

Short Technical Reports

Dual-Function Vector for Protein Expression in Both Mammalian Cells and *Xenopus laevis* Oocytes

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ABSTRACT

Both *Xenopus laevis* oocytes and mammalian cells are widely used for heterologous expression of several classes of proteins, and membrane proteins especially, such as ion channels or receptors, have been extensively investigated in both cell types. A full characterization of a specific protein will often engage both oocytes and mammalian cells. Efficient expression of a protein in both systems have thus far only been possible by subcloning the cDNA into two different vectors because several different molecular requirements should be fulfilled to obtain a high protein level in both mammalian cells and oocytes. To address this problem, we have constructed a plasmid vector, pXOOM, that can function as a template for expression in both oocytes and mammalian cells. By including all the necessary RNA stability elements for oocyte expression in a standard mammalian expression vector, we have obtained a dual-function vector capable of supporting protein production in both *Xenopus* oocytes and CHO-K1 cells at an expression level equivalent to the levels obtained with vectors optimized for either oocyte or mammalian expression. Our functional studies have been performed with hERG1, KCNQ4, and Kv1.3 potassium channels.

INTRODUCTION

Functional analyses of a number of proteins are performed in both *Xenopus laevis* oocytes and mammalian cells. Oocytes and mammalian cells have different advantages and disadvantages as expression systems and can therefore complement each other when investigating a specific protein.

Protein expression in oocytes can be obtained by injection of cRNA generated by in vitro transcription (5,8,10). The 5' and 3' untranslated mRNA re-

gions from *Xenopus* β -globin have been reported to enhance both the stability and translatability of a number of different mRNAs and can result in a very significant increase in the level of expression (3,9). Wallner et al. (13) reported that the *Shaker* potassium channel generated a 4–50 times higher current level in oocytes when the cDNA was flanked by the untranslated β -globin elements from *Xenopus*. The level of expression was increased even further for this *Shaker* channel by altering the sequence just upstream the initiation codon towards a Kozak consensus sequence (6,11). Furthermore, the addition of a 5' cap structure appears to be essential for RNA stability and efficient translation in oocytes (3).

Protein expression in mammalian cells can be obtained by transfecting the cells with DNA (e.g., plasmids) that contains the specific cDNA flanked by a ubiquitous promoter/enhancer and a polyadenylation consensus sequence. When expressing heterologous genes in mammalian cells, it is often crucial to be able to select cells expressing the introduced gene product. Such a selection can be done by including a marker gene, such as an antibiotic selectable gene or a fluorescent marker, in the transfected plasmid.

As the requirements for obtaining expression of heterologous proteins in oocytes and mammalian cells are rather different, the standard procedure for performing functional analysis in these two systems has been to subclone the gene of interest into two different plasmids. Here, we demonstrate a dual-function plasmid-vector, pXOOM (*Xenopus* oocyte or mammalian), that can be used for protein expression in both mammalian cells and *Xenopus* oocytes. This vector not only supports an expression level equivalent to corresponding optimized single-function vectors but also provides expression of a fluorescent and a selectable marker in mammalian cells, making it possible to detect both transiently and stably transfected cells.

MATERIALS AND METHODS

Molecular Biology

Xenopus β -globin sequences [5' un-

translated region (UTR) and 3'UTR] were PCR-amplified from SP64T [kindly provided by P.A. Krieg (7)] and inserted into the mammalian expression vector pNS2n, a custom-designed derivative of pcDNA3neo (Invitrogen, Carlsbad, CA, USA). A polylinker containing *Bam*HI, *Eco*RI, *Hind*III, and *Not*I restriction recognition sites was inserted between the 5' and the 3' UTRs in this plasmid construct and thereby gave rise to pXOOM. hERG1 was excised with *Bam*HI and *Eco*RI from pcDNA3 and inserted in pXOOM, resulting in pXOOM-hERG1. By a similar approach, KCNQ4 and Kv1.3 were subcloned into pXOOM. The integrity of the cloned sequences was confirmed by sequencing.

cRNA for oocyte injection were obtained by linearizing (by *Xba*I) pXOOM-hERG1, pXOOM-KCNQ4, and pXOOM-Kv1.3 and performing in vitro transcription and capping (mCAP mRNA capping kit; Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. The integrity of the transcripts was confirmed by agarose gel electrophoresis, and cRNA was stored at -80°C until injection.

Expression in CHO-K1 Cells or *Xenopus* Oocytes

CHO-K1 cells (ATCC, Manassas, VA, USA) were grown in DMEM supplemented with 10% FCS and 40 mg/L L-proline (all from Invitrogen) at 37°C in 5% CO₂. One day before transfection, 2×10^6 cells were plated in a cell culture T75 flask (Nalge Nunc International, Roskilde, Denmark). Cells were transfected with 1 μ g plasmid, using LIPOFECTAMINE PLUS™ reagent (Invitrogen) according to the manufacturer's instructions. The transfection efficiency of pXOOM constructs (approximately 40%, detected by fluorescence) is similar to the efficiencies obtained with single-function vectors. Patch-clamp analyses for all experiments were performed 48–72 h after transfection.

Stage V or VI oocytes were obtained from mature *X. laevis* frogs and defolliculated enzymatically as described previously (4) before injection with 50 nL cRNA (approximately 10 ng). All injections were performed using a

Nanoject microinjector (Drummond). Oocytes were kept in Kulori medium (90 mM NaCl, 1 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 5 mM HEPES, pH 7.4) at 19°C for 2–7 days before electrophysiological measurements were performed. The Kulori medium was changed once a day.

Electrophysiology of CHO-K1 Cells

Experiments were performed 48–72 h after transfection in whole-cell patch-clamp configuration at room temperature with an EPC-9 amplifier (HEKA Electronics, Lambrecht, Germany). Pipets were pulled from thin-walled borosilicate glass (Modulohm, Copenhagen, Denmark) and had a resistance between 1.5 and 2.5 MΩ. A custom-made perfusion chamber (volume 15 μL) with a fixed AgCl-Ag pellet electrode was mounted on the stage of an inverted microscope. Coverslips with transiently transfected CHO-K1 cells were transferred to the perfusion chamber and superfused with a physiological solution consisting of 140 mM NaCl, 4 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, and 10 mM HEPES (pH 7.4 with NaOH). Pipets were filled with solutions consisting of 144 mM KCl, 10 mM EGTA, and 10 mM HEPES (pH 7.2 with KOH). CaCl₂ and MgCl₂ were added in concentrations calculated (Eq-Cal; BioSoft, Cambridge, UK) to give a free Mg²⁺ concentration of 1 mM and free Ca²⁺ concentrations of 100 nM. No zero current or leak current subtraction was performed during the experiments. Cell capacitance and series resistance were updated before each pulse application. Series resistance values were

between 4 and 10 MΩ, and only experiments where the resistance remained constant during the experiments were analyzed. Current signals were low-pass filtered at 3 kHz and acquired using Pulse software (HEKA Electronics, Mahone Bay, NS, Canada).

Electrophysiology of *Xenopus* Oocytes

Current through expressed hERG1 channels was recorded using a two-electrode voltage-clamp amplifier (Dagan CA-1B, Minneapolis, MN, USA). Electrodes were pulled from borosilicate glass capillaries on a horizontal patch electrode puller and had tip resistance between 0.3 and 2.0 MΩ when filled with 1 M KCl. During the experiments, oocytes were placed in a small chamber (volume 200 μL) connected to a continuous flow system (flow 6 mL/min). The hERG1, KCNQ4, and Kv 1.3 channels were activated by membrane depolarization, and channel activity was measured in Kulori solution consisting of 90 mM NaCl, 1 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 5 mM HEPES, pH 7.4. All experiments were performed at room temperature 72 h after cRNA injection.

RESULTS AND DISCUSSION

Figure 1 displays an illustration of the dual-function vector, pXOOM, that contains all the necessary elements for obtaining expression in both oocytes and mammalian cells. Protein expression in *Xenopus* oocytes can be obtained by using the pXOOM construct

in an in vitro transcription and capping reaction, followed by injection of cRNA into oocytes. RNA synthesis of sense strand RNA is initiated from the T7 bacteriophage promoter and proceeds until run-off at the linearization point. Linearization of the plasmid, which is performed at an appropriate restriction enzyme site located just downstream of the 3' β-globin gene, is done to prevent the T7 RNA polymerase from transcribing the complete plasmid. Antisense RNA can be obtained using the SP6 bacteriophage promoter located in the 3' end of the *cistron*. In mammalian cells, transcription of the gene inserted in the multiple cloning site is initiated by the cytomegalovirus enhancer/promoter and polyadenylated after the polyA consensus sequence located in the 3' end of the 3'UTR. Apart from the elements described above, the pXOOM plasmid also contains a transcription unit where the simian virus 40 enhancer/promoter directs translation of a neomycin resistance and an enhanced GFP fusion gene (*neo-EGFP*). This fusion gene makes it possible both to select for stably transfected cells and to visualize transiently transfected cells by fluorescence microscopy (excitation approximately 500 nm).

For proof of concept, we subcloned hERG1 into pXOOM and performed electrophysiological analyses (Figure 2). hERG1 is a potassium channel predominantly expressed in cardiac tissue (2). hERG1 and the β-subunit KCNE2 (or Mirp1) are the molecular units, which comprise the rapid delayed rectifier current (I_{Kr}) of the heart (1,12). I_{Kr} has a very important role in repolarization of the heart, and mutations in the *herg* gene are associated with the Long QT syndrome type 2 (2). The hERG1 channel exhibits extreme channel block at positive potentials due to C-type inactivation, resulting in a characteristic bell-shaped I-V curve with a maximal amplitude around 0 mV (Figure 2). Oocytes injected with hERG1 cRNA were clamped at -80 mV and subsequently activated by a step protocol as potentials from -80 mV to +50 mV with 10-mV increments. Tail current was recorded at -60 mV. The result of this step/voltage protocol was an activation and subsequent inactivation of hERG1 channels with the expected

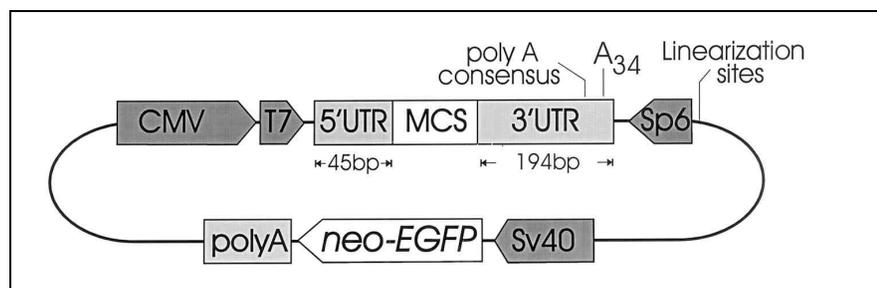


Figure 1. Illustration of the dual-function plasmid vector pXOOM. CMV, cytomegalovirus immediately early enhancer/promoter; T7, T7 RNA polymerase promoter; UTR, β-globin untranslated region; polyA consensus, polyadenylation consensus sequence; SP6, SP6 RNA polymerase promoter; SV40, simian virus 40 enhancer/early promoter; neo-EGFP neomycin resistance-enhanced GFP fusion gene; and polyA, simian virus 40 fragment containing polyadenylation signal.

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bell-shaped I-V curve and a maximal current of $2.1 \pm 0.5 \mu\text{A}$ ($n = 12$) at 0 mV (Figure 2, A and B).

Having confirmed that hERG1 cRNA obtained by in vitro transcription of pXOOM-hERG1 resulted in an appropriate level of hERG1 current in oocytes, pXOOM-hERG1 plasmids were transiently transfected into CHO-K1 cells. The *neo*-EGFP part of the pXOOM plasmid made identification of successfully transfected cells possible by use of fluorescence. Applying the same protocol on the hERG1-expressing mammalian cells as on hERG1 expressing oocytes resulted in an inwardly rectifying current with a maximal amplitude of $242 \pm 117 \text{ pA}$ ($n = 11$) at 0 mV (Figure 2, C and D). The modest current level obtained after expression of hERG1 in CHO-K1 cells means that leak can have a relative high impact on the measured I-V curve. This is observed in Figure 2D, where the inward rectification at positive potentials is not as pronounced in the CHO-K1 cells as in oocytes, as leak adds to the overall current level at potentials far away from -80 mV.

The displayed results show that no difference in kinetics and rectification properties could be observed when hERG1 was analyzed in the oocyte or the mammalian system. The only observed difference after expressing pXOOM-hERG1 in oocytes and mammalian cells was the overall current amplitude. This is to be expected since oocytes with a larger surface area compared to mammalian cells will express more membrane proteins.

To analyze further the functionality of the dual-function vector, we subcloned cDNA from the voltage-gated six transmembrane potassium channels KCNQ4 and Kv1.3 into pXOOM and made electrophysiological characterizations (data not shown). pXOOM-KCNQ4 responded as expected to the applied voltage-protocol with a slowly activating outward current with a current amplitude recorded at +60 mV of $2.6 \pm 0.8 \mu\text{A}$ ($n = 6$) in oocytes and $2.5 \pm 2 \text{ nA}$ ($n = 7$) in CHO-K1 cells (transient transfection). pXOOM-Kv1.3 also responded as expected with a fast activating and a slowly inactivating current with an amplitude recorded at +60 mV of $4.1 \pm 1.9 \mu\text{A}$ ($n = 12$) in oocytes and

$0.99 \pm 0.8 \text{ nA}$ ($n = 8$) in CHO-K1 cells (transient transfection).

To compare the pXOOM dual-function vector with conventional single-function vectors, we transiently transfected hERG1 and Kv1.3 standard plasmid vectors into mammalian cells (data not shown). We obtained a voltage activation no different from what was observed with the pXOOM-hERG1 construct and a current amplitude at 0 mV of $219 \pm 51 \text{ pA}$ ($n = 9$) for hERG1. For Kv1.3, a current amplitude at +60 mV of $1.1 \pm 0.9 \text{ nA}$ ($n = 12$) was observed. These results demonstrate that the insertion of sequences necessary for oocyte expression in conventional mammalian single-function vectors do not compromise the expression level in mammalian systems.

As the oocyte expression cassette is unchanged compared to standard

oocyte vectors, a change in expression level with pXOOM obtained cRNA in oocytes would not be expected. This was confirmed by comparable electrophysiological analyses of Kv1.3 cRNA from the two types of vectors (data not shown).

In conclusion, the results described here demonstrate that it is possible to use a single plasmid vector for protein expression in both *Xenopus* oocytes and mammalian cells. By including a bacteriophage promoter sequence and the *Xenopus* β -globin 5' and 3' stability elements together with a polyA stretch of 34 adenines in a standard mammalian expression vector, it has been possible to obtain a plasmid vector that has a dual-function in being suitable for heterologous expression of proteins in both the oocyte system and in mammalian cells.

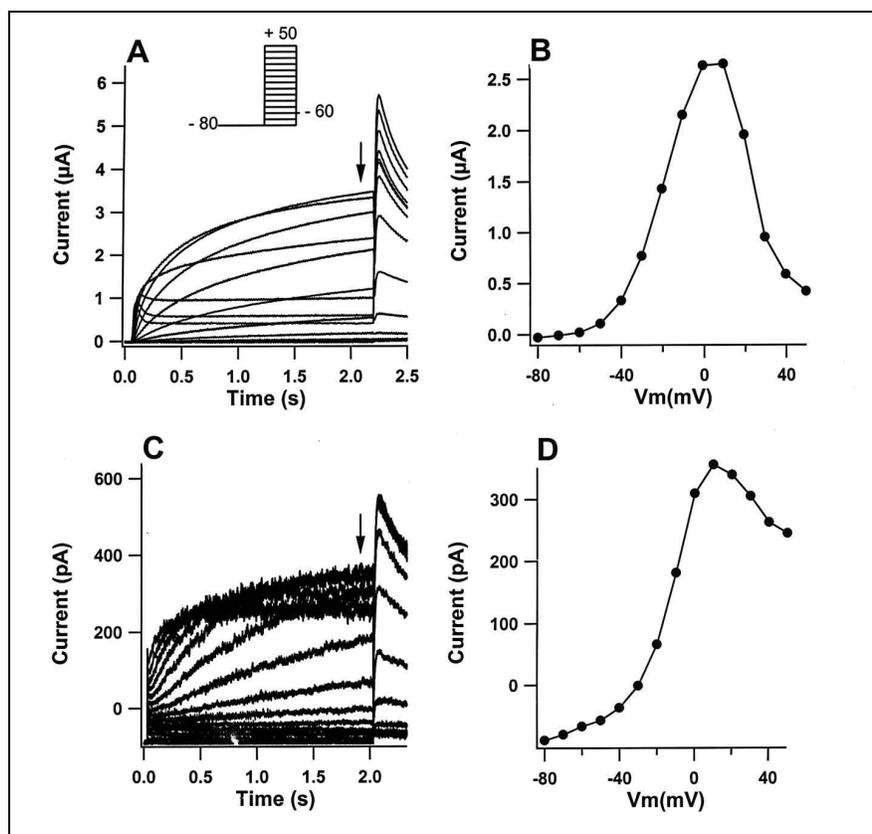


Figure 2. Confirmation of protein expression from pXOOM-hERG1. (A and B) Two-electrode voltage-clamp electrophysiological measurements of hERG1 expressed in *Xenopus* oocytes. (C and D) Whole-cell voltage-clamp electrophysiological measurements of hERG1 expressed in CHO-K1 cells. hERG1 currents were activated either in oocytes (A) or in CHO-K1 cells (C) by 2-s square pulses at potentials ranging from -80 to +50 mV, applied in 10-mV increments (see step protocol inserted in panel A). The corresponding I-V relationship for hERG1 is shown in panel B and D. Currents were measured at the end of the 2-s test pulse, as indicated by arrows. Traces show representative experiments for a number of oocyte experiments ($n = 12$) and CHO-K1 experiments ($n = 11$).

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Simultaneous Cycle Sequencing Assessment of $(TG)_m$ and T_n Tract Length in CFTR Gene

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ABSTRACT

The lengths of the dinucleotide $(TG)_m$ and mononucleotide T_n repeats, both located at the intron 8/exon 9 splice acceptor site of the cystic fibrosis transmembrane conductance regulator (CFTR) gene whose mutations cause cystic fibrosis (CF), have been shown to influence the skipping of exon 9 in CFTR mRNA. This exon 9-skipped mRNA encodes a nonfunctional protein and is associated with various clinical manifestations in CF. As a result of growing interest in these repeats, several assessment methods have been developed, most of which are, however, cumbersome, multi-step, and time consuming. Here, we describe a rapid method for the simultaneous assessment of the lengths of both $(TG)_m$ and T_n repeats, based on a nonradioactive cycle sequencing procedure that can be performed even without DNA extraction. This method determines the lengths of the $(TG)_m$ and T_n tracts of both alleles, which in our samples ranged from TG_8 to TG_{12} in the presence of T_5 , T_7 , and T_9 alleles, and also fully assesses the haplotypes. In addition, the repeats in the majority of these samples can be assessed

by single-strand sequencing, with no need to sequence the other strand, thereby saving a considerable amount of time and effort.

INTRODUCTION

Cystic fibrosis (CF), the most common severe autosomal recessive disease in the Caucasian population, is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene (1,21). Since the genomic DNA sequence of the CFTR gene, with its exon-intron boundaries, was completed by Zielenski et al. (26), the existence of some polymorphic sequences has been detected. Two of these are the dinucleotide $(TG)_m$ repeats and the adjacent polythymidine IVS8-T tract (T_n), both located at the end of intron 8, at the exon 9 splice acceptor site, with the $(TG)_m$ that precedes the T_n . Chu and co-workers (5,6) demonstrated that the shorter the T_n tract, the higher the proportion of CFTR mRNA without exon 9 in the respiratory epithelium, and that the shortest T_5 allele is the main factor causing exon 9 skipping. Subsequent studies demonstrated that, in addition to the effect of the T_n tract, exon 9 skipping is also influenced by the $(TG)_m$ repeat, which thus can explain the partial penetrance of the T_5 allele as a disease mutation (8,19). In particular, the longer the $(TG)_m$ is, the higher the proportion of exon 9 defective CFTR mRNA. This mRNA encodes a nonfunctional protein (5,23) and is associated with variable clinical manifestations in CF (2,4,7,8,12). Growing interest in the involvement of these repeats in the production of variable CF phenotypes has led to the development of several assessment methods, most of which are, however, cumbersome, multi-step, and time consuming.

Before the increased interest in the $(TG)_m$, several methods that were specific for the analysis of the T_n tract alone had been developed. They were usually based on a preliminary PCR amplification of exon 9 and its intronic boundaries and subsequent evaluation of the PCR product (see Discussion section). In addition to these widely used methods, a number of less common methods for the characterization