Short Technical Reports

Bicistronic Vector for the Creation of Stable Mammalian Cell Lines that Predisposes All Antibiotic-Resistant Cells to Express Recombinant Protein

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ABSTRACT

We have developed an improved vector for the stable expression of recombinant protein in mammalian cells. In this vector, designated pCIN, both the recombinant cDNA and the neomycin phosphotransferase selection marker are transcribed from a single promoter element. To facilitate translation of the second open reading frame, the encephalomyocarditis virus internal ribosome entry site has been inserted into the expression cassette immediately before the start codon of this sequence. We report the use of this vector to generate stable cell lines expressing the human 5-HT_{1Da} serotonin receptor and show that following transfection and clonal selection, all ten cell lines characterized express similar and high levels of receptor (1.5–11.9 pmol receptor/mg protein). Use of pCIN should permit the rapid and efficient production of stable mammalian cell lines for the characterization of recombinant protein, as this vector appears to predispose all transfected cells to express such protein.

INTRODUCTION

The expression of recombinant protein in mammalian cell lines is a fundamental technique in modern biology. Such studies have been used to characterize many proteins, including members of the superfamily of 7-transmembrane domain, guanine-nucleotide binding protein (G protein) coupled receptors (6). The stable expression of such receptors has facilitated the identification of selective ligands (21), the understanding of receptor/G protein interaction and the identification of second messenger signaling mechanisms (6,20,23). Indeed these studies have revealed several features of this class of protein not predicted by traditional pharmacological analysis. Many receptors over-expressed in cell lines show constitutive activity, that is, the ability to signal in the absence of ligand. This observation has led to the identification of a class of compounds, termed inverse agonists, which are able to lower this basal level of activity (3,14). However, the study of these and other proteins expressed in stable cell lines is hampered by the technical problems associated with their development.

Standard methods for the creation of a stable cell line involve transfection into a host cell of two expression cassettes: the first for expression of recombinant protein and a second for expression of an antibiotic resistance gene, carried on either two plasmids (co-transfection) (23) or on a single "all-in-one" vector (5). However, following transfection and clonal selection for stable integrants, the proportion of antibiotic resistant clones that also express recombinant protein is rarely more than 30%. This is a result of deletion or inactivation of the expression cassette for the production of recombinant protein during plasmid integration, or in the case of co-transfection, due to integration of only the plasmid carrying the antibiotic selection marker. Furthermore, the level of expression is impossible to predict, is often low and will often decrease during time in culture, as there is no positive selection to maintain the transcriptional activity of the site of vector integration.

The standard model of protein translation that applies to the vast majority of eukaryotic and prokaryotic mRNAs involves ribosome entry at the 5′ cap structure followed by scanning of the mRNA in a 5′ to 3′ direction until the initiation codon is reached with the subsequent initiation of protein translation (13). This model of translation does not appear to apply to the picornaviridae family of viruses, which includes encephalomyocarditis virus (EMCV), foot-and-mouth disease virus and poliovirus. These viruses possess a single-stranded RNA genome of positive polarity containing a 600–1200 nucleotide (nt) 5′ untranslated region (UTR) followed by a single open reading frame (ORF) encoding the viral polyprotein. Gene expression is unusual in that the ribosome is able to recognize and bind directly to a 450–600-nt secondary structure element located within the 5′ UTR termed an internal ribosome entry site (IRES) (8,9). The 5′ UTR contains numerous AUG initiation codons; however, following ribosome entry at the IRES, a number of these are bypassed to allow translation initiation at the authentic AUG (AUG 11 or AUG 12 for EMCV) (8). IRES elements have been removed from their viral setting and linked to unrelated genes to produce polycistronic RNAs. Several reports describe their application in retroviral mediated gene transfer, in the creation of transgenic mice and for the expression of multiple ORFs from a single transcript in mammalian cells (10,11,15).

To facilitate the creation of stable cell lines, we have developed a bicistronic expression vector that predisposes every transfected cell to express recombinant protein and at apparently high levels. This is achieved through inclusion of the EMCV IRES (8) into the expression cassette to permit the translation of two ORFs, the recombinant protein and the neomycin phosphotransferase (NPT II) antibiotic resistance marker, from a single messenger RNA. We demonstrate the use of this vector to express the human 5-$HT_{1D\alpha}$ serotonin receptor in HEK 293 cells.

MATERIALS AND METHODS

Plasmid Constructions

All DNA manipulations were performed using standard methods unless otherwise described (17).

The construction of pCIN is summarized in Figure 1. A synthetic intron (7) was cloned from pOG44 (Stratagene Ltd, Cambridge, UK) between the *Eco*RI and *Sal*I sites of pcDNA3 (Invitrogen, San Diego, CA, USA) to generate pcMV1, followed by deletion of the *Eco*RI site in this vector by digestion with this enzyme and filling the resulting sticky ends with Klenow DNA Polymerase to create pcMV2. The NPT II expression cassette in pcMV2 was

Table 1. Assay of the Ability of the EMCV IRES to Permit Translation of NPT II

Vector	pg NPT II/ µg Protein
no DNA	0.0 ± 0
pΔIRES/5-HT$_{1D\alpha}$	0.0 ± 0.3
pCIN/5-HT$_{1D\alpha}$	4.0 ± 0.4
pcMV1/5-HT$_{1D\alpha}$	59.0 ± 3.8

NPT II assays were performed on cell extracts prepared following transient transfection of expression vector into Cos M6 cells. Results are expressed as the mean of 4 independent assays in pg NPT II per µg of total cellular protein.

excised by digestion with *Xba*I and *Bsm*I with simultaneous insertion of a synthetic linker created following annealing of the oligonucleotides 5′- CTAGGGCGGCCGCGTACGAATTCGATACTCGAGTG-3′ and 5′-CTCGAGTATCGAATTCGTACGCGGCCGCC-3′ encoding the restriction sites *Xba*I, *Not*I, *Eco*RI and *Xho*I, to generate pcMV3. Following this, the NPT II cDNA and the bovine growth hormone polyadenylation sequence were excised from the plasmid pPol2sneobpA (18) by digestion with *Eco*RI and *Xho*I and inserted between the *Eco*RI and *Xho*I sites of pcMV3 to create pcMV4. Finally, the EMCV IRES was amplified using the polymerase chain reaction (PCR) from pIRESβ-geo (Dr. P. Mountford, University of Edinburgh, UK) with the primers 5′-GATCGCGGCCGCACTAGAGGAATTCCGCCCCTCTCC-3′, incorporating *Not*I and *Eco*RI sites (underlined) and 5′-CATGGATATCCCGGGTTGTGGCAAGCT TATCATCGT-3′, containing overlapping *Sma*I and *Eco*RV sites (again underlined). The PCR product was digested with *Not*I and *Eco*RV and cloned between the *Not*I and Klenow-treated *Eco*RI sites of pcMV4 to generate pCIN. The IRES was re-sequenced following PCR.

To validate these vectors, the human 5-HT$_{1D\alpha}$ serotonin receptor cDNA was amplified from pCD-MA6A (4) by PCR with the primers 5′-CCAGAATTCCATGTCCCCACTGAACCAGTCAGCAG-3′ and 5′-CCGAGAATTCCTAGGAGGCCTTCCGGAAAGGG-3′ incorporating *Eco*RI restriction sites at their 5′ termini (underlined). The product was digested with this enzyme and subcloned into the *Eco*RI site of pcMV1 to generate pcMV1/5-HT$_{1D\alpha}$. The cDNA was re-sequenced following PCR. For expression studies, the 5-HT$_{1D\alpha}$ cDNA was further transferred into the *Eco*RI site of pCIN to create pCIN/5-HT$_{1D\alpha}$.

A control vector without the IRES sequence was built by insertion of a *Asp*718-*Sac*II fragment from pcMV1/5-HT$_{1D\alpha}$, encoding the 5-HT$_{1D\alpha}$ cDNA and part of the intron, between the *Asp*718 and *Sac*II sites of pcMV4 (recreating an intact intron) to generate pΔIRES/5-HT$_{1D\alpha}$.

Expression Studies

All cells were maintained at 37°C with 5% CO_2 and 92% humidity, in Dulbecco's modified Eagle Medium/Ham's F-12 (1:1) media, supplemented with 10% fetal calf serum and 4 mM glutamine (all reagents from Life Technologies, Gaithersburg, MD, USA).

For transient transfection, Cos M6 cells, grown to 40% confluence in 500 cm^2 triple flasks, were transfected with 50 µg of plasmid using the DEAE dextran procedure (12). Cell extracts were prepared 48 h post-transfection. Cells were washed twice in Hank's balanced salt solution (HBSS), detached from the plate by incubation in HBSS containing 600 µM EDTA and collected by centrifugation at 200× *g* in a Heraeus Bactifuge® (Heraeus Instruments GmbH, Hanau, Germany). The cell pellet was resuspended in 1 mL of phosphate-buffered saline (PBS) containing 1 mM phenylmethyl sulphonyl fluoride, and the cells lysed by three alternate brief immersions into a dry ice/ethanol bath followed by a 37°C water bath. Cell debris were removed by centrifugation at 500× *g* for 5 min at 4°C in a microcentrifuge (Model 5415C; Eppendorf, Hamburg, Germany) and the supernatant was collected and stored at -80°C for NPT II assay.

For stable transfection, 10 µg of pCIN/5-HT$_{1D\alpha}$ were linearized with *Ssp*I and transfected into HEK 293 cells, plated at 1% confluence in a 10-cm-diameter tissue-culture dish, using the calcium phosphate procedure. Two days following transfection, the growth medium was replaced with medium supplemented with 1 mg/mL G418 (Life Technologies) (19). Following application of antibiotic selection, single colonies were picked and expanded through several passages to provide cells for homogenate preparation. Cells, grown to confluence in 175-cm^2 flasks, were detached by incubation in PBS containing 5 mM EDTA, counted and harvested by centrifugation at 4500× *g* for 35 min [SORVALL® RC-3 Centrifuge; Du Pont (UK) Ltd., Herts, UK]. The cell pellet was re-suspended in assay buffer (100 mM Tris-HCl, 1 mM EDTA and 1% ascorbic acid, pH 7.7) to yield a concentration of 10^7 cells/mL. Cells were disrupted by homogenization, and the homogenates were stored at -80°C for ligand binding studies.

Table 2. Expression of the Human 5-HT$_{1D\alpha}$ Serotonin Receptor in HEK 293 Cells Transfected with pCIN/5-HT$_{1D\alpha}$

Clone	K_D (nM)	B_{max} (pmol/mg)
1	1.5	1.5
2	1.4	2.9
4	1.6	3.2
13	1.7	1.8
14	1.9	3.4
15	2.4	11.9

Saturation analysis with the high-affinity 5-HT$_{1D}$ selective ligand [3H]-5-CT was performed on cell homogenates as described. The equilibrium dissociation constant (K_D) and receptor density (B_{max}) were determined using the curve-fitting program LIGAND. Each value is calculated from a single experiment performed in triplicate.

NPT II Assay

The sandwich enzyme-linked immunosorbent assay (ELISA) for NPT II activity was performed according to the protocol supplied with the NPT II assay kit (CP Laboratories, Bishop's Stortford, Herts, UK), except that the final

incubation of bound streptavidin alkaline phosphatase with *p*-nitrophenolphosphate was performed at room temperature. NPT II levels were determined according to reaction rate using the kinetic mode of the $THERMO_{MAX}$ microplate reader at 405 nm (Molecular Devices, Menlo Park, CA, USA). Four assays were performed on each transfection.

Protein Assay

Protein concentrations were determined with bovine serum albumin as a standard (1). Six independent assays were performed on each cell extract or homogenate.

Radioligand Binding Assay

Saturation experiments were carried out under the following conditions: cell homogenate (11–41 µg protein in 750-µL assay buffer), [^{3}H]-5-carboxytryptamine (5-CT; 0.1–30 nM final concentration, 200 µL; Du Pont NEN, Boston, MA, USA) and unlabeled 5-CT or assay buffer (50 µL) were incubated at 37°C for 30 min. Reactions were terminated by rapid filtration (Brandel 48-well harvester; Semat, Luton, UK) through glass fiber filters (GF/B; Whatman) presoaked in 0.1% polyethyleneimine for 30 min. The filters were washed four times with 1 mL ice-cold wash buffer (100 mM Tris-HCl, 1 mM EDTA, pH 7.6) and placed in vials with scintillation fluid. Bound radioactivity was measured on a liquid scintillation counter (Model 2200CA; Packard, Pangbourne, UK). Under these conditions, specific [^{3}H]-5-CT binding (defined as that inhibited in the presence of 10 mM 5-CT) represented greater than 95% of total binding, while less than 10% of total ligand was bound. The K_D and B_{max} values were calculated using the curve-fitting program LIGAND (16).

RESULTS AND DISCUSSION

Plasmid Construction

To facilitate the creation of stable mammalian cell lines, we have developed a bicistronic expression vector in which both the antibiotic resistance marker and the recombinant protein are under the transcriptional control of a single promoter element. The expression cassette in pCIN consists of the human cytomegalovirus (CMV) major intermediate early promoter/enhancer sequence, followed by a multiple cloning site, a synthetic intron shown to enhance message stability (7), the EMCV IRES followed by the NPT II cDNA, for the selection of stable transformants in the presence of the antibiotic G418, and the bovine growth hormone polyadenylation signal. The remainder of the plasmid consists of

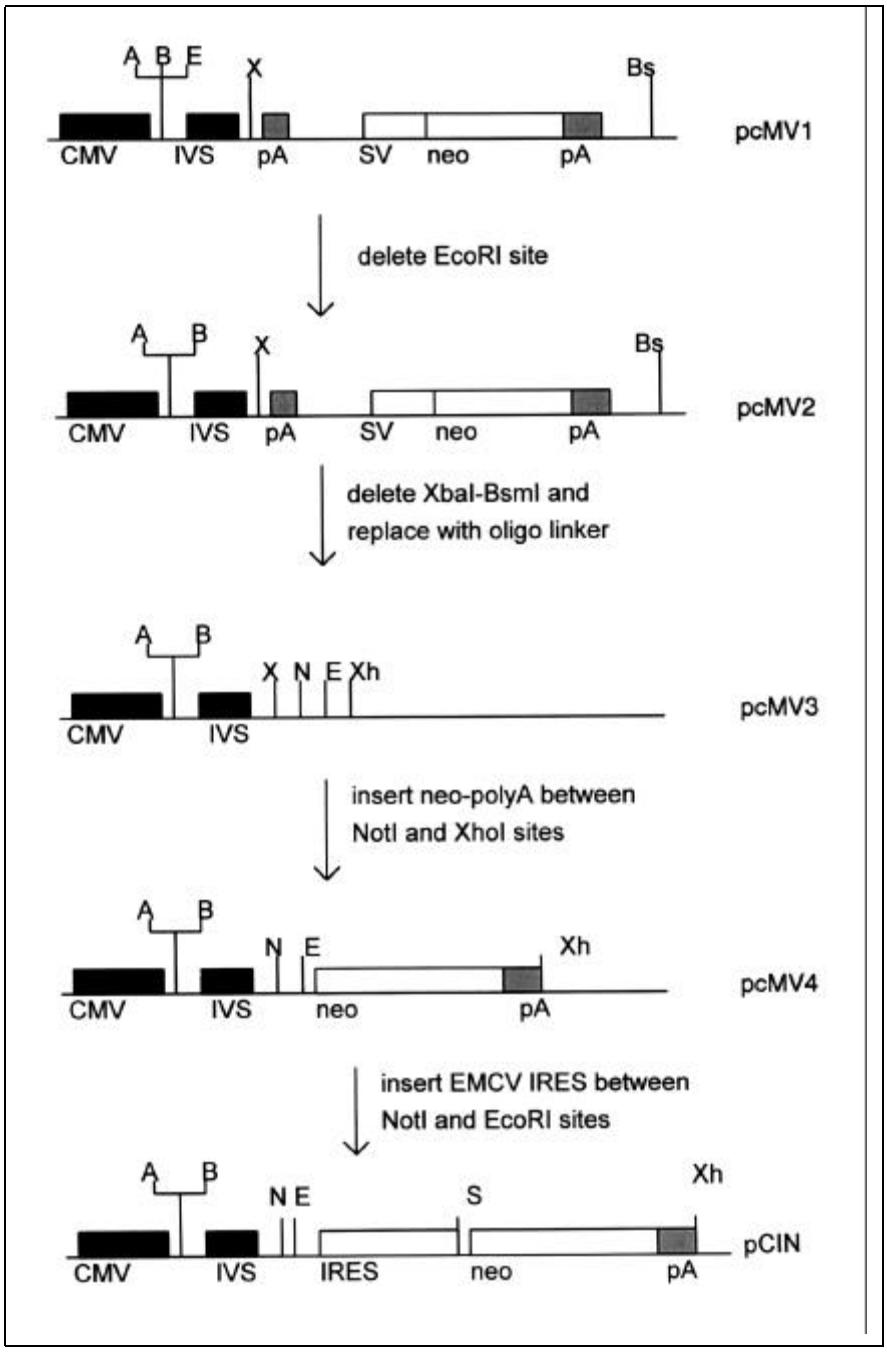

Figure 1. Construction of the expression vector pCIN. A = *Asp*718, B = *Bam*HI, Bs = *Bsm*I, E = *Eco*RI, N = *Not*I, S = *Sma*I, X = *Xba*I, Xh = *Xho*I; CMV = cytomegalovirus intermediate early promoter, IVS = intron, pA = polyadenylation sequences, SV = SV40 early promoter/origin of replication, neo = neomycin phosphotransferase cDNA and IRES = internal ribosome entry site.

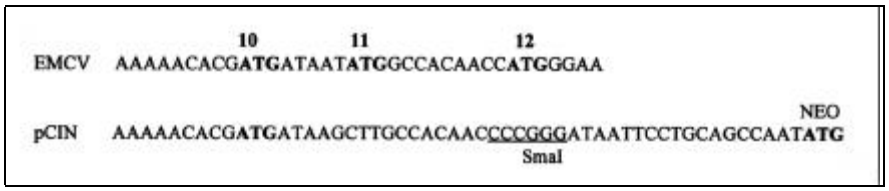

Figure 2. Structure of the junction between the EMCV IRES and the NPT II cDNA in the expression vector pCIN. EMCV ATG 10, 11 and 12 and the neomycin phosphotransferase ATG are indicated.

the *Bsm*I to *Bgl*II region of pcDNA3 (Invitrogen) and encodes the β-lactamase gene to confer ampicillin resistance in *Escherichia coli* and the bacterial ColE1 origin of replication (Figure 1). We also constructed a control vector, pΔIRES, identical to pCIN but lacking the IRES element.

The 5′ UTR (IRES) of EMCV is 718-nt long and contains 10 AUG initiation codons scattered throughout the sequence (8). The major site of initiation for viral polyprotein synthesis is AUG 11, with some initiation at AUG 12 (8). To achieve maximal translation through the IRES, the position of AUG 11 relative to the upstream IRES must be maintained (8,9). However, during the construction of pCIN, it was decided to disable the ability of the IRES to mediate NPT II translation by displacing the start codon 44 nt downstream of AUG 11 (Figure 2). We expect this to result in the generation of cell lines likely to express high levels of recombinant protein, since in order to express sufficient NPT II to allow growth in 1 mg/mL G418, we would select for sites of high gene expression. Furthermore, to prevent inappropriate initiation of protein translation at either EMCV AUG 11 or AUG 12, both of these start codons have been destroyed (Figure 2).

To characterize these vectors, the human 5-$HT_{1D\alpha}$ serotonin receptor cDNA (4) was subcloned into the multiple cloning site of each to generate pCIN/5-$HT_{1D\alpha}$, pΔIRES/5-$HT_{1D\alpha}$ and pcMV1/5-$HT_{1D\alpha}$.

Transient Assay for IRES Function

To demonstrate the ability of the ribosome to enter the messenger RNA through the disabled IRES in pCIN/5-$HT_{1D\alpha}$, a transient expression study was performed. Protein extracts prepared following transfection of this plasmid, pcMV1/5-$HT_{1D\alpha}$ and pΔ–IRES/5-$HT_{1D\alpha}$ into Cos M6 cells were assayed for NPT II using a sandwich

ELISA assay. We found significant NPT II expression in extracts prepared following transfection with pcMV1/5-$HT_{1D\alpha}$ (59 pg NPT II/μg protein; Table 1). In this vector, NPT II expression is under the transcriptional control of the simian virus 40 (SV40) early promoter. However, the plasmid also contains the SV40 origin of replication, which permits episomal replication in Cos M6 cells due to the stable expression of the SV40 large T-antigen in this cell line. Consequently, the high level of NPT II can be attributed to both the plasmid amplification seen in this expression system and the relative strength of the SV40 early promoter. As expected, no NPT II was seen in cells transfected with pΔIRES/5-$HT_{1D\alpha}$, as this vector does not contain the IRES (Table 1). However, we did observe NPT II activity in extracts prepared from cells transfected with pCIN/5-$HT_{1D\alpha}$ (4 pg/μg; Table 1). While the level of NPT II expression is only 7% of that seen when this enzyme is expressed directly from the SV40 early promoter, the disabled IRES is clearly able to permit ribosome entry to lead to translation of this ORF.

Generation of Stable Cell Lines with pCIN

pCIN/5-$HT_{1D\alpha}$ was transfected into HEK 293 cells. Following application of the antibiotic G418 (1 mg/mL), 10 colonies, arising from 10 single cells, were isolated, expanded and characterized for expression of the 5-$HT_{1D\alpha}$ receptor by ligand binding to cell homogenates of the high affinity 5-HT_{1D} agonist radioligand [^{3}H]-5-CT (2). Unlike untransfected cells, all 10 cell lines exhibited specific binding of this ligand. Homogenates harvested from six of these clones displayed an apparently homogenous population of high-affinity, saturable, [^{3}H]-5-CT binding sites (data not shown). The receptor density (B_{max}) was between 1.5 and 11.9 pmol receptor/mg of total cellular protein, with an equilibrium dissociation constant (K_D) for the ligand 5-CT of between 1.5 and 2.4 nM consistent with binding to the 5-$HT_{1D\alpha}$ receptor (Table 2) (4,23). We have examined the stability of expression in four of these cell lines and find no loss of expression following 20 passages in culture.

Since both the 5-$HT_{1D\alpha}$ receptor and the NPT II selection marker are translated from the same bicistronic mRNA, we might expect to see a correlation between the expression level of these two proteins. However, we find no such correlation (data not presented). This observation may imply that individual members of a clonal population are not genetically and biochemically identical; each of the derived cell lines may have different intrinsic resistance to the antibiotic G418 or a differing ability to tolerate the cellular effects caused by overexpression of this receptor. However, it remains a possibility that the level of expression within an individual cell line may be regulated through alterations in the concentration of G418.

Literature reports of the level of expression of the 5-$HT_{1D\alpha}$ serotonin receptor vary from 98 fmol/mg in the human cervical carcinoma HeLa cell line (4) to 4 pmol/mg in mouse LM(tk-) fibroblasts (24) compared with levels of expression in this study of 1.5–11.9 pmol/mg. Similarly, in our laboratory we have generated stable cell lines with many 7-transmembrane receptors and rarely see expression at greater than 2 pmol/mg. Thus, we appear to have succeeded in generating a vector that predisposes transfected cells to express high levels of recombinant protein, presumably due to selection for sites of high transcriptional activity. The implication would be that low expressing cell lines are not isolated, as they produce insufficient NPT II to allow growth in 1 mg/mL G418, although selection with less antibiotic might be expected to result in the identification of clones with a lower level of expression.

There are a number of reports of the development of retroviral vectors containing drug selectable genes under the control of the EMCV IRES including the multiple drug resistance and NPT II selection markers (15,22) and a single report describing the use of such vectors for the derivation of stable mammalian cell lines (10). The vector used in the latter study, pED4 carries dihydrofolate reductase (DHFR) rather than neomycin selection and was used to express the eIF-2α transcription factor in DHFR-deficient CHO cells with the result that all 14 cell lines characterized were shown to express a full-length bicistronic mRNA. However, there is no data presented concerning the number of clones that express eIF-2α protein or the level of expression obtained with the bicistronic DHFR selection vector. In contrast, we have shown that following transfection with pCIN, every cell line characterized expresses recombinant protein and at apparently high levels. Furthermore, the use of neomycin rather than DHFR within a bicistronic mammalian expression vector should greatly increase the versatility of such vectors.

Previously we have attempted to generate stable cell lines expressing the 5-$HT_{1D\alpha}$ receptor using the vector pcDNA3 (Invitrogen) and found a single expressing clone from 19 cell lines screened (unpublished results). The ability of the bicistronic vector to ensure that all antibiotic-resistant clones express recombinant protein saves considerable time and manpower during the generation of a stable cell line, and for certain studies, may eliminate the need for clonal selection to identify expressing cells for analysis. Furthermore, the use of pCIN to generate cell lines predicted to express high levels of recombinant protein would facilitate the preparation of recombinant protein for functional analysis. In particular, the cell lines generated in this study may be used for high-throughput compound screening to identify novel ligands for the human 5-$HT_{1D\alpha}$ serotonin receptor and would allow an examination of constitutive activity and the identification of inverse agonist ligands. We have recently generated CHO/human adenosine A2a, Cos-7/human 5-HT_{5A} serotonin receptor and CHO/human β3-adrenoreceptor expressing cell lines with similar results.

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