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COMPARISON OF BIDIRECTIONAL AND BICISTRONIC INDUCIBLE SYSTEMS FOR CO-EXPRESSSION OF CONNEXIN GENES AND FLUORESCENT REPORTERS

Carthur K. Wan*, Shamim B. Shaikh, Colin R. Green† and Louise F.B. Nicholson

Department of Anatomy with Radiology and the Centre for Brain Research, †Department of Ophthalmology, Faculty of Medical and Health Sciences, University of Auckland,

Auckland 92-019, New Zealand

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(*) Corresponding author: Department of Anatomy with Radiology and the Centre for Brain Research

Faculty of Medical and Health Sciences

University of Auckland

92-019 Auckland, New Zealand

e-mail: c_wan@auckland.ac.nz

fax: (64-9) 923 7484

phone: (64-9) 923 1902
ABSTRACT

Gene expression studies often require inducible co-expression of both a gene of interest and a reporter gene. Fusion of fluorescent reporters can, however, modify protein structure and function. We have generated inducible expression systems for two connexin genes: Cx30 and Cx43. It has been reported recently that reporter fusion to connexins can modify their function. Therefore, we have compared two methods of independent reporter co-expression and examined co-localization with induced connexin expression. Identical levels of connexin expression were observed for both the bidirectional and bicistronic expression systems. In contrast, however, reporter gene expression by the bidirectional promoter provided brighter average fluorescent pixel intensity than expression of a reporter gene in a bicistronic transcript. Moreover, as a result of this difference in reporter expression bidirectional expression systems provided equal or better co-localization between the connexins and reporter gene fluorescence. The results of our study indicate that bidirectional reporter expression provides a robust indicator of transfection and gene expression, and may therefore favor the use of bidirectional over bicistronic reporters in the design of expression systems wherein the gene of interest, such as connexin genes, contains translational motifs or long intronic regions.

Keywords: connexin, reporter gene, expression systems, tetracycline inducible

INTRODUCTION

Tetracycline (tet) inducible systems are widely used for gene expression studies [1-3]. Tet-based control of expression in mammalian cells was first described almost two decades ago [4]. Since
then, the systems have been extensively used and improved. Tet systems are generally comprised of two plasmids, a response plasmid containing the gene of interest and a regulatory plasmid that controls activation of gene expression. The regulatory plasmid expresses a tet or reverse tet transactivator (tTA or rtTA) which is formed from the fusion of a modified tet repressor DNA binding domain (tetR or reverse tetR) and a virion protein 16 (VP16) activator sequence from *Herpes simplex* virus that can stimulate the initiation of transcription. The response plasmid contains seven repeats of a tet operator (tetO) sequence preceding a minimal cytomegalovirus (CMV) promoter sequence which together constitute the tet-responsive tight (TREtight) promoter. The tetR domain of the tTA or rtTA can bind to the tetO only in the absence or presence of tetracycline, or its analogues, respectively. Once bound to the response plasmid the VP16 domain of the transactivator stimulates the CMV promoter to initiate transcription [3, 4].

Tet systems have been optimized to the stage where basal leakiness is generally very low and high levels of induction can be achieved with minimal amounts of tet, or more commonly, its analogue doxycycline (dox). As the system is derived from prokaryotic sources, both the genetic regulatory elements controlling gene expression and the induction agent are generally absent in eukaryotic cells, limiting the possibility of pleiotrophic effects. As such, tet systems enable tightly regulated and transient modulation of expression. Consequently, tet systems are ideal for studies investigating genes that show endogenous temporal patterns of expression, such as connexins (Cxs).

Cxs are a multigenic family of proteins that constitute gap junction channels between cells and are involved in a myriad of physiological processes. While expression systems have been developed previously for certain Cx subtypes, they have been largely constitutive. Previously generated inducible systems have either lacked an *in vitro* reporter of expression [5] or expressed
a fused reporter protein and thus expressed chimeric Cx [6-8]. The co-expression of a reporter gene is often essential as an indicator of vector transfection and gene expression, or as a selectable marker in lieu of antibiotic selection [9]. The terminal fusion of a fluorescent reporter can, however, modify protein structure and function; this has been shown with regard to Cx function and assembly [6, 10, 11]. For investigation of proteins that may be modified by fusion, such as Cxs, reporter genes should be independently translated.

There are two types of response plasmid that can provide co-expression of a reporter without fusion to the protein of interest. One system involves a unidirectional TRETight promoter driving the transcription of a single bicistronic mRNA sequence. The two genes in the mRNA sequence are separated by an internal ribosome entry site (IRES) sequence that enables independent translation of the two genes; the gene of interest and a reporter gene. The alternative system involves a bidirectional TRETight promoter driving the independent transcription and translation of the gene of interest and a reporter gene [12, 13]. The utility of a co-expressed reporter gene ultimately depends on the degree of in situ co-localization to the same cells as the protein of interest. Therefore, in our study we have investigated both potential routes for modulating Cx gene expression in order to determine which method would provide better inducible Cx overexpression, reporter fluorescence and Cx/reporter co-localization. While our study focuses on two Cx paralogs and it is possible the effects reported may be specific to these genes, our observations may well be of value to other studies requiring independent co-expression of a fluorescent reporter.

MATERIALS AND METHODS

Cloning
Plasmid amplification and manipulation were performed using DH5α E. coli (Invitrogen; CA, USA) and DNA was purified from bacterial cultures using a PureLink HiPure Midi Kit (Invitrogen) following the manufacturer’s instructions. Full length cDNA clones of Cx30 and Cx43 were obtained from the TrueClone Human Collection as inserts in the pCMV6-XL4 cloning vector (OriGene; MD, USA).

For bicistronic expression systems, pTRE Tight-IRE S-EGFP was first generated by directionally cloning a fragment containing the internal ribosome entry site (IRES) and EGFP gene from the pIRES-EGFP vector (Clontech; CA, USA) into the MCS of pTRE Tight (Clontech) using BamHI and NotI. Full length Cx30 and Cx43 genes were excised from cloning vectors by sequential digestion with Smal and EcoRI; for Cx43 a partial digest was performed as the gene contained an internal EcoRI restriction site. The MCS of pTRE Tight-IRE S-EGFP digested with Smal and EcoRI and the Cx genes were directionally ligated into the vector generating pTRE Tight-Cx30-IRES-EGFP (bicistronic Cx30 expression system) and pTRE Tight-Cx43-IRES-EGFP (bicistronic Cx43 expression system). Bidirectional expression systems were generated by excising Cx30 and Cx43 from cloning vectors using NotI and ligating the fragments into linearized pBI-TRE Tight-ZsGreen (Clontech), to generate pBI-TRE Tight-Cx30/ZsGreen (bidirectional Cx30 expression system) and pBI-TRE Tight-Cx43/ZsGreen (bidirectional Cx43 expression system) respectively. Restriction digests were performed to ensure correct directionality. The design and major features of the two types of expression system are depicted schematically in Figure 1.

**Fig. 1 near here**

Cell culture and transfection
The K1 subclone of the Chinese Hamster ovary (CHO-K1) cell line, originally obtained from ATCC (Manassas, VA, USA), were cultured in DMEM/F12 with 2 mM L-glutamine (Invitrogen) and 10% v/v FBS. CHO-K1 cells were replated into 96-well imaging plates (BD, Franklin Lakes, NJ, USA) so that they were ~70% confluent on the day of transfection.

Cells were transiently co-transfected with a mixture of both the regulatory pTetOn plasmid (Clontech) and one of the inducible Cx expression plasmids at a molar ratio of 1:1 using Lipofectamine LTX (Invitrogen) as per the manufacturer’s recommendations for this cell line. In brief, for each well to be transfected 100 ng of plasmid mixture was combined with 0.5µL Lipofectamine LTX in 20 µL OptiMEM I Reduced Serum Medium (Invitrogen) and incubated for 25 min before being added directly to the wells.

The following day, transfection media was removed and the cells were fed with fresh growth medium containing 2 µg/ml doxycycline (dox; Clontech). Twenty four hours after the addition of dox, cells were washed with PBS and fixed with 4% PFA. Three wells were independently transfected per expression system examined and a minimum sample of 150 transfected cells were examined per independent transfection.

**Fluorescent immunocytochemistry, confocal imaging and analysis**

Cells were washed with PBS and a blocking buffer (4% v/v NDS/NGS, 1% w/v BSA and 0.1% Triton-X 100 in PBS, pH 7.4) was applied for 30 min at RT. Rabbit anti-Cx30 (Invitrogen; Cat. 712200; 1:250) or rabbit anti-Cx43 (Sigma-Aldrich, MO, USA; Cat. C6129; 1:500) was diluted in 0.5× blocking buffer in PBS and applied to cells for incubation overnight at 4°C. After washing with PBS, Alexa594-conjugated anti-rabbit IGG (Invitrogen; Cat. A21207; 1:1000) was
applied at room temperature for 1 hr. Cells were subsequently washed before the addition of CitiFluor antifade reagent.

For semi-quantitative assessment images were acquired using a Plan-Apochromat 10×/0.45 numerical aperture (NA) dry objective (Carl Zeiss, Germany). For each immunolabeled well, a 9×9 montage of 850 μm × 850 μm images was created, giving a total imaging area of 6.5025 mm². Higher power images were acquired using a Plan-Apochromat 20×/0.8 NA dry objective (Carl Zeiss).

The RGB images were split into their constituent channels. A threshold was applied to each red channel image to create a mask selection of only the highly expressing cells. This mask selection was applied to the original red channel image and measured to derive the mean pixel intensity for cells exhibiting exogenous Cx overexpression. A threshold was then applied to the original red channel image to obtain a mask selection excluding the highly expressing cells. This mask selection was applied to the original red channel image and measured to derive the mean pixel intensity of any endogenous Cx expression present. The same procedure was performed to determine EGFP and ZsGreen fluorescent intensity, relative to background autofluorescence, using the green channel of tile scan montages.

Within the context of our study we have defined co-localization as the appearance of both the Cx gene product, detected by immunolabeling, and the fluorescent reporter protein within the same transfected cells, as this indicated co-expression at the protein level. To determine the proportion of total fluorescent cells that contained both Cx staining and green reporter fluorescence the RGB images were analyzed for co-localization. Channels were split and a threshold was applied to the red and green channels using MacBiophotonics ImageJ. The Co-localization Highlighter tool of the ImageJ suite was used to create an 8-bit grayscale image of co-localized points and a
particle count was performed using the Analyze Particles tool, with a minimum particle size of 50 pixels$^2$ corresponding to CHO-K1 cell size. The merged RGB images were converted to 8-bit grayscale images. A threshold was applied and a particle count performed under the same parameters to give the total number of fluorescent cells. The co-localized points (red and green) were taken as a percentage of the total fluorescent cells (red or green). Statistical analysis was performed by one-way ANOVA with post-hoc testing between the expression systems, taking $p < 0.05$ as the threshold for significance.

RESULTS AND DISCUSSION

In our study we have generated inducible expression systems for both Cx30 and Cx43 that independently co-express a fluorescent reporter gene. Bicistronic expression systems were constructed that transcribe a polycistronic mRNA sequence containing both a Cx gene and EGFP separated by an internal ribosome entry site (IRES). These are referred to as the bicistronic Cx30 expression system and the bicistronic Cx43 expression system. We also generated bidirectional expression systems that transcribe a Cx gene and ZsGreen as separate transcripts under the control of a bidirectional promoter. These are referred to as the bidirectional Cx30 expression system and bidirectional Cx43 expression system. A schematic representation of the two types of inducible expression system is depicted in Figure 1. We examined and compared the bicistronic and bidirectional expression systems to determine which type of system provided better inducible Cx protein expression, reporter protein expression and co-localization, defined as the presence of both proteins in the same cells.
For both bidirectional and bicistronic Cx30 and Cx43 expression systems the transfected cells overexpressing the respective Cx genes exhibited bright fluorescent Cx immunolabeling that was of significantly higher mean pixel intensity than endogenous expression (Fig. 2A). Interestingly, both the bicistronic and bidirectional expression systems resulted in near identical overexpression levels for each of the genes of interest. For the bicistronic and bidirectional Cx30 expression systems, overexpressing cells gave an immunolabeling mean pixel intensity of 65.3±2.5 arbitrary units (AU) and 63.3±2.8 AU respectively. Cells transfected with the bicistronic Cx43 expression system exhibited an immunolabeling intensity of 144.1±1.1 AU compared to 142.3±2.2 AU for the bidirectional Cx43 expression system. This equivalent Cx expression between the two expression paradigms was not necessarily assured due to the differences in the respective promoter structures. The tet operator elements of the bidirectional and bicistronic promoters are largely identical, but in the latter the reverse tetracycline transactivator need only drive expression of a single mRNA. In a bidirectional system the cellular machinery and resources underpinning transcription may be divided between the two independently overexpressed transcripts.

**Fig. 2 near here**

ZsGreen fluorescence was visibly very bright, with the bidirectional Cx30 and Cx43 expression systems giving green fluorescence intensity values of 53.0±2.0 AU and 43.3±1.6 AU respectively (Fig. 2B). This was significantly brighter (p < 0.001) than EGFP green fluorescence when co-expressed with either Cx protein in the bicistronic expression systems (Cx30 system, 22.2±1.0 AU; Cx43 system, 20.5±4.2 AU; Fig. 2B). This may be due to intrinsic differences in fluorescent intensity as ZsGreen is cited as having a relative brightness 117% that of EGFP [14]. However, the magnitude of the greater than two-fold difference in fluorescence observed would
suggest a difference in expression and translation of the reporter gene between the two expression paradigms, even when the difference in relative brightness of the two proteins was taken into consideration.

Low particle counts were observed for detectable EGFP-positive cells for the bicistronic Cx43 expression system, while the bicistronic Cx30 expression system produced consistently detectable numbers of EGFP-positive cells. This finding contrasts with previously reported bicistronic systems for Cx and EGFP expression that have indicated reasonable utility. The difference in observed fluorescence could be due to the differences in promoters driving expression. As previously mentioned, the reported bicistronic expression systems were driven by the constitutive full cytomegalovirus (CMV) promoter. Conversely, the genes in the present study were driven by the TRETight promoter containing only the minimal CMV sequence. Additionally the CMV promoter has been found to exert different expression efficiency contingent upon the cell line used [15]. Other methodological considerations may account for the difference in observed fluorescence as EGFP may be more sensitive to fixation and pH changes. However, care was taken to maintain cells in neutral media during growth and in neutral pH solutions during all immunolabeling experiments.

Beyond those methodological considerations, low levels of EGFP fluorescence within transfected cells, and low numbers of EGFP-positive cells observed using the bicistronic Cx43 expression system, may be due to the nature of internal ribosome entry site dependent (IRES-dependent) translation. The translational efficiency of the second gene in a bicistronic mRNA transcript has been shown to be generally between 20-50% that of the first gene [16]. Furthermore, IRES-dependent translation may also vary depending upon the resultant mRNA structure due to insertion of a gene of interest.
Co-localization, defined as the appearance both the Cx of interest and the fluorescent reporter within the same cells, was compared between the two types of expression system by examining merged images of Cx immunolabeling and green fluorescence from the EGFP or ZsGreen proteins (Fig. 3A). In principle, due to the nature of the two expression paradigms, it would be expected that a bicistronic system would provide a greater degree of co-localization with a reporter gene than a bidirectional system. With the former method, while the genes are expressed as independent proteins, they both derive from a single transcript. Thus adequate, and preferably equal, translation of the latter gene would consequently result in co-localization.

In practice, however, this obligate expression, and consequential robust co-localization, has not been observed in the present study. As a result of the low numbers of detectable EGFP-positive cells in cultures transfected with the bicistronic Cx43 expression system, a low degree of only 9.2±1.1% co-localization was observed (Fig. 3B) and this was significantly lower than the bidirectional Cx43 expression system that showed 77.2±2.2% co-localization (p < 0.001). The bicistronic Cx30 expression system, while producing relatively low average fluorescence intensity within transfected cells compared to the bidirectional systems, did provide effective co-localization of 77.4±2.5%, due to greater numbers of detectable EGFP-positive cells observed through particle counts. This degree of co-localization was not significantly different to the bidirectional Cx30 expression system, which showed 79.9±2.1% co-localization (p > 0.05, Fig. 3B). This observed difference in IRES-dependent reporter translation and subsequent co-localization between bicistronic Cx30 and Cx43 expression systems may be due to differences in sequence structure.
This study used full length cDNA sequences for the expression of Cxs, including untranslated regions (UTRs), rather than purely coding sequence. Intercistronic length has been shown to affect IRES-dependent bicistronic expression with greater intercistronic length adversely affecting translation [17]. Both Cx sequences contain large 3’ UTRs contributing to intercistronic length that may interfere with IRES-dependent translation. The Cx43 gene, in particular, contains a greater than 1.6 kb 3’ UTR, which is twice as long as the Cx30 3’ UTR. Furthermore, an intrinsic IRES sequence is present in the 5’ UTR of Cx43 mRNA [18]; such motifs have not been reported for Cx30 [19]. It has been shown that when multiple IRES sequences are present they may interfere with each other leading to poor translational efficiency [20]. Therefore, the intrinsic IRES site in Cx43 gene could have further contributed to a negative effect on the translation of the reporter gene in the bicistronic Cx43 expression system.

Conversely, the bidirectional system was reliant on an equivalent degree of transcription occurring in both directions upon activation of the promoter. Although it has been reported that the transcriptional regulatory sites in a bidirectional promoter serve to increase expression of both the right- and left-flanking genes [13], equivalent expression was not guaranteed as it has also been previously reported that an imbalance in expression may occur [5]. In fact, bidirectional systems resulted in high levels of co-localization for both Cx genes, equivalent to the bicistronic Cx30 expression system, despite the greater degree of independence between the reporter and the gene of interest.

Consequently, in the design of expression systems where a co-expressed reporter is required consideration should be given to the nature of the genes of interest; particularly, whether elements in the mRNA structure will interfere with the system chosen. We found that, somewhat counter-intuitively, independent bidirectional transcription and translation of the gene of interest
and reporter gene gave better co-localization than co-expression of a reporter from a single bicistronic transcript, and thus, may provide a better in vitro or in vivo indicator of gene expression. This may well be gene dependent and future investigation is needed to determine the mechanisms underlying the effect of Cx43 on bicistronic reporter expression. Nonetheless the selection of reporter paradigms in plasmid design should be made carefully, and for expression of Cx43 we found a bidirectionally structured plasmid provided the better solution.

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Figure 1: Schematic representation of the bidirectional and bicistronic expression system constructs. When doxycycline is added, the reverse tetracycline transactivator (rtTA) binds to the tetracycline inducible (Tet-On) promoter and initiates transcription. In the bicistronic systems a single transcript containing the Cx of interest, internal ribosome entry site (IRES) and EGFP is expressed and translation occurs from this polycistronic transcript. Conversely, in the bidirectional system the Cx of interest and ZsGreen fluorescent reporter are transcribed independently and subsequently translated. The plasmids contained ColE1 origin of replication (Ori) and ampicillin (Amp) resistance gene sequences for cloning.

Figure 2: Comparison between bicistronic and bidirectional expression of (A) the two Cx proteins and (B) the reporter gene. (A1) Representative images showing Cx immunolabeling for the four expression systems. (A2) Cx immunolabeling intensity resulting from induced overexpression by the four different Cx expression systems. Average fluorescent intensity levels of immunolabeling were compared and were not significantly different between the two Cx30 expression systems or between the two Cx43 expression systems ($p > 0.05$, post-hoc testing). (B1) Representative images showing green fluorescence intensity levels in cells transfected with the four expression systems. (B2) Induced green fluorescence intensity, in detectable fluorescent cells, resulting from the different expression systems was compared. Bidirectional systems expressing ZsGreen showed significantly higher average green fluorescence intensity than bicistronic systems expressing EGFP ($p < 0.01$ for all post-hoc tests between the types of system). For all groups $n = 3$, independently transfected cultures; immunolabeling and green fluorescence intensity are expressed as mean pixel intensity in arbitrary units (AU). Scale bar = 10 µm.
**Figure 3**: Comparison of co-localization between the gene of interest and the reporter gene for bidirectional and bicistronic Cx expression systems. (A) Representative images showing the degree of co-localization between Cx immunolabeling and green fluorescence for the four systems. (B) Comparison of co-localization between Cx immunolabeling and green fluorescence for the different Cx expression systems. No significant differences in co-localization were found between the bidirectional Cx30 and bidirectional Cx43 expression systems, or between either bidirectional system and the bicistronic Cx30 expression system ($p > 0.05$). However, the bicistronic Cx43 expression system showed significantly lower co-localization than the other three systems ($p < 0.001$ for all post-hoc tests). For all groups $n = 3$, independently transfected cultures; co-localization is expressed as a percentage. Scale bar = 10 µm.