JOURNAL OF VIROLOGY, Sept. 1996, p. 6054–6059 Vol. 70, No. 9
0022-538X/96/$04.00+0

Inducible Gene Expression by Retrovirus-Mediated Transfer of a Modified Tetracycline-Regulated System

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Received 18 March 1996/Accepted 6 June 1996

The ability to regulate gene expression via exogenous stimuli will facilitate the study of gene functions in mammalian cells. In the present study, we modified the tetracycline-controlled inducible system by the addition of the ligand-binding domain of the estrogen receptor to the carboxy terminus of the tTA transactivator. A single retroviral vector can transduce both the transactivator gene and the gene of interest controlled by the tTA-inducible promoter into mammalian cells. We show that cell lines expressing the transactivator can readily be established and that expression of the gene of interest depends on the removal of tetracycline and the addition of estrogen. By using this system, cell lines with inducible expression of the G protein of vesicular stomatitis virus, a potentially toxic gene product, were established. The combination of a powerful inducible system and retrovirus-mediated gene transfer can not only be used to study gene function but may also be applied in the future to clinical trials in human gene therapy.

The ability to regulate the transcription of transduced foreign genes in mammalian cells and transgenic animals will facilitate the analysis of gene function during embryonic development and cellular differentiation and for studies of gene therapy (1, 22, 24). Gossen and Bujard (11) have demonstrated tight control of gene expression in mammalian cells by using an inducible system based on the Tn*10*-specified tetracycline resistance operon of *Escherichia coli*. In this system, gene expression is regulated by a hybrid transcription factor, tTA, that consists of the transactivation domain of herpes simplex virus VP (10, 23) fused to the carboxy terminus of the tetracycline repressor (tetR). The gene of interest is placed downstream of a minimal promoter derived from the immediate-early gene of cytomegalovirus (CMV) linked to seven tandem copies of the tetR-binding site (tetO). The activation of transcription from this promoter depends strongly on the binding of tTA to the tetO site, a process which is tightly regulated by tetracycline. In the presence of tetracycline, the binding of tTA to the tetO site is blocked and gene expression is silent. The removal of tetracycline results in the binding of tTA and activation of gene expression. Since even low-level concentrations of tetracycline are sufficient to block tTA function and most mammalian cells can tolerate tetracycline to certain extent, this system provides a tightly regulated on/off switch for gene expression in many different cell types (9, 11, 13, 18, 20).

However, in order to use this system, cell lines that stably express tTA must first be established. For this difficult purpose, conventional transfection methods, such as calcium phosphate coprecipitation, may not always be efficient enough for the transfer of the tTA gene into the cell of interest. Furthermore, efficient expression of tTA is toxic to cells probably because of the squelching effect generated from the VP16 transactivation domain in tTA (10, 11, 20). Once cell lines that stably express tTA have been established, the gene of interest under the control of the tetO-containing promoter must be introduced. Thus, the whole procedure to establish such cell lines for inducible gene expression can be tedious and time-consuming. To alleviate some of these problems, we have constructed a hybrid protein, tTAER, containing the ligand-binding domain of the estrogen receptor (ER) fused to the carboxy terminus of tTA, and inserted both the modified tTA gene and the gene of interest controlled by the tetO-containing promoter into a retroviral vector for gene delivery into cells.

When the ligand-binding domains of several members of the steroid hormone receptor family are fused with heterologous proteins, the expression of fusion proteins becomes regulated by the cognate hormone (15). It has been hypothesized that the disruption of the normal functions of the wild-type protein is due to the association of the hormone receptor ligand-binding domain with a complex of cellular proteins, including heat shock protein 90, in the absence of the cognate hormone (15). Hormone binding results in the release of the cellular protein complex from the fusion protein, leading to the restoration of the normal functions of the protein. By placing the ER ligand-binding domain adjacent to the transactivation domain of VP16 in tTAER, it seemed likely that the transactivation function and the potential transcriptional squelching effect of VP16 would come under hormone control. This property of tTAER should alleviate the difficulty in the establishment of stable tTA-expressing cell lines.

To introduce this inducible system into mammalian cells more efficiently, we have incorporated both the tTAER gene and the gene of interest under the control of the tetO-containing inducible promoter into the same retrovirus vector. We show that the complete inducible system can be transferred efficiently into cells via retrovirus infection rather than DNA transfection. Since many mammalian cell lines are refractory to DNA transfection but are accessible to infection by retrovirus, the availability of an efficient viral transduction method for transferring the entire regulatory system ensures that studies of gene functions regulated by this inducible system will not be limited only to those cell types with high transfection efficiencies. Moreover, this inducible system should allow the expression of a toxic gene product in mammalian cells conditionally. Using this system, we have established stable cell lines containing the gene encoding the G protein of vesicular stomatitis

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virus (VSV). High-level expression of VSV G in mammalian cells has been shown to lead to syncytium formation and cell death (26), making it difficult to establish stable cell lines that express this gene. The inducible system described here overcomes this difficulty and allows the inducible expression of VSV G in human cells. Using a similar approach, Paulus et al. recently demonstrated inducible gene expression regulated by tTA (16).

MATERIALS AND METHODS

Plasmid construction. To construct the tTAER gene, a 1-kb *Eco*RI-*Bam*HI DNA fragment containing the tTA gene was isolated from pUHD15-1 (11) and ligated to a 0.95-kb *Bam*HI-*Sst*I DNA fragment containing the ligand-binding domain of the ER from pHE14 (14). To construct pTetO-CAT, a 1.5-kb *Bam*HI-*Hpa*I DNA fragment containing the gene encoding bacterial chloramphenicol acetyltransferase (CAT) from pTKCAT (25) was inserted at the unique *Bam*HI site of pUHG10-3 (11). To construct pTetO-G-1, the 1.6-kb *Bam*HI DNA fragment containing the VSV G gene was isolated from pCMV-G (26) and was inserted at the unique *Bam*HI site of pUHG10-3. To construct pTEPN, the tTAER gene was first inserted at the unique *Bam*HI site of pCMV-Bam (26), thus placing the gene under the control of the immediate-early promoter of CMV to make pCMV-tTAER. The 2-kb *Eco*RI DNA fragment containing the tTAER gene was isolated from pCMV-tTAER and inserted at the unique *Bam*HI site of pLPONL6 (26). To construct pTEPN-CAT, the 1.9-kb *Xho*I-*Xba*I DNA fragment containing the TetO-CAT cassette was isolated from pTetO-CAT and was inserted at the unique *Xho*I site immediately downstream of the neomycin phosphotransferase (*neoR*) gene in pTEPN. Plasmid pTEPN-G was similarly constructed by inserting the 2.1-kb *Xho*I DNA fragment containing the TetO-G cassette isolated from pTetO-G-1 at the unique *Xho*I site in pTEPN.

Cell lines and virus production. The human 293GP kidney cell line expressing the gag and pol proteins of Moloney murine leukemia virus (MoMLV) has been described previously (3). The human HT1080 fibrosarcoma cell line was obtained from American Type Culture Collection. All the cell lines used in this study were maintained in Dulbecco's modified essential medium containing 10% fetal calf serum. Tetracycline was added to the culture medium at a concentration of 1 μg/ml as needed. Phenol red-free Dulbecco's modified essential medium containing 10% charcoal-dextran-treated fetal calf serum and 2 μM 17β-estradiol was used to replace the tetracycline-containing medium for the induction of gene expression.

To generate infectious virus pseudotyped with VSV G, the plasmid construct containing the retroviral vector was cotransfected with pCMV-G into 293GP cells by the calcium phosphate coprecipitation method (12). Forty-eight hours after transfection, the virus harvested was used to infect 293GP cells, and individual G418-resistant colonies were isolated after 2 weeks. The expression of the introduced gene in picked clones was determined as described below, and the clone that produced the highest level of the gene product was then used for the production of the retroviral vector as described above.

Induction of gene expression. G418-resistant HT1080 cells were routinely maintained in medium containing 1 μg of tetracycline per ml and 800 μg of G418 per ml. To remove tetracycline, cells were washed with medium containing no tetracycline, with subsequent incubation at 37°C for 30 min. The washing procedure was repeated a minimum of three times. For induction of gene expression, cells were incubated in phenol red-free Dulbecco's modified essential medium containing 10% charcoal-dextran-treated fetal calf serum and 2 μM 17β-estradiol for 72 h before the cell extract was prepared. The CAT activity was determined by the method of Sleigh (21). The reaction mixture (100 μl) contained 150 mM Tris-HCl (pH 7.8), 1.6 mM chloramphenicol, 90 μM acetyl coenzyme A (Pharmacia), 1 μCi of [^{14}C]acetyl coenzyme A (60 mCi/mmol; Amersham), and 10 μl of cell extracts. The mixture was incubated at 37°C for 60 min, and the labeled chloramphenicol was quantitated by liquid scintillation counting after extraction into the ethyl acetate layer. The protein concentration was determined by the method of Bradford (2).

To detect VSV G expression by immunoblotting, cells were lysed in a buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, and 0.1% sodium dodecyl sulfate. The extract was spotted onto nitrocellulose paper, and VSV G protein was detected by the enhanced chemiluminescence Western blotting (immunoblotting) system (Amersham) with the I1 monoclonal antibody specific for VSV G (3). For the detection of cell surface VSV G expression, cells were stained with the I1 monoclonal antibody and fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G, with subsequent flow cytometric analysis.

Northern (RNA) blot analysis. Total cellular RNAs were isolated by the procedure of Chomczynski and Sacchi (5). Poly(A)$^+$ mRNAs were isolated by the polyATtract mRNA isolation system (Promega). mRNAs were separated on a 1% agarose–2.2 M formaldehyde gel, transferred to a nylon membrane, and hybridized with ^{32}P-labeled probes generated by the random primed DNA labeling kit (Boehringer Mannheim) (19). The probe for the VSV G gene was derived from a 1-kb *Kpn*I fragment of pTetO-G-1. The probe for β-actin was derived from a 2-kb *Pst*I fragment of pUCA1 containing the chicken β-actin cDNA (6).

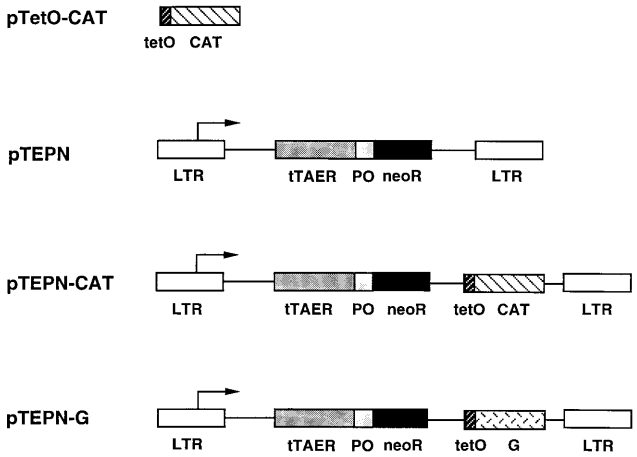

FIG. 1. Schematic illustrations of the plasmids used in this study. tetO, a minimal CMV immediate-early gene promoter linked to seven tandem copies of the tetR-binding site; CAT, the bacterial CAT gene; LTR, the LTR of MoMLV; tTAER, the gene encoding tTAER; PO, the internal ribosome entry site of poliovirus; *neoR*, the gene encoding the neomycin phosphotransferase; G, the VSV G gene. Arrows above LTRs indicate the approximate positions of transcriptional initiation sites. The plasmid maps are not drawn to scale.

RESULTS

Construction and expression of the tTAER gene from a retroviral vector. To overcome the potential squelching effect of tTA, we constructed a gene, designated tTAER, encoding the ER ligand-binding domain fused to the carboxy terminus of tTA. To express this gene, a bicistronic retroviral vector, pTEPN, in which the tTAER gene was followed by the internal ribosome entry site derived from the poliovirus genome and the neomycin phosphotransferase (*neoR*) gene was constructed (Fig. 1). The expression of both genes was under the control of the MoMLV 5′ long terminal repeat (LTR). To test the regulation of the tTAER transactivation function, human HT1080 fibrosarcoma cells were cotransfected with pTEPN and pTetO-CAT, containing the CAT gene controlled by a minimal promoter of the CMV immediate-early gene linked to seven tandem copies of the tetR-binding site (Fig. 1). The CAT activity of transfected cells was determined 48 h after transfection. As shown in Fig. 2, CAT expression was not activated in the presence of tetracycline alone or tetracycline plus the estrogen analog 17β-estradiol, consistent with the model that tTAER cannot bind DNA in the presence of tetracycline. In contrast to the transactivation function of tTA, tTAER failed to activate CAT expression upon the removal of tetracycline from the culture medium. CAT expression was strongly activated only when tetracycline was removed and 17β-estradiol was added to the culture medium. These results demonstrate that the transactivation function of VP16 in tTAER is modulated by the ER ligand-binding domain in *cis* and that the activity of tTAER is under the control of both tetracycline and 17β-estradiol.

To test whether cell lines that stably express tTAER can readily be established, HT1080 cells were infected with the infectious TEPN virus generated from 293GP cells and 17 individual G418-resistant colonies were picked and expanded. To test for tTAER activity, plasmid pTetO-CAT was transfected into these clones and CAT expression was determined 72 h after transfection. As shown in Fig. 3, 16 of these 17 clones produced CAT activity only when tetracycline was removed

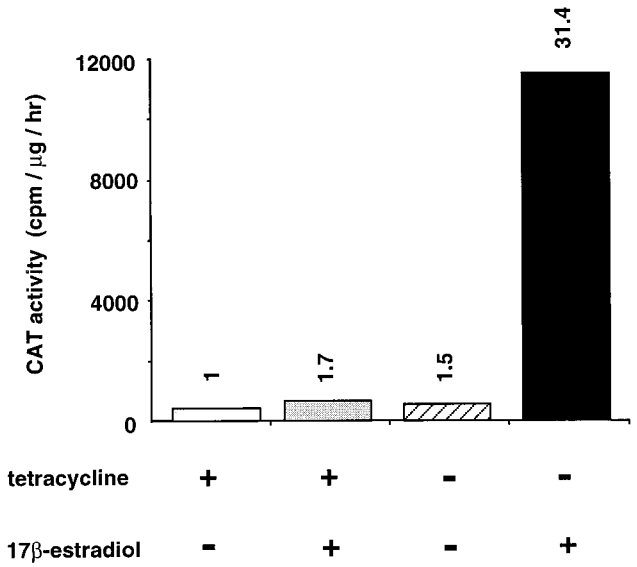

FIG. 2. Inducible CAT gene expression by tTAER. Fifteen micrograms of pTEPN was cotransfected with 15 μg of pTetO-CAT into HT1080 cells by the calcium phosphate coprecipitation method (12). As indicated, tetracycline or 17β-estradiol was (+) or was not (−) added to the culture medium 18 h after transfection, and cells were incubated for an additional 24 h. The CAT assay was performed as previously described (21), and the numbers above the bars of the graph are the relative fold of induction, as determined by normalizing the CAT activity of each condition to that of tetracycline-containing medium, which was arbitrarily set at 1.

and 17β-estradiol was added. The degree of induction varied from 3- to 40-fold (an average of 20-fold). The variation in induction may reflect different levels of tTAER in individual clones due to random retrovirus integration into host chromosomes. These results demonstrate that the addition of the ER ligand-binding domain subjects the transactivation function of tTA to the regulation of estrogen. Moreover, since a majority of the isolated clones expressed tTAER, it suggests that the toxicity associated with stable tTA expression is alleviated by the addition of the ER ligand-binding domain.

Inducible CAT expression using retrovirus-mediated gene transfer. To test whether the complete estrogen-inducible expression system, including the tTAER gene and the target gene controlled by the tetO promoter, can be transduced into mammalian cells with a single retroviral vector, we constructed pTEPN-CAT, containing the TetO-CAT cassette inserted immediately downstream of the *neoR* gene in pTEPN. Infectious TEPN-CAT virus was used to infect HT1080 cells, and 12 G418-resistant clones were picked and expanded. CAT expression in 10 of these 12 clones was activated only upon the removal of tetracycline and the addition of 17β-estradiol (Fig. 4). The induction of CAT expression varied from 8- to 27-fold (an average of 15-fold). The two clones which failed to induce expression had CAT activities close to the background level under all conditions, a result possibly of integration of the retroviral vector into a site unfavorable for gene expression or of mutations introduced into the retroviral genome during the process of reverse transcription. These results demonstrate that this inducible gene expression system can be transduced into mammalian cells with high efficiencies via retrovirus-mediated gene transfer.

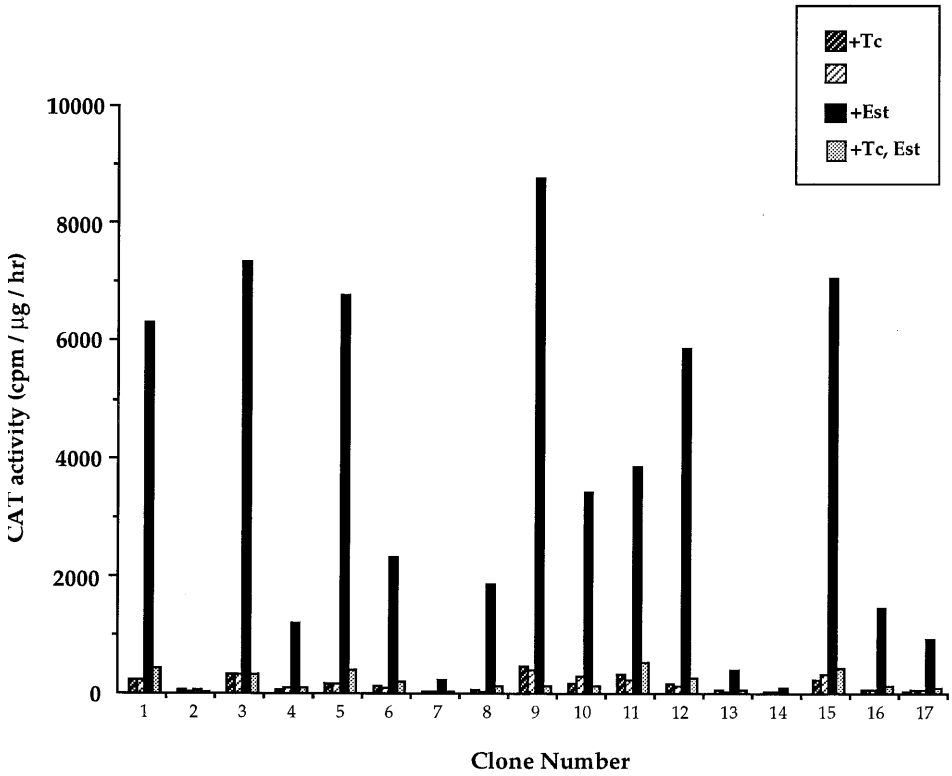

FIG. 3. Inducible CAT expression in stable tTAER-expressing HT1080 cells. Each G418-resistant HT1080 clone was transfected with 15 μg of pTetO-CAT. The induction of CAT expression and the CAT assay were performed as described in the legend to Fig. 2. +Tc, with tetracycline; +Est, with 17β-estradiol; +Tc, Est, with tetracycline and 17β-estradiol.

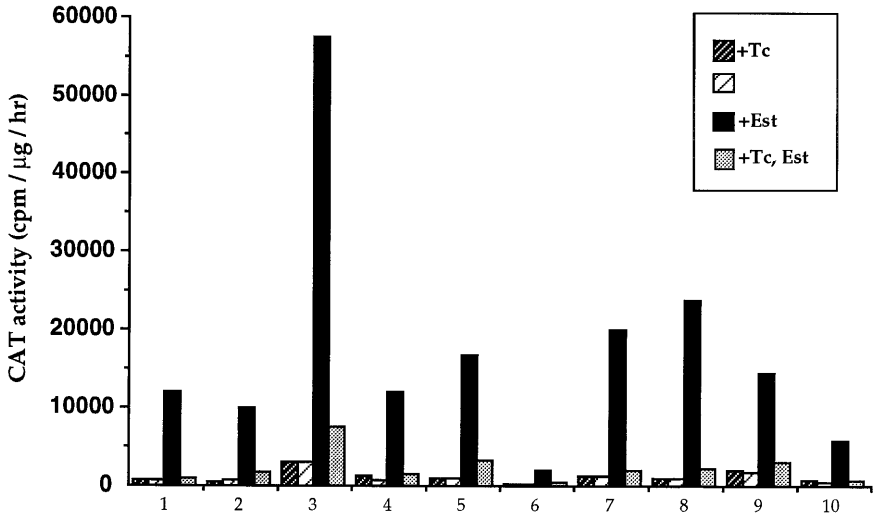

FIG. 4. Inducible CAT expression in TEPN-CAT virus-infected HT1080 cells. The G418-resistant clones derived from TEPN-CAT virus-infected HT1080 cells were incubated in the culture medium containing tetracycline and/or 17β-estradiol, as indicated, for 72 h. Cell extracts were prepared, and the CAT assay was performed as described in the legend to Fig. 2. +Tc, with tetracycline; +Est, with 17β-estradiol; +Tc, Est, with tetracycline and 17β-estradiol.

Inducible expression of the VSV G glycoprotein. The G glycoprotein of VSV is toxic to most of the mammalian cells tested (26). Cell surface expression of VSV G usually results in syncytium formation and cell death. To demonstrate the usefulness of the tTAER inducible system, we tested whether stable cell lines capable of the inducible expression of VSV G can be established with the retroviral vector described above. The CAT gene in pTEPN-CAT was replaced with the VSV G gene, and the infectious retroviral vector was generated from 293GP cells transfected with pTEPN-G and used to infect HT1080 cells. The inducible expression of VSV G with 17β-estradiol was confirmed in 30 of 35 individually derived G418-resistant clones by immunoblotting (data not shown). Cell surface VSV G expression in 5 of these 30 clones was examined further by flow cytometric analysis. As shown in Fig. 5, 72 h after the removal of tetracycline and the addition of 17β-estradiol, all five clones expressed significant levels of VSV G on the cell surface. In contrast, no VSV G expression can be detected on the cell surfaces of these same clones when grown in tetracycline-containing medium.

To determine whether inducible VSV G expression on the cell surface was due to an increase in the level of the transcript

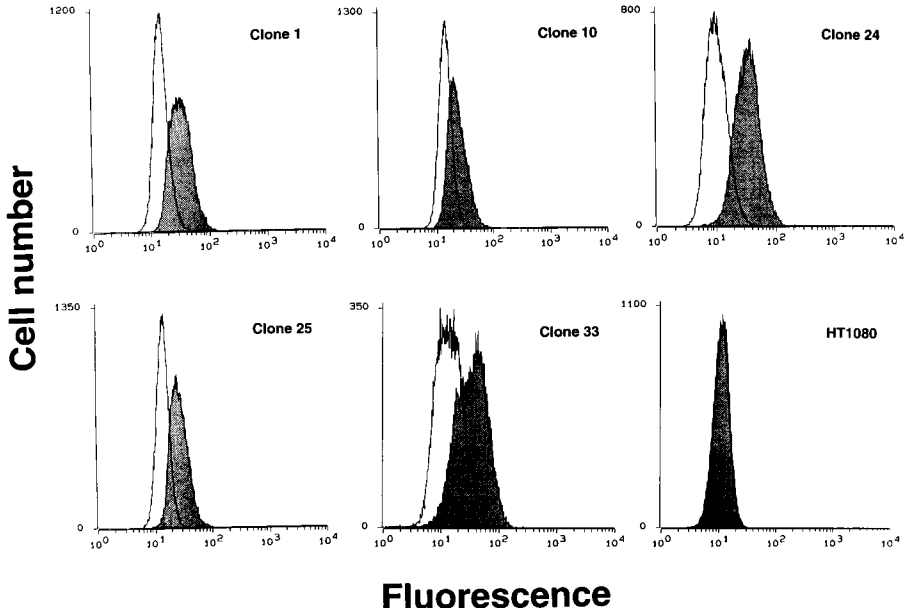

FIG. 5. Flow cytometric analysis of inducible VSV G expression on the cell surfaces of TEPN-G virus-infected HT1080 cells. The G418-resistant clones derived from TEPN-G virus-infected HT1080 cells were incubated in medium containing tetracycline (open) or 17β-estradiol (shaded) for 72 h. Cell surface expression of VSV G was assayed by staining cells with the I1 monoclonal antibody and analyzed by flow cytometry. Horizontal and vertical axes measure fluorescence intensity and cell number, respectively.

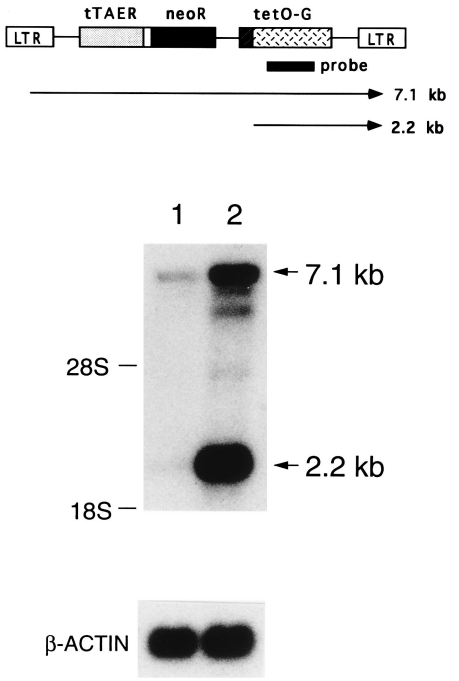

FIG. 6. Northern blot analysis of inducible VSV G mRNA expression. The poly(A)$^+$ RNA from clone 24 of TEPN-G virus-infected HT1080 cells was prepared after 72-h incubation of cells in tetracycline- or 17β-estradiol-containing medium. The poly(A)$^+$ RNA was fractionated on a formaldehyde-agarose gel and transferred to a nylon membrane. The blot was hybridized with a ^{32}P-labeled DNA probe containing the VSV G gene, as indicated in the schematic drawing. For quantitation, the same blot was rehybridized with a chicken β-actin probe as the control for RNA loading. The positions of 28S and 18S rRNAs are shown. The 7.1- and 2.2-kb RNAs correspond to transcripts initiated from the 5′ LTR and tetO-containing promoter, respectively. Lanes: 1, clone 24 with tetracycline; 2, clone 24 with 17β-estradiol.

initiated from the tetO-containing promoter in the TEPN-G virus, mRNA from clone 24 grown in tetracycline- or 17β-estradiol-containing medium was isolated and analyzed by Northern blot analysis. Both the 7.1-kb retroviral genomic transcript initiated from the 5′ LTR and the 2.2-kb transcript initiated from the tetO-containing promoter isolated from cells grown in tetracycline-containing medium were detectable with the probe derived from the VSV G gene (Fig. 6). Besides these two major transcripts, at least three other minor bands, of 6.8, 6, and 4.5 kb, can be observed. Since the 5′ splice donor site of MoMLV was retained in pTEPN-G, these minor transcripts may arise from the use of this splice donor site and downstream cryptic splice acceptor sites. Upon 17β-estradiol induction, the level of the 7.1-kb genomic transcript increases approximately 4-fold, whereas the level of the 2.2-kb transcript increases approximately 28-fold under the same conditions, as determined by densitometric analysis. Cell surface expression of VSV G upon induction can therefore be correlated with an increase in the transcription of the VSV G gene from the tetO-containing promoter. Together, these results demonstrate that cell lines containing genes encoding potentially toxic gene products can readily be established with the inducible system described here.

DISCUSSION

The tetracycline-controlled inducible system for gene expression described by Gossen and Bujard consists of two components, the transcription factor tTA and the inducible promoter responsive to tTA-mediated activation (11). Stable expression of even moderate levels of tTA in mammalian cells proves to be difficult, probably because of the squelching effect of the VP16 domain in tTA (10, 17, 23). Shockett et al. overcame this problem by placing the tTA gene under the control of the tetO-containing promoter, making the expression of tTA itself autoregulatory (20). In the presence of tetracycline, the low level of tTA expressed from the tetO-containing promoter is rendered nonfunctional, allowing stable cell lines that harbor the tTA expression plasmid to be established readily. The removal of tetracycline allows the binding of tTA to the tetO-containing promoter, leading to efficient expression of tTA and the gene of interest. The modification of tTA in the present study represents an alternative approach to overcome the problem of stable expression of tTA in mammalian cells. HT1080 clones that stably express tTAER can readily be established by retrovirus-mediated gene transfer. Since retrovirus infection under the conditions we use usually introduces only one copy of the gene into cells, the relative ease of establishing these cell lines may be attributed to a low level of tTAER expression. In a separate study, stable cell lines derived from DNA transfection of the tTAER gene controlled by the strong CMV immediate-early gene promoter have also been established without difficulties (4). This is in sharp contrast to the observation of Shockett et al. that most of the NIH 3T3 clones established for stable tTA expression demonstrated little or no tTA activity (20). The results in the present study therefore suggest strongly that the addition of the ER ligand-binding domain to tTA reduces the toxicity of tTA in the absence of 17β-estradiol, allowing the establishment of tTAER-expressing clones.

Besides the toxicity of tTA, problems arise in the use of the tTA-based inducible system from the fact that previous methods have required the establishment of cell lines in two steps and only those cell lines with good transfection efficiencies can readily be used. With the system reported here, retrovirus-mediated transfer of both the tTAER gene and the gene of interest overcomes these problems. One concern with such an approach is that the tetO-containing promoter in the retroviral construct may be activated fortuitously by the enhancer in the MoMLV LTR, which functions efficiently in many mammalian cell types. The results in Fig. 4 and 5 clearly indicate that the effect of the LTR enhancer on the tetO-containing promoter, if any, is minimal. This may be due to the fact that except for the TATA box, this promoter contains no other regulatory elements which are required for the LTR enhancer to activate transcription. The fact that transcription from the tetO-containing promoter can be activated by tTAER may be due to the close proximity of the tetO sites to the promoter. Unexpectedly, the levels of mRNA initiated from the 5′ LTR increased upon 17β-estradiol induction. The presence of putative estrogen-responsive elements in the MoMLV LTR may account for the observed increase. Alternatively, the strong transactivation domain of VP16 in tTAER may boost LTR promoter activity upon the binding of tTAER to tetO sites.

The results in Fig. 4 and 6 show that the highest induction level for CAT and VSV G is approximately 30-fold. The inability so far to obtain clones with induction levels higher than 30-fold may be due to several reasons. Since retrovirus-mediated gene transfer delivers only one copy of the gene, the level of tTAER in infected cells may not be sufficiently high to induce gene expression beyond 30-fold. This hypothesis is consistent with our observation that tTAER-expressing cell lines established by DNA transfection have higher levels of gene expression upon induction (4). Alternatively, the transcription initiated from the 5′ LTR may interfere with the transcription

initiated from the internal tetO-containing promoter. This is consistent with previous observations that closely positioned promoters in mammalian genomes can interfere transcriptionally with each other (7, 8). Finally, since the induction of transcription by tTA has been shown to vary in different cell types (13), the relatively low induction levels that we have observed may be a specific feature of the HT1080 cells used in this study. Future studies performed with other cell lines should clarify this point.

Using retroviral vectors instead of DNA transfection to transduce the complete inducible system greatly expands the target cell types in which gene functions can be studied. Using tissue-specific control elements to regulate the expression of the tTAER gene can further limit the inducible system to function only in specific cell types. This is specially important in the setting of gene delivery for the purpose of treating human diseases. Target gene expression in undesirable cell types at the wrong time may lead to detrimental consequences. The use of the vector described here should help to alleviate these potential problems. Moreover, since the concentration of tetracycline has been shown to modulate transcription initiation from the tetO-containing promoter (11), the level of the target gene product can be regulated by controlling the amounts of tetracycline used in the culture medium or in animal models. In summary, the combination of a powerful inducible system and retrovirus-mediated gene transfer described here can not only be used to study gene function but may also be applied in the future to techniques aimed at the clinical application of retroviral gene transfer in human gene therapy trials.

ACKNOWLEDGMENTS

We thank H. Bujard for providing plasmids pUHD15-1 and pUHG10-3 and P. Chambon for providing plasmid pHE14. The 293GP line was obtained from Chiron Viagene.

This work was supported in part by National Institutes of Health grant HD20034 and the Del E. Foundation. A.I. was supported by Kyowa Hakko Kogyo Co., Ltd.

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