The present invention provides a vector comprising Protein Translation Peptide Elongation Factor-1 α promoter and nucleic acids encoding reverse tetracycline controlled transactivator, wherein the expression of said transactivator is under the control of Protein Translation Peptide Elongation Factor-1 α promoter. In addition, the invention provides a method of generating a reverse tetracycline controlled transactivator expression system for inducible tetracycline regulated gene expression comprising: (a) isolation of a DNA fragment encoding the reverse tetracycline controlled transactivator by restriction enzyme digestion, (b) generation of Protein Translation Peptide Elongation Factor-1 α promoter vector, by restriction enzyme digestion, (c) directional cloning of reverse tetracycline controlled transactivator into Protein Translation Peptide Elongation Factor-1 α promoter vector by ligation of 5' EcoRI compatible restriction enzyme overhangs, (d) directional cloning of reverse tetracycline controlled transactivator into Protein Translation Peptide Elongation Factor-1 α promoter vector by Klenow fragment mediated blunt end generation of 3' Bam HI end of DNA fragment encoding the reverse tetracycline controlled transactivator and 3' XbaI end of Protein Translation Peptide Elongation Factor-1 α promoter vector and (e) blunt cloning of partially ligated fragment to produce Protein Translation Peptide Elongation Factor-1 α promoter vector expressing reverse tetracycline controlled transactivator.
FIG. 4A
Mda-7
1 1' 2 2' 3 3' 4 4' 5 5' 6 6' 7 7' 8 8' 9 9'

FIG. 4B
Jun B
1 1' 2 2' 3 3' 4 4' 5 5'

6 6' 7 7' 8 8' 9 9'

Figure 4
This application claims priority and is a continuation-in-part application of U.S. Ser. No. 09/268,303, filed Mar. 15, 1999, the contents of which is hereby incorporated by reference.

The invention disclosed herein was made with Government support under Grant No. CA 35675 from the National Institutes of Health, U.S. Department of Health and Human Services. Accordingly, the U.S. Government has certain rights in this invention.

Throughout this application, various publications are referenced. The disclosures of these publications in their entirety are hereby incorporated by reference in order to more fully describe the state of the art as known to those skilled therein as of the date of the invention described and claimed herein.

BACKGROUND OF THE INVENTION

Since the first report by Gossen and Bujard (Gossen and Bujard, 1992) and subsequent documentation of a variant form (Gossen et al., 1995), the Tetracycline (Tc)-regulated system, has been broadly adopted and is widely acknowledged as the method of choice, in experiments requiring inducible expression of genes of interest. In its originally reported form, the system employs two plasmids. One expressing the tTA or rtTA cDNA (henceforth jointly referred to as TA), a fusion protein of the bacterial Tc-repressor, fused to the C-terminal acidic activation domain of the Herpes Simplex virus (HSV), VP16 transcriptional transactivator. The second plasmid enables cloning of a cDNA of interest downstream of a heptamerized Tc-operator transcription regulatory DNA sequence, fused to a DNA element providing basal promoter activity, derived either from the CMV IE or HSV thymidine kinase promoters. Establishing a cell line having Tc-regulatable expression of the gene of interest involves a two step process. In the first, a cell line stably expressing the TA-cDNA is established and identified by clonal selection and expression analysis through transient transfection with a Tc-responsive reporter. In the second step, the gene of interest cloned under control of the Tc-regulatable element is introduced into the cell line made in the previous step and a second round of selection is performed to identify clones displaying Tc-responsive inducibility of the cDNA(Gossen and Bujard, 1992; Gossen et al., 1995). The Tc-regulated system has effectively overcome several drawbacks seen in earlier systems which showed high basal levels of expression, poor responsiveness and toxicity of the inducing agent. The Tc-inducible system is in addition, able to achieve induction over ranges of several orders of magnitude in a graded manner, responsive to varying levels of inducer. Furthermore, the system is extremely versatile and amenable to several types of modifications, permitting the study of the role of a particular gene, or combinations thereof, in a wide variety of cell types of interest. The potential to use this system in medical applications including gene therapy protocols and pharmacological small molecule screening are areas of active investigation. Its versatility has enabled adaptation to situations requiring inducible gene expression in a tissue specific or generalized manner in animal or plant models, opening new avenues to study gene function in vivo.

The Tc-inducible expression system has been modified in several ways, in attempts to improve performance or tailor it to specific needs. Autoregulatory control was achieved by placing both the tTA as well as exogenous cDNA under control of Tc-operator sequences (Shockett et al., 1995), which reportedly permitted regulation of available tTA levels only on induction and thereby increased overall performance in terms of inducibility and frequency of positive clones obtained. Single plasmid vectors containing the TcA sequence and gene of interest in opposite orientations have been developed to obviate the need for multiple rounds of clonal selection(Baron et al., 1995; Schultz et al., 1996; Weinmann et al., 1994). Overcoming a sometimes considerable barrier of introduction of DNA into transfection recalcitrant cells has been made possible through the development of retroviral vectors for delivery of both components of the system in either a single or combination of two separate viruses (Bohl et al., 1997; Hofmann et al., 1996; Kringlestein et al., 1998; Paulus et al., 1996; Rossi et al., 1998). Several promoters have been used to enable generalized or tissue specific expression of tTA in plants (Weinmann et al., 1994) or animals (Efrat et al., 1995; Fishman et al., 1994; Furth et al., 1994; Hennighausen et al., 1995). Modification of the Tc-operator containing plasmid to reduce leaky expression or reduce the effects of integration site has been attempted. Strategies toward this end include Epstein Barr virus (EBV) replication origin based vectors that are maintained episomally (Jost et al., 1997), modified basal promoters to reduce uninduced expression (Hoffmann et al., 1997) and incorporation of sequences that prevent interference from adjoining elements at the site of integration (Hennighausen et al., 1995; McKnight et al., 1992; Stief et al., 1989).

The original report and several other studies have documented potential pitfalls and have provided troubleshooting strategies using the Tc regulated system (reviewed in Blau and Rossi, 1999; Gossen et al., 1994; Shockett and Schatz, 1996). However, anecdotal evidence non-rigorously documenting failure to establish cell lines that show any significant levels of expression or inducibility of the exogenously introduced gene (Ackland-Berglund and Leib, 1995; Gossen and Bujard, 1995) exists. Drawing upon previous experiences using expression constructs with strong viral promoters based on CMV or SV40 derived sequences, extinction of expression of transactivator function could be a potentially significant factor encountered in the inability to establish Tc-responsive cell lines. This might be of special relevance in cells having a relatively slow growth rate and/or the potential to differentiate, making them particularly sensitive to this phenomenon, since changes in cell physiology could affect the activity of exogenously introduced viral promoter constructs. The time lapsed between establishing the initial TA expressing clone and identification of cell lines inducibly expressing the gene of interest, is of a sufficient duration, during which the host cell possibly stops supporting CMV promoter enhancer expression, resulting in the shutdown of TA expression. Despite the recent introduction of retroviral vectors that enable single step and therefore relatively quick selection of positive clones, several of these also depend on viral promoters for expression of one or more elements and are therefore also prone to similar problems. The construction of a specific retrovirus is in itself time consuming and a not as yet routine procedure in many laboratories, compared to
transfection or electroporation of plasmid DNA into cells. Based on these factors modification of the existing construct for rTA cDNA expression was done by placing it under the regulation of the human Protein Translation Peptide Elongation Factor-1 α promoter (EF-1 α). The gene has a housekeeping function in all cells and has been documented to be expressed to relatively high levels. More importantly, due to its indispensable housekeeping function in all cells, Protein Translation Peptide Elongation Factor-1 α promoter (EF-1 α) expression is consistent from a temporal viewpoint, relatively insulated from changes in cell physiology and is cell type independent (Goldman et al., 1996; Kim et al., 1990; Wakabayashi-Ito and Nagata, 1994). Utilization of this construct in cells lines derived from diverse human tissues enabled the successful construction of Tc-regulatable lines in every case attempted so far. This modified vector will not only be of general utility but will be especially useful in cases where difficulties have been previously experienced in successfully establishing Tc-responsive clones.

SUMMARY OF THE INVENTION

The present invention provides a method of screening pharmacological products using the vector. Finally, this invention provides a method for monitoring inducible gene expression in a tissue specific of generalized manner using the vector.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 Plasmid map of Protein Translation Peptide Elongation Factor-1 α expression construct: The map shows component elements of the vector including the rTA ORF, human EF-1 α promoter, Bovine growth hormone (BGH) polyadenylation (poly A) signal and partial multiple cloning site retained from the vector pCDEF3 (Goldman et al., 1996) after cloning. The Neomycin resistance marker (NeoR) flanked by the SV40 promoter and poly A signal, Ampicillin resistance marker (AmpR) for bacterial propagation and selection and some reference restriction site are also shown.

FIG. 2 Luciferase assay to test activity of the Protein Translation Peptide Elongation Factor -1 α promoter vector. Extracts from human HO-1 melanoma cells transiently co-transfected with the original (bars marked pUHD 17-Ineo) or modified (bars marked EF1p Tet on) rTA expression vectors and the Tc luciferase reporter pUHC 13-3 were quantitated for luciferase activity. These extracts were prepared from cells treated without the inducer (-Dox) or with (+Dox). Treatment with inducer was for 48h as described in materials and methods.

FIG. 3 Luciferase assay to select Tc-inducible clones: Panels show quantitation of luciferase extracts from individual Neomycin resistant clonally isolated cell lines of human prostate (DU-145 and PC3), cervical (HeLa), breast (MCF-7) and melanoma (HO-1) tumor origin. Each stable clone was transiently transfected with the Tc luciferase reporter pUHC 13-3 in the absence (-Dox) or presence (+Dox) of inducer. Extracts prepared from these cells were assayed for luciferase activity to identify clones showing adequate levels of inducibility for each cell type as described in material and methods.

FIG. 4 Northern blot analysis of individual Tc responsive clones expressing regulatable Mda-7 or Jun B cDNAs: Autoradiographic detection of levels of induced RNA message levels expressed in clonally selected cells stably transfected with the Mda-7 (A) or Jun B (B) cDNAs under regulation of Tc, probed with respective radiolabelled cDNA probes after transfer to nylon membranes. Each similarly numbered sample was derived from the same clone without induction [1-17(A) and 1-9 (B)] or after addition of inducer, 1-17(A) and 1-9 (B).


DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method comprising an Protein Translation Peptide Elongation Factor -1 α pro-
motor and nucleic acids encoding reverse tetracycline controlled transactivator, wherein the expression of said transactivator is under the control of Protein Translation Peptide Elongation Factor -1 α promoter. In an embodiment the vector is a plasmid. In another embodiment the vector is as set forth in FIG. 1.

[0018] The present invention further provides a cell comprising the vector set forth above. In an embodiment the cell is from a cell line. In a further embodiment the cell line is HeLa (human cervix), HO-1 (human melanoma), MCF-7 (human breast), PC3 (human prostate) or DU-145 (human prostate).

[0019] The invention also provides an animal comprising the vector set forth above. An embodiment of this invention the vector has been introduced into the animal or an ancestor of the animal at an embryonic stage. The animal includes but is not limited to a mouse.

[0020] This invention also provides an animal which comprises a cell which comprises Protein Translation Peptide Elongation Factor-1 α promoter and nucleic acids encoding reverse tetracycline controlled transactivator, wherein the expression of said transactivator is under the control of Protein Translation Peptide Elongation Factor -1 α promoter.

[0021] The present invention provides a method of generating a method of generating a reverse tetracycline controlled transactivator expression system for inducible tetracycline regulated gene expression comprising: (a) isolation of a DNA fragment encoding the reverse tetracycline controlled transactivator by restriction enzyme digestion (b) generation of Protein Translation Peptide Elongation Factor -1 α promoter vector, by restriction enzyme digestion (c) directional cloning of reverse tetracycline controlled transactivator into Protein Translation Peptide Elongation Factor -1 α promoter vector by ligation of 5′ EcoRI compatible restriction enzyme overhangs (d) directional cloning of reverse tetracycline controlled transactivator into Protein Translation Peptide Elongation Factor -1 α promoter vector by Klenow fragment mediated blunt end generation of 3′ Bam HI end of DNA fragment encoding the reverse tetracycline controlled transactivator and 3′ Xba I end of Protein Translation Peptide Elongation Factor -1 α promoter vector and (e) blunt cloning of partially ligated fragment to produce Protein Translation Peptide Elongation Factor -1 α promoter vector expressing reverse tetracycline controlled transactivator.

[0022] In accordance with the method of the invention, the fragment includes but is not limited to an Eco RI-Bam HI fragment, the mammalian expression vector includes but is not limited to pCDEF3, cloning is at the 5′ Eco RI and 3′ Bam HI of the inserts and the ligation is at the 5′ Eco RI site and the 3′Xba I site of pCDEF3.

[0023] The present invention provides a vector which is directed to providing a consistent cellular expression of the tetracycline repressor in cells. Such a vector may be useful in situations requiring inducible gene expression in a tissue specific or generalized manner in animal or plant models. In one embodiment of the invention, pharmacological products are monitored to determine use in medical applications. In the preferred embodiment monitoring is of the gene changes associated with cellular process such as aging, cancer, development, differentiation and growth.

[0024] More specifically, methods which are well known to those skilled in the art can be used to construct a vector directed to providing a cellular expression of the tetracycline repressor in cells. These methods include in cell culture techniques, northern blotting, enzyme activity analysis, construction of plasmids and sequencing. See e.g., the techniques described in Maniatis et al., 1989, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y. and Ausubel et al., 1989, Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley-Interscience, N.Y.

[0025] As used herein the term “tetracycline controlled transactivator” encompasses a vector expressing a protein that binds and activates transcription of downstream tetracycline induced operator binding elements, only when tetracycline is present.

[0026] This invention provides a method of screening pharmacological products using the vector. Finally, this invention provides a method for monitoring inducible gene expression using the vector.

[0027] This invention is illustrated in the Experimental Details section which follows. This section is set forth to aid in understanding the invention but is not intended to, and should not be construed to, limit in any way the invention as set forth in the claims which follow thereafter.

EXPERIMENTAL DETAILS

[0028] Materials and Methods

[0029] Construction of Plasmids: An ECo RI-Bam HI fragment containing the rTA open reading frame was isolated from PHUD 17-lneo (Gossen et al., 1995). This fragment was cloned directionally into the mammalian expression vector pCDEF3 (Goldman et al., 1996) at the 5′ Eco RI and 3′Xba I sites of the vector multiple cloning site to generate the final construct termed, EF1prTIA. Ligation of the 3′Xba I site of pCDEF3 and the BamHI site of the fragment was possible after Klenow filling the overhangs to make them blunt-ended. This modified vector places the rTA gene under direct transcriptional control of the human polypeptide chain elongation factor-1 alpha promoter (EF-1α). Plasmids expressing the Mda-7 and Jun B cDNAs were constructed in pUHD 10-3 (Gossen and Bujard, 1992) by blunt cloning of isolated cDNA fragments into Klenow filled blunt vector followed by sequence analysis for confirmation.

[0030] Cell culture and derivation of stable cell lines: All cell lines used in this study were grown and maintained under standard conditions as previously described (Giang et al., 1996). Selection of stable clones expressing the rTA cDNA using EF1prTIA was carried out in the presence of 500 to 1000 μg/ml G418 (Life Technologies Inc.) depending on the individual cell line. After the selection period, macroscopic visible colonies were picked, expanded and analyzed for activity by assaying for luciferase activity for rTA expression or by Northern blot analysis of inducible cDNA such as Mda-7 or Jun B respectively.

[0031] Northern blotting: Total cellular RNA was resolved by denaturing formaldehyde agarose gel electrophoresis after isolation of RNA using the RNeasy Kit (Qiagen). Transfer was done onto Hybond nylon membranes (Amer sham) and probed with appropriately labeled cDNA probes for Mda-7 and Jun B.
Luciferase activity analysis: Luciferase assays were performed using a Luciferase Assay Kit (Promega) and quantitation was performed on a Turner Design TD 20/20 luminometer. Equal quantities of RNA were loaded on each gel following spectrophotometric estimation at 260 nm. Normalization of RNA levels between samples was confirmed by visualizing RNA on ethidium bromide stained gels. Normalization of luciferase activity was achieved by quantitating protein and adjusting the amount of extract to a fixed amount of protein.

Results

Construction and Initial Testing of the EF-1a Promoter Based rT A Expression Vector

Details of the cloning steps performed in construction of the EF-1 a promoter rT A (EF1prTA) expression vector is described in materials and methods and FIG. 1. The protein expressed by this cDNA, a mutant form of the original bacterial Tc-repressor (Gossen et al., 1995), binds to and activates transcription of genes downstream of Tc-operator binding elements, only when Tc is present. EF1prTA was transiently co-transfected with the Tc-responsive luciferase reporter plasmid, pUHC 13-3 (Gossen et al., 1995), into HO-1 human melanoma cells to determine if the construct was active. A parallel set of transfections was performed with the original CMV IE based construct, puHD 17-1neo (Gossen et al., 1995) in the absence or presence of 1 μg/ml doxycycline (Dox). Cells were harvested 48 hours after transfection and luciferase activity (FIG. 2) was determined using a luminometric Luciferase assay system (Promega). As previously documented (Gossen et al., 1994; Gossen and Bujard, 1992; Gossen et al., 1995) transient assays poorly reflect the level of inducibility actually obtainable after final selection of stable clones, since basal levels of expression change dramatically once plasmid DNA is integrated into chromatin. The initial experiments clearly demonstrated that the EF1prTA expression vector was functional at comparable levels to the original puHD 17-1neo construct in transient assays. Based on the positive activity obtained, the EF1prTA construct was utilized to establish stable lines expressing rT A in HeLa (human cervical carcinoma), HO-1, (human melanoma) MCF-7 (human breast carcinoma) and PC3 and DU-145 (human prostate carcinoma) cancer cell lines.

Analysis of Stable Cell Lines Expressing the rT A cDNA Under Regulation of the EF-1α Promoter

Cells were transfected with the EF1prTA construct using Superfect transfection reagent (Qiagen) based on standard conditions recommended in the usage protocol. The efficiency of transfection, reflected by the number of clones obtained at the end of the selection period, varied with each cell line. Colonies were selected using Neomycin resistance conferred by the marker present within the construct. For each cell line, twenty-four Neomycin resistant colonies were isolated for further analysis. These individually selected clones were transiently transfected with the Tc-responsive luciferase reporter pUHC 13-3 (Gossen et al., 1995) to determine the presence and level of rT A activity. Some cell lines used in this series of experiments had failed to generate Tc-responsive clones in previous attempts utilizing the CMV IE based construct puHD 17-1neo (Gossen et al., 1995).

Discussion

Inability to support continual strong expression from a given type of promoter, specifically those of viral origin, has been documented for certain cell types (Gorman et al., 1985; Hasegawa et al., 1990; Li et al., 1992; Müller et al., 1995). The efficiency of expression observed in previous attempts utilizing the CMV IE based construct puHD 17-1neo (Gossen et al., 1995) is likely to be a reflection of leaky expression in uninduced conditions due to the transient transfection conditions used in this initial screen. Despite this leakiness, clones with high or low relative levels of inducibility were identifiable in every case and potentially usable cell lines were identified with relative ease.
Rizzino, 1995; Sleigh, 1987). The primary goal of this work is to reduce a significant and hitherto unaddressed variable in successfully establishing Tc-inducible cells. Expression of the Tc-operator expression construct, pUHD 10-3 (Gos sen and Bujard, 1992) or its derivatives, into which the cDNA of interest is usually cloned, is ultimately dependent on expression of the TTA or rtTA gene product. Preventing or avoiding TA-cDNA expression is shut down, during or subsequent to establishing a cell line, a variable that is likely to be cell type associated (Ackland-Berglund and Leib, 1995; Gossen and Bujard, 1995) should considerably enhance success rates. To achieve steady and adequate levels of the TA-cDNA expression, relatively independent of temporal factors, cell-type, cell physiology status and cell passage number, we replaced the CMV IE promoter enhancer with the cellular EF-1α promoter (Goldman et al., 1996; Kim et al., 1996; Wakabayashi-Iio and Nagata, 1994). Experience in using pUHD 17-neo (Gossen et al., 1995) indicated that while activity and inducibility in transient assays using sensitive detection methods with luciferase reporters worked reasonably well, we failed to generate cells showing any level of activity of the gene of interest after clonal selection of individual lines, despite presence of expression construct DNA, in the genome using Southern analysis (data not shown).

Numerous modifications of the basic Tc-regulatable system have been reported in the literature directed toward enhancing performance. Several alternative promoters have been utilized to drive expression of the TA-cDNA. Many of these are based on the requirement for tissue or species specific expression in plants (Weinmann et al., 1994), Drosophila (Bischle et al., 1998) or mice, (Bohl et al., 1997; Dhawan et al., 1995; Faiss et al., 1997; Henninghausen et al., 1995; Hoffmann et al., 1997; Hoewell et al., 1997; Li et al., 1992; Liang et al., 1996; Miller and Rizzino, 1995; Thompson and Myatt, 1997). Another modification of the TA expressing construct involves use of bi- or multi-cistronic plasmid constructs which drives expression, through oppositely oriented promoters, of both TA-cDNA and Tc-operator regulated cDNAs, mainly to circumvent two rounds of transfection of separate plasmids (Baron et al., 1995 Fussenegger et al., 1997; Liang et al., 1996; Schulze et al., 1996; Weinmann et al., 1994). However they are based on one or a combination of viral promoters with accompanying drawbacks mentioned above. Multi-cistronic single retroviral or combinations of two or more retroviruses expressing different components has also been constructed (Bohl et al., 1997; Hoffmann et al., 1997; Kringstein et al., 1998; Paulus et al., 1996; Rossi et al., 1998). These overcome the barrier of gene delivery into cells but again expression is often based on viral promoter sequences, prone to possible shutoff in some cell types. The relatively complex steps involved in making a virus for a given cDNA of interest including the intricate cloning strategies due to large vector size and investment in time, somewhat offsets the advantages they present over classical DNA transfection approaches. Making retroviral vectors is presently restricted to a relatively small proportion of laboratories and safety concerns impose limitations of use in several setups. Therefore, while these vectors hold considerable promise, the likelihood of a major shift over to their usage from widespread DNA transfection approaches may only be in the long term. The relevance of improved plasmid vectors is therefore still strong.

A generally applicable modification to the original TA-expression construct involved expression of both TA-cDNA and exogenous cDNA under regulation of Tc-operator sequences (Liang et al., 1996; Shoketz et al., 1995). The rationale being that, exquisite regulation with very high inducibility could be built into a system when both the activator molecule and the regulatable gene of interest are under control of the same inducer through an autoregulatory loop. Unfortunately, it appears that the high levels of TTA protein produced as a result of induction results in toxic side effects in cells (Gallia and Khalili, 1998; Gossen and Bujard, 1992) most likely due to interference in cellular metabolism by the acid activation domain of the HSV, VP16 protein present in TA-proteins. This could be an additional reason why certain cell types apparently shut down expression of TA-cDNA after extended periods time. Alternatively, cells strongly expressing TA proteins might be at a selective disadvantage, particularly in cells with a long doubling time due to accumulation of toxic levels of TA protein. While we can only speculate about the true reason for the apparent loss of TA expression, it appears that switching over to the EF-1α expression cassette is able to balance out and overcome these problems.

The conclusion is based on observations over periods of time, extending to almost twelve months in the case of certain EF1prTIA cells lines such as those established in HO-1 melanoma. The parental HO-1 EF1prTIA cell line was made and initially analyzed over a period of time (>60 days) before being expanded and frozen for future use. These parental cells were used to establish inducible Jun B and Mda-7 expression (FIGS. 4, A and B) that showed functional levels of TA-expression and inducible properties after being thawed out several months and passage numbers subsequent to when the line had initially been established and frozen. This line and others (FIG. 3) continue to retain Tc-responsive properties and were all maintained in the absence of antibiotic selection, indicating that expression of the rtTA cDNA continued irrespective of lack of positive selective pressure, passage number and time elapsed between introduction and integration of the plasmid DNA and final usage. Overall, following modification of the expression construct for the rtTA cDNA we have demonstrated that it had enabled us to significantly enhance the likelihood of establishing cell lines that are Tc-regulatable. It appeared that positive clones were obtained at higher frequencies than previously reported and that consistent expression and clonal stability over an extended period of time was accomplished. Based on these observations we conclude that the modified EF1prTIA presents a useful reagent with broad applicability in establishing Tc-regulatable cells.

Generation of Transgenic Mice Expressing the rtTA cDNA Under Control of the EF-1α Promoter

Experiments to obtain expression of the rtTA protein in all tissues of mice, utilizing transgenic technology, are presently in progress. The EF-1α gene and its promoter are ubiquitously expressed in all animal tissues and is therefore a suitable expression system to achieve this goal. A transgenic expression cassette, consisting of the human EF-1α gene promoter linked to the rtTA cDNA has already been constructed and functionally tested in rat, mouse and human cell lines (described in the literature as rEF1prTIA (Gopalkrishnan et al., Nuc. Acids Res. 27:4775-4782, 1999)
and references therein). Standard procedures in the generation of transgenic mouse lines was performed. In brief, microinjection of pEF1ptTA into pronuclei of fertilized mouse eggs was carried out and these were implanted into pseudo-pregnant female mice. These manipulations resulted in a final litter of three mice which were analyzed for presence of the transgene in genomic DNA, derived from tail-tip samples by genomic Southern blot analysis using a radioactively labeled rTA cDNA probe. This analysis revealed that one of the three founder mice was positive for the transgene since it displayed an appropriate sized band as detected by autoradiography. This founder, a female, has been subsequently crossed with wild type male mice to generate F1 progeny. Analysis of tail-tip DNA from the F1 generation has permitted us to determine whether the founder possess the capacity to transmit the transgene. Southern blot analysis of tail-tip DNA from 15 F1 generation indicated that eight (8) mice were positive for the transgene, confirming that the original founder animal had the capacity to transmit the inserted gene. Subsequent to our successful generation of transgenic mouse lines, we are presently in a position to breed additional animals and begin extensive expression analysis of the transgene to determine level of expression. This will be carried out on F1 or later generation mice, while maintaining the original founder until we are certain that stable expressing lines can be generated from progeny for future use and distribution. These mouse lines can be used to generate mice that can inductively express specific genes under regulation of tetracycline to study the in vivo effect of specific genes in animals or screen for anti-tumoral or other pharmacological effects of drugs or small molecules.

Construction of pEF1ptTA, An Expression Vector Expressing tTA, Tetracycline Repressor Under Regulation of the EF-1α Promoter for Significantly Increasing Success in Establishing Stable Cell Lines With Consistent Expression.

An EcoRI-BamHI fragment containing the tTA open reading frame was isolated from pUHD 15-1. This fragment was cloned directionally into the mammalian expression vector pCDE3F at the 5′ EcoRI and 3′ XbaI sites of the vector multiple cloning site to generate the final construct, termed EF1ptTA. Ligation of the 3′ XbaI site of pCDE3F and the BamHI site of the fragment was possible after Klenow filling the overhangs to make them blunt-ended. This modified vector places the tTA gene under direct transcriptional control of the human EF-1α promoter. The construct was confirmed by restriction enzyme and DNA sequencing functional testing in rodent and human cell lines is presently underway and will be performed essentially as described for pEF1ptTA. Compared to the earlier construct (pEF1ptTA) wherein gene expression is induced in the presence of the inducer (tetracycline or doxycycline), the present construct is active in the absence of tetracycline or doxycycline and gene expression is shut-down in the presence of these reagents. Both plasmids may be used in setting up inducible gene expression systems in cell lines or mice and the choice will be dependent on whether one desires to grow cells in the presence or absence of the chemical agent.

References


[0066] 20. Henninghausen, L., Wall, R. J., Tillmann, U., Li, M., and


promoter of the human elongation factor-1 alpha gene."

1. A vector comprising:
   a) a human Protein Translation Peptide Elongation Factor-1 α promoter;
   b) a nucleic acid encoding a tetracycline controlled transactivator, wherein the expression of said transactivator is under the control of the promoter;
   c) a tetracycline inducible operator binding element under the control of the nucleic acid encoding the transactivator, and
   d) a gene of interest under the control of the promoter.
2. The vector of claim 1, wherein the vector is a plasmid.
3. The vector of claim 1, wherein the vector is as set forth in FIG. 1.
4. An isolated cell comprising the vector of claim 1.
5. The cell of claim 4, wherein the cell is from a cell line.
6. The cell of claim 5, wherein the cell line is HeLa (human cervix), HO-1 (human melanoma), MCF-7 (human breast), PC3 (human prostate) or DU-145 (human prostate).
7. The cell of claim 4, which consistently expresses tetracycline repressor.
8. A cell comprised of Protein Translation Peptide Elongation Factor-1 α promoter and nucleic acids encoding reverse tetracycline controlled transactivator, wherein the expression of said transactivator is under the control of Protein Translation Peptide Elongation Factor-1 α promoter.
10. The animal of claim 9, wherein the animal is a mouse.
11. A method of generating a reverse tetracycline controlled transactivator expression system for inducible tetracycline regulated gene expression comprising:
    a) isolation of a DNA fragment encoding the reverse tetracycline controlled transactivator by restriction enzyme digestion.
    b) generation of Protein Translation Peptide Elongation Factor-1 α promoter vector, by restriction enzyme digestion;
   c) directional cloning of reverse tetracycline controlled transactivator into Protein Translation Peptide Elongation Factor-1 α promoter vector by ligation of 5' EcoRI compatible restriction enzyme overhangs;
   d) directional cloning of reverse tetracycline controlled transactivator into Protein Translation Peptide Elongation Factor-1 α promoter vector by Klenow fragment mediated blunt end generation of 3' Bam HI end of DNA fragment encoding the reverse tetracycline controlled transactivator and 3' Xba I end of Protein Translation Peptide Elongation Factor-1 α promoter vector; and
   e) blunt cloning of partially ligated fragment to produce Protein Translation Peptide Elongation Factor-1 α promoter vector expressing reverse tetracycline controlled transactivator.
12. The method of claim 11, wherein the fragment of 11(a) is an Eco RI-BAM HI fragment.
13. The method of claim 11, wherein the mammalian expression vector of 11(b) is pCDEF3.
14. The method of claim 11, wherein the cloning of 11(a) is at the 5' Eco RI and 3' BAM HI sites.
15. The method of claim 11, wherein the ligation of 11(c) is at the 5' Eco RI site of pCDEF3.
16. The method of claim 11, wherein the ligation of 11(d) is at the 3' Xba I site of pCDEF3.
17. A vector generated by the method of claim 11.
18. A method for screening for an anti-tumor drug which comprises administering to a transgenic non-human animal a drug wherein the animal inducibly expresses or repression expression of a gene of interest under regulation of tetracycline or doxycycline and wherein the gene of interest is associated with cancer, and determining whether the animal develops a tumor thereby screening for an anti-tumor drug.
19. A method for expressing a gene of interest which comprises contacting the cell of claim 4 with an inducer of the tetracycline inducible operator binding element so as to cause the cell to express the gene of interest.
20. The method of claim 19, wherein the inducer is tetracycline or doxycycline.