Construction and Characterization of a Hybrid Mouse Mammary Tumor Virus/Murine Leukemia Virus-Based Retroviral Vector

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Mouse mammary tumor virus (MMTV)-based vectors are characterized by low titers. In an effort to transfer MMTV-specific regulation of gene expression to a more efficient murine leukemia virus (MLV) vector, we have replaced the complete 3′ U3 region of MLV with the complete U3 region of MMTV. Virus titers were not significantly affected by this modification, there was no impairment of reverse transcription and integration, and after infection of cells, the MMTV promoter is duplicated and translocated to the 5′ long terminal repeat, resulting in glucocorticoid-regulatable RNA expression.

A consequence of the reverse transcription of the retroviral genomic RNA into a double-stranded DNA molecule in the infected cell is the duplication of this U3 region, located at the 3′ end of the viral RNA, and the juxtapositioning of this second copy to the 5′ end (for a review, see reference 10). The U3 region carries the retroviral promoter and enhancer elements which control gene expression from the provirus. This property was original exploited by Yu and coworkers (26) to construct retroviral vectors in which 299 bp of the murine leukemia virus (MLV) U3 region, carrying the two 72-bp repeat enhancer elements, were deleted, creating self-inactivating vectors. Such self-inactivating vectors carry, after infection and reverse transcription, a single functioning internal heterologous promoter which controls the expression of the linked therapeutic gene.

Double-copy vectors in which a promoter and a gene are inserted into the 3′ long terminal repeat (LTR) of the vector have also been constructed. Upon infection of cells with the vector, the expression cassette is present twice in each LTR (7). The promoters of a number of viral or cellular genes have been inserted into the MLV U3 region, either in addition to or in place of the virus enhancer, so that the expression of genes carried by such vectors is placed under heterologous-promoter transcriptional control in the infected cell (2, 3, 18, 25). The earliest attempt to insert a heterologous enhancer into the U3 region of an MLV provirus involved the use of a 335-bp Sau3A fragment of the U3 region of the mouse mammary tumor virus (MMTV) LTR, though this was in the context of a complete biologically active provirus rather than a retroviral vector (16).

The expression of MMTV is controlled by a number of factors, including glucocorticoid hormones, and the promoter of this retrovirus has been extensively exploited in transgenes, including those of therapeutic relevance (24), for conditional expression (5). Retroviral vectors based on MMTV would be required for vector provirus integration (4), were replaced with those of MMTV.

To test this hypothesis, we constructed a hybrid MMTV/MLV vector based on the MLV-derived pBAG retroviral vector (17). This vector carries a β-galactosidase (β-gal) gene that is expressed from the MLV promoter as well as a neomycin gene that is expressed from the simian virus 40 (SV40) promoter (Fig. 1A). The pBAG vector was chosen since even though the titers are lower than with contemporary vectors, the presence of a prokaryotic origin of replication in the vector allows facile recloning of integrated proviruses for later molecular characterization.

The U3 region of this vector was deleted by a PCR-mediated approach (20). This strategy ensured that the inverted repeat at the 5′ border of the U3 region was left intact and also that the promoter of MMTV U3 region of MMTV was inserted by PCR using specific primers carrying SacII and MluI restriction enzyme cleavage sites and inserted into the 3′ U3-deleted MLV-based vector plasmid (Fig. 1B). The β-gal gene was introduced into the vector plasmid in such a way as to ensure that its expression would be driven by the 5′ MLV U3 promoter in the context of the plasmid after transfection (Fig. 1C), whereas it would be placed under the transcriptional control of the MMTV U3 glucocorticoid response element) into the 3′ LTR U3 region of MLV at the expense of the MLV enhancer was previously shown to be incompatible with recombinant virus production (16). Only when part of the MMTV U3 region was additionally inserted into the 3′ MLV U3 region could recombinant virus be obtained, although this virus showed a serious reduction in infectivity and was not stable, undergoing frequent rearrangements. To circumvent these problems, Overhaus and Fan (16) had to insert MMTV U3 fragments into both the 3′ and 5′ MLV LTRs. Again, only constructs with additional MMTV sequences in both LTRs gave functional virus, and no virus could be recovered when the MLV enhancer sequences were replaced by MMTV sequences. This instability may have been due to the ability of this virus to undergo more than one round of infection. For this study, we reasoned that a stable retroviral vector might be obtained if all of the MLV vector U3 sequences except for the inverted repeat (IR) located at the 5′ end of the U3 region, which required for vector provirus integration (4), were replaced with MMTV.

Mouse mammary tumor virus (MMTV)-based vectors are characterized by low titers. In an effort to transfer MMTV-specific regulation of gene expression to a more efficient murine leukemia virus (MLV) vector, we have replaced the complete 3′ U3 region of MLV with the complete U3 region of MMTV. Virus titers were not significantly affected by this modification, there was no impairment of reverse transcription and integration, and after infection of cells, the MMTV promoter is duplicated and translocated to the 5′ long terminal repeat, resulting in glucocorticoid-regulatable RNA expression.
promoter in infected cells after successful promoter conversion following reverse transcription (Fig. 1D).

The hybrid vector plasmid pMMTV-BAG and the original pBAG vector plasmid, both of which carry an internal neomycin resistance gene constitutively expressed from an internal SV40 promoter, were introduced into the amphotropic packaging cell line PA317 (12), and the resultant retroviral vector particles were used to infect a number of cell lines. The titer of transducing particles obtained from a population of MMTV-BAG vector virus-producing cells (Fig. 2, solid bars) was slightly reduced in comparison to that obtained from a population of the parental BAG vector-producing cells (Fig. 2, cross-hatched bars) when assayed on Rat-2 or EJ cells but was similar as judged by infection of CrFK cells and even slightly increased on NIH 3T3 cells. These cell lines were chosen for infection studies since it is known that the MMTV promoter is active in these cells, a factor of importance for later expression studies. The titers obtained contrast favorably with the previously reported 500-fold reduction in titer using MLV virus with 3' and 5' MMTV insertions in the presence of dexamethasone (16). One explanation for this may be the higher transcriptional efficiency of the 5' MLV LTR in transfected packaging cells compared to the relatively poor transcriptional efficiency of the MMTV promoter even in the presence of dexamethasone.

To confirm that the transduced cells had acquired the vector

FIG. 1. Construction of the hybrid vector pMMTV-BAG. (A) Shown is a schematic representation of the pBAG (17) vector carrying the β-gal marker gene (cross-hatched box), the SV40 promoter driving expression from the neomycin resistance gene (checked box), as well as the origin of replication (ori) from pBR322 (hatched box). The complete U3 region except the IR of the 3' LTR within the BAG vector was deleted by a PCR-mediated approach (for the complete cloning strategy, please contact the corresponding author). (B) Deletion of the U3 region was confirmed by sequencing from the ClaI site into the U5 region. The U3 region of MMTV was amplified by PCR with the plasmid pBG102 (a plasmid containing the 3' LTR from Mtv 2 [21]) as the template and primers carrying the MluI and SacII extension. (C and D) The product was digested with SacII and MluI and ligated to the SacI- and MluI-digested vector to give the plasmid pMMTV-BAG (8,377 bp), in which the β-gal gene is under the transcriptional control of the MLV promoter after transfection (C) and is under the control of the MMTV promoter after infection (D) (for further details, see reference 20). The positions of primers specific for the MMTV U3 region (P1, 5'-GACCACAGCCCACTTCTCTTACA-3' [21] or MLV R sequences (P4, 5'-GCGCCAGTCCTCCGATTGA-3') together with a primer specific for the MLV packaging region (P2, 5'-GGTCCGCCAGATACAGAGCTAGTTA-3') or the β-gal gene (P5, 5'-TTCATCCACCACACATACAGCC-3') are shown (D), as are the expected sizes of the PCR products that can be obtained by using these primers and the MMTV (labelled fragment A) and MLV (labelled fragment B) probes that can be used to detect them.
constructs and to verify that the MMTV promoter was now present in the 5' LTR of the vector provirus in these cells, DNA was prepared from noninfected and infected cell populations of Rat-2, EJ, and NIH 3T3 cells and analyzed by PCR. Primers specific for either the MMTV U3 (P1 and P3) or MLV R (P4) sequences in combination with a second primer complementary to the packaging region (P2) or the β-gal gene (P5) present in all three constructs were used for these analyses (Fig. 1D). The PCR products were hybridized to MMTV U3- or MLV-specific probes (Fig. 1D, labeled fragments A and B, respectively). DNA prepared from cells infected with the MMTV-BAG virus gave PCR products of 1.2 and 1.6 kb after amplification with the P1-P2 and P3-P2 primer pairs, respectively. These products hybridized, as expected, to both MMTV U3-specific (Fig. 3A, lanes 1, 3, 5, 7, 9, and 11) and MLV-specific (Fig. 3B, lanes 1, 3, 5, 7, 9, and 11) hybridization probes. In contrast, the primer pair P4-P5 gave a PCR product specific (Fig. 3B, lanes 1, 3, 5, 7, 9, and 11) hybridization with a 387-bp MLV LTR probe (data not shown). Although MMTV promoter activity is restricted to a few cell types in vivo (19), this promoter is active in many cell types in vitro, and the ability of glucocorticoid hormones to stimulate expression from this promoter has been extensively utilized in many cell types, including fibroblasts (9). To examine the effect of glucocorticoid hormone on β-gal expression in MMTV-BAG-

![Graph](image)

**FIG. 2. Titer is not appreciably altered by replacement of U3 sequences.** Rat-2, NIH 3T3, EJ, and CrFK cells were infected with MMTV-BAG (solid bars) and BAG (cross-hatched bars) produced from stably transfected populations of PA317 cells, as previously described (8). Each bar represents the average of data from three independent experiments, and error bars (indicating the standard error of the mean) are shown.

![Diagram](image)

**FIG. 3. PCR analysis of infected-cell DNA for the presence of MMTV-BAG sequences.** Genomic DNA (1 μg) was amplified by PCR using 40 pmol of each primer specific for the MMTV U3 region or MLV R sequences and of a primer specific for the MLV packaging region or the β-gal gene. PCRs were performed under the following reaction conditions: 1 min at 94°C, 2 min at 50°C, and 3 min at 68°C for 35 cycles. The PCR product was separated on a 0.8% agarose gel, transferred to Zetaprobe membranes (Bio-Rad), and hybridized to 32P-labeled probes. Promoter conversion was detected by using two primers specific for the MMTV U3 region (P1 and P3) in combination with a primer specific for the MLV packaging region (P2). An MLV R region-specific primer (P4) was also used in combination with a primer specific for the β-gal gene (P5). Filters were hybridized against a 0.9 kb MMTV U3-specific PstI fragment (A) or an MLV-specific PCR fragment (B), as shown in Fig. 1D. Lanes 1, 7, and 13, infected Rat-2 cells; lanes 2, 8, and 14, noninfected Rat-2 cells; lanes 3, 9, and 15, infected NIH 3T3 cells; lanes 4, 10, and 16, noninfected NIH 3T3 cells; lanes 5, 11, and 17, infected EJ cells; lanes 6, 12, and 18, noninfected EJ cells.

### Table 1. Dexamethasone-inducible expression of β-gal in transduced cells

<table>
<thead>
<tr>
<th>Infecting virus and glucocorticoid status</th>
<th>β-Gal activity (lu/mg) in infected cell lines</th>
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<tbody>
<tr>
<td></td>
<td>NIH 3T3</td>
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<tr>
<td>BAG</td>
<td></td>
</tr>
<tr>
<td>Dex</td>
<td>1.14 × 10⁶</td>
</tr>
<tr>
<td>+ Dexamethasone</td>
<td>1.73 × 10⁶</td>
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<tr>
<td>Fold induction</td>
<td>1.5 D</td>
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<tr>
<td>MMTV-BAG</td>
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<tr>
<td>Dex</td>
<td>1.25 × 10⁷</td>
</tr>
<tr>
<td>+ Dexamethasone</td>
<td>4.16 × 10⁷</td>
</tr>
<tr>
<td>Fold induction</td>
<td>3.3 D</td>
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a Total cell protein was extracted from populations of infected cells that had previously been selected in G418-containing medium, and 5 μg was analyzed for β-gal enzymatic activity by using the Galacto-Light kit according to the manufacturer’s instructions (Tropix, Bedford, Mass.).

b – Dex, without dexamethasone; + Dex, with dexamethasone.

c lu, light units.

d ND, not detectable.
infected cells, enzyme activity was determined, either by using a light-emitting substrate in a photometric assay or by histochemical staining. All of the tested infected cell populations showed expression of β-gal in the absence of glucocorticoid. However, addition of the synthetic glucocorticoid dexamethasone resulted in enhanced expression (Table 1), as expected, from the MMTV promoter (5). Levels of induction were between 3- and 40-fold, depending on the cell type; the best induction was observed in CrFK cells (Table 1), one of the few cell lines known to be permissive for MMTV replication (reference 21 and references cited therein). In contrast, levels of induction from the MLV promoter were maximally 1.6-fold. This low level of glucocorticoid induction of murine C-type promoters has been reported previously (11). Histochemical staining of Rat-2 cells infected with MMTV-BAG grown in the presence or absence of dexamethasone revealed that more cells express the β-gal enzyme when grown in the presence of dexamethasone than when grown in the absence of this hormone (data not shown). The hormone response element of MMTV has been delineated to the region between positions −50 and −202 (for a review, see reference 5); thus, the lower (two- to threefold) levels of dexamethasone induction of virus-specific RNA and reverse transcriptase activity in the supernatant previously reported by Overhauser and Fan (16) may be explained by the fact that only part of this element (positions −117 to −202) was included in the previously transferred 335-bp U3 fragment.

The MMTV promoter should be especially well suited for the expression of genes in the mammary gland in vivo. To investigate this, we analyzed cells explanted from the mammary gland of a pregnant mouse and established in monolayer culture as well as the pregnant-mouse mammary gland in vivo for expression of the β-gal reporter gene after infection with the two vectors (Fig. 4). Although the MLV promoter in the BAG virus gave better levels of expression than the MMTV-BAG in infected primary mammary cells in cell culture, the in vivo expression levels from the MMTV promoter were consistently higher than those from the MLV promoter (BAG virus).

The MMTV-BAG retroviral vector carries a procaryotic origin of replication (Fig. 1), allowing the recloning of the integrated provirus from genomic DNA. Genomic DNA was isolated from the population of MMTV-BAG-infected Rat-2 cells; digested with the restriction enzyme Asp700, which does not cleave within the vector provirus; ligated; and used for electroporation of Escherichia coli. After selection on kanamycin-containing medium, a number of plasmids carrying integrated proviruses were obtained. Restriction enzyme mapping combined with Southern blotting confirmed that the MMTV

![Graph](image-url)

**FIG. 4.** Expression of β-gal in infected mammary cells. Primary mammary epithelial cells were prepared from 10-day-pregnant BALB/c mice and infected with either the MMTV-BAG (MMTV.) or BAG virus. For in vivo experiments, the MMTV-BAG or BAG virus was injected directly into the mammary gland of a 10-day-pregnant BALB/c mouse; 5 days later, protein extracts were prepared and analyzed for β-gal activity, using the Galacto-Light kit according to the manufacturer’s instructions (Tropix, Bedford, Mass.). Each bar represents the average of data from multiple independent experiments, and error bars (indicating the standard error of the mean) are shown. RLU, relative light units.

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**FIG. 5.** Analysis of structure and expression of the hybrid provirus from transduced cells. (A) Sequencing of the junction regions of a recloned provirus. Sequencing was carried out with an automatic sequencer (ABI 373a; Applied Biosystems). The reactions were performed with a dye terminator cycle sequencing kit (Applied Biosystems) according to the manufacturer's instructions. (B) Electrophoresis of the same DNA. Each lane was loaded with 1 μg of Escherichia coli plasmid DNA and 10 pmol of a primer pair, one of which was complementary to the LTR region of MMTV (S1, 5′-CCACAAGTCGGA-3′) and the other of which was complementary to the U5 region of MLV (S2, 5′-TGGCTGCTTCTC-3′). Shown are the sequences at the U3-R junction of the 50 and 89 nt fragment. (A) 202 (for a review, see reference 5); (B) 117 to 202. (C) The MMTV-BAG retroviral vector carries a procaryotic origin of replication (Fig. 1), allowing the recloning of the integrated provirus from genomic DNA. Genomic DNA was isolated from the population of MMTV-BAG-infected Rat-2 cells; digested with the restriction enzyme Asp700, which does not cleave within the vector provirus; ligated; and used for electroporation of Escherichia coli. After selection on kanamycin-containing medium, a number of plasmids carrying integrated proviruses were obtained. Restriction enzyme mapping combined with Southern blotting confirmed that the MMTV

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U3 region had replaced that of MLV (data not shown). Sequencing of the junction between the rat flanking sequence and at the 5′ end of the provirus revealed that the SacII restriction site and the MMTV U3 sequences inserted into this site were intact, as expected. The MLV IR was also present, but it had been shortened by 2 bp, as expected due to processing during the integration event (4). Similarly, the sequence at the border between the MMTV U3 and MLV R regions in the 5′ LTR revealed that the MMTV U3, MluI restriction site, and MLV R sequence were also intact (Fig. 5A).

Finally, we investigated whether the initiation site of RNA transcribed from the MMTV promoter carrier in the U3 region is maintained after insertion of the MluI restriction enzyme site, since it is known that the spacing between the TATA box in the promoter and the transcription initiation site is critical. The RNA initiation site was determined by S1 analysis. Total RNA from infected cells grown in the presence and absence of dexamethasone or from noninfected cells was prepared and hybridized to a probe protected a fragment of 90 nucleotides (Fig. 5B, lanes 1 and 2). The probe protected a fragment of 90 nucleotides (Fig. 5B, lanes 1 and 2), placing the site of transcription initiation 1 bp upstream of the probe protected a fragment from S1 digestion (Fig. 5B). The probe protected a fragment of 90 nucleotides (Fig. 5B, lanes 1 and 2), and the TATA box within the MMTV promoter and the classical R-U5 border by 6 bp. A clear induction of the signal could be observed in RNA derived from cells grown in the presence of the synthetic glucocorticoid hormone dexamethasone (lane 1) compared to that from cells grown in the absence of dexamethasone (lane 2). This confirms the data obtained by enzymatic assay for β-gal (Table 1).

In summary, the hybrid MMTV/MLV vector behaves as expected at the molecular level throughout the whole transduction cycle, and in the infected cell, expression of the integrated provirus is controlled by the newly inserted MMTV U3 promoter rather than the original MLV promoter. The MMTV promoter is preferentially active in mammary epithelial cells and a few additional cell types. This has been attributed to a mammary-specific regulatory region located in the MMTV U3 region between positions −1166 and −739 (5, 13, 14). This region was not included previously in the MLV constructs with MMTV U3 sequences inserted in the 3′ and 5′ LTRs (16). We have established transgenic mice carrying the recloned MMTV-BAG provirus and are currently determining the expression spectrum of the hybrid MMTV/MLV virus. Preliminary data suggest that expression from these vectors is limited to that of MMTV, suggesting that the hybrid MMTV/MLV vector may be a useful high-titer alternative to the previously described MMTV vector systems for further analysis of the viral life cycle.

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ADDENDUM IN PROOF


REFERENCES


