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A Replication-Competent Promoter-Trap Retrovirus

JOSÉE COULOMBE,[1,2] YVONNE AVIS,[2] AND DOUGLAS A. GRAY[1,2,3]*

Departments of Biochemistry[1] and Medicine,[3] University of Ottawa, and Ottawa Regional Cancer Centre,[2] Ottawa, Ontario, Canada K1H 8L6

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A promoter-trap retrovirus has been constructed in which a promoterless polyomavirus middle T antigen gene was inserted in the U3 region of the long terminal repeat of a replication-competent Moloney murine leukemia virus. The resulting virus, designated PyT, was used to infect mouse mammary glands in situ. As expected, mammary tumors appeared in some infected animals. These tumors were found to contain PyT proviruses of the predicted structure. From one such tumor, the PyT provirus and surrounding sequences from the integration site were cloned. The provirus was found to have integrated adjacent to the promoter of a novel mouse gene (TRAP1) that was expressed at low levels in various mouse tissues. These data show that the PyT retrovirus provides a sensitive means of detecting active promoters in vivo.

Promoter-trap strategies have proven to be very effective for cloning genes that are active at various stages of development. Frequently this has been done in embryonic stem cells, which when reintroduced into blastocysts generate chimeric embryos that can be bred to identify developmental defects (2, 5, 6, 15, 17). By using a promoter-trap strategy, one can considerably increase the efficiency of insertional mutagenesis because there is selection for insertions into genes as opposed to "junk" DNA. This enrichment decreases the number of embryonic stem cell clones to be screened for a phenotype. The enrichment of the promoter-trap has been estimated to be 100- to 1,000-fold (2). Promoter traps also facilitate the cloning of the integration site in that the transgene is located within the gene to be cloned.

Two types of promoter-trap vectors have been used for insertional mutagenesis in embryonic stem cells. The first has a promoterless reporter gene in a plasmid vector which is electroporated into cells (6, 15). The second type makes use of retroviral vectors (5, 17, 18), which have a number of advantages over plasmid vectors. The integration of proviral DNA into the genome is less disruptive than the integration of naked DNA and therefore eliminates the possibility of rearrangements which can inactivate the promoter. Retroviruses also have a certain affinity for DNase-hypersensitive sites which are found near promoter regions (13, 16), therefore increasing the chances of integration within a gene. Finally, as we have shown here, retroviral vectors allow promoter-trap experiments to be performed in vivo.

Different variations of a retroviral promoter-trap vector have been used (5, 17, 18). In one experiment, a promoterless drug resistance gene was inserted in the U3 region of the long terminal repeat (LTR) so that upon proviral integration, the indicator gene was positioned 30 bp away from genomic sequence (18). The presence of foreign DNA in this region of the LTR did not alter the ability of the virus to replicate (18). This strategy had the advantage that the virus was replication competent. A second group introduced a reporter gene preceded by a splice acceptor site, in place of the viral structural genes (5). This increased the probability that the reporter gene would be expressed (expression of the reporter occurs upon insertion in the gene anywhere after the first splice donor; such strategies are more accurately described as gene traps) but rendered the virus incapable of replicating. Because the comparatively low titer of defective viruses would limit the number of insertion events that we could screen in vivo, we modeled our strategy on the replication-competent promoter-trap virus (18).

We chose to identify promoters in the mouse mammary gland. This gland is unusual in that most of its development takes place postnatally (the breast remains rudimentary until puberty, whereupon the ductal structure of the gland expands; during pregnancy, the mammary gland reaches full development [4]), and it is likely that there is a program of gene expression that accompanies development. The mammary gland is also ideal for a retroviral promoter-trap strategy in that its epithelium is topologically continuous with the exterior of the animal and can be infected by introduction of virus through the nipple.

Obviously it is not possible to perform in vivo experiments using retroviruses that incorporate genes conferring resistance to cytotoxic drugs that would kill the animal. We therefore chose to include a promoterless oncogene as the marker gene in our experiments. Mouse mammary epithelium has been shown to be very susceptible to transformation by the polyomavirus middle T antigen (PyMT). Transgenic mice carrying this viral gene under the control of the mouse mammary tumor virus (MMTV) promoter (MMTV LTR) develop multifocal mammary tumors around the age of 2 months (8). The apparent single-hit kinetics of PyMT transformation of mouse mammary epithelium suggested that PyMT would be an appropriate reporter gene for an in vivo promoter-trap retrovirus. Because retroviruses require that the cells be in a replicative stage to integrate (3), we chose to initiate infections at puberty, when the mammary gland undergoes ductal expansion (4). Here we report the construction of PyT, a replication-competent retrovirus incorporating a promoterless PyMT gene, its use in infection of pubertal mouse mammary glands, and the subsequent identification of a cellular promoter active in that tissue.

MATERIALS AND METHODS

Retroviral vector and virus production. The PyMT sequence, a *Hin*dIII-to-*Eco*RI fragment from the MMTVMT vector (a gift from W. Muller; 8), was inserted in pLTR12 (12), which contains one Moloney murine leukemia virus (MoMuLV) LTR with an engineered *Bam*HI site in place of the *Sau*3A site at position 95. The insert was ligated to the vector by blunt ligation. The remainder of the MoMuLV provirus was ligated to the modified pLTR12 vector as an

* Corresponding author. Mailing address: Ottawa Regional Cancer Centre, 501 Smyth Rd., Ottawa, Ontario, Canada K1H 8L6.

*Eco*RI-*Cla*I fragment from pMov3 (gift of R. Jaenisch). This positioned the PyMT coding sequence in the 3′ LTR.

The PyT construct was cotransfected with a neomycin resistance-encoding plasmid (pgk-neo [10]) in NIH 3T3 cells by the $CaPO_4$ method (modification of the method of Graham and van der Eb [7]), and clones were selected in G418. Producer clones were tested for viral titer by an XC assay (14). For the nipple injections, the supernatant from a plate of confluent cells was cleared of cells by passing it through a 0.2 μm-pore-size filter. For cell infection, supernatants were prepared as described above and were transferred to NIH 3T3 or NMuMG cells with 8 μg of Polybrene (Sigma) per ml.

PyMT expression. 293T cells (plated at 5×10^5/60-mm-diameter dish the day before) were transfected transiently, using $CaPO_4$, with various amounts of a plasmid encoding the PyMT sequence under the control of the *pgk* promoter. Cells were harvested 24 h later in sample buffer (4% sodium dodecyl sulfate [SDS], 5% β mercaptoethanol, 20% glycerol, 120 mM Tris [pH 6.8], 1 mg of bromophenol blue per ml).

Approximately 5 million NMuMG cells were infected with PyT at a multiplicity of infection near 2. Forty-eight hours later, the cells were plated in 0.3% agarose, and colonies were isolated after 4 weeks. Proteins were extracted from confluent 60-mm-diameter dishes in 200 μl of sample buffer.

For Western blot (immunoblot) analysis, 1/20 of the protein lysate was loaded on an SDS–10% polyacrylamide gel and transferred to a nitrocellulose membrane (Costar). The PyMT antibody was the monoclonal antibody Ab815.

Nipple injections. Nipple injections were performed by a modification of the method of Wang et al. (19). Four-week-old BALB/c female mice were anesthetized with Avertin (10 g of tribromoethanol [Aldrich] in 10 ml of *tert*-amyl alcohol [BDH], diluted in saline) at 0.4 mg/g of body weight. Freshly isolated virus, to which 80 μg of Polybrene (Sigma) per ml was added, was injected directly in the nipple with a 30-gauge needle. Approximately 0.05 to 0.1 ml (5×10^4 to 1×10^5 CFU) was injected into each nipple.

Tumor analysis and cloning of integration sites. Tumors were surgically removed, and DNA was extracted with 400 μg of proteinase K per ml in SDS buffer (75 mM NaCl, 25 mM EDTA, 10 mM Tris [pH 7.6], 1% SDS) at 37°C overnight. Following two phenol-chloroform extractions and ethanol precipitation, 10 μg of DNA was digested with *Eco*RI and was run on a 1% agarose gel. The DNA was transferred to Hybond-N membrane (Amersham). The PyMT sequence was radioactively labeled by using random oligonucleotide primers (Boehringer). For the construction of the library, 50 μg of DNA was digested with *Eco*RI, and 12- to 20-kb fragments were isolated on a 5 to 20% potassium acetate gradient. The size-selected DNA was ligated to λDASHII arms digested with *Eco*RI (Stratagene) and packaged by using a Gigapack extract (Stratagene). A total of 500,000 plaques were screened, and 6 positives were plaque purified. Phage DNA was digested with various enzymes and was run on 1% agarose gel for Southern blot analysis.

Subcloning and sequencing. The genomic DNA flanking the provirus was subcloned into pBluescript (Stratagene) by using *Eco*RI and *Kpn*I sites. DNA was sequenced by using a Pharmacia T7 sequencing kit with either the Pharmacia Universal primer or a primer located in the LTR. The sequence was analyzed by using the DNASTAR program.

Northern (RNA) analysis. Total RNA was isolated by using LiCl, and mRNA was prepared by using oligo(dT) columns (BDH column, Bethesda Research Laboratories resin). Three micrograms of poly(A)$^+$ RNA was run on 1% agarose formaldehyde gel and transferred to a Hybond-N membrane (Amersham). An *Eco*RI-*Nhe*I genomic fragment flanking the 5′ LTR was labeled as before.

Promoter assay. An *Nhe*I fragment which included all of the cloned 5′ genomic sequence (except for 220 bp at the 5′ end) and the first 31 bp of the 5′ LTR was subcloned into the *Xba*I site of the pCAT-enhancer vector (Promega). Five micrograms of the so-called TRAP-CAT vector was cotransfected in 293 cells, together with an equal amount of a plasmid encoding *lacZ* (pgk-lacZ; gift of M. W. McBurney), using the $CaPO_4$ method. Twenty-four hours later, the cells were harvested in 0.25 M Tris (pH 7.8) and lysed with two rounds of freeze-thaw (dry ice-37°C). The samples were divided into two, one for each enzyme assay, and one half (for the chloramphenicol acetyltransferase [CAT] assay) was heated at 65°C for 10 min. Samples for both assays were centrifuged in a refrigerated microcentrifuge for 10 min. The supernatants were assayed for protein concentration by using a microassay from Bio-Rad. For the β-galactosidase assay, 10 μg of protein was mixed with 800 μl of assay buffer (100 mM $NaPO_4$ [pH 7], 1 mM MgS_4O, 10 mM β-mercaptoethanol) and 200 μl of *o*-nitrophenyl-β-D-galactopyranoside (4 mg/ml; BDH). Samples were analyzed on a Beckman DU 640 spectrophotometer, using the manufacturer's kinetics/Time program, at 420 nm. Five micrograms of protein was used to assay for CAT activity by using a fluorochrome-based kit (FMBIO Tangerine Fast CAT kit; Hitachi). The products were separated by thin-layer chromatography and analyzed on a fluoroimager (Hitachi), using the manufacturer's FMBIO (version 4.04) software.

RESULTS

In vitro analysis. The PyMT sequence was inserted in the 3′ LTR of an MoMuLV vector (Fig. 1A). If viral replication proceeded normally, upon integration of the provirus, the PyMT sequence would be duplicated in the 5′ LTR and would be positioned 95 bp 3′ to genomic sequences.

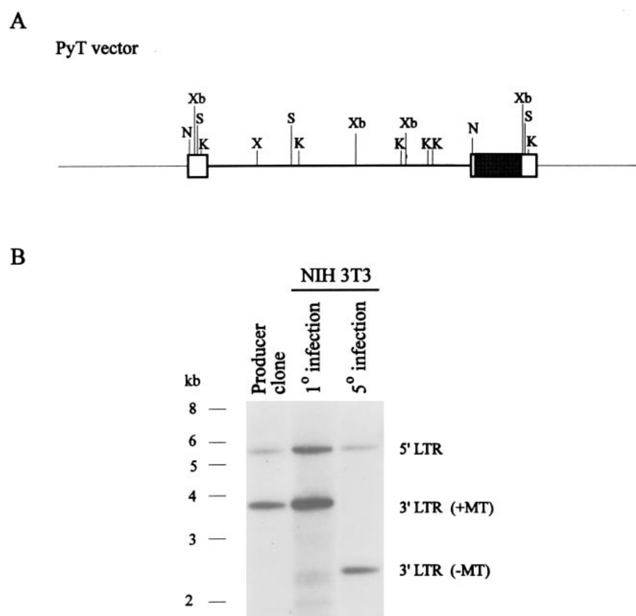

FIG. 1. (A) Schematic representation of the PyT retroviral promoter-trap DNA vector. The open box represents the LTR, and the cross-hatched box represents the PyMT sequence. Thin lines represent plasmid sequence. K, *Kpn*I; N, *Nhe*I; S, *Sst*I; Xb, *Xba*I; X, *Xho*I. (B) Southern blot of a PyT producer clone and PyT-infected NIH 3T3 cells. NIH 3T3 cells were infected with PyT and were fed fresh medium 24 h later. Forty-eight hours postinfection, the supernatant of the infected cells was used to infect fresh NIH 3T3 cells. This cycle was repeated for several passages. DNA was isolated from infected cells (48 h postinfection) and digested with *Xba*I for Southern blot analysis. The probe was MoMuLV LTR.

Before proceeding to in vivo experiments, we tested the replication of the virus in cell cultures. The PyT vector was transfected into NIH 3T3 cells, and 24 producer clones were isolated. The best clones gave a titer of 10^6 CFU/ml as determined by XC assay (14). The integrity of the provirus was confirmed by Southern blot analysis of PyT-infected NIH 3T3 cells. Bands of the predicted size were detected in digests with *Sst*I (not shown) and *Xba*I (Fig. 1B). The stability of the virus was tested for two selected producer clones, again by Southern blot analysis of PyT-infected cells. The virus was found to be stable for three rounds of infection when passaged through NIH 3T3 cells at 48-h intervals. After several passages, the PyMT sequences were lost (Fig. 1B, 5° infection), most likely by a recombination event.

The expression of PyMT was also verified in culture. PyMT was expressed transiently in 293T cells from the *pgk* promoter, and the protein was detected by Western blotting using whole lysate (Fig. 2A). The amount of PyMT detected correlated with the amount of plasmid introduced. Expression of PyMT by promoter trap was tested by infecting mammary epithelial cells (NMuMG) with PyT and selecting for growth in soft agarose. Expression of PyMT by 12 random clones was verified by Western blotting (Fig. 2B). Most clones (10 of 12) showed detectable levels of the PyMT protein. Clones 3 and 8 either did not express PyMT and grew in soft agarose as the result of unrelated events or expressed PyMT at levels that were sufficient for the phenotypic change but below the level of detection of the Western blot.

Mammary gland infections. Four-week-old BALB/c female mice were used for the mammary gland injections because this

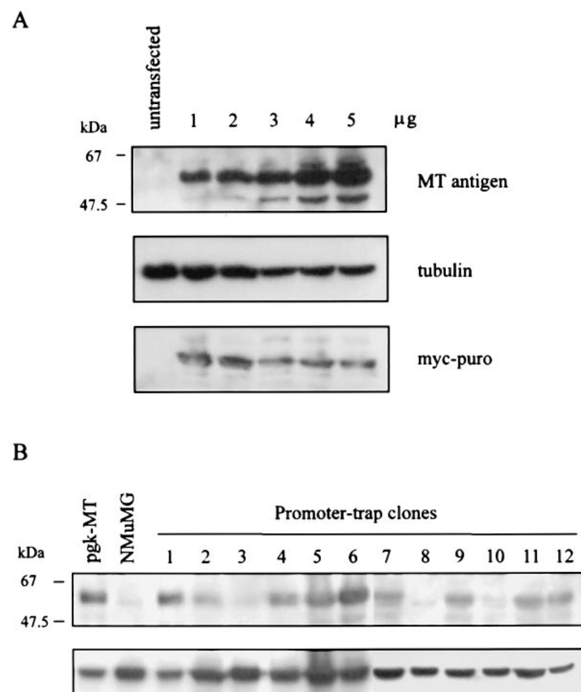

FIG. 2. Western blot analysis of PyMT expression. (A) Transient expression of PyMT in human 293T cells. The amounts of DNA used are indicated. Tubulin was used as a loading control. myc-puro, a fusion between six copies of a human Myc epitope and the puromycin protein, served as an internal control for the transfections. (B) Expression of PyMT by promoter trap. pgk-MT is a stable clone used as a positive control for PyMT expression. NMuMG is the parental cell line for the promoter-trap clones (negative control). Promoter-trap clones 1 to 12 were derived from colonies that grew in soft agarose after infection with PyT. The loading control is tubulin.

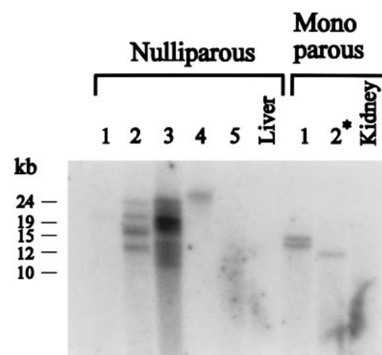

FIG. 3. Southern blot of *Eco*RI-digested tumor DNA, probed with the PyMT sequence. Each band represents one proviral integration site. Tumors from two different animals (nulliparous and monoparous) are shown, with normal liver or kidney DNA as controls. Each number represents a different mammary tumor except for 2*, which represents a spleen tumor.

is a period during which the mammary epithelium proliferates to form the ducts (4), proliferation being a requirement for retroviral infection (3). A summary of the tumors obtained is shown in Table 1. A total of 20 animals were injected, most in all 10 glands. The first tumor was detected in an animal 6 weeks postinfection. This animal eventually developed two additional visible tumors and was sacrificed (Fig. 3, nulliparous). One tumor mass was quite large and turned out to be a mixture of two or three tumors (as evidenced by blot analysis [Fig. 3]). To ensure activation of mammary gland-specific promoters, animals that were free of tumors at 6 weeks postinfection were mated. One mouse developed a tumor 3 weeks later. The tumor stopped growing after about a week, and the animal was sacrificed (Fig. 3, monoparous). A second tumor was observed on the spleen and was saved for analysis. A third mouse developed a tumor a week after weaning her first litter (7 weeks postmating). The animal was sacrificed a week later, and a second tumor was found on the colon; this tumor was also saved for analysis. No other tumors were detected among the rest of the injected animals, and they were sacrificed after 5 months.

TABLE 1. Summary of tumors found in PyT-infected animals

Mouse	No. of mammary tumors	Time of detection (wk postinfection)	Other tumor
Nulliparous	5	6	None
Monoparous	1	9 (3 wk postmating)	Spleen
Weaning	2	13 (7 wk postmating)	Colon

A second set of 19 mice were infected in the same way with a replication-competent MoMuLV containing a different DNA sequence in place of PyMT. No tumors were detected after 10 weeks.

Analysis of the tumors. All tumors were analyzed for the presence of the PyT provirus by Southern blot analysis (Fig. 3). *Eco*RI was chosen because it did not have any recognition site in the provirus and could therefore reveal the number of integrated proviruses (one *Eco*RI band equals one integrated provirus). The presence of the virus was confirmed in all but the three tumors from the mouse that developed tumors after weaning (Fig. 3). The spleen tumor found in the second mouse (Fig. 3, lane 2*) was independent of the mammary tumor, as indicated by their different patterns of provirus integration site. This finding suggests that PyT was replicating and getting into the circulation. This assertion was also supported by the observation that the tumor-free mice eventually showed symptoms of MoMuLV infection (9), including enlarged thymuses.

Cloning of the integration site and analysis of the genomic flanking sequence. We chose to focus on the mammary tumor that arose in the pregnant mouse (Fig. 3, monoparous). A genomic library was constructed from size-selected *Eco*RI-digested tumor DNA and probed with the LTR sequence. Two different clones corresponding to the two integrations sites were isolated. This was confirmed by reprobing the Southern blot shown in Fig. 3 with genomic sequences from these two clones sequentially. Each clone showed one band corresponding to one of the two integration sites seen on the initial probing with PyMT (data not shown). Figure 4A shows the map of the genomic clone corresponding to the promoter trap. This clone was selected based on both sequencing and Northern blot analysis data (see below). Also shown are the sizes of the 5′ and 3′ genomic flanks as mapped by Southern blot analysis using *Eco*RI in combination with either *Kpn*I or *Xho*I (Fig. 4A).

Genomic sequence from the 5′ end of the integration site was used to probe a Southern blot containing NIH 3T3 cell DNA digested with three different enzymes (Fig. 5). The restriction enzyme pattern of our clone is consistent with a single-copy gene, as evidenced by the single intense band detected in *Eco*RI and *Sst*I digests (Fig. 5). In addition, the genomic probe detected an extra band in an *Eco*RI digest of genomic

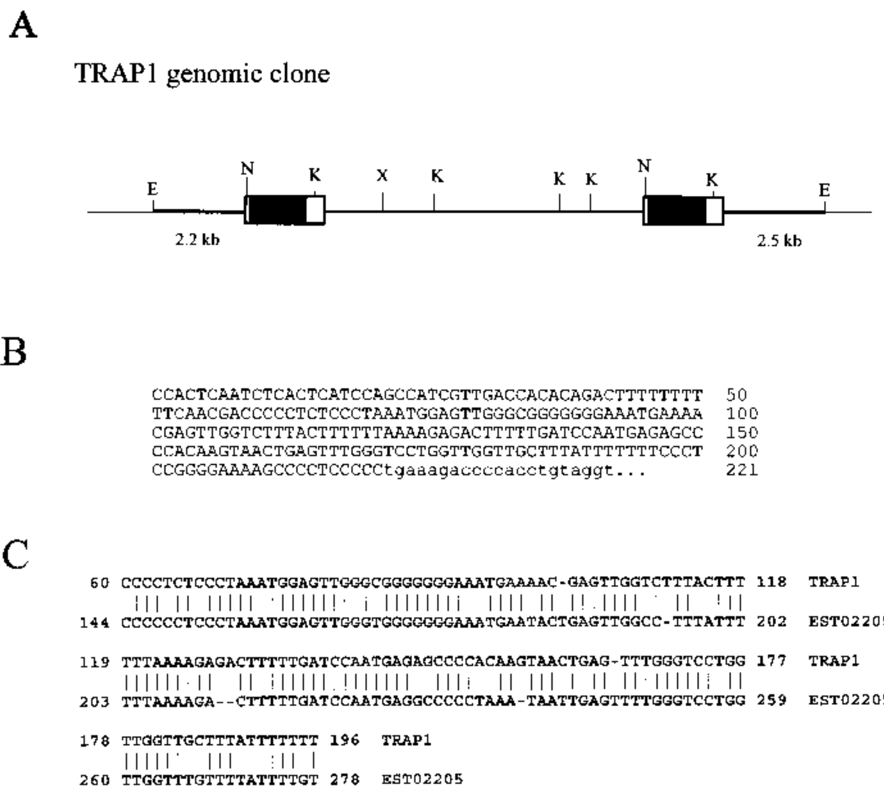

FIG. 4. (A) Schematic representation of the TRAP1 genomic clone. The open box represents the LTR, and the cross-hatched box represents the PyMT sequence. The thick line represents genomic sequence, and the thin line represents the phage arms. E, *Eco*RI; K, *Kpn*I; N, *Nhe*I; X, *Xho*I. (B) Sequence data from the genomic DNA proximal to the 5′ LTR. Numbers refer to the genomic sequence (capital letters). Lowercase letters represent LTR sequence. (C) Alignment of TRAP1 and EST02205 as obtained from GenBank. There is 85% identity between these sequences.

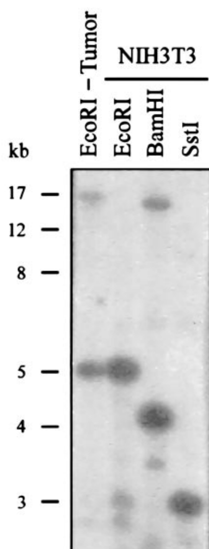

FIG. 5. Southern blot of tumor DNA digested with *Eco*RI and of NIH 3T3 cell DNA digested with *Eco*RI, *Bam*HI, or *Sst*I. The probe was the genomic fragment 5′ of the proviral integration site. Comparison of tumor DNA and NIH 3T3 DNA digested with *Eco*RI confirmed that the probe was derived from the integration site.

DNA from the tumor corresponding to the disrupted allele. These data confirmed that the cloned sequences were from the site of proviral integration.

Northern blot analysis of various mouse tissues was used to test whether the genomic DNA flanking the provirus contained transcribed sequences. Using RNA from breast tissue of virgin and pregnant mice, we could at the same time determine whether the trapped gene was expressed specifically during mammary differentiation. Using poly(A)$^+$ RNA and the genomic probe used in the analysis described above, we detected a low-abundance transcript of approximately 4.5 kb which was present in all tissues tested (Fig. 6). Other transcripts of lower abundance were also detected. The major transcript was present in equal amounts in pregnant and virgin breast tissue. Testis contained high levels of this transcript together with two additional transcripts not detected in the other tissues.

Sequence was obtained from both ends of the genomic clone. A 220-bp sequence adjacent to the 5′ LTR (Fig. 4B) showed no similarity to any mouse sequence in the GenBank database but revealed strong similarity to a partial human cDNA sequence, previously isolated from human brain as expressed sequence tag (EST) (1) (Fig. 4C). The sequence identity between TRAP1 and EST02205 was 85% over 136 bases, ending at the 3′ limit of the 278-base EST02205. It is possible that further similarity exists with the remainder of the mRNA from which EST02205 was derived.

The TRAP1 promoter. Because retroviruses tend to integrate near the 5′ ends of genes, we decided to test for the presence of the TRAP1 promoter in the 2.2-kb genomic frag-

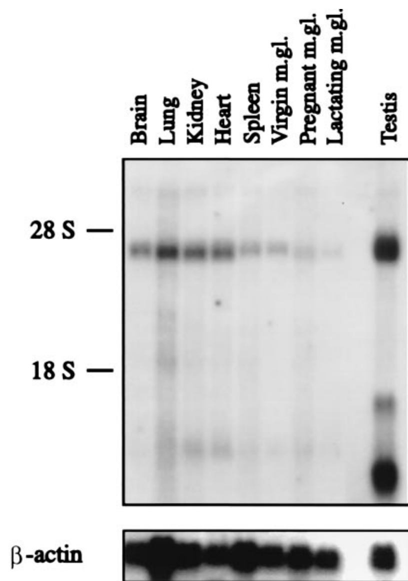

FIG. 6. Northern blot [poly(A)$^+$ RNA] of various adult mouse tissues. The probe used was the genomic fragment 5′ of the retroviral integration site. β-Actin was used as a loading control. m.gl., mammary gland.

ment 5′ of the integration site. This fragment was subcloned in front of the bacterial CAT gene in a vector containing the simian virus 40 (SV40) enhancer. The vector was assayed for CAT activity by transient transfection in the human 293T cell line. Figure 7 shows an example of the thin-layer chromatograph (Fig. 7A) and the CAT activity (Fig. 7B) reported as percentage of the substrate converted (the percent conversion was calculated from nonsaturating amounts of samples). There was a 75-fold difference between the percent conversion of the putative promoter (TRAP+) and that of the vector alone (average of three experiments). The transfection efficiency did not vary more than twofold as calculated by β-galactosidase activity.

To demonstrate that the TRAP1 promoter did drive the expression of PyMT in the tumor, the 5′ junction fragment of the provirus (which included the genomic sequence used in the CAT assay described above and the start of the provirus up to the *Xho*I site in the *gag* sequence) was transfected transiently into 293 cells, and PyMT was detected by Western blot analysis of whole lysates (Fig. 8). The amount of PyMT detected paralleled the amount of plasmid added, indicating that the PyMT sequence in the LTR was expressed from the TRAP1 promoter.

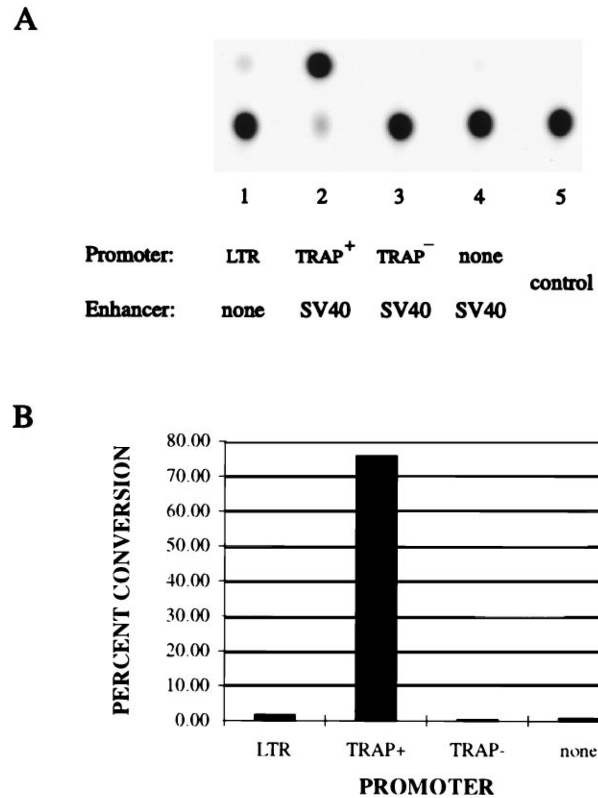

FIG. 7. (A) Thin-layer chromatograph of a typical CAT assay (the modified fluorescent substrate generates only one acetylated product). Lane 1 represents a positive control, a plasmid encoding CAT under the control of the MMLV LTR (gift of Ninan Abraham; this plasmid does not contain the SV40 enhancer). TRAP$^+$ and TRAP$^-$ are plasmids containing the TRAP1 genomic fragment subcloned in front of CAT in the positive and negative orientations, respectively. Lane 4 represents the vector alone, with the SV40 enhancer but no promoter. In lane 5, no protein was added to the assay. The amount of converted substrate in lane 5 was used as the background for the calculation of the conversion. (B) CAT activity shown as percentage of converted substrate. Samples are the same as in panel A.

DISCUSSION

From 20 animals that were infected in the mammary glands with PyT, we collected a total of 10 tumors, 8 of which appeared at sites and with gross morphology consistent with a mammary origin. Six of ten tumors were found to contain PyT proviral DNA. One animal developed three distinct tumors that were found to be negative for the PyT provirus. We currently cannot explain the origin of these tumors, which arose in a strain (BALB/c) of low spontaneous tumor incidence (11). In contrast, no tumor formed in animals infected with a similar replication-competent virus containing irrelevant and nonfunctional sequences (3a).

The fact that a number of tumors have more than one integrated provirus indicates that the efficiency of mammary infection by nipple injection can be very high. It is unlikely that multiple *Eco*RI bands of roughly equal intensity represent reinfection events during the growth of the tumor; rather, these bands represent proviral content in the cell that initiated tumor formation. It is difficult to estimate the efficiency of the promoter trap in vivo because one cannot accurately estimate the number of target cells in the mammary gland (cycling cells to which the virus has access) or the number of infection events for the PyT retrovirus prior to the loss of the marker PyMT sequences.

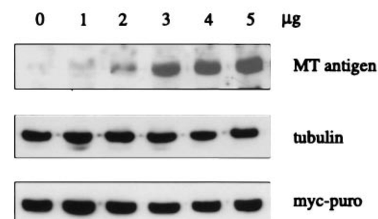

FIG. 8. PyMT expression from the TRAP1 promoter. The Western blot shows transient expression of PyMT from the TRAP1 promoter in 293T cells. The amounts of DNA used are indicated. Tubulin was used as a loading control. myc-puro, a fusion between six copies of a human Myc epitope and the puromycin protein, served as an internal control for the transfections.

The isolation and characterization of one of the promoter-trap integration sites allowed us to clone what appears to be a novel mouse gene that we called TRAP1. We base this conclusion on several lines of evidence. First, a fragment (2.2 kb) from this gene hybridized to a 4.5-kb transcript on a Northern blot of various mouse tissues. This gene was expressed at low levels, as the transcript was detected only in poly(A)$^+$ RNA. Second, the same fragment of genomic DNA was used to probe a Southern blot of normal DNA digested with several enzymes (Fig. 5), and the pattern obtained was consistent with a single-copy gene. These data make it unlikely that the transcripts detected with the TRAP1 genomic probe are derived from some repetitive element present in the probe. Finally, genomic sequence adjacent to the 5′ LTR showed 85% identity over 136 bases to the previously identified human brain partial cDNA EST02205. Transcripts were detected by the TRAP1 probe in the mouse brain (Fig. 6); our results are therefore consistent with TRAP1 genomic sequences corresponding to the murine homolog of the gene from which the human EST02205 was derived.

Northern blot analysis showed that TRAP1 was expressed at comparable levels in all adult tissues tested. The tumor from which TRAP1 was isolated was detected during pregnancy, but its early appearance (at 3 weeks postmating) exceeded expectations based on previous transgenic mouse studies in which PyMT was driven by the MMTV promoter (8). In these studies, PyMT-induced mammary tumors had a 4-week latency. For this reason, and in light of the low abundance of the TRAP1 mRNA transcript, we favor the hypothesis that relatively low levels of PyMT were expressed from the TRAP1 promoter. This level of expression resulted in the appearance of a tumor 9 weeks postinfection, which coincidentally corresponded to 3 weeks postmating. There is no evidence that the TRAP1 gene is transcriptionally regulated during gestation or that the gene is involved in mammary differentiation.

Northern blot analysis also showed that the TRAP1 promoter is active in normal mammary tissue and was not activated from a silent state by the retroviral enhancer. To avoid the possible activation of silent promoters through juxtaposition of the MMTV enhancer, these elements could have been eliminated from the virus by deletion or mutation, but such alterations would likely have resulted in lower viral titers. We chose to make as few alterations to the MMTV sequences as possible so that the titer (and hence the number of insertion events) would be optimal for in situ infections, for which the volume of the inoculum is necessarily limited.

It is an expectation of our promoter-trap strategy that integration of the PyT provirus will occur immediately downstream of an active promoter; intervening sequences beyond a certain length would be expected to contain AUG codons that would impede the translation of the PyT marker gene. We therefore thought it likely that the 2.2-kb 5′ genomic fragment cloned from the integration site would contain a promoter element. This prediction was confirmed in experiments where the 5′ genomic sequences were attached to a CAT reporter gene (Fig. 7). The activity of this promoter was found to be higher than that of the MoMuLV LTR, but the comparison has the caveat that the TRAP1 promoter was aided by a distal SV40 enhancer element, whereas the MoMuLV LTR was not (it did, however, contain nascent LTR enhancer elements). Our expectation (based on Northern analysis) was that the TRAP1 promoter would be weak. Either its activity is greatly elevated by the SV40 enhancer or the 2.2-kb fragment is devoid of neighboring negative elements that exist in the mouse genome (or both). We have also shown that the TRAP1 promoter can drive the expression of PyMT (Fig. 8).

We have shown that PyT, a replication-competent retrovirus, can be used to introduce a promoterless oncogene into the genomes of mouse cells in the intact animal. The data presented herein demonstrate that this virus will infect a sufficient number of cells in a target tissue to cause the formation of a tumor. Further, from cloning a provirus from one such tumor, we have demonstrated the presence of a cellular promoter immediately adjacent to the proviral LTR as predicted. We are therefore confident that PyT can be used to screen for active promoters in murine tissues or cells, either in vivo or in vitro. By producing the virus in an amphotropic cell line, it may also be possible to infect nonmurine cells or tissues, although we have yet to test this prediction.

ACKNOWLEDGMENTS

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