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Expression of the Avian Na,K-ATPase Subunits in *Dictyostelium discoideum*

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Abstract. This study explored whether *Dictyostelium discoideum* can be used to express the avian Na,K-ATPase, a heterodimeric membrane protein. *Dictyostelium* was able to express mRNAs encoding the avian Na,K-ATPase subunits. However, *Dictyostelium* expressed avian Na,K-ATPase protein when only when a *Dictyostelium* consensus ribosomal binding sequence, AAAATAAA, was inserted in front of the open reading frames of the α_1- and β_1-subunit cDNAs and the first eight codons following the start-translation codons were changed to *Dictyostelium* preferred codons. These modified mRNAs appeared to be much less stable than the forms that were not readily translated. *Dictyostelium* could express the avian β-subunit alone but only expressed the α_1-subunit when the β_1-subunit was co-expressed. Subunit assembly occurred in cells expressing both α_1- and β_1-subunits. The bulk of the exogenously expressed sodium pump subunits remained in an intracellular compartment, presumed to be the endoplasmic reticulum. *Dictyostelium* exported little or no Na,K-ATPase or free β-subunit to the plasma membrane.

Key words: Na,K-ATPase — membrane proteins — *Dictyostelium* — codon usage — mRNA turnover — expression systems — slime mold

Introduction

The Na,K-ATPase, the sodium pump, is a heterodimeric membrane protein minimally composed of α and β subunits (Jørgensen & Andersen, 1988). It transports sodium ions out of cells in exchange for extracellular potassium ions at the expense of ATP in virtually all animal cells. This activity leads to the establishment and maintenance of Na^+ and K^+ gradients across the plasma membrane, gradients that are essential for nutrient uptake, regulation of intracellular Ca^{2+} concentration, osmolarity and pH, and for electrical excitability in excitable tissues. Although significant progress has been made in understanding structure-function relationships in the sodium pump during the last decade, many questions remain. Expression of cloned DNAs encoding the Na,K-ATPase in an appropriate host is an important step toward an understanding of structure-function relationships of the pump. *Dictyostelium discoideum* is a primitive eukaryotic organism that is not a member of the animal kingdom and does not rely upon sodium ion gradients in the ways that animal cells do. In its vegetative form, *Dictyostelium* grows as single amoebae. In the laboratory, it can be grown in suspension cultures with a rapid growth rate (8–11 hr doubling time), making it suitable for large-scale production for biochemical analyses (*see* Jung & Williams, 1997). The genetics, cell biology and development of *Dictyostelium discoideum* have been extensively studied. Unlike animal cells, *Dictyostelium discoideum* has no endogenous Na,K-ATPase, yet unlike fungi, plants, and most eukaryotic organisms, *Dictyostelium discoideum* has no cell wall. These features make *Dictyostelium discoideum* an attractive organism to consider for expression of the Na,K-ATPase. Here, we report the results of our attempts to express the avian Na,K-ATPase in *Dictyostelium.*

Materials and Methods

DNA Preparation and Transfection

The α_1- and β_1-subunit cDNAs of the avian Na,K-ATPase were subcloned to the Bgl II site of the expression vectors pB18 (Sun &

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Devreotes, 1991) and pJK1 (Pitt et al., 1992) (gifts of Dr. Peter Devreotes, The Johns Hopkins University). The mRNA expression levels of cDNAs in pJK1 are higher than for the same cDNAs in pB18. However, each *Dictyostelium* amoeba can only incorporate one copy of pJK1. Thus, one of the subunit cDNAs had to be in the pB18 vector when the α_1- and β_1-subunits were co-expressed.

To enhance translational efficiency, nucleotide-directed mutagenesis was employed to delete the 5′-end untranslated regions of the cDNAs, to insert a *Dictyostelium* consensus ribosomal binding sequence, AAAATAAA, in front of the open reading frames and to change the first eight codons following the start ATG to *Dictyostelium*-preferred ones (Sharp & Devine, 1989). For the α_1-subunit cDNA, the nucleotide sequence ATG GGG AAG GGG GCT GGA AGA GAC AAG was changed to sequence ATG GGT AAG GGT GCC GGT AGA GAT AAG. For the β_1-subunit cDNA, the nucleotide sequence ATG GCC CGC GGG AAG GCC AAG GAC GGC was changed to ATG GCC CGT GGT AAG GCC AAG GAT GGT. The plasmid DNAs were introduced into *Dictyostelium discoideum* (Ax3 strain) by electroporation. The wild type Ax3 strain was kept axenically in HL-5 medium at 22°C. The positive clones expressing either or both Na,K-ATPase subunits were selected in the presence of 10–20 μg/ml G418 and identified by immunoblot analyses or antibody binding assays.

RNA and Protein Blot Analyses

Total RNA was extracted with RNAZol B (Biotecx Laboratories, Houston, TX) according to manufacturer's recommendations. 20 μg total RNA per lane was fractionated by electrophoresis in 1.2% agarose-formaldehyde gels and blotted onto Gene Screen Plus nylon membranes (Biotechnology Systems, Boston, MA). Membranes were hybridized overnight at 42°C with ^{32}P-labeled α_1- or β_1-subunit cDNA probes prepared by the random priming method (Life Science, Amersham, UK). The composition of the hybridization medium was 5X SSPE, 50% formamide, 5X Denhardt's and 1% SDS.

Total protein from microsomal preparations was extracted in detergent solution containing the protease inhibitors leupeptin, benzamidine, and aprotinin, each at 5 μg/ml and freshly added PMSF at 0.17 mg/ml. Extracted proteins were fractionated by electrophoresis in 10% polyacrylamide-SDS gels and blotted onto PVDF membranes. Immunoblots were probed with monoclonal antibody as the primary antibody and alkaline phosphatase-conjugated second antibody. Monoclonal antibody α5 (Lebovitz, Takeyasu & Fambrough, 1989) was used to detect avian α1-subunit; monoclonal antibody β29, which recognizes denatured avian β1-subunits (Renaud, Inman & Fambrough, 1991), was used to detect the avian β1-subunit. Antibody binding was detected by chemiluminescense, according to the manufacturer's suggested procedures (Tropix, Bedford, MA).

Antibody Binding Assays

The ^{125}I-labeled monoclonal antibody specific for the avian Na,K-ATPase β_1-subunit (Easton antibody; Wolitzky & Fambrough, 1986) was used to screen and quantify avian β_1-subunit expression. The cells were fixed with 1% formaldehyde prior to permeabilization with 0.25% saponin. The cells were incubated in antibody binding medium at 22°C for 45 min, washed three times with ice-cold Ca^{2+}, K^+-free Hanks' solution in mM): NaCl 150, $MgCl_2$ 0.05, Na_2HPO_4 0.2, and NaH_2PO_4 0.4 and solubilized in 0.5 N NaOH. Radioactivity was quantified with a gamma-counter (Beckman Gamma 5500, Fullerton, CA). Specific binding was defined as the difference between total binding and nonspecific binding. Total binding medium contained 0.5 μg/ml ^{125}I-labeled antibody plus 5% horse serum in Ca^{2+}-free Hanks' solution. Nonspecific binding medium was identical to total binding medium except contained a 50-fold excess of unlabeled antibody. For cells expressing the avian β1-subunit, nonspecific binding was less than 20% of total binding.

For immunofluorescence microscopy, monoclonal antibodies α7C and β24 were used to detect avian α1- and β1-subunits respectively. Cells grown on coverslips were fixed and permeabilized as for ^{125}I-antibody binding, described above. Fixed and fixed plus permeabilized cells were labeled with monoclonal antibody followed by FITC-labeled goat anti-mouse secondary antibody. Labeled cells were examined with a Zeiss Axiophot microscope equipped for epifluorescence microscopy.

The monoclonal antibodies used in this study are available from the Developmental Studies Hybridoma Bank, URL http://www.uiowa.edu/~dshbwww/ and from the authors' laboratory.

Results

The Na,K-ATPase α_1 and β_1 mRNA But Not Protein Expression were Detected from the Avian cDNAs Expressed in *Dictyostelium*

We first tried to express the unmodified avian α_1- and β_1-subunit cDNAs in *Dictyostelium*. Northern analyses revealed high levels of both α_1- and β_1-subunit mRNAs expressed from the pJK1 vector (Fig. 1*A* and *B*). We also detected the mRNAs expressed from the pB18 vector by longer exposure of the blot (*data not shown*). The differences in mRNA sizes between the mRNAs from chicken kidney and those expressed in *Dictyostelium* from the vectors are due to the different sizes in the 5′- and 3′-untranslated regions. The same blot was probed with *Dictyostelium* G protein β-subunit cDNA (Fig. 1*C*) to reveal the relative loading of total RNA in each lane. In contrast to the analyses of the mRNAs levels, neither α_1- nor β_1-subunit protein was detected by protein immunoblot analyses (Fig. 2). Possible explanations for this discrepancy are (1) the mRNAs were not efficiently translated, or (2) the subunits were not stable in *Dictyostelium*.

Selective Use of the *Dictyostelium*-Preferred Codons Allowed Expression of the α_1- and β_1-Subunit Polypeptides

Figure 3 shows that *Dictyostelium* expressed avian α_1- and β_1-subunit polypeptides when *Dictyostelium* preferred codons were used for the first eight amino acids of each subunit (*see* Materials and Methods). Protein immunoblot analyses detected the α_1-subunit polypeptide only from the clone that was cotransfected with the β_1-subunit cDNA, not from clones transfected with the α_1-subunit cDNA alone (Fig. 3*A*). One plausible explanation is that the β_1-subunit stabilized the α_1-subunit, and unassembled α_1-subunits were quickly degraded. Even in cells co-expressing the α_1- and β_1-subunits, multiple

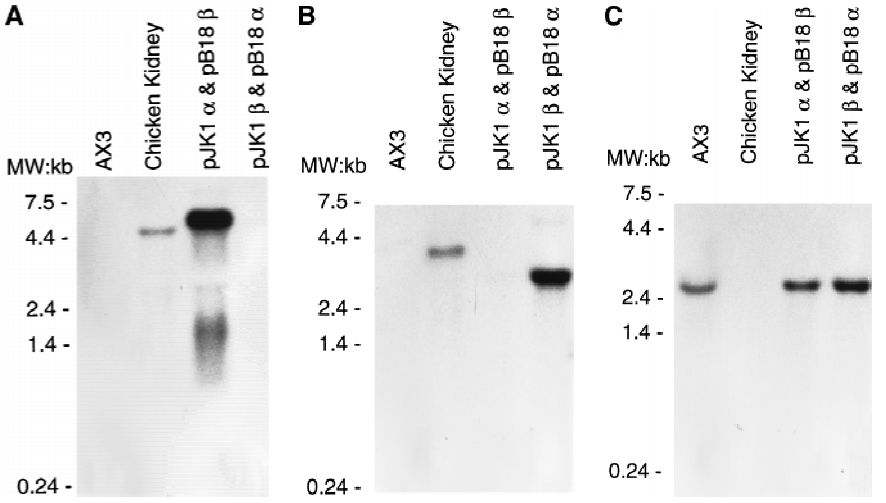

Fig. 1. Northern analyses of mRNAs expressed from the unmodified avian cDNAs in *Dictyostelium*. 20 μg total RNA was loaded in each lane as follows: lane (1) RNA from parental AX3 strain of *Dictyostelium,* (2) RNA from adult chicken kidney; (3) RNA from *Dictyostelium* cells transfected with pJK1 α (the avian Na,K-ATPase α1-subunit cDNA cloned into the vector pJK1) and pB18β (the avian Na,K-ATPase β_1-subunit cloned into the vector pB18); (4) RNA from *Dictyostelium* cells transfected with PJK1β and pB18α. (*A*) The blot was probed with ^{32}P-labeled chicken α_1-subunit cDNA. (*B*) The same blot was probed with chicken β_1-subunit cDNA. (*C*) The same blot was probed with *Dictyostelium* G protein β subunit cDNA. Each expression construct contained a consensus *Dictyostelium* ribosome binding site upstream of the Na,K-ATPase coding sequence.

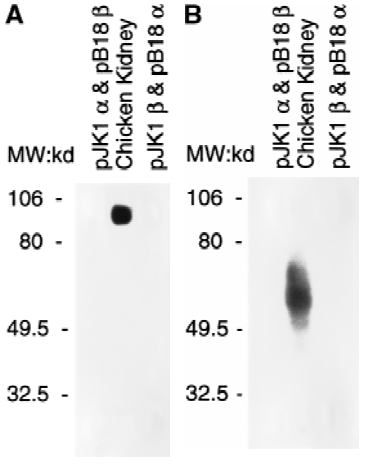

Fig. 2. Immunoblot analyses detected no Na,K-ATPase subunit expression in *Dictyostelium* from the unmodified avian cDNAs. Microsomal fractions were solubilized in SDS-PAGE sample buffer and subjected to SDS-PAGE and transfer to nitrocellulose membrane. Lane (1) extract from *Dictyostelium* cells transfected with pJK1α and pB18β plasmids; lane (2) extract from chicken kidney; lane (3) extract from *Dictyostelium* cells transfected with pJK1β and pB18α. (*A*) The blot was probed with the antibody α5 to the chicken α_1-subunit. (*B*) The same blot was probed with antibody β29 to the chicken β_1-subunit.

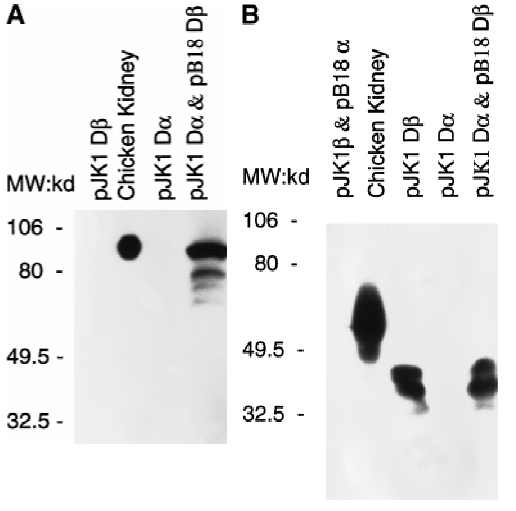

Fig. 3. Immunoblot analyses of sodium pump subunit expression from the cDNAs in which the *Dictyostelium*-preferred codons were used. pJK1 Dβ means that the β_1-subunit cDNA whose 5′-end was modified for use of *Dictyostelium*-preferred codons was subloned to the vector pJK1. The same form of nomenclature is used for the other expression constructs. (*A*) The blot was probed with antibody α5, specific for the chicken α_1-subunit. (*B*) The blot was probed with antibody β29, specific for the chicken β_1-subunit.

bands were seen in immunoblots probed with α_1-subunit specific antibody, possibly representing degradation intermediates of the α_1 subunit polypeptide. However, the major α_1-subunit produced by *Dictyostelium* had the apparent molecular weight expected for the full-length α_1-subunit (Fig. 3*A*).

Dictyostelium could express the β_1-subunit polypeptide without co-expression of the α_1-subunit (Fig. 3*B*). The β_1-subunit appeared in the immunoblots as a set of bands with apparent molecular weights greater than the apparent molecular weight of the protein core (35 kD) but smaller than that of the β_1-subunit extracted from

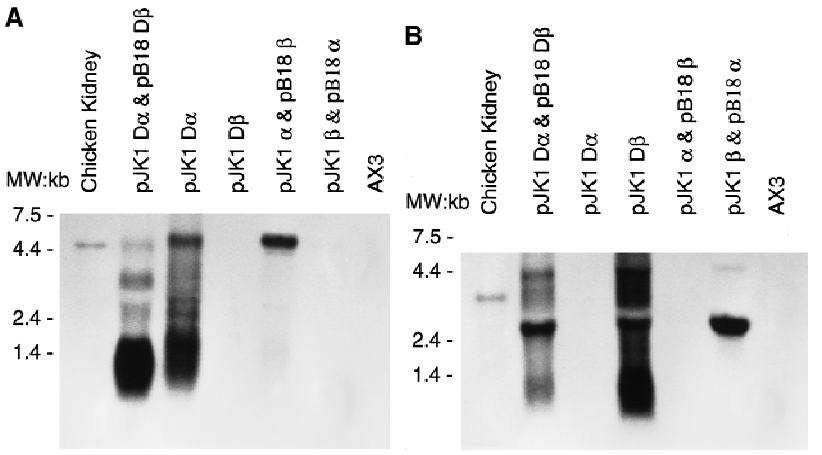

Fig. 4. Northern analyses of RNAs from *Dictyostelium* strain AX3, chicken kidney, and *Dictyostelium* cells transfected with the indicated plasmids. Vectors pJK1 and pB18 carrying the avian Na,K-ATPase α1 or β1 cDNA modified to contain *Dictyostelium*-preferred codons and consensus ribosomal binding sequences are denoted pJK1Dα, pJK1Dβ, pB18Dα and pB18Dβ. Vectors containing unmodified avian cDNAs are denoted pJK1α, pJK1β, or pB18α and pB18β. (*A*) The blot was probed with chicken α1-subunit cDNA. (*B*) The blot was probed with chicken β1-subunit cDNA.

chicken kidney. Such heterogeneity is also seen when avian β-subunits are expressed in tissue cultured mammalian cells, where the heterogeneity is due to different levels of N-glycosylation. It is most likely that differences in the number of N-linked oligosaccharides post-translationally added to the β_1-subunit account for the heterogeneity seen in *Dictyostelium*. To examine whether the expressed β_1-subunit was correctly folded, a monoclonal antibody against the native conformation of the β_1-subunit was used in ^{125}I-labeled antibody binding assays (Wolitzky & Fambrough 1986) and in immunofluorescence microscopy of fixed, permeabilized cells (Hamrick, Renaud & Fambrough, 1993). Both assays detected high levels of β_1-subunit expression in cells transfected with the modified 5′ end cDNA clone, the quantitative assay showing 5.2 ± 0.2 fg β_1-subunit expressed per cell. These data suggest that the β_1-subunits were properly-folded in *Dictyostelium*.

The Translated α_1 and β_1 mRNAs Showed Decreased Stability

To our surprise, we observed evidence of decreased stability of the α_1- and β_1-subunit mRNA, especially the α_1-subunit mRNA, after their 5′-ends were modified so that these mRNAs could support translation in *Dictyostelium* (Fig. 4). It is not clear whether the decreased stability was due to the deletions of the untranslated regions or to translation-coupled mRNA degradation.

Subunit Assembly of Na,K-ATPase α_1- and β_1-Subunits in *Dictyostelium*

Subunit assembly of the α- and β-subunits of the sodium pump can be analyzed by immune precipitation of β-subunits from detergent extracts of expressing cells, followed by assay for the presence of the α-subunit in the immune precipitate (Fambrough et al., 1994). To determine whether α-β-subunit assembly occurred in *Dictyostelium* cells expressing both Na,K-ATPase subunits, we prepared detergent extracts and precipitated with anti-β-subunit monoclonal antibody β24 coupled to Sepharose beads. The immunobeads were washed to remove unbound material and the bound proteins were analyzed by SDS-PAGE and immunoblotting. As shown in Fig. 5, the immune precipitates contained both β-subunits and full-length α-subunits (approximately 100 kD). Thus, we conclude that the α- and β-subunits of the avian Na,K-ATPase do assemble when co-expressed in *Dictyostelium* amoebae.

No Surface Expression Was Detected

^{125}I-labeled antibody binding, ^{3}[H]ouabain binding, and $^{86}Rb^{+}$ uptake assays were employed to determine whether *Dictyostelium* delivered β-subunits or the assembled, functional Na,K-ATPase to the cell surface. However, none of these assays yielded positive results. In seeking optimal conditions for surface expression of the avian sodium pump, we grew cells in media with various Na^{+} and K^{+} concentrations, we varied pH, and we varied culture temperature through the range tolerated by *Dictyostelium* cells. We also grew cells in medium containing concentrations of ouabain sufficient to block sodium pump function, hypothesizing that sodium pump function might somehow be detrimental to growth. None of these maneuvers resulted in significant expression of sodium pump activity or β-subunit epitope expression at the cell surface.

Discussion

Elucidation of structure-function relationship of the Na,K-ATPase has proven to be difficult. One problem has been that most expression systems have significant levels of endogenous Na,K-ATPase, complicating the

design of experiments and analysis of results. Vertebrate Na,K-ATPase has been expressed in yeast, which has no Na,K-ATPase (Horowitz et al., 1990) and SF9 insect cells, which have a low level of Na,K-ATPase (DeTomaso et al., 1993). Several interesting discoveries have been reported from these two expression systems (Blanco et al., 1994*a,b;* DeTomaso, Blanco & Mercer, 1994; Eakle et al., 1994; Pedersen, Rasmussen & Jorgensen, 1996), and yeast expression seems particularly promising for future studies. Although *Dictyostelium* was an attractive alternative expression host, only a few foreign glycoproteins have been successfully expressed in *Dictyostelium* (Jung & Williams, 1997). The major aim of the present study was evaluation of *Dictyostelium discoideum* as an expression host for Na,K-ATPase.

We have found that *Dictyostelium* can express avian Na,K-ATPase subunits when a consensus ribosomal binding sequence was inserted in front of the open reading frames and the first eight codons were changed to *Dictyostelium*-preferred codons. To our knowledge, this is the first report of *Dictyostelium* used to express an exogenous heteromeric membrane protein. Disappointingly, although subunit assembly occurred (Fig. 5), *Dictyostelium* did not export the expressed Na,K-ATPase to the cell surface. The reason remains to be determined. Dingermann (1995) and Cohen, Knecht & Lodish (1996) reported that the human m2 muscarinic receptor and rat glucose transporter, respectively, could be functionally expressed in *Dictyostelium*, and Chaumont, Loomis & Chrispeels (1997) showed that *Dictyostelium* delivered an *Arabidopsis* plasma membrane aquaporin to its cell surface. However, to the best of our knowledge, there has been no report that *Dictyostelium* delivered an exogenous heteromeric membrane protein to the cell surface.

Our studies also revealed that *Dictyostelium* did not express the α_1-subunit polypeptide unless the β_1-subunit polypeptide was also expressed, suggesting that the unassembled α_1-subunit was quickly degraded in *Dictyostelium* (Fig. 3*A*). These data are consistent with the hypothesis that one function of the β_1-subunit is stabilization of the α_1-subunit. In contrast to α_1-subunit expression, *Dictyostelium* expressed the β_1-subunit without co-expression of the α_1-subunit (Fig. 3*B*). Moreover, the β_1-subunit was N-glycosylated and sufficiently folded to display a conformation-sensitive epitope present on correctly folded β1-subunits, but it was not transported to the plasma membrane. It is not clear why *Dictyostelium* more readily expressed the β_1-subunit. We hypothesize that in the environment of the endoplasmic reticulum the β_1-subunit is more readily accommodated by the *Dictyostelium* proteins that participate in protein folding and therefore acquires a stable, folded state; the α-subunit folds with more difficulty and perhaps less innate stability and is therefore more subject to degradative mechanisms involved in quality control in the endoplasmic reticulum.

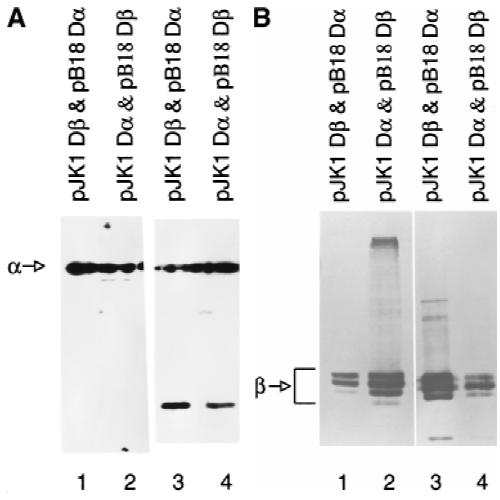

Fig. 5. Subunit assembly of the avian Na,K-ATPase in *Dictyostelium,* demonstrated by co-precipitation and immunoblotting. Immunobeads bearing antibodies to the avian β-subunit were used to isolate β-subunits from detergent extracts of *Dictyostelium* cells co-expressing avian Na,K-ATPase α- and β-subunits. The detergent extracts (lanes 1 and 2) and the immune precipitates from these extracts (lanes 3 and 4) were analyzed by SDS-PAGE, followed by immunoblotting with anti-α-subunit monoclonal antibody α5 (*A*) or anti-β-subunit monoclonal antibody β-29 (*B*). The 100 kD bands in the blot probed with the α5 represent the full-size avian α-subunit. The α-subunit bands in lanes 3 and 4 (*A*) represent α-subunits that coprecipitated with the avian β-subunits, signifying α-β subunit assembly had taken place in *Dictyostelium.*

During the course of our studies we learned of a number of unsuccessful attempts to express membrane proteins in *Dictyostelium.* However, to our knowledge, none of these has been reported in the literature. It is hoped that the observations reported here will be useful to those who are contemplating or involved in similar studies.

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