JOURNAL OF VIROLOGY, Nov. 1987, p. 3454–3462
0022-538X/87/113454-09$02.00/0

Vol. 61, No. 11

Transcription from a Spleen Necrosis Virus 5′ Long Terminal Repeat Is Suppressed in Mouse Cells

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Received 18 May 1987/Accepted 22 July 1987

To determine the block(s) to spleen necrosis virus (SNV) replication in mouse cells, we studied the expression of a dominant selectable marker, *neo*, or a gene whose product is easily assayed, the chloramphenicol acetyltransferase (*cat*) gene, in SNV-derived and murine leukemia virus-derived vectors. Using transient (CAT) and stable (Neo^r phenotype) transfection assays, we showed that the SNV promoter was used in mouse cells only when the 3′ SNV long terminal repeat (LTR) was absent. Infection of mouse cells with recombinant SNV viruses was 1% as efficient as infection of permissive dog (D17) cells. The SNV proviruses in mouse cells appeared normal by Southern blot analysis, indicating that their integration probably occurred by normal mechanisms. S1 nuclease analyses of Neo^r mouse cell clones, each harboring a single recombinant SNV provirus, showed that the selected (internal) promoter was active, but that the 5′ SNV LTR promoter was not. However, in the rare ($<10^{-6}$) Neo^r colonies in which expression of the 5′ LTR was selected, both promoters were active. Thus, the block to SNV infection of mouse cells is at least at two levels; one is a 100-fold-decreased efficiency at some step(s) up to and including integration, and the other is at transcription.

Retroviral gene expression is controlled at many levels. In cells permissive for retroviral replication, regulation is often at the level of transcription, determined by tissue-specific promoters or enhancers (or both) present in the viral long terminal repeat (LTR), by viral proteins, and by host cell chromatin structure (for a review, see reference 50). For example, enhancers in both avian leukosis virus and murine leukemia virus (MLV) LTRs influence levels of virus production in particular tissues or cell types (for a review, see reference 50). In addition to the *cis*-acting effect of the viral LTR, human and bovine retroviruses encode *trans*-acting regulatory proteins which enhance transcription from the LTR (3, 5). Expression from both endogenous and exogenously acquired retroviral genomes also appears to be regulated in some cases by chromatin structure surrounding the proviral integration site (for a review, see reference 50).

In semi- and nonpermissive cells, the regulation of retroviral gene expression can occur at any step(s) in the life cycle. The most common block to replication is the absence of cell surface receptors for the virus (4, 20, 25, 32, 48, 51). However, there are also postentrance controls of retroviral replication. For example, (i) the replication of N- or B-tropic MLVs is inhibited between entrance and integration by the *Fv-1* locus in mouse cells (25, 27–29, 32, 45); (ii) both internal and LTR promoters can be suppressed in proviruses from spleen necrosis virus (SNV)- and MLV-derived vectors in rat cells (10–12); (iii) the gag polyprotein is not properly processed in Rous sarcoma virus-infected rat and mouse cells (6, 15); and (iv) the replication of MLV is inhibited at multiple steps in murine teratocarcinoma cells (1, 14, 17, 26, 28, 33, 38, 44, 46). In some cases, the host flanking sequences appear to be involved in the suppression (1) or activation of retroviral expression in nonpermissive cells (40).

SNV, a retrovirus of the avian reticuloendotheliosis species, replicates in chicken and dog cells but not in mouse cells. To determine which step(s) of the retroviral life cycle was blocked in SNV infection of mouse cells, we studied gene expression from SNV- and MLV-derived vectors in mouse cells. Previously, we used similar vectors to determine the block(s) to SNV replication in rat cells (8). SNV infection of rat cells (a semipermissive host) proceeds normally from virus binding through proviral integration. The promoter efficiency of the 5′ SNV LTR, as measured by the chloramphenicol acetyltransferase (CAT) assay and by a Neo^r phenotype, is similar to the MLV LTR in rat cells. Thus, the block(s) to SNV replication in rat cells is posttranscriptional. In contrast, we find that there are at least both pretranscriptional and transcriptional blocks to SNV replication in mouse cells.

MATERIALS AND METHODS

Nomenclature and abbreviations. All SNV-based recombinant vectors were derived from a molecular clone of SNV. A molecular clone of REV-A (pSW253) was used as the source for helper virus.

Plasmid DNAs constructed in our lab contain a "p" to distinguish them from virus derived from those plasmids [e.g., ME111(Rev) denotes a virus stock derived from cotransfection of pME111 and Rev-A helper virus DNAs].

neo is the aminoglycoside 3′ phosphotransferase gene from the bacterial transposable element Tn*5* (30). When the *neo* gene is expressed in eucaryotic cells, it confers resistance to the drug G418 (43). The neomycin-transforming unit (NEO TU) titer of a virus stock is the number of cells converted to a G418^r phenotype per milliliter of virus.

Stocks of amphotropic murine leukemia virus (Am-MLV; 21, 39) were derived by transfection of NIH 3T3 cells with a molecular clone of Am-MLV strain 4070A (prA8; 41; kindly provided by J. Sorge).

Plasmids. All plasmids used in this study were constructed by standard techniques (34). A brief description of each vector is listed below.

pJD215 (see Fig. 3A; 9) is a SNV-derived vector in which the *neo* gene is expressed from the 5′ LTR. *neo* transcripts end in the 3′ SNV LTR.

pJE118 (see Fig. 3B) is a vector in which the *cat* gene is expressed from the SNV LTR. pJE118 was derived from

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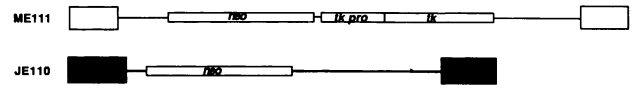


VIRUS	NEO TU/ ml	
	DOG	MOUSE
ME111(REV)	2×10^5	$0 (\leq 10^2)$
ME111(Am-MLV)	1×10^4	$0 (\leq 10^2)$
JE110(REV)	1×10^2	$0 (\leq 10^{-3})$
JE110(Am-MLV)	1×10^5	3×10^4

FIG. 1. NEO TU titers of pseudotyped viruses on dog and mouse cells. Large open boxes represent SNV LTRs, and shaded boxes represent Ha-MSV-derived LTRs. Thin straight lines represent SNV-derived sequences internal to the LTRs. Heavy straight lines represent Ha-MSV-derived sequences (7) internal to the LTRs. The rectangular box marked *neo* represents the bacterial Tn5-derived aminoglycoside 3′ phosphotransferase gene (30). The rectangular box marked *tk pro* represents the herpes simplex virus thymidine kinase gene promoter. The rectangular box marked *tk* represents the herpes simplex virus thymidine kinase-coding region. *Neo*-containing SNV-derived or MLV-derived genomes were rescued from nonproducer (helper virus-free) dog cell clones with Rev-A or Am-MLV helper virus as previously described (8). The NEO TU titer of each stock was determined in parallel on dog (D17) and mouse (NIH 3T3) cells. These results are representative of data from virus stocks derived and assayed at three different times. There was a 10-fold variation in the titers of virus stocks derived by superinfection [i.e., the NEO TU titers of JE110(Rev) ranged were between 10^2 to 10^3 per ml]. However, the relative NEO TU titer was approximately the same [i.e., the ratio of the NEO TU titer of ME111(Rev) to JE110(Rev) was always between 500 and 1,000]. The data from dog cells have been previously reported (8).

pME111 (see below) by replacing the *neo* gene with the *cat* gene and then deleting both the *tk* promoter and *tk* coding sequences by an *Sma*I deletion.

pJE129 (see Fig. 3A; 9) is a vector in which the *neo* gene is expressed from the SNV LTR. pJE129 was derived from pJD215 by removing the Rev E.

pJE133 (see Fig. 3B; pREV133 in reference 8) is a vector in which the *cat* gene is expressed from the SNV LTR. *cat* transcripts are terminated by the polyadenylation signal from simian virus 40 (SV40).

pJE120 (see Fig. 3B; pMLV120 in reference 8) is a vector in which the *cat* gene is expressed from the Harvey (Ha)-MSV LTR. *cat* transcripts are terminated by the polyadenylation signal from SV40.

pSV2CAT (see Fig. 3B; 16) expresses the *cat* gene from the SV40 early promoter. The transcription termination signals are within the same SV40 sequences used in pJE133 and pJE120 (see above).

pSV2neo (see Fig. 3A; 43) is a *neo*-containing vector with the SV40 early promoter 5′ to the *neo* gene. The transcription termination signals are within SV40 sequences 3′ to the *neo* gene.

pJE110 (Fig. 1; pMLV110 in reference 8) is a Ha-MSV-based vector that contains the *neo* gene expressed from the 5′ LTR.

pME111 (see Fig. 1, 2, 3A, 4, 7, and 8; pREV111 in reference 8; pME111 in reference 10) is an SNV-based vector that contains the *neo* gene expressed from the 5′ LTR and the herpes simplex virus thymidine kinase gene (*tk*) expressed from the *tk* promoter.

pME123 (Fig. 2 and 4; 10) is an SNV-based vector in which the *neo* gene is expressed from an internal herpes simplex virus thymidine kinase gene (*tk*) promoter. The *tk* promoter lies within a 0.5-kilobase (kb) *Bam*HI-to-*Bgl*II fragment (49).

pME129 (Fig. 2, 3A, and 4; 11) is an SNV-based vector in which the *neo* gene is expressed from the SV40 early promoter. The SV40 early promoter is present on an *Nde*I-to-*Hin*dIII fragment from SV2neo (43). This *Nde*I-to-*Hin*dIII fragment contains pBR322-derived sequences from *Nde*I to *Pvu*II and SV40 sequences from *Pvu*II to *Hin*dIII.

pME149 (see Fig. 2, 3A, 4, and 6; 12) is an SNV-based vector in which the *neo* gene is expressed from an MLV promoter. The MLV promoter is present on a 0.5-kilobase-pair (kbp) *Rsa*I-to-*Rsa*I fragment from a molecular clone of Ha-MSV, H1 (7).

pME136 (Fig. 3A; 9) is an MLV-based vector in which the *neo* gene is expressed from a recombinant 5′ MLV LTR. pME136 was derived from AFVXM (kindly provided by M. Kriegler). The 5′ LTR of pME136 contains Abelson MLV-derived sequences up to the *Kpn*I site (50) and Ha-MSV-derived sequences 3′ to the *Kpn*I site. The 3′ LTR has Moloney MLV-derived sequences up to the *Kpn*I site and Abelson MLV-derived sequences 3′ to the *Kpn*I site.

pME151 (Fig. 2A and 5; 12) is an AFVXM-based vector in which the *neo* gene is expressed from the SNV promoter. The SNV promoter is present on a 0.4-kbp *Eco*RI-to-*Ava*I fragment from pSW210 (49). This *Eco*RI-to-*Ava*I fragment contains all of the SNV U3.

CAT assay. The CAT assay was done essentially as previously described (16). NIH 3T3 cells were treated for 1 h with 60 mM chloroquine before transfection. Plasmid DNA (5 to 20 μg) was transfected in a 100-mm dish by the calcium phosphate method (18). These DNA amounts were in the linear range for uptake and expression in mouse cells. Extracts were made 48 h after transfection. Equal amounts of protein (25 to 100 μg), as determined by the Bradford assay (2), were assayed from each cell extract. These

VIRUS	NEO TU/ml	
	DOG	MOUSE
ME111 (REV)	1.5×10^6	$0 (\leq 10^6)$
ME123 (REV)	2×10^5	150
ME129 (REV)	4×10^5	325
ME149 (REV)	7×10^5	9×10^3
ME151(Am-MLV)	3×10^3	2×10^3

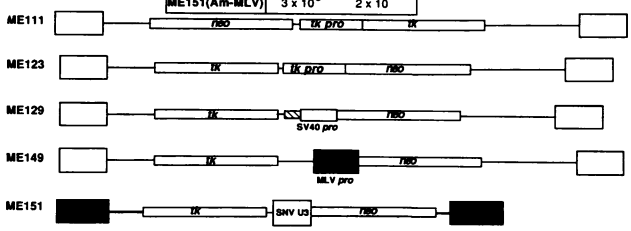
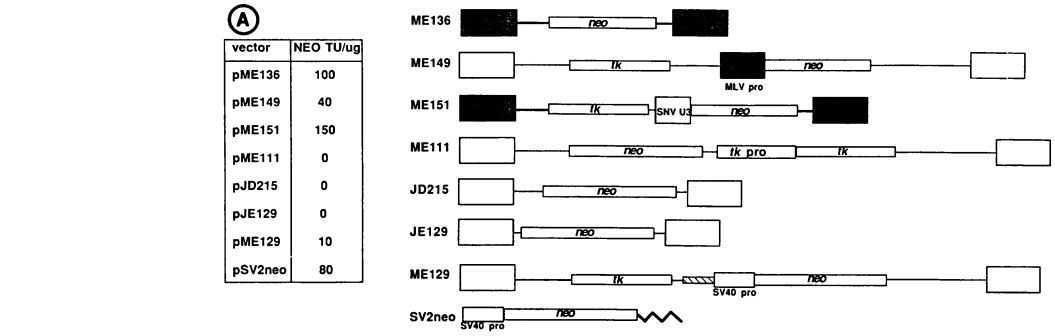


FIG. 2. NEO TU titers ot SNV-derived viruses with different internal promoters and a MLV-derived virus with a SNV internal promoter. The box marked SV40 *pro* represents the SV40 early region promoter. The hatched box in pME129 represents pBR322 sequences from *Nde*I to *Pvu*II. The shaded box marked MLV *pro* represents the U3 and 5′ 30 bp of R from Ha-MSV. The box marked SNV U3 represents the entire SNV U3. Mouse and dog cells were infected in parallel. Virus stocks were derived by cotransfection of CEF with pME111, pME123, pME129, or pME149 DNAs and Rev-A helper virus DNA or by superinfection with Am-MLV of Neo^r dog cells transfected with pME151. See the legend to Fig. 1 for a description of symbols not mentioned here. These results are averages from at least two transfections or superinfections. Virus titers varied fourfold from transfection to transfection [i.e., the NEO TU titers of ME111(Rev) were 0.5×10^6 to 2.0×10^6 per ml].



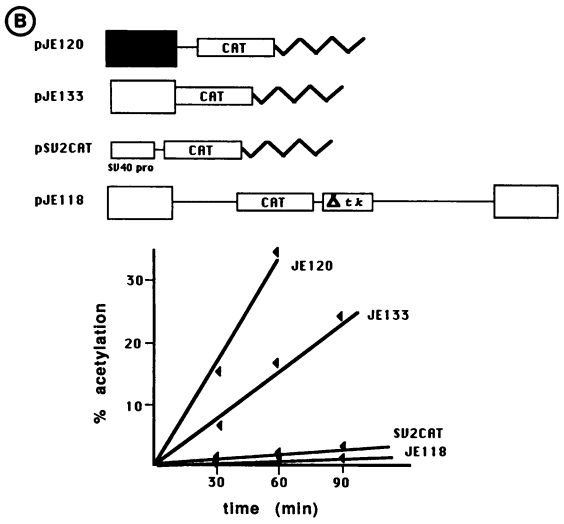


FIG. 3. The SNV promoter is not active in mouse cells when adjacent to a 3′ SNV LTR. (A) Mouse cells were transfected with *neo*-containing vectors, and the concentration of NEO TU per microgram was determined. Results are averages from three separate transfections. The variation in NEO TU per microgram was twofold, and the relative difference in NEO TU per microgram between any two vectors was always the same. (B) Mouse cells were transfected with *cat*-containing vectors, and the CAT activity was determined 48 h after transfection. The box marked CAT represents the *cat* gene (16). The box marked SV40 pro represents the SV40 early promoter. pJE118 contains a deletion of the herpes simplex virus *tk* gene (Δ *tk*). pJE133 was previously called pREV133 (8). The wavy line represents the SV40 polyadenylation signal. These results are averages from at least three separate transfections. See the legends to Fig. 1 and 2 for an explanation of symbols not described here.

amounts of protein gave a linear increase in acetylation with time for each transfected DNA.

Stable transfection assay. NIH 3T3 cells were transfected with various *neo*-containing plasmid DNAs by the dimethyl sulfoxide-polybrene method (31). At 1 to 2 days after transfection, G418 was added to a concentration of 400 μg/ml. Fresh selective medium was added every 3 days thereafter. Transfected DNA, 0.01 to 10.0 μg, gave a linear dose response.

Cells. Chicken embryo fibroblasts (CEF; from embryos from SPAFAS, Inc.), D17 (dog) cells, and D17-C3 (Rev helper cells) were grown as previously described (10, 49). NIH 3T3 and BALB/3T3 cells were grown in Temin-modified Eagle minimal essential medium with 7% calf serum. BALB/3T3 cells were kindly provided by R. Risser.

Neo^r NIH 3T3 cell clones containing ME111, ME123, ME129, ME149, or ME151 proviruses were isolated in the following manner. NIH 3T3 cells were infected with 10-fold dilutions of ME111(Rev), ME123(Rev), ME129(Rev), ME149(Rev), or ME151(Am-MLV). Selection for Neo^r NIH 3T3 cells began 24 h after infection by the addition of 400 μg of G418 (GIBCO Laboratories) per ml. After G418 selection, individual Neo^r colonies on plates with 1 to 50 Neo^r colonies were isolated by using cloning cylinders, trypsinized, transferred to cloning wells, and expanded.

Virus. Virus stocks were made by three methods.

(i) Superinfection of helper-free D17-derived cell clones. Helper-free Neo^r D17 cell clones containing either a JE110 or an ME111 provirus were infected with stocks of Rev-A or Am-MLV. Five days after infection, supernatants were collected and subjected to three freeze-thaw cycles before the infection of fresh cells.

(ii) Cotransfection. CEF on 60-mm dishes were cotransfected with SNV-derived vector and Rev-A helper virus DNAs in a ratio of 5 μg to 0.2 μg by using the dimethyl sulfoxide-polybrene method (31). Virus was harvested 5 days after transfection.

(iii) Superinfection of transfected cells. D17 cells were transfected with pME151 and selected for Neo^r. Neo^r transfectants were superinfected with Am-MLV, and the supernatants were harvested 5 days after infection. Before infection of fresh cells, the virus stocks were subjected to three freeze-thaw cycles.

Stocks of wild-type SNV were quantified by cytopathic effects on CEF by endpoint dilution (47).

Infections were done in the presence of 50 μg of polybrene per ml.

Nucleic acid analyses. S1 nuclease analyses were done essentially as previously described (9, 36). The 5′-end-labeled probe (pME134) for the analysis of ME111 and ME123 proviruses and the 5′-end-labeled probe (pSV2neo) for the analysis of ME129 proviruses have been previously described (11). The 5′-end-labeled probe for the analysis of ME149 proviruses was made by subcloning the *Rsa*I-to-*Rsa*I fragment of the Ha-MSV LTR into the *Sma*I site of pUC12(pME142). pME142 was digested with *Bam*HI, treated with calf intestinal phosphatase (Boehringer Mannheim Biochemicals), and then digested with *Pvu*I. After gel purification, the fragment was 5′ end labeled by using T4 polynucleotide kinase. The probe used for the analysis of ME151 proviruses was made from pME145. pME145 contains the entire U3 of SNV (*Eco*RI to *Ava*I of pSW210; 49) and the *neo* gene (*Bgl*II to *Hin*dIII) cloned into pGem1 (Promega Biotec). pME145 was digested with *Pvu*II and then treated with calf intestinal phosphate. After gel purification,

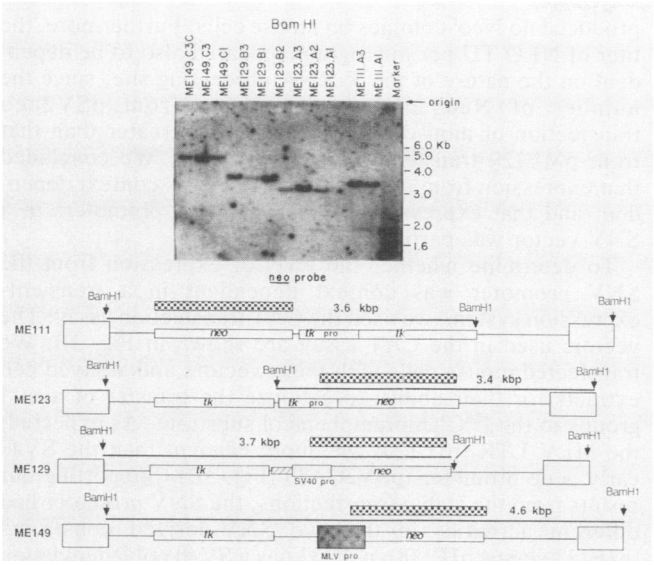


FIG. 4. Southern blot analysis of SNV-derived proviruses from infected mouse cell clones. Genomic DNAs were isolated from individual cell clones, digested with *Bam*HI, and separated in a 1.2% agarose gel. After transfer, the nitrocellulose filter was hybridized with a 1.3-kbp ^{32}P-labeled *neo* probe. Diagrams of each provirus, the *Bam*HI sites in each provirus, and the expected size for a *neo*-hybridizing fragment are shown. The *neo* probe is shown by a checkered box, and the *Bam*HI fragment the *neo* hybridizes to is denoted by a line between the appropriate *Bam*HI sites.

the blunt ends were 5′ end labeled by using the protocol of Maniatis et al. (34). Hybridizations were done at 47 to 52°C for 16 h.

RNAs homologous to the *tk* promoter (pJE189), the SV40 promoter region (pJE194), and the MLV promoter (pJE188) were generated by treatment of linearized pGem-3-derived plasmids with T7 polymerase according to the protocols of the manufacturer (Promega Biotec). pJE189 contained the *tk* promoter region present in pME123 (*Bam*HI-to-*Bgl*II fragment) subcloned into the *Bam*HI site of pGem-3. pJE194 contained the SV40 early promoter present in pME129 (*Hin*dIII site 5′ to SV2neo-derived sequences to *Bgl*II site in *neo*) subcloned into pGem-3. To make pJE190, pGem-3 was digested with *Hin*dIII and *Bam*HI. The linearized pGem-3 was ligated to the SV40 promoter-containing fragment. pJE188 contained the MLV promoter from pME142 (*Eco*RI-to-*Bam*HI fragment) subcloned into pGem-3.

Primer extension analyses were done as previously described (9, 35). Primers (Pr2 is a 20-mer, University of Wisconsin Biotechnology Center) were 5′ end labeled by using T4 polynucleotide kinase. Pr2 is homologous to sequences within the SNV LTR from 0.531 and 0.550 kbp relative to the 5′ end of the provirus.

Genomic and unintegrated linear viral DNAs were isolated and analyzed as previously described (19, 23, 24, 42). Quantification of the relative amounts of defective and replication competent virus was done as previously described, by using unintegrated linear viral DNA (8). Genomic DNAs were digested with restriction enzymes and separated on 1.0 to 1.5% agarose gels. Radiolabeled probes (13) used to analyze the structure of proviruses were (i) the *neo* gene (*Hin*dIII to *Hin*dIII, from pME110; 7) without plasmid sequences, (ii) the *tk* coding region (*Bgl*II to *Bam*HI from SW227; 49) without plasmid sequences, and (iii) the SNV LTR (without plasmid sequences).

RESULTS

Mouse cells are not permissive for SNV infection. We infected mouse cells (NIH 3T3) and CEF in parallel with SNV, at multiplicities of infection of 10 and 1 IU per cell, respectively. Five days after infection, the titers of progeny virus were determined by assay of cytopathic effects on CEF (47). Supernatants from SNV-infected mouse cells produced no cytopathic effects on CEF (data not shown). Progeny virus titers from supernatants of SNV-infected CEF were 10^7 IU/ml (data not shown). This block to SNV replication in mouse cells was not the result of restriction by the *Fv-1* locus (24, 27–29, 32, 45), since SNV-infected BALB/3T3 cells also produced no detectable progeny virus (data not shown). Thus, the block(s) to SNV replication in mouse cells is very profound and probably occurs during the first round of infection.

SNV-derived genomes pseudotyped with SNV or Am-MLV proteins and MLV-derived genomes pseudotyped with SNV proteins do not replicate in mouse cells. If the only block to SNV replication in mouse cells resulted from the lack of cell surface receptors for the SNV *env* gene product, SNV genomes pseudotyped in MLV proteins should efficiently form proviruses in mouse cells. Earlier work (8) showed that

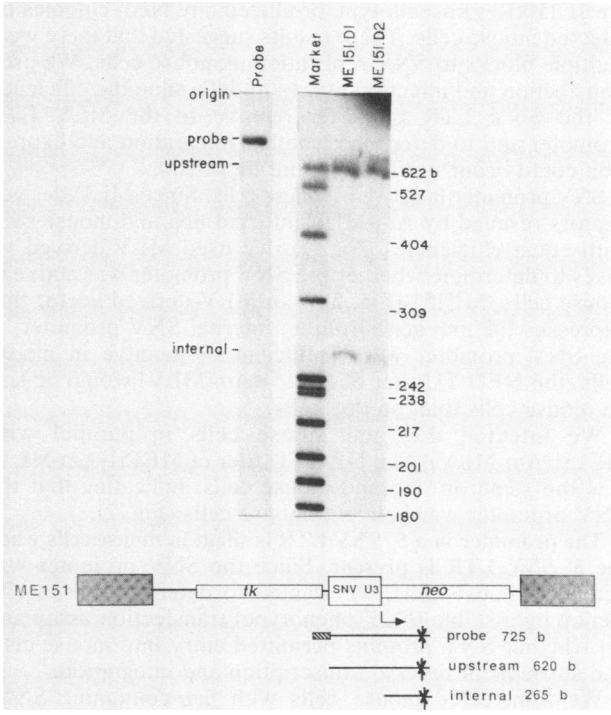


FIG. 5. S1 nuclease analysis of RNA from two ME151-infected mouse cell clones. S1 analysis was done as previously described (9, 36). The probe used was a 725-bp fragment from pME145. pME145 was cut with *Pvu*II and was 5′ end labeled with T4 polynucleotide kinase. RNA isolated from two Neo^r ME151-containing NIH 3T3 cell clones (ME151.D1 and ME151.D2) was hybridized with the probe at 50°C for 16 h. After digestion with S1 for 0.5 h at 37°C, S1 nuclease-resistant fragments were separated on a 6% polyacrylamide–8.3 M urea gel. The S1 nuclease-resistant fragments expected and their respective sizes are shown. The hatched box represents 100 bp of pUC12-derived sequence which is not homologous to sequences present in the provirus. The dark line represents sequences present in the probe which are homologous to sequences in provirus-derived transcripts. Asterisks denote ^{32}P-5′ end labels. b, Bases.

these pseudotypes could be made at a high efficiency when SNV genomes were rescued by Am-MLV proteins and were effective in mediating SNV infection of dog and rat cells.

SNV-derived (ME111; Fig. 1) or MLV-derived (JE110; Fig. 1) proviruses containing *neo* were rescued from helper virus-free D17-derived cell lines by superinfection with Rev-A or Am-MLV. The NEO TU titer of these pseudotyped viruses was determined on dog (D17) and mouse (NIH 3T3) cells (Fig. 1). There was only a threefold difference in the NEO TU titer of JE110(Am-MLV) on dog and mouse cells (1×10^5 and 3×10^4 NEO TU/ml, respectively), indicating that this virus is capable of replicating with a similar efficiency in both species for all steps in the replicative cycle through integration and expression. However, surprisingly, infection of mouse cells with a SNV-derived genome packaged in Am-MLV proteins [ME111(Am-MLV)] produced no Neo^r colonies. This result indicated that at least one block to SNV replication in mouse cells is after entrance, since the presence of Am-MLV *env* protein should permit internalization of the virion. Infection with JE110(Rev) also produced no Neo^r colonies on mouse cells. In this case, the lack of Neo^r colonies could be the result of the absence of cell surface receptors for SNV proteins on mouse cells. The lack of Neo^r colonies from ME111(Rev)-infected mouse cells is not surprising, given that neither the ME111(Am-MLV) nor the JE110(Rev) pseudotype produced any Neo^r colonies on infected mouse cells. These results suggested that there were multiple blocks to SNV replication in mouse cells. We used transfection and infection assays to determine the efficiency of the SNV LTR promoter relative to the MLV LTR promoter and to determine whether integration and expression could occur normally in murine cells.

SNV promoter is active in mouse cells. Since MLV-derived vectors rescued by Am-MLV-infected dog and mouse cells with equal efficiencies (Fig. 1), we used MLV-derived viruses to determine whether the SNV promoter was active in mouse cells. ME151 (Fig. 2) is an MLV-derived vector that expresses the *neo* gene from an internal SNV promoter. If the SNV promoter were inefficient or inactive in mouse cells, the NEO TU titer of ME151(Am-MLV) would be less on mouse cells than on dog cells.

We infected dog and mouse cells in parallel with ME151(Am-MLV). The NEO TU titer of ME151(Am-MLV) was the same on dog and mouse cells, indicating that the SNV promoter was active in mouse cells (Fig. 2).

The promoter in a 5′ SNV LTR is silent in mouse cells when the 3′ SNV LTR is present. Since the SNV promoter was active in mouse cells, we wanted to determine (i) its efficiency by a stable (Neo^r phenotype) transfection assay and (ii) whether SNV proteins permitted entry into mouse cells and subsequent reverse transcription and integration.

We transfected mouse cells with *neo*-containing SNV-derived or MLV-derived vectors and determined the number of NEO TU per microgram of transfected DNA (Fig. 3A). When *neo* was expressed from an MLV promoter in either an SNV vector (pME149) or an MLV vector (pME136), or from an SNV promoter in an MLV vector (pME151), the numbers of Neo^r colonies per microgram were similar (40 to 150 Neo^r colonies per μg of transfected DNA). However, when *neo* was expressed from a 5′ SNV LTR and a 3′ SNV LTR was present, there were no Neo^r colonies (pME111, Fig. 3A). This lack of *neo* expression from transfected pME111 DNA was not the result of the *tk* gene, SNV sequences 3′ to *tk* and 5′ to the 3′ LTR, or the 400 bp of SNV sequences just 5′ to the *neo* gene, since SNV-derived vectors (pJD215 and pJE129) without these sequences also produced no Neo^r colonies on mouse cells. Furthermore, the titer of NEO TU per microgram appeared also to be dependent on the nature of the 3′ RNA processing site, since the number of Neo^r colonies resulting from pSV2neo transfection of mouse cells was eightfold greater than that from pME129 transfection of mouse cells. We concluded that expression from the SNV promoter was context dependent and that expression of some internal promoters in a SNV vector was partially inhibited.

To determine whether the level of expression from the SNV promoter was context dependent in a transient-expression system, we used the CAT transfection assay. The vectors used in the CAT assay are shown in Fig. 3B. We transfected mouse cells with these vectors and assayed cell extracts for their ability to catalyze the transfer of acetyl groups to the [^{14}C]chloramphenicol substrate. As expected, the MLV LTR (pJE120) was more efficient than the SV40 early gene promoter (pSV2CAT) (Fig. 3B). Supporting our results from the stable transfections, the SNV promoter had different activities in the two SNV-derived constructs (pJE133 versus pJE118). pJE133 has a SV40 polyadenylation signal 3′ to *cat* and produced more *cat* activity than pSV2CAT, whereas pJE118 has a SNV LTR 3′ to *cat* and produced less CAT activity than pSV2CAT.

Since expression from the SNV 5′ LTR was reduced when the 3′ SNV LTR was present, even if SNV could integrate efficiently in mouse cells, SNV replication would be inhibited at the level of expression.

SNV-derived proviruses are integrated normally, although at a reduced efficiency, in mouse cells. To determine whether SNV could form a normal provirus in mouse cells, we infected mouse cells with recombinant SNV viruses. These viruses expressed *neo* from the SNV LTR (ME111) or from one of three internal promoters (*tk* in ME123, SV40 early in ME129, and MLV in ME149; Fig. 2) which should be active in mouse cells. Viruses were derived from cotransfection of CEF with DNA of each vector and Rev-A helper virus and were assayed for their respective NEO TU titers on dog and mouse cells. The NEO TU titers of these viruses on mouse cells correlated with their promoter strength, as determined by the CAT and *neo* transfection assays (i.e., ME149 > ME129 > ME123; Fig. 3; data not shown). However, the NEO TU titer of ME149(Rev) was almost a factor of 100 less on mouse than on dog cells even though it contained a strong (MLV) promoter. Analysis of genomic DNA 2 days after infection with wild-type SNV also indicated that SNV proviral formation was at least 100-fold less efficient in mouse cells relative to dog cells (data not shown). This result indicated that the steps in SNV infection of mouse cells up through integration and expression are approximately a factor of 100 less efficient than in a permissive cell.

Neo^r mouse cells infected with ME111 were very rare. These Neo^r mouse colonies arose at a frequency of less than or equal to 10^{-6} relative to dog cells infected in parallel. Mouse cells infected with JD215 and JE129 (Fig. 3A) also rarely produced Neo^r colonies on mouse cells, indicating that SNV sequences 3′ to the *neo* gene and 5′ to the 3′ LTR in ME111 were not involved in the suppression of *neo* expression in mouse cells (data not shown).

To determine whether integration of the SNV proviruses in mouse cells was normal, we picked individual cell clones and analyzed the proviruses by Southern blotting. The DNAs from two or three cell clones of each type (ME111, ME123, ME129, and ME149) were digested with *Bam*HI, separated by electrophoresis in a 1.2% agarose gel, transferred to a nitrocellulose filter, and hybridized with various

probes. The results from a filter which was probed with *neo* (Fig. 4) indicated that the 3′ ends of ME123 and ME149 proviruses were intact and that two-thirds of the internal region of ME111 and ME129 proviruses were intact. *Bam*HI restriction enzyme-digested genomic DNAs from mouse cell clones infected with ME111 and ME129 were hybridized with a LTR probe to determine whether the 3′ LTR was intact. In both clones, a 1.6-kbp band was seen which corresponded to an intact 3′ region (data not shown). *Sst*I restriction enzyme-digested genomic DNAs from all eight mouse cell clones were hybridized with an LTR probe to determine whether the 5′ LTR was intact. The results of these experiments showed that all proviruses contained an intact 5′ LTR, that there was a single recombinant proviruses in each cell clone, and that there was no Rev-A helper virus present in any of these clones (data not shown). These proviruses, thus, appeared normal and were apparently integrated by normal mechanisms.

There are no detectable steady-state accumulations of transcripts from the 5′ SNV LTR when an internal promoter is selected. Whereas the reduced efficiency of SNV infection of mouse cells was partially (by a factor of 100) the result of events from absorption through integration, the results from the *neo* and *cat* transfections indicated that a postintegration event was also involved. If an additional block to SNV replication were transcription from the 5′ LTR, there should be no detectable transcripts initiated at the 5′ LTR in cell clones with intact SNV proviruses. To test this hypothesis, we used S1 nuclease analyses to determine the steady-state levels of RNA in two cell clones each of mouse cells infected with ME111, ME123, ME129, ME149, and ME151.

The steady-state RNAs from two Neo^r ME151 (a MLV-derived vector)-infected mouse cell clones (ME151.D1 and ME151.D2) were analyzed by the use of a 5′-end-labeled probe that had 620 bases of sequence homologous to the ME151 provirus (Fig. 5). If transcripts were correctly initiated at the cap site in the SNV U3, a 265-base protected fragment would be produced after S1 nuclease digestion. A 620-base protected fragment was expected from transcripts initiated within the MLV LTR (upstream). In both cell clones analyzed, S1 nuclease-resistant bands of 265 and 620 bases were seen (Fig. 5), demonstrating that the SNV U3 was a functional promoter in mouse cells and confirming that the unselected MLV LTR was functional too.

A similar analysis was done on RNA isolated from two cell clones each of Neo^r ME123-, ME129-, and ME149-infected mouse cells. Data from two ME149 cell clones (ME149.C1 and ME149.C3) are shown in Fig. 6. Transcripts initiated at the MLV cap site should produce a 30-base S1 nuclease-resistant fragment, whereas any transcripts initiated upstream of the MLV sequences should give a 500-base protected fragment. Two bands of approximately 30 bases were seen in RNA from both cell clones which corresponded to initiation(s) near the MLV cap site. However, there was no 500-base S1-resistant fragment, indicating that there were no upstream-initiated transcripts. (Occasionally, there was a 138-base band in all lanes where probe was added. This 138-base band was also present in the lane with probe alone [without S1 treatment]. When present, this band did not vary much in intensity with different RNAs or different RNA concentrations.) Similar results were also obtained with two cell clones each of ME123 and ME129; there were no upstream-initiated transcripts (data not shown).

To show that the upstream-initiated RNAs could be detected under the hybridization and S1 nuclease conditions used, we cloned the appropriate regions (see Materials and Methods) into pGem-3. Hybridization of T7-generated RNAs homologous to the upstream-initiated transcripts for each type of provirus and probe (ME123, ME129, and ME149) protected the correct-size band (data not shown), indicating that if the transcripts were present in RNAs from each cell clone, it would have been detected under the conditions used. Thus, for all six of the intact SNV-derived proviruses selected for expression from an internal promoter, there were no stable RNA transcripts from the 5′ SNV LTR.

S1 nuclease analysis was also done for two rare Neo^r ME111-infected mouse cell clones (ME111.A1 and ME111.A3; Fig. 7). The 5′-end-labeled probe was homologous to sequences near the internal (*tk*) promoter. Bands of 500 and 50 bases were expected for transcripts initiated at the upstream (SNV 5′ LTR) and internal (*tk*) promoters, respectively (Fig. 7). The S1 nuclease-resistant fragments seen after hybridization of the probe with RNA from ME111.A1 and ME111.A3 mouse cell clones were of the expected sizes (i.e., 500 bases [upstream transcripts] and 50 bases [*tk* promoter-initiated transcripts]). This result was in contrast to the results with SNV-derived proviruses in which the internal promoter was selected (i.e., there were no upstream transcripts in ME123, ME129, and ME149 cell clones). In the rare ME111 proviruses, when the selected promoter (5′ SNV LTR) was active, so was the unselected (internal) promoter.

Since all the cell clones analyzed contained a single

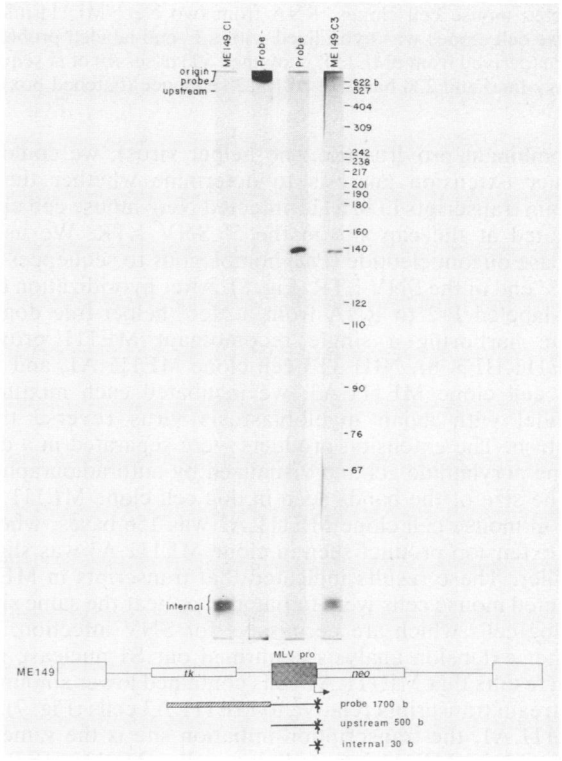


FIG. 6. S1 nuclease analysis of RNA from two ME149-infected mouse cell clones. The probe used contained 500 bases (b) of sequence from Ha-MSV (heavy line) and 1.2 kb of pUC12-derived sequences (hatched box). The probe was 5′ end labeled and was hybridized with 10 to 30 μg of total RNA from two Neo^r ME149-containing NIH 3T3 cell clones (ME149.C1 and ME149.C3). S1 conditions and fragment separations were done as described in the legend to Fig. 5.

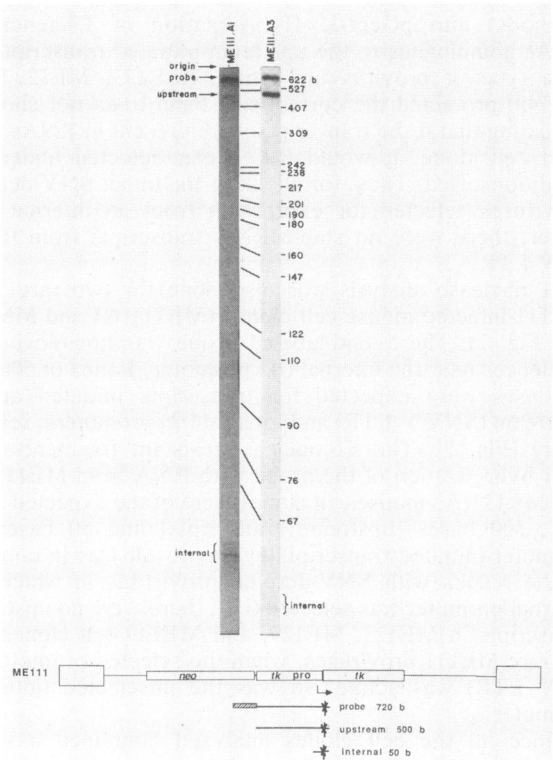


FIG. 7. S1 nuclease analysis of RNA from two Neo^r ME111-infected mouse cell clones. RNA from two Neo^r ME111-infected mouse cell clones was hybridized with a 5′-end-labeled probe. The probe (derived from pME134) contained 500 bases (b) of *tk* sequence (heavy line) and 220 bases of pBR322 sequence (hatched box).

recombinant provirus (i.e., no helper virus), we could use primer extension analysis to determine whether the upstream transcripts in ME111-infected Neo^r mouse cell clones initiated at the cap site in the 5′ SNV LTR. We used a 20-base oligonucleotide (Pr2) homologous to sequences near the 3′ end of the SNV LTR (Fig. 8). After hybridization of 5′-end-labeled Pr2 to RNA from a Neo^r helper-free dog cell clone harboring a single recombinant ME111 provirus (ME111.HF3, 8), NIH 3T3 cell clone ME111.A1, and NIH 3T3 cell clone ME111.A3, we incubated each mixture in parallel with avian myeloblastosis virus reverse transcriptase. The extension products were separated in a denaturing acrylamide gel and visualized by autoradiography.

The size of the bands seen in dog cell clone ME111.HF3 and in mouse cell clone ME111.A1 was 156 bases, whereas the extension product seen in clone ME111.A3 was slightly smaller. These results indicated that transcripts in ME111-infected mouse cells were initiated at or near the same site as in dog cells which are permissive for SNV infection. This primer extension analysis confirmed our S1 nuclease mapping results that ME111.A1 cells contained lower amounts of upstream transcripts relative to ME111.A3 cells (Fig. 7). For ME111.A1, the transcription initiation site is the same one detected in ME111-infected dog cells (ME111.HF3) and SNV-infected CEF (data not shown). The site for transcription initiation in ME111.A3 cells appears to be slightly different, indicating that this virus may be mutant. Finally, in both ME111 cell clones there was a 50- to 100-fold decrease in the amount of stable RNAs per microgram of total RNA initiated at the 5′ SNV LTR in mouse cells versus that in dog cells.

These analyses showed that there were no detectable stable RNAs initiated from an upstream promoter (5′ SNV LTR) in six mouse cell clones harboring an intact recombinant provirus which had been selected for expression from an internal promoter. However, in the rare cases when an upstream promoter (the SNV 5′ LTR in ME111-infected mouse cell clones (ME111.A1 and ME111.A3) was active, so was the internal promoter.

DISCUSSION

To characterize the block(s) to SNV replication in mouse cells, we used recombinant SNV- and MLV-derived vectors. Our results showed that there are at least two blocks to SNV replication. One was a decreased efficiency of infection through integration. Another was reduced accumulations of steady-state RNAs from the SNV LTR promoter when a SNV LTR was 3′ to the SNV promoter.

Absorption through integration of SNV vectors by SNV proteins is less efficient in mouse cells than in dog cells. Using SNV-derived vectors expressing *neo* from various promoters, we determined that SNV infection of mouse cells was approximately 1% as efficient as infection of dog cells (ME149; Fig. 2). The four vectors we used expressed *neo*

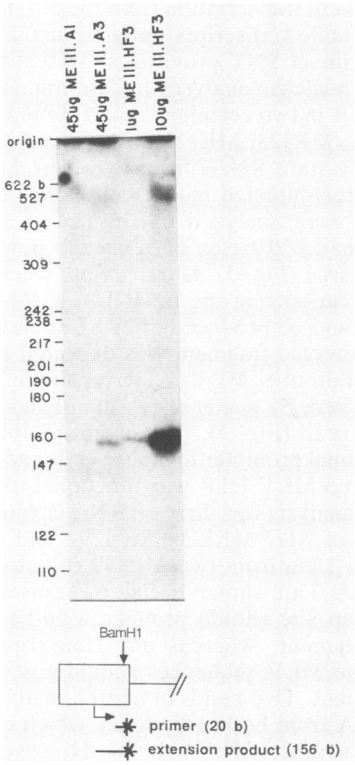


FIG. 8. Analysis of the 5′ transcription initiation site for Neo^r ME111 proviruses in mouse and dog cells. A 5′-end-labeled 20-mer (Pr2) homologous to sequences from 0.531 to 0.551 kbp from the 5′ end of a SNV provirus was hybridized with RNA from a Neo^r helper-free ME111-infected dog cell clone (ME111.HF3) and mouse cell clones ME111.A1 and ME111.A3. After incubation with avian myeloblastosis virus reverse transcriptase, the extension products were separated in a 6% polyacrylamide–8.3 M urea gel. The extension products were visualized by autoradiography. A schematic of a SNV LTR region homologous to the primer and the expected size of the cDNA from transcripts initiated at the cap site in the SNV LTR are shown. These data are indicative of data obtained in two similar experiments. b, Bases.

from the SNV LTR (ME111), HSV *tk* promoter (ME123), SV40 early promoter (ME129), and MLV U3 (ME149). Promoter strength, determined by transfection assays (Fig. 3), correlated with the NEO TU titer of each virus except ME111 (see below). Since the MLV promoter is a strong promoter in mouse and dog cells (7), we concluded that the ratios of NEO TU titers of ME149 in mouse cells relative to dog cells represented the efficiency of SNV infection up through integration. This decreased efficiency could be the result of a low efficiency of binding of the SNV *env* to the receptor, a low number of SNV *env* receptors, or inefficient internalization, reverse transcription, or integration.

Expression from the SNV LTR is context dependent. The low NEO TU titer of ME111(Rev) on mouse cells was surprising, since the SNV promoter was a strong promoter in transfection or infection assays of mouse cells when *neo* or *cat* was expressed from a SNV promoter that was not flanked by a 3′ SNV LTR (Fig. 2 and 3). Thus, the SNV promoter was an efficient promoter when transcripts which initiated from it were terminated at a heterologous sequence (SV40 polyadenylation sequences or 3′ MLV LTR). In fact, the SNV 3′ LTR may also inhibit expression from heterologous, linked promoters (pME129 versus SV2neo; Fig. 3A).

This "communication" between promoters and sequences necessary for 3′ end formation has also been seen for the U1 RNAs. Replacement of the U1 promoter with heterologous promoters (22, 37) results in transcripts which terminate downstream of the U1 3′ end signal and are polyadenylated. Also, deletion of the U1 3′ end signal results in transcripts which terminate at cryptic consensus sites downstream of the deletion but are not polyadenylated (37).

The decreased efficiency of infection and expression of SNV in mouse cells explained the lack of Neo^r colonies seen after infection of mouse cells with JE110(Rev) and ME111 (Am-MLV) pseudotyped viruses. Infection of mouse cells with viruses containing Rev proteins and a Rev genome resulted in an efficiency of infection of 1% [see relative NEO TU titer of ME149(Rev) on dog versus mouse cells, Fig. 2]. However, the efficiency of infection of mouse cells relative to dog cells with a virus containing Rev proteins and a MLV genome [JE110(Rev)] was 0.1%. The additional 10-fold decrease might be caused by lower efficiencies of reverse transcription or of integration (or both). For ME111(Am-MLV), the low NEO TU titer on mouse cells relative to dog cells was caused by lack of steady-state accumulation of RNA initiated from the 5′ SNV LTR when the 3′ SNV LTR was present. As with the JE110(Rev) pseudotype, heterologous interactions of MLV proteins with the SNV genome during reverse transcription and integration might also have contributed to the low NEO TU titer of ME111(Am-MLV) on mouse cells.

Transcripts from the 5′ SNV LTR were not detected in intact recombinant SNV proviruses selected for an internal promoter. Since the ability of SNV *neo*-containing viruses to transform mouse cells to a Neo^r phenotype was much less than on dog cells, we wanted to determine whether SNV-derived proviruses were intact in mouse cells. To this end, we analyzed individual Neo^r mouse cell clones of ME111, ME123, ME129, and ME149 by Southern blotting (Fig. 4 and data not shown). There were no gross alterations in any of the proviruses we analyzed.

Using S1 nuclease mapping, we analyzed the stable transcripts from two of each of the SNV-derived proviruses (ME111, ME123, ME129, and ME149; Fig. 6 and 7 and data not shown) and two of the MLV-derived proviruses in which *neo* was expressed from an internal SNV U3 (ME151; Fig. 5). In both cell clones of ME151-infected mouse cells which were selected for expression from the internal SNV promoter, there were stable transcripts initiated from the MLV LTR and the SNV U3 promoters (Fig. 5). In contrast, when a promoter internal to the SNV LTRs was selected for expression (ME123, ME129, and ME129), only internally initiated transcripts were detected (Fig. 6 and data not shown).

S1 nuclease analysis of the transcripts in the rare Neo^r ME111-infected mouse cell clones showed that an upstream (selected) and a internal (unselected) promoter were active (Fig. 7). In both cell clones analyzed, the upstream transcripts initiated at sites at or near the transcription initiation site in the SNV LTR.

Neo^r mouse cells infected with ME111(Rev) arose at a efficiency of 10^{-6} relative to dog cells. We can account for 100-fold-decreased efficiency caused by events up through integration. Transcription from a 5′ SNV LTR adjacent to a 3′ SNV LTR must, therefore, be decreased by a factor of 10,000. In the rare mouse cell clones (ME111.A1 and ME111.A3) in which the 5′ SNV LTR was active, expression was reduced by a factor of at least 100 relative to a single recombinant SNV-derived provirus in dog cells (Fig. 8). If the ME111 proviruses were not variants, then this level of transcription probably represents the threshold level of expression from the 5′ SNV LTR to form a Neo^r SNV-derived provirus in mouse cells. If, in contrast, Neo^r ME111-infected mouse cell clones represent mutant viruses (as is probably the case for ME111.A3) or mutant cells, then these clones represent mutants which have overcome the lack of expression seen when an SNV 5′ LTR is adjacent to a 3′ SNV LTR.

ACKNOWLEDGMENTS

We thank J. Couch, S. Hinz, and J. Rein for technical assistance. We thank Joe Dougherty, Celine Gelinas, Tom Gilmore, Janet Mertz, Nito Panganiban, and Bill Sugden for their comments on the manuscript.

This work was supported by Public Health Service research grants CA-22443 and CA-07175. J.E.E. is supported by National Institutes of Health predoctoral training grant CA-09135. H.M.T. is a American Cancer Research Professor.

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