (8) from the herpes simplex type I virus (see Figs. 1 and 2). In these and all other constructions with the tk gene, the 3' RNA processing sequences have been removed.

pME111-2 (Fig. 4) was made by inserting a 2.8 HindIII fragment from the fourth putative intron of turkey c-rel into a HindIII site at the 3' end of the neo gene in pME111. pME111-1 has a 0.4 kbp XbaI deletion of sequences in the pME111-2 insert.

pME149 (Fig. 1) was constructed by deleting the tk promoter from pME123 and replacing it with the MLV U3 region (9). The MLV U3 region included 50 bp from the end of env at its 5' end (the RsaI site at 7763) and 32 bp of R at its 3' end (the RsaI site at 8296).

pME139, pME140, and pME151 (Fig. 1) were based on the MLV vector AFVXM (10, kindly provided by Michael Kriegler). pME139 contains the neo gene-tk promoter-tk gene fragment from pME111 (Fig. 1) inserted into the polylinker of AFVXM. pME140 contains the tk gene-tk promoter-neo gene fragment from pME123 (Fig. 1) inserted into the polylinker of AFVXM. pME151 was constructed by replacing the tk promoter in pME140 with the SNV U3 region from the AvrII site at 7691 to the AvaI site at the U3-R border.

In vitro TK assay.

The in vitro TK assays were done essentially as described (2,11,12). Pools of 50-100 clones were transfered to 35 mm dishes and grown for two to four days in the presence of either HAT medium or G418 plus 5 x 10^{-5} M thymidine. Cells were washed twice with phosphate buffered saline, suspended in 300 µl 50µM Tris-HCl (pH 7.4), 5 mM β-mercaptoethanol, 5µM dThd, disrupted in a Branson sonifier, and clarified by centrifugation. The relative amount of protein in each sample was determined using the Bradford assay (13). Dilutions of a concentrated sample of uninfected BRL TK^- cell extract and bovine serum albumin served as standards. Equal amounts of protein from each sample (typically about 50 µg) were incubated at 37°C with TK assay buffer (2,12), and aliquots were removed at 1, 2, and 3 hrs to determine the conversion of [^{3}H]dThd to [^{3}H]TMP by the ability to bind to DE81 paper. The amount of radioactivity bound to DE81 paper when no protein was added to the reaction was subtracted from each point, and the amount of TK activity in

each extract was determined by measuring the slope of the DPMs bound to DE81 paper plotted against time (hrs) of incubation.

Isolation of nuclei and DNaseI digestion.

Nuclei were isolated and treated with DNaseI essentially as described (14,15). Nearly confluent plates of cells were trypsinized, scraped, and washed twice with 5% sucrose, 85 mM KCl, 5 mM PIPES (pH 7.0), and 1mM EDTA (Y1 buffer + EDTA). Nuclei were isolated by Dounce homogenization with an A pestle (four strokes) in Y1 buffer plus 0.5% Nonidet P-40 at 4°C, followed by centrifugation at 1 g for 8 min. The nuclei were washed twice with Y1 buffer and resuspended at a concentration of 1 mg/ml. One-tenth volume of 10 mM $CaCl_2$ and various amount of DNaseI (Worthington Biochemicals; 0 to 2 µg/ml) were added, and the nuclei were incubated at 37°C for 10 min. The digestion was stopped by the addition of an equal volume of a solution containing 1% SDS, 0.6 M NaCl, 20 mM Tris-HCl pH 7.4, 10 mM EDTA, and 400 µg/ml proteinase K (Sigma). The DNA was then isolated, digested with restriction enzymes, run on gels, blotted, and hybridized to labelled probes as described (6). Probes were made from DNA fragments with ^{32}P-dTTP and ^{32}P-dCTP by the method of Feinberg and Vogelstein (16).

RESULTS

MLV vectors show less suppression than the analogous SNV vectors.

To test the generality of suppression of promoters in retrovirus vectors, we constructed infectious retrovirus vectors based on an MLV genome that had the same selectable genes and the same internal promoter as the SNV vectors we had previously tested (1,2). pME139 and pME140 are analogous to pME111 and pME123, respectively (Fig. 1), except that pME111 and pME123 are SNV-based, while pME139 and pME140 are MLV-based. All four of these plasmids contain the *neo* gene, the HSV-1 *tk* gene, and the HSV-1 *tk* promoter.

Virus was made from the SNV-based vectors by co-transfecting CEF cells with plasmid vector DNA and helper virus (Rev-A) DNA. Virus was obtained from MLV-based vectors by transfecting D17 cells, selecting for NEO^R cells, and infecting these cells with either amphotrophic MLV 4070A (5) or with Rev-A. Virus from both SNV- and MLV-based vectors was then used to infect Buffalo rat liver TK^- cells (BRL TK^-), and the cells were selected for the NEO^R phenotype, the TK^+ phenotype, or both. Rat cells were used for these experiments because they can be efficiently infected by both SNV and MLV (17). Thus, we could make a direct comparison of the amount of suppression in the same cell type.

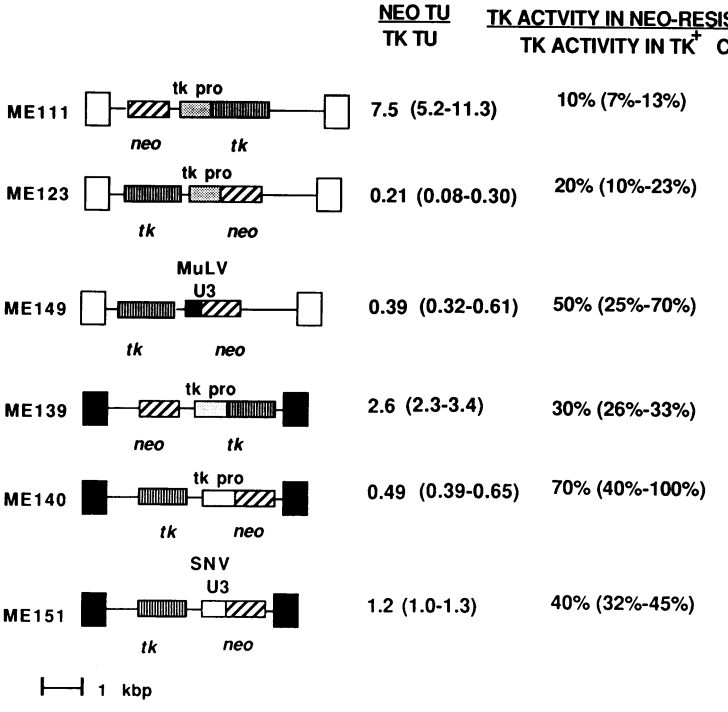

Figure 1. Structure, transforming activity, and suppression of expression in retrovirus vectors with an internal promoter.

ME111, ME123, and ME149 are SNV-based, while ME139, ME140, and ME151, are MLV-based. Open boxes represent the SNV LTRs, filled boxes represent the MLV LTRs of AFVXM (10 and Michael Kriegler, personal communication), and lines represent viral sequences. All other inserts are labelled in the figure.

The NEO TU (transforming units) are the number of cells transformed by infection from a NEO^S phenotype to a NEO^R phenotype. The TK TU are the number of cells transformed by infection from a TK^- phenotype to a TK^+ phenotype. The NEO TU and TK TU were determined in parallel infections of BRL TK^- cells. The average value of four consecutive experiments is given, followed by the range of values. The TK activity in NEO^R cells is expressed as a percentage of the TK activity in TK^+ cells. Thymidine kinase assays were done on standardized cell lysates as described in Materials and Methods. Determinations were done on pools of TK^+ and NEO^R clones which were derived in parallel. The average value of different experiments is given, followed by the range of values of all experiments. The determination of thymidine kinase activity in parallel pools of TK^+ and NEO^R clones was done four times for ME111, five times for ME123, three times for ME149, twice for ME152, three times for ME139, nine times for ME140, and three times for ME151.

Suppression was first measured in a phenotypic assay in which we compared the number of colonies that could be transformed to a NEO^R and TK^+ phenotype to the number that could be transformed to NEO^R or TK^+ alone. As

we previously described (1,2), the number of colonies transformed to both NEO^R and TK^+ was 2% to 20% of the number of colonies transformed to NEO^R alone or TK^+ alone after infection with the SNV-based MEl11 or MEl23. However, after infection with the MLV-based MEl39 or MEl40, the number of colonies transformed to both NEO^R and TK^+ was 60%-100% of the number of colonies transformed to the TK^+ phenotype alone (for MEl39) or the NEO^R phenotype alone (for MEl40, data not shown).

We previously described an assay for suppression which is more sensitive than the phenotypic assay (2). In this assay the amount of suppression is measured by the ratio of the amount of thymidine kinase (TK) activity in cells selected for the NEO^R phenotype to the amount of TK activity in cells selected for the TK^+ phenotype. This assay measures the amount of activity of the non-selected gene (TK activity in NEO^R cells) compared to the activity of that gene when it is selected (TK activity in TK^+ cells). Thus, this assay measures suppression in cells in which the promoter still expresses the gene at a level above the threshold for survival in selective media (2).

In MEl11- and MEl23-infected cells, the amount of TK activity in cells selected for NEO^R is 10% and 20%, respectively, of the amount of TK activity in cells selected for TK^+ (Fig. 1). In contrast, in cells infected with the analogous MLV-based vectors, MEl39 and MEl40, the amount of TK activity in cells selected for NEO^R is 30% and 70%, respectively, of the amount of TK activity in cells selected for TK^+ (Fig. 1). Therefore, comparing MEl11 to MEl39 and MEl23 to MEl40, we found that the promoters in the MLV-based vectors were less supressed by selection for expression of the other promoter than were the promoters in the analogous SNV-based vectors.

The different level of suppression between the MEl40-infected cells and the MEl23-infected cells was not the result of a difference in the helper virus used because the same results were obtained when MEl40 was pseudotyped with an Rev-A helper and MEl23 was pseudotyped with an amphotrophic MLV helper virus (data not shown).

Vectors with both the MLV U3 and the SNV U3 regions show intermediate amounts of suppression.

To determine whether a vector with both SNV and MLV promoters efficiently expressed genes transcribed from both promoters, we constructed an SNV-based vector with the tk and neo genes and the MLV U3 region as the internal promoter (MEl49, Fig. 1). The TK activity in MEl49-infected NEO^R cells was 50% of the TK activity in MEl49-infected TK^+ cells, whereas in the MEl23- and MEl40-infected cells the TK activity in NEO^R cells was, repectively, 20%

and 70% of the TK activity in TK^+ cells (Fig. 1). Thus, comparing vectors with similiar structures (that is, tk as the 5' gene and neo as the 3' gene), the SNV vector where the MLV U3 was the internal promoter (ME149) was less suppressed than the SNV vector where the tk promoter was the internal promoter (ME123), but it was more suppressed than the MLV vector where the tk promoter was the internal promoter (ME140).

To test the amount of suppression in another vector that contained both SNV and MLV promoters, we constructed an MLV-based vector with the tk and neo genes that used the SNV U3 region as the internal promoter (ME151, Fig. 1). In ME151-infected cells the TK activity in NEO^R cells was 40% of the TK activity in TK^+ cells, whereas with the SNV-based ME123 this ratio was 20%, and with the MLV-based ME140 this ratio was 70%. Therefore, like the SNV vector with the MLV U3 region as the internal promoter, the amount of suppression in cells infected with the MLV vector with the SNV U3 region as the internal promoter was intermediate between the SNV vector with the tk promoter and the MLV vector with the tk promoter.

The amount of suppression is not correlated with the strengths of the promoters in the vectors.

We used the ratio of NEO TU to TK TU for each virus (Fig. 1) as an estimate of the differences in promoter strengths between the LTR and the internal promoter. Although the ratio of NEO TU to TK TU cannot be equated with promoter strength in absolute numerical terms, because it is an indirect measurement, in a previous study (2) we found that steady-state RNA levels were correlated with enzyme activities in our retrovirus vectors. By this assay in BRL cells, the SNV LTR gives 5 to 7.5 times more colonies than the tk promoter (ME123 and ME111), while the MLV LTR gives 2 to 2.6 times more colonies than the tk promoter (ME140 and ME139). The SNV LTR gives 1.2 to 2.6 times more colonies than the MLV LTR (ME151 and ME149). However, comparing viruses of similiar structures (the tk gene as the 5' gene and the neo gene as the 3' gene), we found that the amount of suppression was greatest in ME123-infected cells, intermediate in ME149- and ME151-infected cells, and lowest in ME140-infected cells (Fig. 1). Thus, the amount of suppression is not correlated with the differences between the strengths of the LTR promoter and the internal promoter in each virus. Rather, the amount of suppression is dependent on the particular pair of promoters in the vector.

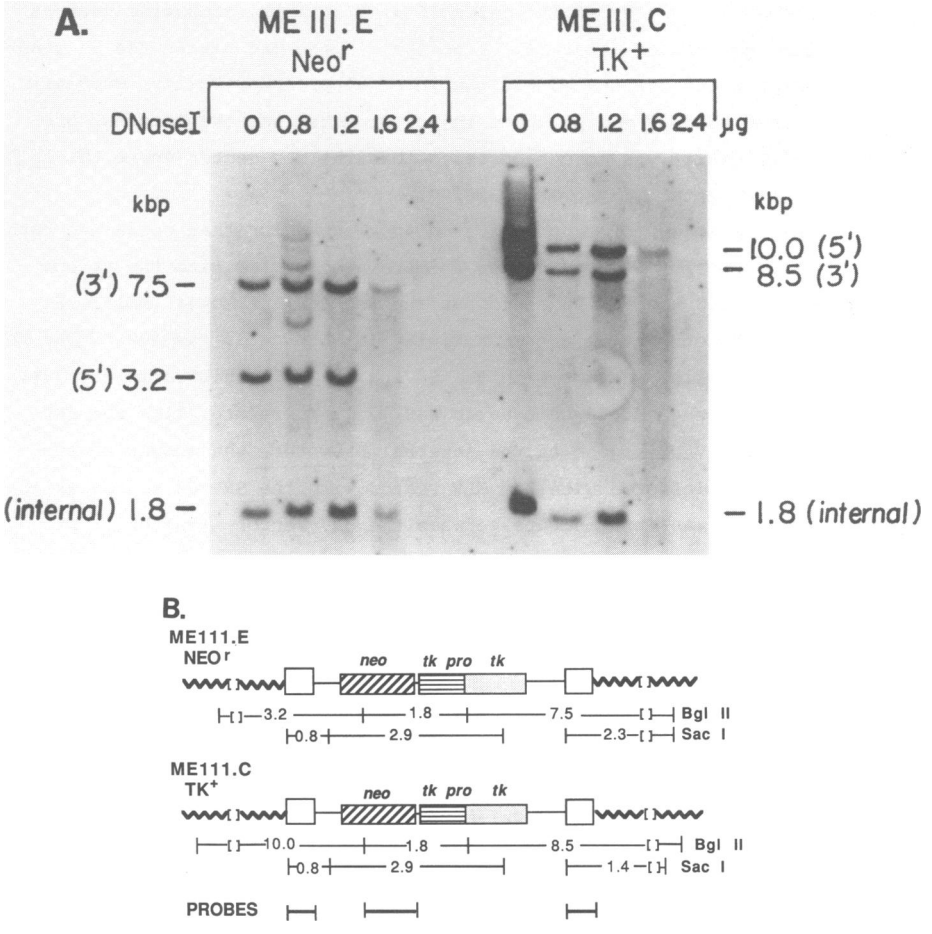

Figure 2. DNaseI sensitivity of MElll-infected cell clones selected for either NEO^R or TK^+.

A. Nuclei were isolated from the rat cell clones MElll.E (selected for NEO^R) and MElll.C (selected for TK^+) and were treated with different concentrations of DNaseI for 10 min. DNA was isolated, digested with BglII, separated by electrophoresis, and blotted to nitrocellulose. The filter was simultaneously hybridized to a radioactive fragment from the BglII site in neo to the 3' end of the neo gene and with a radioactive fragment from the SNV LTR. The amount of DNaseI used in each aliquot of nuclei is listed above the lane. The position of the restriction fragment in the provirus (5', 3', or internal) is listed next to the band.

B. Restriction enzyme cleavage site maps of the integrated proviruses in MElll.E and MElll.C. The wavy lines represent cellular DNA, the open boxes represent LTRs, and the lines represent viral sequences. The BglII and SacI fragments from each clone are listed below the provirus. The assigment of the bands containing the LTRs was done by hybridization with probes that distinguish them (data not shown). The probes used in panel A are shown.

Suppression correlates with the DNaseI sensitivity of the chromatin in the promoter regions of proviruses.

Regions of chromatin containing transcriptionally active promoters often show increased sensitivity to digestion with DNaseI (18). To determine if suppression of the non-selected gene in SNV-based vectors correlated with differences in the chromatin structure around the promoters of the selected and the non-selected genes, differences in sensitivity to digestion by DNaseI in different regions of integrated MElll-proviruses were assayed. We isolated intact nuclei from clones of rat cells that had been infected with a helper-free stock of MElll and selected for either TK^+ or NEO^R phenotypes. The nuclei were treated with various amounts of DNaseI, and the DNA was extracted and digested with the restriction endonuclease BglII. After Southern blotting, the filter was probed with a mixture of labelled neo and LTR fragments.

The restriction endonuclease BglII cuts MElll proviruses once in the neo gene and again at the junction of the tk promoter and the tk gene (Fig. 2B). Thus, BglII digestion of DNA from a cell clone that contains an MElll provirus will produce a 5' junction fragment which contains the 5' LTR, an internal fragment which contains the tk promoter, and a 3' junction fragment which contains the 3' LTR. Specifically, BglII digestion of DNA of cell clone MElll.E (selected for NEO^R) gave a 3.2 kbp band corresponding to the 5' side of the provirus, a 1.8 kbp internal fragment, and a 7.5 kbp band corresponding to the 3' side of the provirus (Fig. 2). BglII digestion of DNA of cell clone MElll.C (selected for TK^+) gave a 10 kbp band corresponding to the 5' side of the provirus, a 1.8 kbp internal fragment, and a 8.5 kbp band corresponding to the 3' side of the provirus (Fig. 2).

In cell clone MElll.E (NEO^R), the band containing the 5' LTR was more sensitive to DNaseI digestion than was the band containing the tk promoter or the band containing the 3' LTR, that is, the 3.2 kbp band disappeared at lower concentrations of DNaseI than did the 1.8 kbp band or the 7.5 kbp band (Fig. 2A). On the other hand, in clone MElll.C (TK^+) the band containing the tk promoter was more sensitive to DNaseI digestion than was the band containing the 5' LTR, that is, the 1.8 kbp band disappeared at lower concentrations of DNaseI than did the 10 kbp band (Fig. 2A).

Reprobing this same filter with an alpha-globin-specific fragment showed that the chromatin around this unrelated gene had the same DNaseI sensitivity in both cell clones, and that the absolute difference in the density of the bands between different lanes was partially due to unequal loading of the

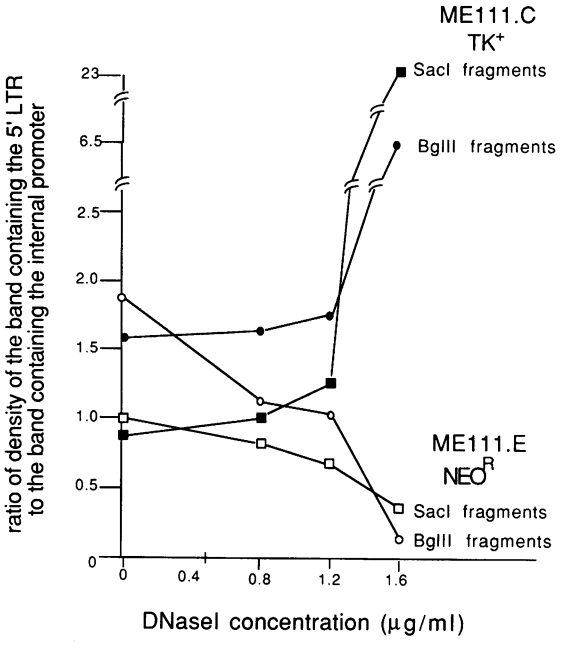

Figure 3. Relative DNAseI senstivity of different regions of the proviruses.

Nuclei were isolated from MElll.C (filled symbols) or MElll.E (open symbols) and were treated with different concentrations of DNaseI as described in Materials and Methods and in the legend of Figure 2. The DNA was digested with either BglII (circles) or SacI (squares) and hybridized to the radioactive fragments shown in Figure 2, panel B. Multiple exposures of the films were scanned by densitometry. Each data point represents the ratio of the density of the 5' band (which contains the 5' LTR) to the internal band (which contains the tk promoter). The 3' bands were also scanned, but they are not included in this Figure. MElll.C is TK^+ and NEO^S, while MElll.E is TK^- and NEO^R.

gel (data not shown). Thus, to compare the relative DNaseI sensitivity of the bands in each lane, we determined the ratio of the 5' LTR-containing band to each of the other bands at different DNaseI concentrations. The region of the MElll provirus that was most DNaseI sensitive depended on whether the cell clone was selected for expression of the 5' gene or selected for expression of the 3' gene (Fig. 3 and data not shown). In the clone selected for NEO^R (MElll.E) the ratio of the density of the band containing the 5' LTR to the density of the band containing the tk promoter decreased as the DNaseI concentration was increased. However, in the clone selected for TK^+ (MElll.C), the ratio of the density of the band containing the 5'

LTR to the density of the band containing the tk promoter increased as the DNaseI concentration was increased (Fig. 3).

We also digested DNA from the DNaseI treated nuclei with the restriction enzyme SacI. SacI digests MEl11 near the 5' border of U3, between the LTR and the neo gene, and in the tk gene. Thus, with the probes illustrated in Figure 2B, three bands are obtained--an internal band containing the 5' LTR; an internal band containing the neo gene, the tk promoter, and most of the tk gene; and a junction fragment containing the 3' LTR (Fig. 2B). Therefore, because both the 5' LTR and the tk promoter are contained on internal proviral restriction fragments, SacI digestion of the DNaseI-treated nuclei allows us to assign the DNaseI sensitivity to regions of the provirus rather than to the adjacent cellular DNA.

The relative intensities of the SacI bands when the nuclei were treated with different concentrations of DNaseI showed that in the clone selected for expression of the neo gene (MEl11.E), the band containing the 5' LTR was more sensitive to digestion than was the band containing the internal promoter (Fig. 3). In contrast in the cell clone selected for expression of the tk gene (MEl11.C), the band containing the 5' LTR was less sensitive to DNaseI digestion than was the band containing the internal promoter (Fig. 3). Thus, the SacI digest confirms the BglII digest that the chromatin region around the promoter selected is more sensisitve to DNaseI digestion than the chromatin region around the non-selected promoter.

DNaseI treatment of nuclei from cell clones infected with MEl11 and selected for either NEO^R or TK^+ phenotypes was repeated for two other cell clones (one selected for NEO^R and one selected for TK^+). The DNaseI sensitivity of the chromatin around the proviruses for these clones was similar to that for the clones shown in Figures 2 and 3. That is, the NEO^R clone showed increased sensitivity to DNaseI digestion around the 5' LTR region of the provirus compared to the sensitivity to DNaseI digestion of the internal region of the provirus (data not shown). Moreover, the TK^+ clone showed increased sensitivity to DNaseI digestion in the internal region of the provirus compared to the 5' LTR region of the provirus (data not shown).

On the other hand, the sensitivity of the band containing the 3' LTR showed no concordance with phenotype. In one out of two NEO^R clones the band containing the 3' LTR was sensitive to DNaseI digestion, while in both TK^+ clones the band containing the 3' LTR was sensitive to DNaseI digestion (data not shown). However, because the 3' LTR is on a junction fragment

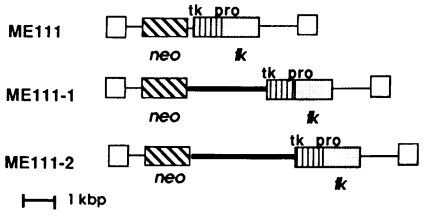

Figure 4. Transforming activities of MEl11, MEl11-1, and MEl11-2. Symbols are the same as in Fig. 1, except that the thick line represents sequences from the middle of an intron of turkey c-rel. In MEl11-1 the c-rel sequences are 2.1 kbp, and in MEl11-2 the c-rel sequences are 2.8 kbp. The TK TU are the number of cells per ml of virus transformed from a TK^+ to a TK^- phenotype by infection. The NEO TU are the number of cells per ml of virus transformed from a NEO^S to a NEO^R phenotype by infection. The TK + NEO TU are the number of cells per ml of virus transformed from TK^- and NEO^S phenotypes to both TK^+ and NEO^R phenotypes by infection.

with both BglII and SacI digestions, the DNaseI sensitivity may be 3' to the provirus, rather than in the 3' LTR itself.

Additional sequences between the promoters in MEl11 do not reduce the amount of suppression.

We inserted sequences from the middle of a large intron of the turkey c-rel gene between the neo gene and the tk promoter in MEl11 to determine if the amount of suppression was dependent on the distance between the promoters. MEl11-1 and MEl11-2 are, respectively, 2.1 kbp and 2.8 kbp larger than MEl11 (Fig. 4). Southern blot analyses of unintegrated viral DNAs of MEl11-1 and MEl11-2 showed that these viral genomes were the correct size (M. Emerman, Ph.D. thesis, University of Wisconsin-Madison, 1986). We tried, but were unable to construct viruses with larger inserts because of the packaging limits of SNV vectors (Gelinas and Temin, Proc. Natl. Acad. Sci. USA, Dec., 1986).

Several different assays were used to measure the amount of suppression. First, we counted the number of colonies transformed to a NEO^R phenotype, a TK^+ phenotype, and a NEO^R and TK^+ phenotype by infection with virus stocks prepared from pMEl11, pMEl11-1, and pMEl11-2. We found that the number of colonies transformed to both phenotypes was at least ten times lower than the number transformed to one phenotype alone for all three viruses (Fig. 4).

In addition, we compared the amount of TK activity in NEO^R cells to the TK activity in TK^+ cells in pools containing 50-100 clones transformed by

each virus. We found that the amount of suppression was about the same in each case (12% for MElll, 8% for MElll-1, 10% for MElll-2). We also picked five cell clones selected for each phenotype, measured the amounts of TK and G418-phosphotransferase activities in each clone, and compared these activities to those in MElll-infected cell clones selected for TK^+ or NEO^R. The results showed that cells infected with MElll-2 had no greater ability to express the non-selected gene than those infected with MElll (data not shown). Thus, the amount of suppression was the same whether the distance between the promoters was 2.4 kbp (MElll) or 5.2 kbp (MElll-2).

DISCUSSION

We have further characterized the phenomenon of suppression of promoters in retrovirus vectors. We found that the amount of suppression depends on the particular pair of promoters in the vector, does not depend on the distance between the promoters, and correlates with the relative DNaseI sensitivity of the chromatin surrounding the promoters. The measurements of suppression in proviruses described here were performed in rat cells infected with SNV- and MLV-based vectors. These proviruses depend on cell-coded gene products for transcription of their genes. Thus, the phenomenon of gene suppression described here is a cellular phenomenon as well as a retroviral phenomenon, and it may be relevant to the expression of chromosomal genes as well as genes introduced into cells by retrovirus vectors.

Suppression is less pronounced in MLV-based vectors and SNV-based vectors with the MLV U3 region.

Several other groups have designed retrovirus vectors with an internal promoter (19-26) based on an MLV genome. None have reported the phenomenon of gene suppression that we described with SNV-based vectors, although 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. Nonetheless, their results appeared inconsistent with the amount of suppression that we described previously with SNV-based vectors. This report explains this discrepancy between our previous results and the results with MLV-based vectors with internal promoter. We show here that promoters in vectors that contain the MLV U3 region (either in the LTR or as the internal promoter) are less suppressed by selection for expression of the other promoter than the SNV-based vectors with an internal *tk* promoter.

Possible mechanism of suppression.

Systems have been described in which the expression of a strong promoter

inhibits expression of a downstream promoter in the same orientation by the physical interference of transcription through a downstream promoter (27-29). This phenomenon has been called "promoter occlusion" in prokaryotes (27) and "transcriptional interference" in eukaryotes (28). Transcriptional interference cannot fully account for promoter suppression because the amount of suppression is independent of the relative strengths of the promoters in the vector (Fig. 1), and because the region around the 3' promoter is DNaseI insensitive when the 5' gene is selected (Fig. 2). However, transcriptional interference of the LTR transcript through the internal promoter does explain why the 3' gene is always more suppressed when the 5' gene is selected than the 5' gene is suppressed when the 3' gene is selected (Fig. 1--compare MEl11 with MEl23 and MEl39 with MEl40).

One mechanism consistent with the data is based on observations that transcription in eukaryotes is influenced by DNA topology (30-32). We hypothesize that transcription from a promoter in the newly integrated provirus precludes the efficient use of the other promoter because the establishment of a transcriptional complex at one promoter changes the nearby chromatin such that other transcriptional complexes are not efficiently formed. In proviruses that show a high amount of suppression, our finding that, unlike chromatin nearby the selected promoter, the chromatin around the non-selected promoter is relatively insensitive to digestion with DNaseI is consistent with this hypothesis. This process would be promoter-specific because different promoters bind different transcription factors that might distort to different extents the chromatin surrounding these promoters. Alternatively, the chromatin topology might be more important for certain promoter-specific transcription factors than for others. That a retroviral promoter induces a transcriptionally unfavorable topology on another gene was demonstrated in another system (33,34). In addition, the chromatin region around an integration site could influence how one or both of the promoters is transcribed (35-37).

Implications for retrovirus vectors.

Retrovirus vectors that express two genes are of two types. In one type, one gene is transcribed from the LTR and the other gene is transcribed from an internal promoter. In the other type, both genes are transcribed from the LTR and one of the genes is translated from a spliced transcript. Vectors with an internal promoter avoid the problems associated with sequences in the intron that can interfere with splicing the message for the second gene (37,38, Dougherty and Temin, submitted). We have constructed

retrovirus vectors with at least six different internal promoters. We find that the amount of suppression is not wholly predictable. Furthermore, the amount of suppression varies from cell clone to cell clone (1), and it can vary in different pools of clones infected with the same virus (Fig. 1).

In vectors which show a high amount of suppression, the suppression can not be relieved by increasing the distance between the LTR and the internal promoter by 2.8 kbp (Fig. 4) nor by constructing the vector such that the internal promoter directs transcription in the opposite orientation to that of the LTR (M. Emerman, Ph.D. thesis, University of Wisconsin-Madison, 1986). Our experiments were done in rat cells, a cell type semi-permissive for SNV replication (40). The amount of suppression may be different in other cell types.

ACKNOWLEDGMENTS

We thank J. Couch, S. Hellenbrand, J. Rein, and B. Rhode for technical assistance, M. Groudine for suggestions on DNaseI digestions, M. Kriegler for unpublished plasmids, and J. Dougherty, J. Embretson, C. Gelinias, T. Gilmore, J. Ross, and A. Panganiban for helpful comments on the manuscript.

This work was supported by Public Health Service grants CA-22443 and CA-07175 from the National Institute of Health. M.E. was supported by Public Health Service training grant CA-09135-10. H.M.T. is an American Cancer Society research professor.

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