Gene expression from transcriptionally disabled retroviral vectors

(in vitro mutagenesis/internal promoter/hypoxanthine phosphoribosyltransferase gene)

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ABSTRACT Retroviral vectors are used for the efficient transfer of foreign genes into mammalian cells. We report here the construction of murine retrovirus-based vectors carrying the full-length cDNA for human hypoxanthine phosphoribosyltransferase (HPRT; EC 2.4.2.8) and from which the enhancer sequences, the "CAAT box," and the "TATA box" in the long terminal repeats (LTRs) have been deleted. After infection of HPRT-deficient rat cells by the vectors, transcriptional activity from the 5' LTR was undetectable and expression of the HPRT cDNA was dependent on an internal promoter. Removal of the LTR regulatory signals increased HPRT gene expression from an internal promoter, indicating interference between the two sets of transcriptional signals. Such disabled vectors may reduce the likelihood of undesirable genetic changes through insertional mutagenesis in cells infected with retroviral vectors.

Retroviral vectors have become important and useful tools for studying mammalian gene transfer and expression (1-9). In these vectors, genes transduced into recipient cells are generally expressed from promoter, enhancer, and other regulatory signals contained within the viral long terminal repeat (LTR) sequences. Since retroviruses and their derived vectors integrate into random sites in the host genome (10-12), and since the transcriptional regulatory elements of the LTRs are relatively strong, deleterious effects on flanking cellular genes such as the interruption of essential genes or the altered regulation of growth-regulatory genes (13-16) may result. Furthermore, LTR-driven gene expression may not demonstrate tissue specificity in vivo (17), necessitating the use of internal, tissue-specific promoters. However, transcription from the 5' LTR has already been shown under some conditions to interfere with the regulation of gene expression from downstream promoters (18, 19).

To approach these problems of gene expression by retroviral vectors, Yu et al. (20) constructed a self-inactivating (SIN) vector in which the enhancer and the "CAAT box" were deleted from the LTRs but the "TATA box" was retained. Although transcription from the 5' LTR in SIN vector-infected cells decreased, it was not completely eliminated, thereby providing continued potential for altered regulation of cellular genes and possible aberrant expression from internal promoters in the vector. We report here the properties of a vector more completely transcriptionally disabled by the deletion of regulatory elements in the LTRs, including the TATA box, the CAAT box, and the enhancer elements. Expression of a transduced gene from such a disabled vector is dependent on an internal promoter introduced upstream from the gene to be expressed. In the present study, we demonstrate regulated expression of a transduced gene, in this case the cDNA for human hypoxanthine phosphoribosyltransferase (HPRT) (21), from an internal human metallothionein (MT-IIa) promoter (22) or human cytomegalovirus (hCMV) immediate early promoter (23). We also show that the absence of the regulatory elements in the LTRs increases HPRT gene expression from an internal promoter, providing further evidence for promoter interference.

MATERIALS AND METHODS

Cell Culture and Viruses. The packaging lines PA12 (24) and PA317 (25) were obtained from I. Verma (The Salk Institute, San Diego, CA) and A. D. Miller (Fred Hutchinson Cancer Center, Seattle, WA), respectively. Both packaging lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. The HPRT- rat cell line 208F (26) was maintained in α minimal essential medium (α-MEM) supplemented with 10% fetal bovine serum. HPRT- cells were selected in medium containing 100 μM hypoxanthine, 0.3 μM aminopterin, and 50 μM thymidine (HAT). Neomycin-resistant cells were selected in medium containing G418 (400 μg/ml).

To produce transmissible virus, vector DNA was cotransfected with pSV2neo (38) DNA into packaging cells by the calcium phosphate coprecipitation method (27). Single colonies were isolated by selection in G418-containing medium and expanded into mass cultures. To assay for virus, aliquots of cell-free culture medium from each producer line were applied to cells in the presence of Polybrene (4 μg/ml). Selection was applied 24 hr later and colonies were isolated or stained and counted after 2 weeks.

To rescue HPRT-expressing virus, MA helper virus (28) was applied to cells containing provirus. One week after MA virus infection, culture medium was assayed for HPRT virus on 208F cells as described above.

Site-Specific in Vitro Mutagenesis and Retroviral Constructs. To construct a vector expressing HPRT cDNA from an internal promoter, an 840-base-pair (bp) HindIII-BamHI fragment containing the promoter for the human metallothionein MT-IIa gene, obtained from M. Karin (University of California, San Diego), was inserted immediately upstream from the HPRT cDNA in the Moloney murine leukemia virus (MoMLV)-based vector pPLM (29). We called this construct pLMTPL. Specific deletions were introduced into pLMTPL by site-specific in vitro mutagenesis (30). The "TATA box" (5' AATATAA 3'), at -25 to -30 bp from the mRNA cap site, the "CAAT box" (5' CCAAT 3'), at -78 to -82 bp from the mRNA cap site, and the splice-donor site (5' GAGGTTA 3') adjacent to the 5' LTR were deleted directly by site-specific mutagenesis using oligonucleotides spanning

| Abbreviations: HAT, hypoxanthine/aminopterin/thymidine; hCMV, human cytomegalovirus; HPRT, hypoxanthine phosphoribosyltransferase; LTR, long terminal repeat; MoMLV, Moloney murine leukemia virus; SIN vector, self-inactivating vector; c14, colony-forming unit(s) |

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each region. To delete the MoMLV enhancer, an Xba I site 15 bp upstream from the enhancer region was constructed by site-specific mutagenesis. A second Xba I site is located 31 bp downstream from the enhancer. The region between the two Xba I sites was deleted by digestion with Xba I and religation. The resulting recombinant containing the fully deleted 3' LTR was called pRMH.

In constructs pNHP-1 and pNHP-2, a 600-bp HincII-Sau3A fragment spanning the promoter for the immediate early gene of hCMV was inserted immediately upstream from the human HPRT cDNA. A Cla I-Sma I fragment containing the 5' LTR of MoMLV and the neomycin-resistance gene derived from the construct pN2 (7) was inserted upstream from the hCMV promoter. The deleted 3' LTR of pNHP-2, including the enhancer, CAAT box, and TATA box deletions, was derived from the construct pRMH, while the intact 3' LTR in pNHP-1 was derived from the construct pLPLM.

DNA and RNA Analysis. Samples of total cellular DNA extracted as described (31) were digested with restriction enzymes according to the supplier's recommended conditions, and the restriction fragments were separated by electrophoresis in 1% agarose gels. DNA was transferred to nitrocellulose according to the method of Southern (32), hybridized with nick-translated probe, and washed under high-stringency conditions as described (31).

Poly(A)^+ RNA samples were prepared as described (31) and subjected to electrophoresis in 1.2% agarose/2.2 M formaldehyde gels, transferred to nitrocellulose, and hybridized to nick-translated probe. For induction of the metallothionein promoter, subconfluent cells were incubated for 8 hr in the presence of 1 μM cadmium sulfate before isolation of cellular RNA.

HPRT Enzyme Assays. HPRT activity was measured by the incorporation of [3H]hypoxanthine into inosine monophosphate (IMP), using 40-μl samples of cell lysate derived from 2 × 10^6 cultured cells, according to published methods (33).

RESULTS

Generation of Recombinant Retroviruses with Deletions in LTRs. A retroviral vector, pLMTPL, was constructed in which the promoter of the human metallothionein MT-II_A gene was inserted immediately upstream from the human HPRT cDNA (Fig. 1). To abolish transcription from the viral LTRs completely, several deletions were introduced into the U3 region of the 3' LTR of pLMTPL by site-specific in vitro mutagenesis. These deletions include the hexanucleotide 5'-AATAAA 3' (TATA box) located at -25 to -30 bp from the cap site, the pentanucleotide 5'-CCAAAT 3' (CAAT box) at -78 to -82 bp, and a segment at -150 to -356 bp encompassing the 75-bp tandem repeats comprising the LTR enhancers (2). Deletions made in the 3' LTR will appear in both LTRs after one cycle of viral replication (Fig. 2). The splice-donor site adjacent to the 5' LTR was also deleted to prevent aberrant splicing events between the splice-donor site and any cryptic splice-acceptor sites present in inserted DNA. This disabled vector is called pRMH (Fig. 1).

To generate infectious virus, we transfected the pRMH construct into the packaging line PA12. Virus produced was assayed by infection of HPRT^+ rat 208F cells, followed by quantitation of the number of HAT-resistant colonies. The highest titer obtained from a PA12 producer of RMH virus was 10^6 HAT-resistant colony-forming units (cfu) per ml, titered on 208F cells.

Lysates from individual HAT-resistant colonies of 208F cells infected by RMH were tested for HPRT activity by an isoelectric-focusing gel assay (35). The cDNA expressed in the vector RMH encodes a mutated HPRT protein with a new and unique epitope at its C-terminus (29). This mutated protein has an altered isoelectric point so that its enzymic activity can be easily distinguished from wild-type human or rodent HPRT activities by the isoelectric-focusing assay. All HAT-resistant rat 208F colonies expressed the mutated human HPRT activity, indicating the production of transmissible HPRT virus in the transfected PA12 producer line (data not shown).

Analysis of DNA of HAT-Resistant Cells. To determine the presence, organization, and copy number of the newly introduced RMH vector, high molecular weight DNA from three infected, HAT-resistant 208F clones designated MT1.

![Fig. 1. Structure of retroviral constructs. Open boxes represent LTRs divided into U3, R, and U5 regions (see Fig. 2). Hatched boxes represent the promoter of the human metallothionein MT-II_A gene (MT) or the hCMV immediate early gene (HCMV). Stipped boxes represent the cDNA for human HPRT. Shaded boxes represent the neomycin-resistance gene (NEO). Triangles indicate the deleted sequences described in the text and Fig. 2. S, Sst I; H, HindIII.](image-url)

![Fig. 2. Scheme illustrating the transfer of deletions in the 3' LTR to the 5' LTR. Double wavy lines represent cellular DNA flanking the provirus. Open boxes containing the U3-R-U5 (U, unique; R, repeated) sequences are viral LTRs, and X represents the inserted foreign sequences. S.D. indicates the splice-donor site of MoMLV. Hatched boxes represent deletions produced by site-specific mutagenesis, and the precise nucleotide sequence of each deletion is indicated according to positions on the MoMLV genetic map (34). Triangles indicate areas with deleted sequences. Transcriptional regulatory elements were deleted from the 3' LTR of the retroviral construct, and the construct was introduced into a packaging line by transfection. Transcription starts at the 5' LTR and proceeds to the termination signal in the 3' LTR, resulting in a transcript with the regulatory sequences deleted from its U3 region. The RNA is packaged and the virus is used for infection. In infected cells, viral transcript is reverse-transcribed into double-stranded DNA by virion-associated reverse transcriptase. During this process, the U3 region of the RNA is used as a template for the synthesis of the U3 region in both LTRs. The deletions are therefore duplicated in both LTRs after one round of retroviral replication.](image-url)
MT4, and MT5 was prepared. The DNA was digested with various restriction enzymes and examined by Southern blot analysis. The restriction enzyme Ssr I cuts once within each LTR (Fig. 1) and the presence of the 4.7-kilobase (kb) LTR–LTR fragment is therefore diagnostic for an intact proviral structure. The expected fragment of 4.7 kb was detected in all three MT clones after Southern blot hybridization with a MoMLV-specific probe (Fig. 3A). Additional blotting studies after digestion of the DNA with Hind III, which cuts twice in the provirus (Fig. 1A), revealed the presence of a common 1.4-kb fragment and two junctional fragments of variable sizes (data not shown), confirming the expected single and independent integration sites in the three cell lines.

To test for the transfer of the deletions from the 3' LTR to the 5' LTR of the provirus, the DNA of infected 208F cells was cut with Nhe I and Xho I. Nhe I cuts once in each LTR, upstream from the retroviral enhancer sequences. Digestion with Xho I, which cuts the provirus once in the HPRT cDNA, plus Nhe I should produce two fragments, 2.7 kb and 2.1 kb in length, from vectors containing LTRs without deletions (Fig. 3B Lower). If, on the other hand, the 217-bp deletion is transferred from the 3' LTR to the 5' LTR, a 1.9-kb fragment would be expected in place of the 2.1-kb fragment. As predicted, a 1.9-kb fragment was detected in all three MT clones (Fig. 3B Upper), indicating that the 3' LTR modifications had been transferred to the 5' LTR.

Transcriptional Analysis of Cells Infected with the RMH Vector. To determine the transcriptional activity of both the 5' LTR and the metallothionein MT-II A promoter, poly(A)+ RNA from the three clones was isolated and examined by blot hybridization analysis (Fig. 4A). A transcript initiated in the 5' LTR would be expected to be 4.8 kb long. No RNA transcripts of this length were detected in the three clones (Fig. 4A, lanes 2–4). Instead, a 3.3-kb RNA transcript, consistent with initiation at the internal metallothionein MT-II A promoter, was observed. Overexposure of the film failed to detect any transcripts longer than 3.3 kb. The 1.5-kb RNA probably represents endogenous host-cell HPRT transcripts, since it is also present in noninfected cells (data not shown). RNA isolated from infected cells incubated with LMTPL virus, containing an intact, functional 5' LTR, was also analyzed. As expected, a 4.8-kb transcript initiated in the viral 5' LTR would be detected by infection of rat 208F cells followed by HAT selection. About 105 MA virus infection units were used to infect separate cultures containing 5 x 105 cells of each of the three clones. After 1 week, 1 ml of culture medium was assayed on 208F cells for the presence of HPRT virus. No HAT-resistant colonies were observed in the medium of any of the clones, whereas under identical conditions, more than 105 HAT-resistant colonies were detected from cells previously infected with LMTPL viruses and rescued with the same amount of MA helper virus. Therefore, by the two criteria of RNA blot analysis and virus rescue, it is apparent that the transcriptional activity of the 5' LTR of the RMH provirus has been inactivated.

The human metallothionein promoter can be induced by heavy metals (22). To test for regulated expression of the human HPRT cDNA from the internal MT-II A promoter, MT1 cells were exposed to cadmium for 8 hr before the isolation of RNA and blot analysis. The amount of human HPRT RNA in induced cells increased about 3-fold relative to the RNA from uninduced cells (Fig. 4B). Furthermore, metabolic labeling of uninduced and induced cells with [35S]methionine and determination of the level of human HPRT protein produced in each MT clone by immunoprecipitation with polyclonal rabbit anti-peptide antibody to the mutated human HPRT (29) revealed a 3- to 5-fold increase in the amount of HPRT protein (data not shown).

**Promoter Interaction: Interference of Expression of the HPRT cDNA by an Intact 5' LTR.** To determine whether the

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**Fig. 3.** Southern blot analysis of the proviruses. Ten to twenty micrograms of restriction enzyme-digested genomic DNA isolated from uninfected or infected rat 208F cells was loaded on each lane. The 32P-labeled probe used for hybridization was isolated from pLPL as a BamHI–Ssr I fragment (3) and recognizes most of the coding region of the env gene and the LTRs of MoMLV. (A) DNA digested with Ssr I. Lanes 1: rat 208F DNA. Lanes 2-4: MT1, MT4, and MT5 DNA. Lane 5: molecular size marker showing the position of the 4.7-kb fragment. (B) DNA digested with Nhe I and Xho I. Lane 1: molecular size markers of 2.1-kb and 2.7-kb fragments. Lane 2: rat 208F DNA. Lanes 3-5: MT1, MT4, and MT5 DNA.

**Fig. 4.** Blot hybridization analysis of poly(A)+ RNA prepared from infected and uninfected rat 208F cells. The filter was probed with the 32P-labeled human HPRT cDNA isolated from p4aA8 (21). (A) Lanes: 1, LMTPL-infected cells; 2–4, MT1, MT4, and MT5. Four to seven micrograms of poly(A)+ RNA was loaded on each lane. Lane 1 was exposed for 4 hr and lanes 2 to 4 were exposed for 24 hr to visualize the transcript initiated from the MT-II A promoter. (B) RNA was prepared from MT1 cells that had been infected in the absence (lane 1) or presence (lane 2) of 5 μM cadmium sulfate. Five micrograms of poly(A)+ RNA was loaded on each lane.

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absence of the regulatory elements in the 5' LTR has any effect on expression from the downstream promoter, we constructed two vectors, pNHP-1 and pNHP-2, containing the promoter for the immediate early gene of hCMV linked to the cDNA for the human HPRT gene (Fig. 1) and also expressing the neomycin-resistance gene from the 5' LTR promoter. The hCMV promoter has been shown to be severalfold more active than either the simian virus 40 early promoter or the Rous sarcoma virus LTR (36). The construct pNHP-1 has an intact 3' LTR, whereas pNHP-2 has the 75-bp tandem repeats, the CAAT box, and the TATA box deleted from its 3' LTR. The neomycin-resistance phosphotransferase gene was inserted immediately downstream from the 5' LTR to facilitate selection of infected cells. The splice-donor site of MoMLV was retained in both constructs to ensure the presence of a complete packaging signal for MoMLV and to examine the possibility that the low virus titers from RMH virus-producer cells may have been due to deletion of this site in the RMH vector.

Producer clones were generated by cotransfection of each construct with pSV2neo DNA into the PA317 packaging line and selected for G418-resistant clones as described in Materials and Methods. To assay for the presence of virus, supernatant from producer cells was then applied to NIH 3T3 cells or 208F cells and the infected cells were selected in G418-containing medium. Table 1 shows that G418 selection of the NHP-2 virus-infected cells resulted in no surviving colonies. However, about 10^5 HAT-resistant cfu/ml were detected when the same producer line was assayed on 208F cells, indicating HPRT gene expression. This titer is similar to that obtained from a producer line for the RMH virus, and therefore the low titers of virus obtained from producer lines of disabled vectors seem not to be due to the deletion of the 5' splice-donor site.

To confirm the complete absence of transcription from the 5' LTR, 208F cells were infected with limiting dilutions of each virus to ensure single-copy integration. About 200 HAT-resistant colonies were pooled and expanded into mass cultures. Each culture was then superinfected with MA helper virus as above. As shown in Table 1, infectious HPRT virus (8 x 10^2 cfu/ml) was recovered from NHP-1 virus-infected cells, whereas no HPRT virus was recovered from NHP-2 virus-infected cells. These results, obtained by using an extremely sensitive assay for packageable, LTR-initiated viral transcripts, show that the 5' LTR in the NHP-2 provirus is completely inactivated, and expression of the HPRT cDNA is therefore solely under the control of the internal hCMV promoter.

The HPRT enzymic activity was measured in cell extracts prepared from pooled 208F cells infected with either the NHP-1 or the NHP-2 virus. The HPRT activity in NHP-2 virus-infected cells consistently showed an ~2-fold increase over the activity in NHP-1 virus-infected cells (Fig. 5). Cells infected with the LPL virus (3), in which the human HPRT cDNA is under the control of a fully intact and functional 5' LTR, showed a 7-fold higher HPRT activity than that found in the NHP-1-infected cells (Fig. 5). To test the possibility that this result was promoter-specific, we replaced the hCMV promoter in pNHP-1 with the intact Rous sarcoma virus LTR, also known to contain a relatively strong promoter (37). The HPRT enzymic activity in cells infected with this virus was about one-seventh as great as the HPRT activity in LPL virus-infected cells (data not shown). Thus, although deletion of the transcriptional elements from the 5' LTR allows slightly increased expression from potentially strong internal promoters, both of the internal promoters we tested gave levels of expression far less than those obtained for gene expression directly from the 5' LTR.

**DISCUSSION**

The growing use of retroviral vectors for the delivery of genes into mammalian cells has highlighted several features of these vectors that tend to compromise their efficiency, safety, and proper regulation of transduced genes. Since retroviruses integrate into random sites in the host genome, they can induce mutation either by interrupting essential genes or by introducing promoters, enhancers, or other regulatory elements near flanking cellular genes, causing their inappropriate expression. Furthermore, attempts to provide tissuespecific regulatory sequences in retroviral vectors may also be complicated by the fact that the presence of powerful regulatory elements in the LTRs may interfere with the functions of other tissue-specific signals.

In the SIN vectors of Yu et al. (20), a deletion in the 3' LTR removes the CAAT box and most of the enhancer regions but leaves the TATA box intact. A low level of transcription was detected from the 5' LTR in cells infected with such a vector, suggesting that either the enhancers of the simian virus 40 internal promoter were able to activate the enhancerless LTR or that the TATA box (alone or together with flanking viral or cellular sequences) acted as a weak promoter. To eliminate such remaining transcriptional activity, we constructed retroviral vectors in which the transcriptional signals in the LTRs had been more completely deleted. By using site-specific in vitro mutagenesis, we deleted only the transcriptional signals in the 3' LTR, preserving those LTR sequences required

**Table 1. Analysis of virus production and rescue**

<table>
<thead>
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<th>Experiment</th>
<th>Cell line</th>
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<tr>
<td></td>
<td>208F</td>
<td>HAT</td>
<td>8 x 10^3</td>
<td>0</td>
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*Virus was titered by infection of rat 208F cells or NIH 3T3 cells and selection in HAT- or G418-containing medium. Results are expressed as HAT- or G418-resistant cfu/ml.

**FIG. 5.** Effect of the LTR deletions on expression of the human HPRT cDNA from the hCMV promoter. Data represent HPRT activity in extracts from uninfected rat 208F cells (○) or cells infected with NHP-1 (●), NHP-2 (●), or LMTPL (□) and selected for HAT-resistance (resistant colonies were pooled). The amount of [3H]inosine monophosphate bound to the origin of a PE1-cellulose sheet after chromatography is plotted as a function of time of incubation. Equal amounts of protein in each extract were used for each assay. The assay has been repeated five times, and the absolute amount of HPRT activity varied from experiment to experiment, but the relative amounts of HPRT activity were within 10% of each other.
for polyadenylation of viral transcripts and efficient proviral integration. These disabled vectors can infect cells, integrate into host genomes, and express the transduced HPRT gene from internal promoters. In the present study, no transcripts consistent with initiation from the 5' LTR were detected in cells infected with the disabled vectors, and no infectious HPRT virus was recovered from infected cells by the extremely sensitive virus-rescue assay. Thus, the deletion of the TATA box in our disabled vectors may account for the total absence of promoter activity of the 5' LTR when compared with the previously described (20) SIN vectors.

The low titers (10^2 cfu/ml) obtained with the totally disabled vectors can be partially explained by the order-of-magnitude lower titers determined on 208F cells compared with NIH 3T3 cells (Table 1), although even titers of 10^2 cfu/ml are much lower than those obtained from packaging cells transfected with retroviral constructs containing an intact 3' LTR. A possible explanation is that recombination occurs between the 5' LTR and 3' LTR during transfection, resulting in replacement of the functional 5' LTR with the nonfunctional 3' LTR in some of the integrated DNA copies. Viral titers produced from such cells would be markedly reduced. We expect that the transcriptional activity of the 5' LTR of our disabled vectors should remain fully functional in transfected packaging cells prior to viral replication, and that the deletions in the 3' LTR do not interfere with functions such as viral RNA packaging, DNA replication, or integration of proviral DNA.

Expression of HPRT from the internal hCMV promoter increases about 2-fold when the transcriptional regulatory sequences in the LTRs are deleted. It is possible that the absence of these sequences from the U3 region of the transcript stabilizes viral transcripts, resulting in an increase of HPRT activity, or that transcriptional activity from the internal promoter increases in the absence of transcription from the upstream LTR. In either case, our results suggest that it is useful to inactivate transcription from the 5' LTR of a retroviral vector in order to obtain more efficient expression from internal promoters.

It remains to be determined if such disabled retroviral vectors are less likely to cause aberrant regulation of expression of flanking cellular genes. We do not address here the safety issue of insertional mutagenesis by retroviral integration into essential cellular genes, and only through the development of efficient site-specific targeting methods could such cell-damaging events be prevented.

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