Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 10057–10062, September 1996
Biochemistry

Generation of packaging cell lines for pseudotyped retroviral vectors of the G protein of vesicular stomatitis virus by using a modified tetracycline inducible system

SHIN-TAI CHEN*, AKIHIRO IIDA†‡, LIN GUO†, THEODORE FRIEDMANN*, AND JIING-KUAN YEE†§

†Department of Pediatrics, Miller Building, Room 109, City of Hope National Medical Center, Duarte, CA 91010-3000; and *Center for Molecular Genetics, Department of Pediatrics, University of California at San Diego, La Jolla, CA 92093

Communicated by Morris E. Friedkin, University of California at San Diego, La Jolla, CA, June 10, 1996 (received for review April 17, 1996)

ABSTRACT **We have previously shown that the G protein of vesicular stomatitis virus (VSV-G) can be incorporated into the virions of retroviruses. Since expression of VSV-G is toxic to most mammalian cells, development of stable VSV-G packaging cell lines requires inducible VSV-G expression. We have modified the tetracycline-inducible system by fusing the ligand binding domain of the estrogen receptor to the carboxy terminus of a tetracycline-regulated transactivator. Using this system, we show that VSV-G expression is tetracycline-dependent and can be modulated by β-estradiol. Stable packaging cell lines can readily be established and high-titer pseudotyped retroviral vectors can be generated upon induction of VSV-G expression.**

Retroviral vectors derived from Moloney murine leukemia virus have been used extensively to transduce genes into mammalian cells (1, 2). However, problems such as the inability to infect quiescent cells, low virus titers, and relatively poor infectivity in human cells severely limit the application of retroviral vectors in gene transfer. To overcome some of these problems, we have generated retroviral vectors pseudotyped with the G protein of vesicular stomatitis virus (VSV-G) (3–5). We have shown that the pseudotyped retroviral vectors have an expanded host range and are able to infect many cell types with a greater efficiency than traditional amphotropic retroviral vectors (3–6). Moreover, the pseudotyped virus can be concentrated by ultracentrifugation to titers greater than 10^9 colony forming units/ml (4). These unique properties of the VSV-G pseudotyped virus not only extend the use of retroviral vectors for genetic studies in previously inaccessible species, but also facilitate more efficient preclinical and clinical studies of the potential for human gene therapy.

However, the generation of stable packaging cell lines for pseudotyped retroviral vectors has been difficult because of the toxicity of VSV-G in most mammalian cells (4, 5). To overcome this problem, we have modified the tetracycline-inducible system (7) to regulate the expression of VSV-G. In that system, a chimeric transcription factor, tTA, was produced by combining the repressor of *Escherichia coli* tetracycline-resistance operon (tetR) and the activation domain of virion protein 16 (VP16) of herpes simplex virus (HSV). This chimeric transactivator can regulate gene expression from a minimal promoter linked to multiple copies of tetO, the binding site for the tetracycline repressor. In the absence of tetracycline, tTA binds the tetO site and activates transcription from the tetO-containing promoter. In the presence of tetracycline, interaction of tTA with the tetO is prevented by its binding to tetracycline, thereby prevents transcription.

While the expression of several genes has been reported to be inducible using this system (7–11), establishment of cell lines stably expressing tTA has been difficult, presumably because the activation domain of VP16 is capable of squelching general cellular transcription when expressed in large quantities (7, 11–13). To relieve this effect, we constructed a fusion protein, tTAER, containing the ligand-binding domain of estrogen receptor (ER) fused to the carboxy terminus of tTA. Linkage of the ligand-binding domain of a steroid hormone receptor to an unrelated protein has led to hormone-dependent function of the fusion protein (14). In the present study, we demonstrate that the ER ligand-binding domain in tTAER blocks the transactivation function of tTA in the absence of the estrogen analog β-estradiol, thus reducing the putative squelching effect of VP16. Under these conditions, we were able to efficiently establish cell lines expressing high-level tTAER. We have constructed a plasmid containing the VSV-G cDNA under the control of the tetracycline-inducible promoter and introduced it into tTAER-expressing cell lines. With high efficiency, we obtained stable clones demonstrating hormone-inducible VSV-G expression. Using this approach, we have established stable packaging cell lines capable of producing VSV-G pseudotyped retroviral vectors with high titers.

Yang *et al.* (15) have reported the use of the originally described tetracycline-inducible system to establish producer cell lines capable of generating a specific VSV-G pseudotyped retroviral vector. These producer cell lines were established by first introducing the retroviral vector into cells stably expressing the gag and pol proteins, followed by introducing the VSV-G gene under the control of the tetracycline-inducible promoter. Thus, these cell lines can generate the specific pseudotyped retroviral vector, but are not suitable for general use as packaging cell lines. In our present studies, we report the development of packaging cell lines for the production of any VSV-G pseudotyped retroviral vector. In addition, the successful establishment of stable cell lines expressing VSV-G suggests that the modified tetracycline-estrogen inducible system may be applicable for the conditional expression of other potential toxic genes in mammalian cells.

MATERIALS AND METHODS

Plasmid Construction. To construct tTAER, a 1-kb *Eco*RI–*Bam*HI DNA fragment containing the tTA gene was isolated from pUHD15-1 (7) and ligated to a 0.95-kb *Bam*HI–*Sst*I DNA fragment containing the ER ligand-binding domain from pHE14 (16). To construct pCMV-tTAER, a 1.95-kb *Eco*RI fragment containing the complete tTAER gene was inserted at the unique *Bam*HI site in pCMV-Bam (5). To construct phyg-CMV-tTAER, a 2.0-kb *Bam*HI–*Hin*dIII DNA fragment

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: VSV-G, G protein of vesicular stomatitis virus; HSV, herpes simplex virus; VP16, virion protein 16; ER, estrogen receptor; TK, thymidine kinase; CMV, cytomegalovirus; FCS, fetal calf serum; lux, luciferase.

‡Present address: Tokyo Research Laboratories, Kyowa Hakko Kogyo Co., Ltd., Machidashi, Tokyo 194, Japan.
§To whom reprint requests should be addressed.

containing the gene encoding hygromycin B phosphotransferase (17) under the control of the HSV thymidine kinase (TK) promoter was isolated from pTK-hyg (J.-K.Y., unpublished data) and inserted at the unique *Not*I site immediately upstream of the cytomegalovirus (CMV) immediate early gene promoter in pCMV-tTAER. To construct pTetO-G-1, a 1.6-kb *Bam*HI DNA fragment containing the VSV-G gene was isolated from pCMV-G (5) and inserted at the unique *Bam*HI site in pUHG10-3 (7). To construct pTetO-G-2, a 2.3-kb *Bam*HI DNA fragment containing the gene encoding puromycin-*N*-acetyltransferase (18) under the control of the HSV TK promoter was isolated from pTK-pur (J.-K.Y., unpublished data) and inserted at the unique *Bgl*II site downstream from the VSV-G gene in pTetO-G-1. To construct pLTK-FIX, a 1.5-kb *Eco*RI DNA fragment containing the canine factor IX cDNA from pLNCdFIX (19), a 0.2-kb *Xba*I DNA fragment containing the HSV TK promoter linked to four copies of the BIII enhancer of the tyrosine aminotransferase gene from ptat-TKCAT (J.-K.Y., unpublished data) (20), and a 7-kb *Xho*I–*Sal*I DNA fragment from LPONL (5) were ligated together.

Cell Culture. Rat 208F, human 293GP, and HT1080/LSHL cells have been described (4, 5). NIH 3T3 cells were obtained from American Type Culture Collection. All cell lines except 293GP/tTAER and 293GP/tTAER/G cells were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM)/10% fetal calf serum (FCS). The 293GP/tTAER cells were maintained in DMEM containing 10% FCS, 1 μg/ml tetracycline, and 800 μg/ml hygromycin. The 293GP/tTAER/G cells were maintained in DMEM containing 10% FCS, 1 μg/ml tetracycline, 1 μg/ml puromycin, and 800 μg/ml hygromycin. For selection, 400 μg/ml G418, 800 μg/ml hygromycin, and 1 μg/ml puromycin were used, respectively. For induction of VSV-G expression, the tetracycline-containing medium was removed, and the cells were washed twice with DMEM followed by incubation in DMEM for at least 30 min between the washes. The cells were then maintained in DMEM/10% FCS containing β-estradiol at a concentration of 2 μM.

Virus Production. To generate the pseudotyped virus from the producer cells, the cells were grown in tetracycline-containing medium to a confluence of ≈90%. The cells were then washed and the medium was changed to tetracycline-free medium with or without β-estradiol as described above. The pseudotyped virus was collected at different times as indicated in the text and the titer of the virus was determined in 208F cells in G418-containing medium. To detect the presence of replication-competent retrovirus, the virus stocks were amplified in NIH 3T3 cells for 2 weeks followed by the marker rescue assay in HT1080/LSHL cells as described (5).

DNA Transfection and Detection of the Luciferase Activity. DNA transfection was performed by calcium phosphate coprecipitation (21). To detect the luciferase (lux) activity, cell extracts were prepared by three cycles of freeze–thawing in 0.1 M potassium phosphate/1 mM dithiothreitol (pH 7.8), followed by centrifugation to remove the cell debris. The lux activity was determined as described (22).

Selection of 293GP/tTAER Clones. To establish stable tTAER-expressing clones, 293GP cells were transfected with phyg-CMV-tTAER and hygromycin-resistant colonies were picked and expanded. To screen for tTAER expression, the 293GP/tTAER clones were transfected with pUHC13-3 (7) with tetracycline or β-estradiol and the lux activity was determined 48 hr after transfection. The clone that generated the highest induction level of the lux activity was chosen for the introduction of pTetO-G-2 for inducible VSV-G expression.

Selection of 293GP/tTAER/G Clones. To establish stable 293GP/tTAER/G clones, 293GP/tTAER cells were transfected with pTetO-G-2 and puromycin-resistant colonies were picked and expanded. To screen for inducible VSV-G expression, ≈1×10^5 cells derived from each clone were harvested after 48 hr incubation in medium containing either 1 μg/ml tetracycline or 2 μM β-estradiol. The cells were lysed in 25 μl buffer containing 50 mM Tris·HCl (pH7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, and 0.1% SDS. One microliter of extract was spotted on a nylon membrane and VSV-G protein was detected by the ECL Western blotting system (Amersham) with the I1 monoclonal antibody specific for VSV G (a gift of John Holland, University of California at San Diego). Expression of VSV G on cell surface was determined by reacting the cells with monoclonal antibody I1, followed by staining with the fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulins (Biosource International, Camarillo, CA), and analyzed by flow cytometry as described (4). For the detection of the VSV-G mRNA, total RNA was isolated by the procedure of Chomczynski and Sacchi (23) and the mRNA was isolated using the polyATract mRNA isolation system (Promega). The mRNA was separated on a 2.2 M formaldehyde/1% agarose gel and transferred to a nylon membrane. The membrane was hybridized with ^{32}P-labeled probes prepared by the random primed DNA labeling kit (Boehringer Mannheim). The probe for VSV-G was derived from a 1.6-kb *Bam*HI DNA fragment of pCMV-G containing the VSV-G gene (5). The probe for β-actin was derived from a 2-kb *Pst*I DNA fragment of pUCA1 (24).

RESULTS

Construction and Expression of tTAER. To reduce the squelching effect of the VP16 transactivation domain in tTA, we constructed a chimeric gene tTAER encoding the ligand-binding domain of ER fused to the carboxy terminus of tTA. To express tTAER, we constructed pCMV-tTAER containing the tTAER gene under the control of the promoter of the CMV immediate early gene (Fig. 1). To test the transactivation function of tTAER, 293GP cells were cotransfected with pCMV-tTAER and pUHC13-3 containing the firefly *lux* gene under the control of a minimum promoter linked to seven tandem copies of tetO (7). Although removal of tetracycline or addition of tetracycline plus β-estradiol activated *lux* expression, maximum *lux* expression was observed only after simultaneous removal of tetracycline and addition of β-estradiol (Fig. 2). The fact that tTAER requires β-estradiol for its maximum activity suggests that the VP16 transactivation function and its presumed squelching effect are regulated by the ER ligand-binding domain in tTAER. This property may allow

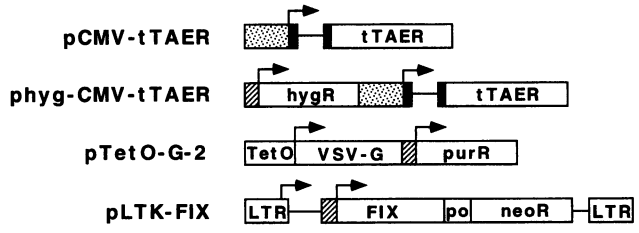

FIG. 1. Schematic representation of the organization of the plasmids. Solid boxes and lines in pCMV-tTAER and phyg-CMV-tTAER represent the exons and the intron of the rabbit β-globin gene, respectively. Stippled boxes represent the promoter of the CMV immediate early gene. Hatched boxes represent the HSV TK promoter. tTAER, the gene encoding tTAER; hygR, the gene encoding hygromycin B phosphotransferase; purR, the gene encoding puromycin-*N*-acetyltransferase; neoR, the neomycin phosphotransferase gene; TetO, the minimum CMV immediate early gene promoter linked to seven tandem copies of the tetR binding site; VSV-G, the gene encoding VSV-G; LTR, the long terminal repeat of Moloney murine leukemia virus; FIX, the canine factor IX cDNA; po, the internal ribosome entry site of poliovirus. Arrows indicate the approximate locations of the transcription initiation sites and the direction of transcription. The figure is not drawn to scale.

more efficient isolation of stable tTAER-expressing cell lines in the absence of β-estradiol. The observed transactivation function of tTAER in regular medium or medium containing tetracycline plus β-estradiol remains unclear.

Establishment of Packaging Cell Lines for VSV-G-Pseudotyped Retroviral Vectors. To establish stable cell lines for inducible VSV-G expression, the tTAER gene was introduced into 293GP cells expressing the gag and pol proteins (5). For this purpose, the hygromycin-resistant gene under the control of the HSV TK promoter was inserted into pCMV-tTAER to produce phyg-CMV-tTAER (Fig. 1). 293GP cells were transfected with phyg-CMV-tTAER, and 30 hygromycin-resistant colonies were picked and expanded.

To test for stable tTAER expression, cells derived from these clones were transfected with pUHC13-3 with tetracycline or β-estradiol and the lux activity was determined 48 hr after transfection. All 30 clones responded to β-estradiol induction with increased lux activities, ranging from 2- to 90-fold when compared with that in tetracycline-containing medium (data not shown). This result suggests that tTAER is less toxic than tTA, and stable cell lines expressing tTAER can readily be established. A 293GP/tTAER clone which produced the highest inducible lux activity was selected for establishing the packaging cell lines.

To establish the packaging cell lines, the 293GP/tTAER cells were transfected with pTetO-G-2 containing the VSV-G gene under the control of the inducible tetO-containing promoter and the puromycin-resistant gene as a selectable marker (Fig. 1). Seventy puromycin-resistant colonies were picked, and approximately one-third of these clones failed to survive serial passage even in the presence of tetracycline, probably due to high basal level VSV-G expression. Inducible VSV-G expression by β-estradiol was confirmed in 34 clones using an immunoblotting assay as described in *Materials and Methods* (data not shown).

To confirm inducible VSV-G expression, mRNAs from the parental 293GP/tTAER cells and two 293GP/tTAER/G clones (clones 13 and 21) were analyzed by Northern blot analysis. The VSV-G mRNA in clone 13 was detectable in the presence of tetracycline (Fig. 3, lane 3). Upon β-estradiol induction, the level of the VSV-G mRNA increased dramatically (compare lanes 3 and 4). In contrast, the VSV-G mRNA in clone 21 was undetectable in the presence of tetracycline (lane 5), but became detectable upon β-estradiol induction (lane 6). Consistent with the levels of the VSV-G mRNA, β-estradiol induction of clone 13 led to severe cytopathic effects and cell death within 4 days, whereas the cell morphology of clone 21 remained relatively normal under the same condition.

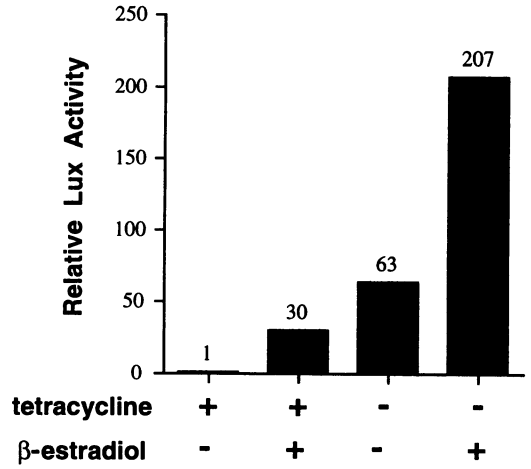

FIG. 2. Inducible *lux* gene expression by tTAER in 293GP cells. Fifteen micrograms of pUHC13-3 was cotransfected with 15 μg of pCMV-tTAER into 293GP cells by calcium phosphate coprecipitation (20). Tetracycline and/or β-estradiol was added to the culture medium 18 hr after transfection as indicated, and the cells were incubated for an additional 40 hr. The numbers above the bars of the graph represent the relative fold induction by normalizing the lux activity of each condition to that of tetracycline-containing medium, which was arbitrarily set as 1. The data are the average of two independent experiments.

Inducible Production of VSV-G Pseudotyped Retroviral Vectors. To demonstrate inducible production of the pseudotyped retroviral vectors, cells derived from clones 13 and 21 were infected with a retroviral vector LTK-FIX containing both the canine factor IX cDNA and the neomycin-resistant gene under the control of the HSV TK promoter (Fig. 1). The G418-resistant colonies were pooled and the level of VSV-G expression was determined. As shown in Fig. 4, cell surface VSV-G expression was undetectable in LTK-FIX virus-infected clone 21 cells in the presence of tetracycline and was induced upon the removal of tetracycline and the addition of β-estradiol. In contrast, a significant level of VSV-G expression was detected in the LTK-FIX virus-infected clone 13 cells even in the presence of tetracycline, and the level of VSV-G was increased further upon β-estradiol induction. The levels of cell-surface VSV-G in the presence of tetracycline or β-estradiol is consistent with the levels of VSV-G mRNA in these two clones under the same conditions (Fig. 3).

The LTK-FIX virus produced from the infected clone 13 and 21 cells was harvested following 48 hr incubation in tetracycline- or β-estradiol-containing medium, and the virus titer was determined in 208F cells. As shown in Table 1, virus production increased in both clones upon β-estradiol induction. However, despite the fact that clone 13 expressed significantly more VSV-G upon induction, the virus titers from both clones were similar. This may be due to the cytopathic effect generated from overexpression of VSV-G in clone 13 cells upon β-estradiol induction (see below). The observation that clone 13-derived cells generated ≈20-fold more virus than clone 21-derived cells in the presence of tetracycline is consistent with the higher basal levels of VSV-G expressed in clone 13 cells under the uninduced condition (Figs. 3 and 4).

Because β-estradiol induces not only virus production but also VSV-G accumulation that inevitably will lead to cell death, it is important to determine the duration of virus production from the producer cells upon induction. Culture medium from pooled LTK-FIX virus-infected clone 21 cells was collected over a period of 16 days and the virus was titered in 208F cells. As shown in Fig. 5*A*, the virus titers remained at a low but constant level in the presence of tetracycline for the entire period. In contrast, induction with β-estradiol led to a gradual increase in virus titers. No cytopathic effect was

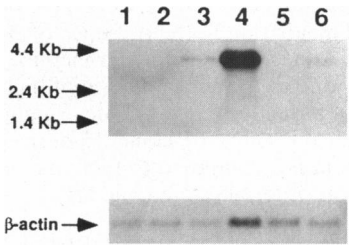

FIG. 3. Northern blot analysis of inducible VSV-G mRNA expression. The poly(A)$^+$ RNA from 293GP/tTAER (lanes 1 and 2), 293GP/tTAER/G clone 13 (lanes 3 and 4), and clone 21 (lanes 5 and 6) were prepared 36 hr after incubation of the cells in tetracycline (lanes 1, 3, and 5) or β-estradiol (lanes 2, 4, and 6) containing medium. The poly(A)$^+$ RNA was fractionated on a formaldehyde-agarose gel, transferred to a nylon membrane, and hybridized with a ^{32}P-labeled VSV-G cDNA probe. For quantitation, the same blot was rehybridized with a chicken β-actin probe. The size of the RNA standards (BRL) is indicated.

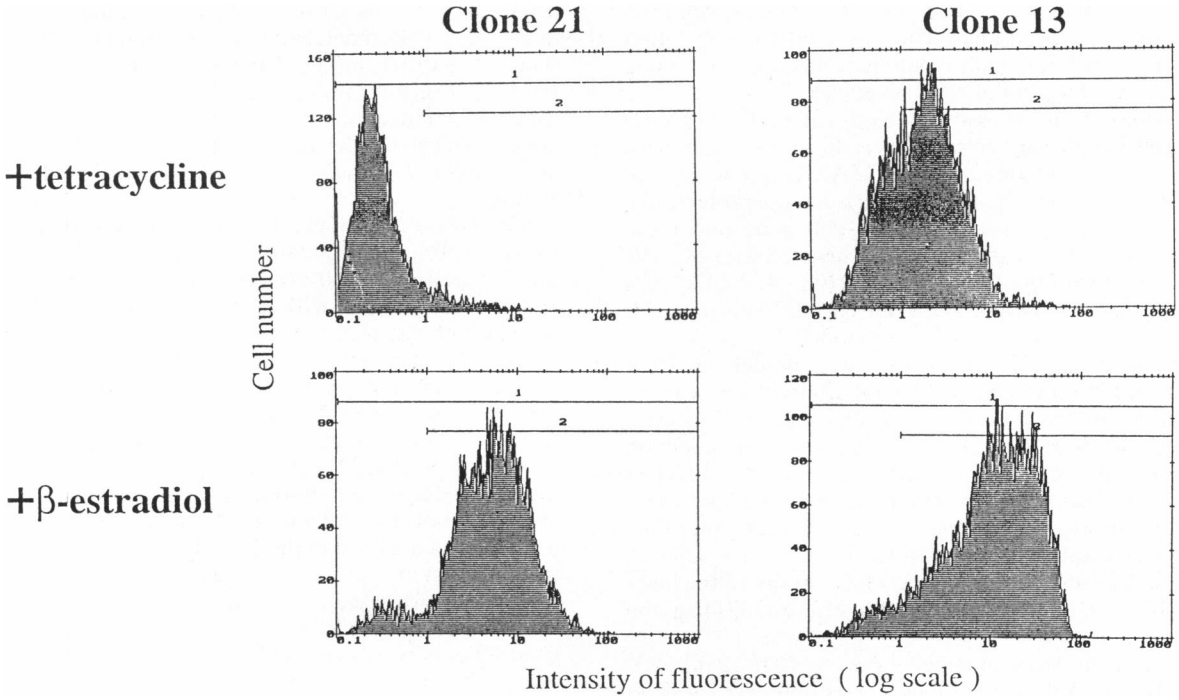

FIG. 4. Flow cytometric analysis of inducible VSV-G expression in the pooled LTK-FIX virus-infected 293GP/tTAER/G clones. The pooled G418-resistant colonies derived from the LTK-FIX virus-infected clone 13 and 21 cells were incubated in tetracycline- or β-estradiol-containing medium for 48 hr. Cell surface expression of VSV-G was assayed by staining the cells with the I1 monoclonal antibody and analyzed by flow cytometry.

observed until 2 weeks after β-estradiol induction, a delay that may be attributed to the relatively low level of VSV-G expression in clone 21-derived cells (Figs. 3 and 4). Interestingly, in the absence of β-estradiol, the viral titers increased from 10^3 colony forming units/ml to 4×10^6 colony forming units/ml over a period of two weeks after the removal of tetracycline (Fig. 5*A*). Mass cell death did occur after 3-week incubation in this medium, and it was accompanied by a reduction in virus titers (data not shown). The reason for the dramatic increase in virus titers remains unclear at the present time (see *Discussion*). To determine whether the increase in virus titers was due to the presence of helper virus contamination, the virus stocks collected from day 14 and day 16 after β-estradiol induction were amplified in NIH 3T3 cells followed by a marker rescue assay (5). No helper virus was detected using this assay (data not shown).

Similar procedures were used to determine the duration of virus production from the pooled LTK-FIX virus-infected clone 13 cells (Fig. 5*B*). The virus titers increased ≈100-fold 2 days after β-estradiol induction. However, the titers decreased with prolonged incubation in the presence of β-estradiol, and this reduction was accompanied by an increase in dead cells. The is probably due to the accumulation of high-level VSV-G after β-estradiol induction in this clone. Similar to the clone 21-derived cells, the titers of clone 13-derived cells in the absence of β-estradiol continued to increase for up to 8 days after the removal of tetracycline (Fig. 5*B*).

Table 1. Inducible generation of the pseudotyped LTK-FIX virus

Cell line	Virus titer,* cfu/ml: + Tetracycline, − β-estradiol	Virus titer,* cfu/ml: − Tetracycline, + β-estradiol	Fold induction
Clone 13	2.7×10^3	6.0×10^4	22
Clone 21	1.4×10^2	6.6×10^4	471

cfu, Clony forming units.
*The virus was harvested 48 hr after β-estradiol induction and titered in 208F cells.

The LTK-FIX virus titer from individual clones of the infected clone 21 cells has also been determined (Table 2). Removal of tetracycline and addition of β-estradiol for 60 hr resulted in an increase in the virus titer exceeding 3–4 orders of magnitude. Similar results were obtained with clone 13-derived cells (data not shown). We have also used these two packaging cell lines to generate other retroviral vectors and obtained similar titers as reported here (data not shown). These results demonstrate that clones 13 and 21 can serve as packaging cell lines for the production of VSV-G pseudotyped retroviral vectors.

DISCUSSION

Due to the toxicity of VSV-G, the preparation of high-titer VSV-G pseudotypes until now has been limited to a transient transfection procedure using 293GP cells (5). Although useful for small-scale virus production, this method cannot be extended to large-scale production of clinical-grade pseudotyped retroviral vectors. To overcome this difficulty, we have modified the tetracycline-inducible system for the expression of VSV-G conditionally. It has been postulated that the difficulty in establishing stable tTA-expressing cell lines is due to the squelching effect of tTA (7). In this study, we demonstrate that the addition of the ER ligand-binding domain to tTA overcomes this difficulty and allows the efficient establishment of stable tTAER-expressing clones.

Using tTAER to regulate VSV-G expression, we have established two stable packaging cell lines, clone 13 and clone 21, that seem particularly useful for pseudotyped virus production. In both cell lines, VSV-G expression is inducible with β-estradiol. The fact that clone 13 expresses detectable amounts of VSV-G in the presence of tetracycline and can be maintained for more than 6 months in culture indicates that human 293 cells can tolerate low-level VSV-G expression. However, despite higher levels of VSV-G in clone 13 than that in clone 21 upon induction, the amounts of virus generated from both clones were similar (Table 1 and Fig. 5). This may

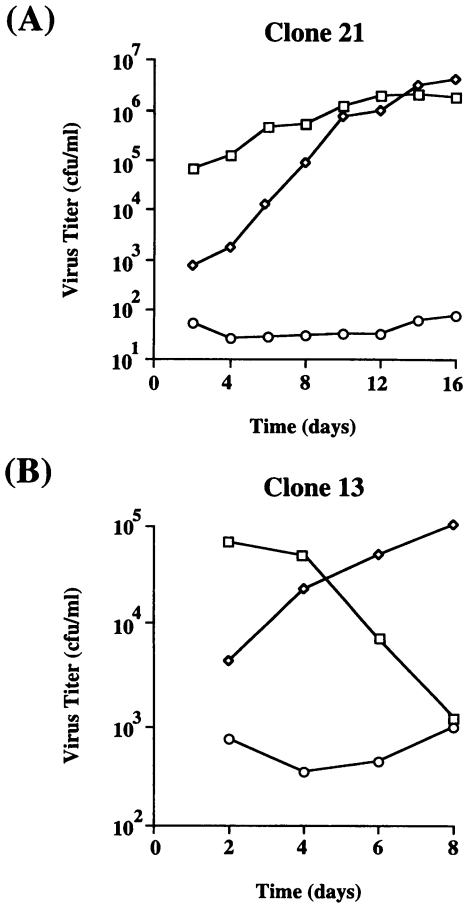

FIG. 5. Time course of virus production from the pooled LTK-FIX virus-infected 293GP/tTAER/G clones. Cells derived from clone 21 (*A*) or clone 13 (*B*) were infected with the LTK-FIX virus. The G418-resistant colonies were pooled and incubated in DMEM (◇), DMEM plus tetracycline (○), or DMEM plus β-estradiol (□) for the period indicated. The medium of the pooled cells was changed every 48 hr, and the titer of the accumulated virus was determined at the time indicated by infecting 208F cells and selecting in G418-containing medium.

be attributed to the cytopathic effect observed in clone 13 after β-estradiol induction, an effect probably caused by the relatively high-level VSV-G expression. The toxicity of VSV-G is most likely the result of its expression on the cell surface, which leads to syncytia formation. The levels of VSV-G expression in clones 13 and 21 correlate well with the degree of the cytopathic effect in these two clones after β-estradiol induction. This observation suggests that a lower level of VSV-G expression such as that in clone 21 cells may have the advantage of allowing the producer cells to survive for prolonged periods after β-estradiol induction, thereby producing more virus from the producer cells.

Table 2. The virus titers generated from independently isolated clones of LTK-FIX virus-infected clone 21 cells

Clone	Virus titer,* cfu/ml	
	+ Tetracycline, − β-estradiol	− Tetracycline, + β-estradiol
1	81	5.9×10^5
2	36	5.2×10^5
3	100	2.7×10^5
4	24	2.4×10^5
5	111	0.8×10^5
6	71	2.0×10^5
7	24	0.9×10^5
8	34	5.7×10^5

cfu, Colony forming units.
*The virus was harvested 60 hr after β-estradiol induction and titered in 208F cells.

We have shown that virus titers increase dramatically in the pooled G418-resistant colonies containing the LTK-FIX virus in the absence of tetracycline and β-estradiol (Fig. 5). Although 293GP cells display a highly transformed phenotype and have lost the property of contact inhibition, a moderate increase in cell number cannot account for the observed 3- to 4-order of magnitude increase in virus titers over a period of 16 days. An alternative explanation might involve reinfection of the producer cells by the produced virus, leading to an increase in provirus copy number per cell. Reinfection with retroviruses is usually inhibited in retrovirus producer cells by the high intracellular levels of the retrovirus envelope protein which saturates the retroviral receptor. The receptor for VSV may include phosphatidyl serine, phosphatidyl inositol, and/or GM3 ganglioside (25, 26), all of which are abundant components of plasma membrane. The abundance of these molecules may prevent receptor saturation, thereby allowing reinfection to occur. This process could increase provirus copy number in the producer cells and lead to increased virus production. Alternatively, the increasing virus titer may result from VSV-G accumulation in the producer cells during prolonged incubation due to contaminating β-estradiol-like substance in DMEM or serum we used, thereby making more VSV-G available to be encapsidated into the virus particles. Despite these possibilities, the precise mechanisms for the dramatic increase in virus titers in the absence of β-estradiol remain unclear.

The ability to generate high-titer retroviral vectors makes these two clones suitable for the mass production of VSV-G pseudotyped retroviral vectors. We have used pLTK-FIX to compare virus production by the previously described transient transfection method and by the stable packaging cell lines reported here. We observed no significant difference in virus titers with the two methods (data not shown). Thus, the current approach may have the advantage of avoiding the time-consuming step to identify high producer clones. The packaging cell lines established in this study should facilitate large-scale production of clinical-grade virus. Furthermore, the relative ease of establishing stable clones expressing tTAER indicates that this system will have advantage for inducible expression of the *tet* regulatory element and other potentially toxic gene products.

We thank Dr. H. Bujard for providing plasmids pUHD15-1, pUHC13-3, and pUHG10-3; and Dr. 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 Webb Foundation. A.I. is supported by Kyowa Hakko Kogyo Co., Ltd.

1. Miller, A. D. (1992) *Nature (London)* **357,** 455–460.
2. Mulligan, R. C. (1993) *Science* **260,** 926–932.
3. Emi, N., Friedmann, T. & Yee, J. K. (1991) *J. Virol.* **65,** 1202–1207.
4. Burns, J. C., Friedmann, T., Driever, W., Burrascano, M. & Yee, J. K. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 8033–8037.
5. Yee, J. K., Miyanohara, A., LaPorte, P., Bouic, K., Burns, J. C. & Friedmann, T. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 9564–9568.
6. Lin, S., Gaiano, N., Culp, P., Burns, J. C., Friedmann, T., Yee, J. K. & Hopkins, N. (1994) *Science* **265,** 666–669.
7. Gossen, M. & Bujard, H. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 5547–5551.
8. Furth, P. A., Onge, L. S., Boger, H., Gruss, P., Gossen, M., Kistner, A., Bujard, H. & Hennighausen, L. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 9302–9306.
9. Howe, J. R., Skryabin, B. V., Belcher, S. M., Zerillo, C. A. & Schmauss, C. (1995) *J. Biol. Chem.* **270,** 14168–14174.
10. Resnitzky, D., Gossen, M., Bujard, H. & Reed, S. I. (1994) *Mol. Cell. Biol.* **14,** 1669–1679.

11. Shockett, P., Difilippantonio, M., Hellman, N. & Schatiz, D. G. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 6522–6526.
12. Gill, G. & Ptashne, M. (1988) *Nature (London)* **334,** 721–724.
13. Ptashne, M. & Gann, A. A. F. (1990) *Nature (London)* **346,** 329–331.
14. Mattioni, T., Louvion, J. & Picard, D. (1994) *Methods Cell Biol.* **43,** 335–352.
15. Yang, Y., Vanin, E. G., Whitt, M. A., Fornerod, M., Zwart, R., Schneiderman, R. D., Grosveld, G. & Nienhuis, A. W. (1995) *Hum. Gene Ther.* **6,** 1203–1213.
16. Kumar, V., Green, S., Staub, A. & Chambon, P. (1986) *EMBO J.* **5,** 2231–2236.
17. Gritz, L. & Davies, J. (1983) *Gene* **25,** 179–188.
18. Lacalle, R. A., Pulido, D., Vara, J., Zalacain, M. & Jimenez, A. (1989) *Gene* **79,** 379–384.
19. Roman, M., Axelrod, J. H., Dai, Y., Naviaux, R. K., Friedmann, T. & Verma, I. M. (1992) *Somatic Cell Genet.* **18,** 247–258.
20. Boshart, M., Weih, F., Schmidt, A., Fournier, R. E. K. & Schutz, G. (1990) *Cell* **61,** 905–916.
21. Graham, F. L. & Van der Eb, A. J. (1973) *Virology* **52,** 456–467.
22. de Wet, J. R., Wood, K. V., DeLuca, M., Helinski, D. R. & Subramani, S. (1987) *Mol. Cell. Biol.* **7,** 725–737.
23. Chomczynski, P. & Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159.
24. Cleveland, D. W., Lopata, M. A., MacDonald, R. J., Cowan, N. J., Rutter, W. J. & Kirschner, M. W. (1980) *Cell* **20,** 95–105.
25. Mastromarino, P., Conti, C., Goldoni, P., Hauttecoeur, B. & Orsi, N. (1987) *J. Gen. Virol.* **68,** 2359–2369.
26. Conti, C., Mastromarino, P., Ciuffarella, M. G. & Orsi, N. (1988) *Arch. Virol.* **99,** 261–269.