



The p1D4-hrGFP II expression vector: A tool for expressing and purifying visual pigments and other G protein-coupled receptors

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ABSTRACT

The heterologous expression of membrane proteins such as G protein-coupled receptors can be a notoriously difficult task. We have engineered an expression vector, p1D4-hrGFP II, in order to efficiently express visual pigments in mammalian cell culture. This expression vector is based on pIRES-hrGFP II (Stratagene), with the addition of a C-terminal 1D4 epitope tag for immunoblotting and immunoaffinity purification. This vector employs the CMV promoter and hrGFP II, a co-translated reporter gene. We measured the effectiveness of pIRES-hrGFP II in expressing bovine rhodopsin, and showed a 3.9- to 5.7-fold increase in expression as measured by absorbance spectroscopy as compared with the pMT vector, a common choice for visual pigment expression. We then expressed zebrafish RH2-1 using p1D4-hrGFP II in order to assess its utility in expressing cone opsins, known to be less stable and more difficult to express than bovine rhodopsin. We show a $\lambda_{280}/\lambda_{MAX}$ value of 3.3, one third of that reported in previous studies, suggesting increased expression levels and decreased levels of misfolded, non-functional visual pigment. Finally, we monitored HEK293T cell growth following transfection with pIRES-hrGFP II using fluorescence microscopy to illustrate the benefits of having a co-translated reporter during heterologous expression studies.

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1. Introduction

G protein-coupled receptors (GPCRs) are integral membrane proteins known to be composed of seven alpha helical transmembrane domains which activate in response to a wide variety of extracellular stimuli, and couple to heterotrimeric GTP-binding proteins (Bockaert and Pin, 1999). These receptors are of significant pharmacologically relevance, being targets for many drugs of economic importance (Tyndall and Sandilya, 2005). Visual pigments, which initiate the critical first step in the biochemical cascade of vision (Dell'Orco et al., 2009), are among the best-

studied members of the GPCR superfamily due to the fact that they can be isolated from retinal tissue, or expressed heterologously *in vitro*. These methods, along with site-directed mutagenesis approaches, can be used to investigate visual pigment structure and function (Kono et al., 2005; Nickle et al., 2006; Takenaka and Yokoyama, 2007), which in turn can provide insight into diseases of the visual system that arise from impaired visual pigment function, such as *retinitis pigmentosa* (Senin et al., 2006; McKibbin et al., 2007).

An issue that arises when attempting to express membrane proteins using heterologous systems is how to generate sufficient amounts of protein with which to perform functional analyses. GPCRs are notoriously difficult to express due to the complex folding, trafficking and modifying mechanisms associated with the translation of membrane proteins. This leads to significant amounts of

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protein that is misfolded, stranded in the ER, moved to unsuitable transport vesicles, or missing post-translational modifications required for proper function (Sarramegna et al., 2003). Bovine rhodopsin is still the only vertebrate visual pigment with a high-resolution crystal structure (Palczewski et al., 2000; Park et al., 2008; Scheerer et al., 2008) because large amounts of the native protein can be obtained from cow retina; other GPCRs with resolved crystal structures include squid rhodopsin (Murakami and Kouyama, 2008) as well as the β_1 - and β_2 -adrenergic receptors (Warne et al., 2008; Rasmussen et al., 2007). Since the majority of visual pigments and GPCRs in general cannot be extracted at such high levels in their native state, finding competent expression systems is a top priority for studies requiring functionally active proteins.

Over the past decade, experiments probing the structure and function of visual pigments have resorted to heterologous expression systems in order to generate visual pigment samples (Ablonczy et al., 2006; Yokoyama et al., 2008). Most expression systems involve an expression vector being transiently transfected into one of a number of different mammalian cell types, including COS-1 (Matsumoto et al., 2006), HEK293 (Kuwayama et al., 2005), and HEK293T (Parry et al., 2004), using lipid-based transfection reagents. While this method is usually successful, even large-scale transfections are sometimes unable to produce serviceable visual pigment samples (Pointer et al., 2007; Davies et al., 2007).

Heterologous expression systems are not just dependent on cell type, but also on the expression vector used to transfect the cells. Many early expression studies of visual pigments employed the pMT expression vector, featuring the adenovirus major late (AML) promoter. This choice was likely made because various versions of pMT had successfully expressed large proteins with post-translational modifications (Bonthron et al., 1986; Kaufman et al., 1989), suggesting it could handle the expression of complex membrane proteins. As pMT use became more frequent for visual pigment studies, newer versions were engineered containing the 1D4 epitope, which consists of nine C-terminal residues of bovine rhodopsin (TETSQVAP-A) recognized by the 1D4 monoclonal antibody (Molday and MacKenzie, 1983), to streamline immunoaffinity purification procedures (Franke et al., 1988; Tsutsui et al., 2007). Other promoters have since been used for visual pigment expression, such as the combination of the SV40 early promoter with the R segment and part of the U5 sequence of the long terminal repeat of human T-cell leukemia virus type 1 (Kayada et al., 1995; Kojima et al., 2008), and the cytomegalovirus (CMV) promoter (Reeves et al., 2002). To date, no systematic comparison of promoters driving expression of visual pigments has been performed.

Other heterologous systems that rely on non-mammalian cells, including *Escherichia coli*, yeast, and insect cells, have been used regularly to express other GPCRs (Sarramegna et al., 2006), but only sparingly to express visual pigments (Mollaaghababa et al., 1996; Klaassen et al., 1999). These systems often strive for large-scale production of GPCRs at the expense of protein function, which can suffer due to differences in membrane composition and post-translational mechanisms of the host cells (Stanasila

et al., 1998; Opekarova and Tanner, 2003). Ultimately, mammalian cells provide the most authentic cell environment for vertebrate GPCRs and are often the system of choice when attempting functional characterizations of a protein of interest, even though the yields produced with mammalian cells tend to be lower than those of other systems (Junge et al., 2008). This fact emphasizes the importance of continuing to improve the efficiency of mammalian cell-based heterologous expression systems.

In this study, we use the pIRES-hrGFP II expression vector (Stratagene) to express bovine rhodopsin at higher levels than the pMT expression vector, while utilizing the co-translated hrGFP II reporter gene to evaluate transfection efficiency and protein expression levels. We also engineer p1D4-hrGFP II, containing the pIRES-hrGFP II backbone with the 1D4 epitope added as a C-terminal tag, allowing for *in vitro* expression of any visual pigment or GPCR of interest. We then express zebrafish RH2-1 using p1D4-hrGFP II to demonstrate its ability to efficiently express cone opsins, notably less stable than rhodopsins (Ramon et al., 2009). This expression vector provides a more efficient tool for researchers that employ a mammalian cell culture for expression of visual pigments and, presumably, other GPCRs.

2. Materials and methods

2.1. Vector construction

The pIRES-hrGFP II expression vector (Stratagene) has a cytomegalovirus (CMV) promoter and the humanized recombinant GFP II (hrGFP II) as a reporter gene, co-translated alongside the inserted gene due to an internal ribosome entry site (IRES). This co-translated reporter does not fuse with the protein of interest, insuring no functional deviation as a result of reporter expression. hrGFP II is a version of GFP modified to contain codons preferred in highly expressed human genes, and has been subjected to random mutagenesis to create a brighter variant. IRES allows for transcription initiation in the middle of the primary mRNA transcript, bypassing the usual 5' cap recognition requirement (Jang et al., 1990).

We first compared expression levels of pIRES-hrGFP II to pMT using bovine rhodopsin, the most highly studied and expressed visual pigment. An EcoRI/NotI double digestion of pIRES-hrGFP II and pMT4 (Franke et al., 1988) was performed to excise the bovine rhodopsin gene from pMT and ligate it into pIRES-hrGFP. We also modified pIRES-hrGFP II to easily accept any visual pigment or GPCR of interest and express it in frame with a C-terminal 1D4 epitope tag, as many GPCR immunoaffinity purification and immunohistochemistry protocols tend to rely on the 1D4 monoclonal antibody (Tsutsui et al., 2007; Oprian et al., 1987). This was not necessary for our pIRES-hrGFP II vs. pMT comparison because the 1D4 epitope is in fact the C-terminus of bovine rhodopsin. Primers were designed to amplify a 400 bp fragment of bovine rhodopsin in pIRES-hrGFP II, containing the last 9 amino acids and stop codon of bovine rhodopsin (1D4 epitope); an EcoRI site followed by a guanine nucleotide was also added to the 5' end

of the forward primer (Table 1). This fragment was cloned into the pJET1 cloning vector from the GeneJET cloning kit (Fermentas). An EcoRI/NotI double digestion of the resulting pJET1-1D4 cloning vector produced a 45 bp fragment (EcoRI-G-1D4-Stop Codon-NotI). This fragment was cloned into the multiple cloning site of pIRES-hrGFP II to create p1D4-hrGFP II (Fig. 1).

The zebrafish cone opsin RH2-1 was selected as a test for p1D4-hrGFP II because cone opsins have been found to be less stable than rhodopsins (Ramon et al., 2009), and because it had been previously expressed using pMT (Chinen et al., 2003). RNA was extracted from zebrafish eyes using the RNeasy Mini Kit (Qiagen) and cDNA libraries were generated using the SMART cDNA Library Construction Kit (BD Biosciences). A forward primer was designed to amplify the coding sequence of zebrafish RH2-1 along with an established reverse primer (Table 1). The RH2-1 gene was cloned into the pJET1 cloning vector where it was re-amplified using primers that added BamHI and EcoRI restriction sites to its 5' and 3' ends, respectively, but leaving out the third nucleotide of the last codon and the entire stop codon (Table 1). This fragment was cloned into the pJET1 cloning vector, digested via BamHI/EcoRI double digestion, and ligated into p1D4-hrGFP II. The omission of the third nucleotide of the last codon of RH2-1 and the addition of a guanine base 5' of the 1D4 epitope results in the reading frame of the EcoRI restriction site to code for Asn-Ser (AAT-CCG) instead of Glu-Phe (GAA-TTC), as the former pair of residues is less likely to affect the function of an expressed GPCR. Maxipreps were generated with the PureLink HiPure Plasmid Maxiprep Kit (Invitrogen), and multiple cloning sites of all expression vectors were confirmed via sequencing.

2.2. Visual pigment expression, purification & detection

Expression vectors (12 µg per 10-cm plate) were transiently transfected into cultured HEK293T cells using Lipofectamine 2000 (Invitrogen). Protein expression was monitored via the hrGFP II reporter, co-translated with visual pigments, using a Leica MZ16F Fluorescence Stereomicroscope. Cells were harvested 48 h after transfection, washed three times with harvesting buffer (PBS, 10 µg/mL aprotinin, 10 µg/mL leupeptin) and incubated in 5 µM 11-cis-retinal to regenerate visual pigments. Samples were then solubilized in solubilization buffer (100 mM NaCl, 1 mM CaCl₂, 50 mM Tris pH 6.8, 1% dodecylmaltoside) and immunoaffinity purified with the 1D4 monoclonal antibody essentially as previously described (Han et al.,

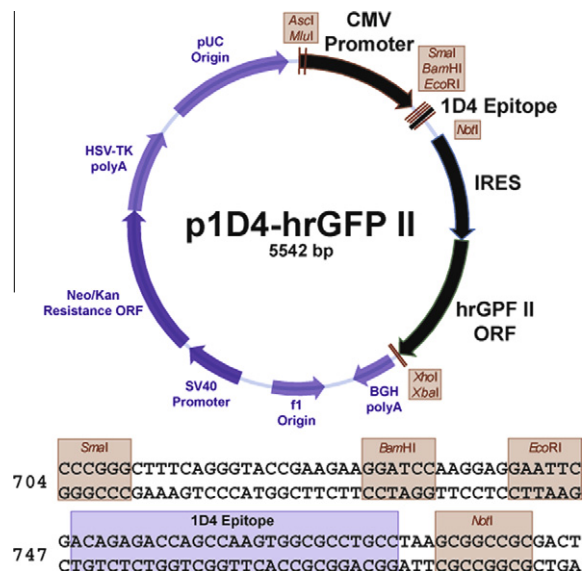


Fig. 1. Physical map of the p1D4-hrGFP II expression vector, derived from pIRES-hrGFP II. Important features include the CMV promoter, C-terminal 1D4 epitope tag (TETSQVAPA), internal ribosome entry site (IRES) for transcription initiation in the middle of the primary mRNA transcript, and hrGFP II reporter gene. Proteins of interest can be ligated into the multiple cloning site between unique BamHI and EcoRI restriction sites.

1996; Chang et al., 2002). Western blots employed the mouse 1D4 monoclonal antibody (University of British Columbia) as a primary antibody and a sheep anti-mouse antibody linked to horseradish peroxidase (Amersham Biosciences) as a secondary antibody; the ECL plus Western blotting detection system (Amersham Biosciences) was used for detection. The UV-visible absorption spectra of solubilized cell lysates or purified visual pigments were recorded at 25 °C using a Cary4000 double-beam spectrophotometer (Varian). Difference spectra of solubilized cell lysates were calculated by subtracting light-bleached absorbance spectra from respective dark spectra; this differential absorbance is then used as an estimate of functional visual pigment concentration.

3. Results and discussion

In this study, we show that the pIRES-hrGFP II expression vector can express visual pigments at high levels compared to pMT in mammalian cell culture as well as utilize the hrGFP II reporter gene to monitor expression levels of a

Table 1

List of primer sequences used in this study.

Primer	Sequence (5'–3')	Purpose
1D4F	GAATTCGACAGACCAGCCAAGTGCG	Amplify 400 bp of bovine rhodopsin, with 1D4 epitope
1D4R	CGCTACAGACGTTGTTGTCTTCAAG	
RH2-1F	ATGAACGGGACAGAAGGGAGCAACTTCTAC	Amplify zebrafish RH2-1 from eye cDNA
RH2-1R ^a	GGAGCACTGAATAGGCAGAT	
BamRH2-1F	CGCCACCCGGATCCATGAACGGGACAGAAGGGAGC	Add restriction sites to zebrafish RH2-1
EcoRH2-1R	CCGGAATTCGACGAGACACAGAGGACACCTC	

^a RH2-1R was previously used to amplify zebrafish RH2-1 (Chinen et al., 2003).

protein of interest. We also engineer the p1D4-hrGFP II expression vector, containing the pIRES-hrGFP II backbone with the 1D4 epitope added as a C-terminal fusion tag, allowing for efficient heterologous expression of any visual pigment or GPCR of interest. This was demonstrated by the efficient expression of zebrafish RH2-1, a cone opsin that is less stable than bovine rhodopsin.

3.1. Expression of bovine rhodopsin by pIRES-hrGFP II

Bovine rhodopsin was expressed in HEK293T cells using either the pMT or pIRES-hrGFP II expression vector, in order to assess the ability of pIRES-hrGFP II to express visual pigments *in vitro*. Each expression vector was used to transfect four 10-cm diameter culture plates. Dark and light-bleached spectra were taken for each sample of cell lysate following solubilization and data was averaged for

each expression vector. Difference spectra were recorded for each sample, with the pIRES-hrGFP II sample showing on average approximately 3.9 times more absorbance than that from pMT (Fig. 2A). Cell lysates from each expression vector were then immunoaffinity purified using 1D4 monoclonal antibody-coupled resin. Dark spectra were recorded for both expression vectors, with pIRES-hrGFP II showing approximately 5.7 times more absorbance than pMT (Fig. 2B). These results suggest the pIRES-hrGFP II expression vector is responsible for a significant increase in functional visual pigment synthesis (3.9- to 5.7-fold) in HEK293T cells compared to pMT. The yield for the entire pIRES-hrGFP II expression system was approximately 6.4 μg of purified bovine rhodopsin per 10-cm plate of HEK293T cells, compared to 1.2 μg per plate using pMT (Table 2). This is also an improvement from a previous system using pMT to express bovine rhodopsin in COS-1 cells

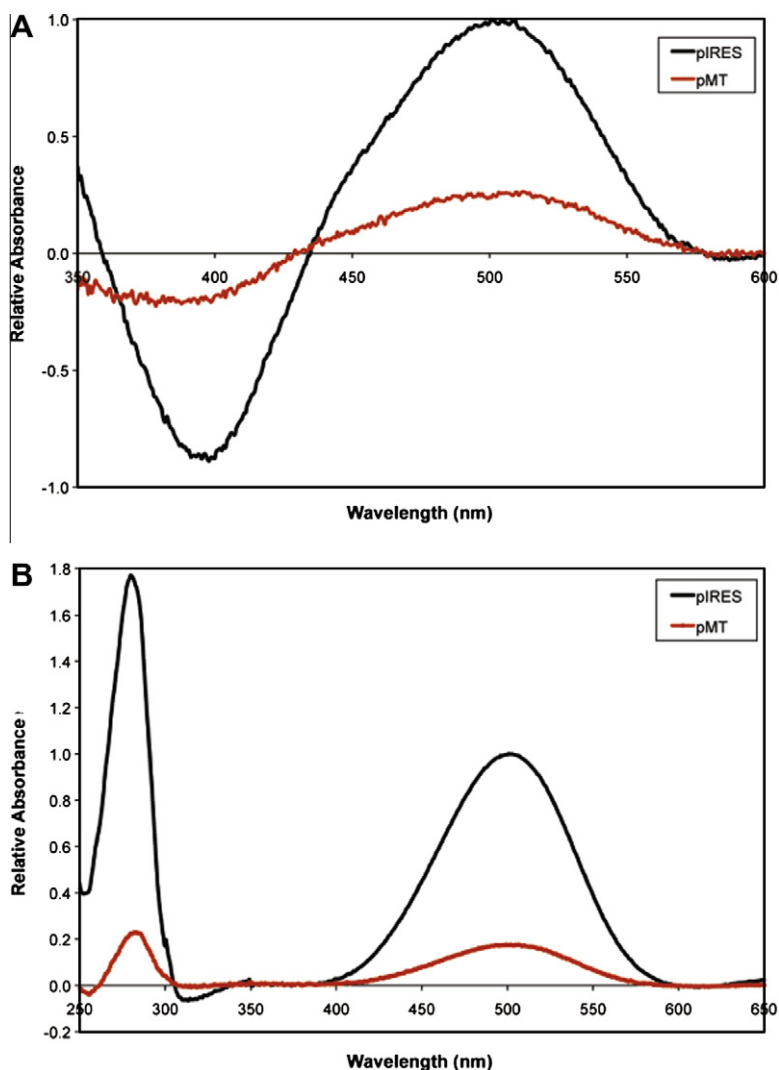


Fig. 2. Absorbance spectrophotometry comparing the expression of bovine rhodopsin in HEK293T cells using either the pIRES-hrGFP II or pMT expression vector. (A) A 3.9-fold increase in absorbance is seen from difference spectra of solubilized lysate samples derived from cells expressing pIRES-hrGFP II compared to pMT. (B) A 5.7-fold increase in absorbance is seen from dark spectra of purified protein samples derived from cells expressing pIRES-hrGFP II compared to pMT.

Table 2

A comparison of features between pIRES-hrGFP II and pMT.

Vector	Promoter	Antibiotic resistance	Yield
pIRES-hrGFP II	Cytomegalovirus (CMV)	Kanamycin	6.4 µg/plate
pMT	Adenovirus major late (AML)	Ampicilin	1.2 µg/plate

(Oprian et al., 1987). A Western blot was performed on solubilized cell lysate samples from both pIRES-hrGFP II and pMT to verify the difference in visual pigment concentration. A notable increase in band intensity was seen for samples generated from pIRES-hrGFP II compared to pMT (results not shown).

3.2. Expression of zebrafish RH2-1 by p1D4-hrGFP II

Bovine rhodopsin was immunoaffinity purified using its endogenous C-terminal 1D4 epitope. In order to efficiently tag other proteins of interest, the nine amino acid 1D4 epitope sequence (TETSQVAPA) was inserted at the unique EcoRI restriction site of the pIRES-hrGFP II expression vector, creating the p1D4-hrGFP II expression vector. To test the potency of this new expression vector, the coding sequence of zebrafish RH2-1 was amplified from zebrafish eye cDNA and ligated into its multiple cloning site. This expression vector was used to transfect twenty-four 10-cm diameter culture plates of HEK293T cells, which were harvested, solubilized and purified as described above. The p1D4-hrGFP II expression vector was able to generate a sample of zebrafish RH2-1 with a maximum absorbance of 0.041 and a ratio of protein absorbance to visual pigment absorbance (A_{280}/A_{MAX}) of 3.3 (Fig. 3).

The absorbance ratio (A_{280}/A_{MAX}) is often used as a measure of purity and an indicator of functional protein con-

tained in a visual pigment sample (Karnik et al., 1988; Bhattacharya et al., 1992), with A_{280} indicating total protein content and A_{MAX} , the absorbance at λ_{MAX} , indicating the amount of functional visual pigment contained in the sample. Previous experiments expressing cone opsins with pMT have produced absorbance ratios ranging from 2.9 to 26, with the range of fish RH2 opsins being 4.8–11.6 (Matsumoto et al., 2006; Chinen et al., 2003; Kawamura and Yokoyama, 1998). These values suggest that the absorbance ratio of 3.3 from zebrafish RH2-1 expressed using p1D4-hrGFP II is among the best ratios found for cone opsins, and is particularly impressive among fish RH2 opsins. The low absorbance ratio in our study indicates a high overall yield and a low proportion of misfolded, non-functional visual pigment. The increased efficiency in expressing an RH2 opsin, along with the recent expression of a novel zebrafish opsin (Morrow et al., in preparation), suggests that p1D4-hrGFP II is an ideal candidate for expressing opsins and GPCRs, even those that are less stable than bovine rhodopsin.

3.3. Comparison of pIRES-hrGFP II and pMT

The difference between the pIRES-hrGFP II/p1D4-hrGFP II and pMT most likely to cause the difference in their overall expression efficiency is the promoter sequence, with the former pair being equipped with the cytomegalovirus (CMV) promoter and the latter containing the adenovirus major late (AML) promoter. A previous study that compared promoters driving specific antibody response induction through replication-defective adenovirus-based inoculation showed a significantly greater response using CMV compared to AML (Ambriovic et al., 1997); another noted that gene expression in synoviocytes was 6–10 times higher when driven by the CMV promoter compared to the AML promoter (Goossens et al., 2000). Considering that

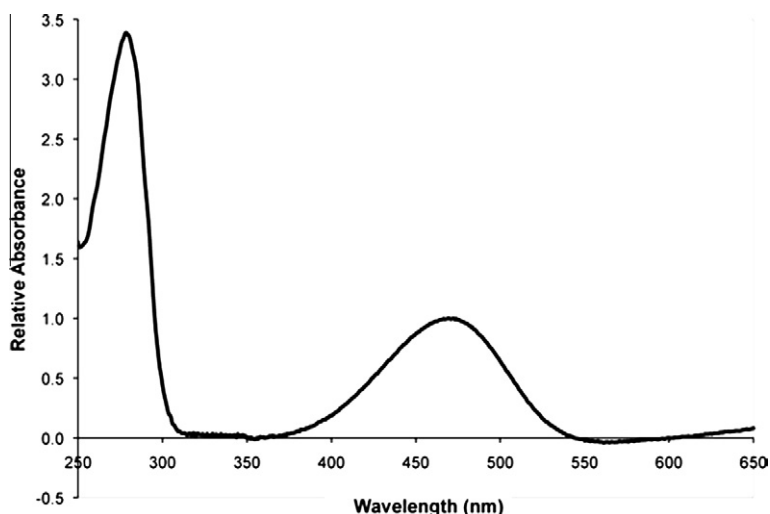


Fig. 3. Dark spectrum of zebrafish RH2-1 cone opsin expressed in HEK293T cells using the p1D4-hrGFP II expression vector. Maximal absorbance was approximately 0.041 with a λ_{MAX} value of 469 nm. A comparison between all protein (λ_{280}) and visual pigment (λ_{MAX}) expression was made, with a value ($\lambda_{280}/\lambda_{MAX}$) of 3.3. This is a significant decrease from previous studies that have expressed zebrafish RH2-1 (Chinen et al., 2003), suggesting an increase in expression and purification efficiency.

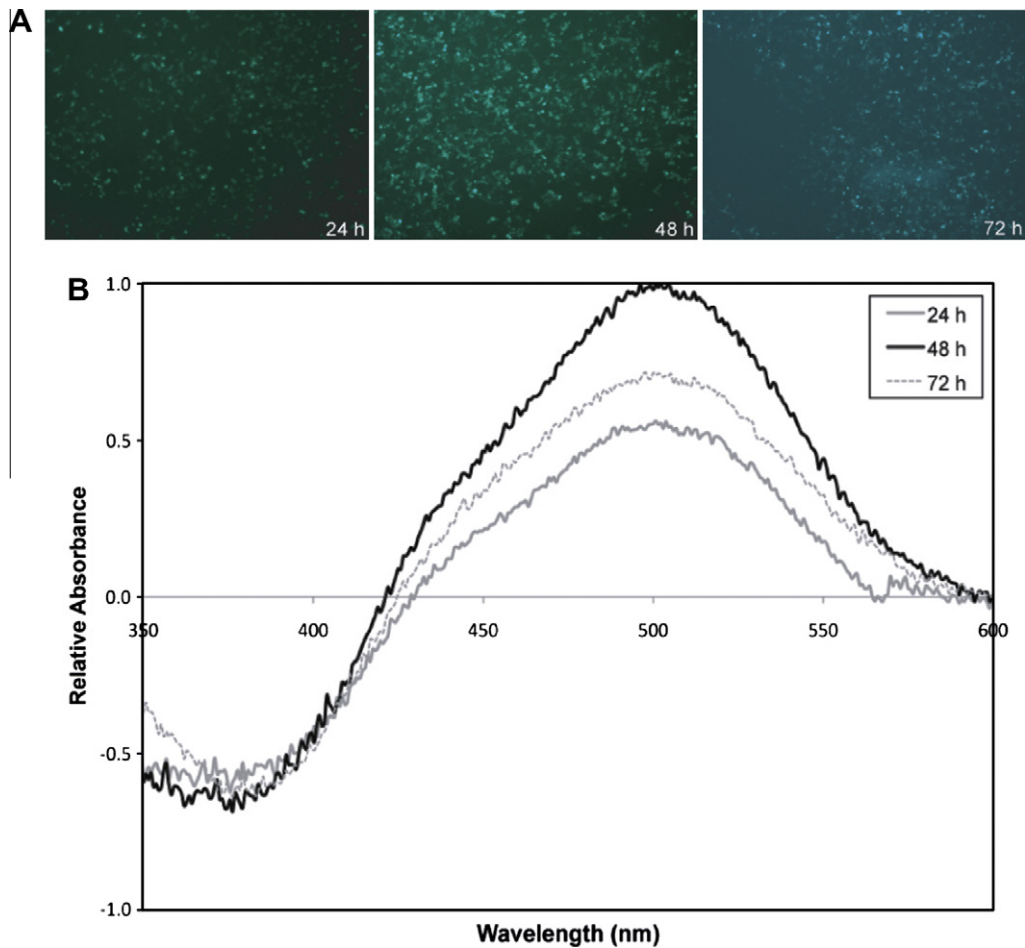


Fig. 4. Fluorescence microscopy of HEK293T cells expressing bovine rhodopsin and the hrGFP II reporter after transfection with the pIRES-hrGFP II expression vector. (A) Reporter activity was measured at 24, 48, and 72 h post transfection with the most widespread fluorescence detected at 48 hpt. (B) Resulting difference spectra from solubilized cell lysates show that increased fluorescence leads to increased absorbance.

many recent promoter comparisons no longer include the AML promoter in their studies (Arita et al., 2008; Chen et al., 2008), it would seem that switching to the CMV promoter is a logical step to increase expression levels in heterologous expression studies.

3.4. The hrGFP II gene as a co-translated reporter

An additional feature of the p1D4-hrGFP II expression vector is the hrGFP II reporter gene that is co-translated with a visual pigment due to the presence of an internal ribosome entry site (IRES), allowing transcription initiation in the middle of the primary mRNA transcript, bypassing the usual 5' cap recognition requirement. hrGFP II has been modified from the original GFP of *Renilla reniformis* (Ward and Cormier, 1979) to contain codons preferred in highly expressed human genes. Additionally, hrGFP II is a GFP variant that was subjected to random mutagenesis to generate greater fluorescence intensity. In this study, the hrGFP II reporter was used to determine the most appropriate time to harvest after transfection. Fluorescent microscopy

showed the most fluorescence around 48 h post transfection, with less fluorescence captured at both 24 and 72 h post transfection in both magnitude and surface area (Fig. 4).

There are several other potential benefits to having a reporter gene such as hrGFP II co-translated alongside a gene of interest. For instance, the reporter could be used to screen for highly expressing colonies from which to start stable cell lines expressing a GPCR of interest. It could also be used to sort positive transfection events using fluorescent-activated cell sorting (FACS), a technique that has previously been employed to sort fluorescing HEK293 and HEK293T cells (Wu et al., 2007; Cheng and Solomon, 2008). While these types of benefits provide new opportunities for researchers involved in the heterologous expression of visual pigments or other GPCRs, it is also possible that hrGFP II translation could be limiting the production of the protein of interest. Therefore, if desired, this reporter gene can be easily removed from both the pIRES-hrGFP II and p1D4-hrGFP II constructs through a NotI/XhoI double digestion.

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