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Expression plasmids and production of EGFP in stably transfected *Acanthamoeba*

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Abstract

New plasmids containing the TATA-Binding Protein (TBP), TBP Promoter Binding Factor (TPBF) or Glyceraldehyde Phosphate Dehydrogenase (GAPDH) gene promoters from *Acanthamoeba castellanii* are described. The promoters for *Acanthamoeba* TPBF and GAPDH genes were used to drive constitutive expression of enhanced green fluorescent protein (EGFP) in stably transfected *Acanthamoeba*. Based initially on fluorescence microscopy and SDS PAGE analysis of EGFP, both promoters produce robust expression of EGFP, with the highest level obtained from the GAPDH gene promoter in cells grown in low concentrations of neomycin G418. Purification of EGFP from lysates of 22-ml cultures by conventional chromatography yielded approximately 1.1 milligrams of EGFP, a value that extrapolates to 50 milligrams per liter of cell culture. The results suggest that *Acanthamoeba* is a useful cost-effective system for the production of recombinant proteins.

Keywords

Acanthamoeba; *A. castellanii*; protein expression; EGFP; promoter; RNA polymerase II; aminoglycoside; stable transfection; over-expression; GAPDH; TPBF; TBP

Introduction

A variety of systems exist for the production of recombinant proteins including *E. coli*, *Bacillus* sp., *Saccharomyces cerevisiae*, *Pichia pastoris*, *Dictyostelium discoideum*, insect cells and mammalian tissue culture cells [1–8]. Each of these has benefits as well as disadvantages for a given application, and choice is dictated by criteria such as convenience, cost, levels of expression required and the presence or absence of accurate post-translational modifications. In general, no single system can be guaranteed to suit every application. An example is the well documented expression of some proteins in *E. coli* as inclusion bodies [9], and considerable effort has gone into finding ways to solubilize such mis-folded proteins [10]. Similarly, in many of these systems, post-translational modifications may be inappropriate resulting in expression of inactive protein, although this latter problem has been partly circumvented using humanized yeasts [4].

We recently described plasmids for the stable transfection of *Acanthamoeba castellanii* [11]. In those experiments, only the weak TBP gene promoter was used to drive expression of EGFP or neomycin phosphotransferase in growing cells. Subsequent experiments using expression

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of mRFP driven by the TPBF gene promoter suggested that high levels of monomeric red fluorescent protein (mRFP) expression could be serendipitously obtained (unpublished data). In the present report, the promoters for TPBF and GAPDH were used to produce new expression vectors and to drive constitutive expression of EGFP in stably transfected *Acanthamoeba*. The TPBF promoter produces moderate levels of EGFP expression, whereas the GAPDH promoter drives high levels of EGFP expression. The results establish *Acanthamoeba* as an efficient and cost-effective system for protein over-expression.

Materials and Methods

Construction of plasmids

All plasmids used here were constructed using the general methods described previously [11], and their structures confirmed by sequencing. Plasmid pEBMCS was constructed by removing a BglIII - XbaI fragment from p-110EGFP [11] and replacing it with a synthetic multiple cloning site (Figure 1). Plasmid pTPBF-EGFP was constructed using PCR fragments derived from the TPBF promoter (−475 to +66) [12] and the EGFP gene [13]. BglIII and NdeI sites were added at the respective 5' and 3' ends of the TPBF promoter. NdeI and XhoI sites were added at the respective 5' and 3' ends of the EGFP gene.

The 734 base pair promoter fragment for GAPDH was obtained by PCR of *Acanthamoeba* genomic DNA, during which a BglIII site was incorporated at the 5' end, and an NdeI site added at the 3' end which extends as far as the GAPDH ATG initiation codon. This too was ligated with the EGFP fragment to pEBMCS digested with BglIII and NdeI.

The sequence of GAPDH was obtained from the Baylor *Acanthamoeba* genomic sequence database (http://www.hgsc.bcm.tmc.edu/projects/microbial/microbial-detail.xsp?project_id=163), and the position of the first in-frame methionine determined by comparison to *Acanthamoeba* GAPDH EST (Genbank) sequences and by BLAST searches. The *Acanthamoeba* GAPDH gene is apparently unique in the *Acanthamoeba* genome and its coding region is highly conserved when compared to GAPDH genes from other species (not shown).

Growth and transfection of *Acanthamoeba*

Acanthamoeba were grown in 22-ml shake cultures in vented conical shake flasks at 28 degrees C, 200 rpm [14]. Transfections were performed as described previously [11], with the exception that selection was initially performed at 10 µg neomycin G418/ml and increased to 50 µg/ml when growth was apparent. The Neomycin concentration was subsequently reduced for some experiments as noted in **Results**.

Preparation of lysates and EGFP purification

Cells were collected by centrifuging at 3000 rpm for 2.5 min at room temperature in Eppendorf tubes and used without washes. For larger scale volumes, cells were centrifuged at 5000 rpm for 10 minutes at 4°C in a Sorvall SA 600 rotor and washed once in column buffer (CB) containing 50 mM KCl (20 mM Tris pH7.9, 0.1 mM EDTA, 50mM KCl, 0.25 mM PMSF and 0.1 mM protease inhibitor TPCK). Cells can be stored at −20°C for at least two weeks prior to processing, but they lose viability when frozen in this manner.

Small scale lysates using approximately one ml of starting culture were prepared by resuspending 1×10^6 cells in 600 µl of CB containing 50 mM KCl and 0.2% Igepal (formerly Triton X-100). Cells were allowed to stand on ice for 5–10 minutes, during which time they lyse without additional manipulation. After cell lysis was complete, as determined microscopically, the mixture was centrifuged at 10,000 rpm for 15 minutes at 4°C to remove

insoluble debris. In all cases, all of the visible EGFP was present in the soluble fraction, with none in the pellets (not shown). Total protein concentrations were determined by the method of Bradford [15], and by absorbance at 280 nm. EGFP concentrations were determined by absorbance measurements at 489 nm ($\epsilon_{489}=55,000 \text{ M}^{-1} \text{ cm}^{-1}$) [16], and by fluorescence measurements using excitation at 489 nm and emission at 509 nm using an Hitachi f-4500 Fluorescence Spectrophotometer.

For EGFP purification, cells from a 22-ml shake culture grown to a density of 6×10^6 cells/ml were harvested by centrifugation and lysates were prepared as for the small scale procedure, except cells were lysed in 10 ml CB containing 50 mM KCl and 0.2% Igepal and centrifuged for 15 minutes at 10000 rpm. Solid ammonium sulfate was added slowly to give 40% saturation, and allowed to stand for 30 minutes on ice. The mixture was centrifuged at 10,000 rpm for 15 minutes and the small precipitate discarded. The supernatant was applied to a 1 ml butyl Sepharose column and washed with 10 ml of CB containing 1200 mM ammonium sulfate. EGFP was subsequently eluted with CB, and dialyzed overnight against the same buffer. The dialysate was passed over a one ml BioRex 70 column and the flowthrough applied to an HR5/5 MonoQ FPLC column. EGFP was eluted using a 10 ml linear gradient from 50 mM KCl to 1M KCl. Fractions containing EGFP were easily identified by their iridescent green color, and were analyzed by SDS PAGE.

SDS PAGE and Western analysis

SDS PAGE and Western analysis of lysates were performed using standard methods [17]. Antibody against EGFP was from Molecular Probes. For silver stained gels of crude lysates, 3.2 μg protein was loaded per lane, and for Western analysis of lysates, 0.8 μg protein was loaded per lane.

Microscopy

Micrographs were obtained using a Sony DXCS 500 camera mounted to a Zeiss axiovert 40 CFL microscope equipped with an appropriate light source and filters. All images were obtained with a $20 \times$ objective.

Results

In order to explore the possibility of using *Acanthamoeba* to express recombinant proteins in useful amounts, several new plasmids were generated (Figure 1). All of these retain the neomycin phosphotransferase gene driven by the TBP promoter [11], but the EGFP expression cassette was first replaced by a multiple cloning site to facilitate subcloning (pEBMCS). In addition, approximately 160 bp of vector sequences upstream of this site were removed in order to reduce the overall size of the plasmid. The promoters for three genes, TBP, TPBF and GAPDH were then added separately to generate pEBMCSTBP, pEBMCSTPBF and pEBMCSGAPDH respectively (Figure 1). The TPBF and GAPDH promoter fragments used were chosen on the basis of preliminary experiments which showed them to be robust promoters, but that shorter versions produce far less EGFP expression (not shown).

The promoters for two genes, TPBF and GAPDH, were used to construct the plasmids pTPBFEGFP and pGAPDHEGFP (Figure 1) in order to drive expression of EGFP in stably transfected *Acanthamoeba*. Photomicrographs of cells transfected with these plasmids are shown in Figure 2.

Both the TPBF and GAPDH gene promoters drive considerable levels of EGFP expression in stably transfected cells (Figure 2), particularly in comparison to the TBP gene reported previously [11], but the GAPDH promoter is clearly the more robust of the two. Interestingly,

EGFP expression from the GAPDH promoter is highest in cells that had been selected at 50 μ g neomycin/ml, but then passaged into media containing 10 μ g neomycin/ml (Figure 2). The TPBF promoter does not show this characteristic (Figure 2).

Fluorescence microscopy provides a facile way to screen constructs for efficiency, however it can only provide a general measure of EGFP expression. In order to determine the relative levels of EGFP expressed from the TPBF and GAPDH promoters, whole cell lysates were prepared from transfected cells and wild type cells and analyzed by SDS-PAGE (Figure 3A). Each of the transfected cell lines produce EGFP at a level at least comparable to many endogenous proteins, and in sufficient quantity that a distinct 26 kDa band, absent in wild type extracts, is clearly visible on the stained gel. In accord with the fluorescence intensities of transfected cells shown in Figure 2, cells transfected with the GAPDH promoter construct and grown at 10 μ g neomycin G418/ml produce by far the greatest amount of EGFP, which is expressed at a greater level than any endogenous protein (Figure 3A, lane 2).

In order to confirm unambiguously that this band indeed corresponds to EGFP, lysates were analyzed by SDS-PAGE followed by Western blotting and EGFP was detected with anti-EGFP antibody. As shown in Figure 3B, all the lysates produce a robust EGFP signal, which migrates to the same position as the band highlighted in Figure 3A. Here too, GAPDH cells grown at 10 μ g neomycin/ml produce the strongest signal. In contrast, no signal is detected in lysates from wild-type cells nor cells transfected with a construct expressing mRFP (Figure 3). It should be mentioned that miniscule amounts (0.8 μ g total protein) of extract were used for this Western analysis, since in preliminary experiments routine amounts of protein (10–20 μ g) resulted in complete over-saturation of the reagents used for Western analysis.

The absolute amounts of EGFP obtained from lysates of each cell line shown in figure 2 were measured using fluorescence spectroscopy (Table 1). Quantification of EGFP in lysates provides the same general result as the microscopy and Western analyses, but clearly shows that the GAPDH gene promoter drives a far greater level of EGFP expression than the TPBF gene promoter, and this is particularly striking when cells are grown at 10 μ g neomycin/ml, where EGFP is present as approximately 6% of the total cell protein (250 μ g total EGFP) derived from 1 ml of culture. In contrast, The TPBF promoter produces about one tenth this amount under both cell growth conditions.

In order to further explore the usefulness of this system, EGFP was purified by conventional methods from a lysate derived from GAPDH-EGFP transfected cells grown at 10 μ g neomycin G418/ml. Lysates were sequentially fractionated by ammonium sulfate precipitation, hydrophobic interaction chromatography, passage over BioRex 70 and MonoQ FPLC, as described in Materials and Methods. The proteins eluted by a linear salt gradient from the MonoQ column were analyzed by SDS PAGE and stained with coomassie blue (Figure 4). Although the EGFP eluting from the MonoQ column is not homogeneous, it is by far the most abundant species, and the peak fractions are a vivid iridescent green (Figure 4).

To quantify the amount of EGFP isolated, protein concentrations were estimated by Bradford assay, absorbance at 280 nm and 489 nm and by fluorescence. These measurements suggest that approximately 1.1 mg of EGFP was purified with 46% yield from lysates of a 22-ml cell culture containing 14 mg total protein. Not accounting for losses during purification and EGFP in other side fractions, this extrapolates to 50 mg EGFP per liter of cell culture, an amount that compares favorably even to *E. coli* expression systems.

Discussion

Acanthamoeba has several features which lend it to recombinant protein production. It is easily and continuously grown axenically in simple low cost media [18;19], it can be easily lysed by

detergent, facilitating lysate production, its plasmids are stably maintained over a period of at least several months, and it can be induced to differentiate into dormant cysts which retain transfected plasmids for at least three years when stored at room temperature. While the stability of transfected cell lines was initially interpreted to mean that transfected DNA became integrated, subsequent experiments suggest that transfected DNA is episomal. In support of this idea, DNA can be isolated from transfected cell lines and used to transform *E. coli*. Isolation of plasmid from transformed *E. coli* and subsequent characterization demonstrated that it is identical to the original plasmid (not shown). In plasmids that contain two, or even three copies of the TBP gene, we have never observed aberrant expression of reporter genes suggesting that possible homologous recombination within these plasmids is not problematic.

In this report the previously uncharacterized promoters for TPBF and GAPDH were used to drive expression of EGFP. Both promoters produce robust expression of EGFP, providing a set of plasmids for expression of recombinant proteins at reasonably defined levels; the previously studied TBP gene produces low level expression [11], the TPBF gene promoter produces a moderate level of expression, and the GAPDH promoter produces very high levels. The choice of these promoters will be useful depending on the experimental design. For example, a pull-down type of experiment would be best served by the TBP gene promoter, whereas the GAPDH promoter would be best if simple over-production of a given protein is the goal.

No promoters from *Acanthamoeba* have previously been studied *in vivo*, and we have found that a greater than expected amount of upstream DNA is required for full expression from either the TPBF or GAPDH promoters. The GAPDH promoter used here contains 734 bp upstream of the ATG initiation codon, and the TPBF promoter contains 541 bp of DNA upstream of the ATG initiation codon. If the GAPDH promoter is deleted to -400 relative to the ATG start codon, or if the TPBF promoter is deleted to -341 relative to the ATG start codon, severe losses in potency result (not shown). Similarly, an actin gene promoter containing sequences to -400 is very poor at driving EGFP expression (not shown). Although no information is available regarding which elements are important in the GAPDH promoter, the region of the TPBF promoter between -341 and -541 contains four TPE elements that have been shown to bind *in vitro* the transcription factor TPBF, suggesting that the TPBF gene is autoregulated [12; 20].

The GAPDH gene promoter was chosen on the basis of the high number of GAPDH cDNAs found in an *Acanthamoeba* EST database, suggesting that the mRNA abundance is very high even though the genomic copy appears to be unique. The high level of EGFP expression driven by the GAPDH promoter obtained in the present experiments are consistent with this observation, and suggest that the GAPDH promoter is among the strongest in *Acanthamoeba*.

In contrast, TPBF mRNA is quite low in abundance, and is difficult to detect by primer extension [21], and its gene product TPBF is also low abundance, making it rather surprising that the TPBF promoter supports such high levels of EGFP expression. It seems likely that in the context of the transfection plasmids used here, the TPBF promoter is grossly mis-regulated, perhaps due to missing regulatory elements derived from the coding portion or 3' UTR of the natural TPBF gene.

The stable *Acanthamoeba* transfection system is based on selection with neomycin G418, which is a potent but complex inhibitor of translation [22–24]. When cells transfected with pGAPDHEGFP are grown with 50 µg neomycin/ml, and subsequently passaged to medium containing 10 µg neomycin/ml (or no Neomycin), there is a substantial increase in growth rate and in the amount of EGFP expression (Figure 2, and results not shown). One possible

explanation for this is that in the presence of neomycin, all translation is inhibited to some extent, resulting in a balance between how much of a given protein can be produced and the neomycin concentration. When all constraints on translation are removed, in some cases at least, cells can produce as much protein as the translation machinery can achieve.

Purification of EGFP produced by *Acanthamoeba* transfected with pGAPDHEGFP using conventional methods produced 1.1 mg of EGFP from 14 mg of crude lysate total protein with a yield of approximately 46%. Extrapolation of this result suggests that, at a minimum, a yield of 50 milligrams EGFP per liter of cell culture can be obtained. While it is not known if other proteins will be expressed at this efficiency, it is noteworthy that the EGFP used here was codon optimized for human (not *Acanthamoeba*) expression [13] suggesting that other proteins will be well expressed. In either case it is clear that significant amounts of active EGFP are produced by *Acanthamoeba*, and that this will be an attractive system for the expression of recombinant proteins.

Improvements to the current plasmids can readily be envisioned in order to increase expression further. Similarly, the use of protein tags such as Strep-tag, hexa-histidine or the HA epitope [25–27] are being tried in order to improve the simplicity and efficiency of purification schemes. In preliminary experiments using an HA-His₆-tagged version of *Acanthamoeba* TBP, we found that several endogenous proteins bind to Nickel columns, suggesting that this may not be the best choice of tag. In contrast, immunoprecipitation of HA-tagged TBP expressed in *Acanthamoeba* was very effective at purifying TBP (EB unpublished data). Here the main consideration is expense, rather than efficiency.

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Figure 1.

Plasmids used in this study. Plasmid pEBMCS contains a multiple cloning site as indicated. Plasmids pEBMCSTBP, pEBMCSTPBF and pEBMCSGAPDH contain the promoters from the *Acanthamoeba* TBP, TPBF and GAPDH genes respectively. Plasmids pGAPDHEGFP and pTPBFEFGFP contain the GAPDH promoter and an EGFP expression cassette or the TPBF promoter and an EGFP expression cassette respectively. All plasmids shown contain an ampicillin resistance gene (Amp) for growth in *E. coli*, and the gene for neomycin phosphotransferase (Neo) downstream from a copy of the TBP gene promoter (TBP) for selection in *Acanthamoeba*. For consistency, the promoter fragments are numbered here in reference to the ATG start codon, instead of the transcription start site.

Figure 2.

Expression of EGFP driven by the TPBF and GAPDH gene promoters. Photomicrographs of *Acanthamoeba* cells transfected with either the pGAPDH-EGFP or pTPBF-EGFP expression vectors. Cells were grown to a density of $5-7 \times 10^6$ cells/ml with the indicated concentrations of neomycin G418 ($\mu\text{g/ml}$). The GAPDH-EGFP cells grown at 10 μg neomycin/ml had previously been selected at 50 μg neomycin/ml. All fluorescent images were obtained using a 0.5 second exposure with a 20 $\times$ objective.

Figure 3.

SDS PAGE and Western analysis of transfected cell lysates. **A.** Silver stained gel of lysates from wild type cells (WT) or cells transfected with the indicated plasmids. All lanes contain 3.2 μ g of lysate. Lanes 2 and 4 are derived from cells grown at 10 μ g neomycin/ml. Lanes 3 and 5 are derived from cells grown at 50 μ g neomycin/ml. The position of EGFP on the gel is indicated. TPBF mRFP lysates are from a cell line expressing mRFP, and serve as a negative control. **B.** Western analysis of transfected cell lysates. Lysates derived from cells as in A., were run on a 12.5% polyacrylamide gel, transferred to nitrocellulose and probed with an anti-EGFP antibody. 0.8 μ g of whole cell lysate was run on each lane.

Figure 4. Purification of EGFP from GAPDH-EGFP transfected cell lysates. **A.** Photomicrograph of cells used for the EGFP purification. A 1/7.5 second exposure was used. **B.** Photograph of the MonoQ purified EGFP illuminated with a UV transilluminator. **C.** Coomassie Blue stained SDS PAGE analysis of fractions gradient-eluted from a MonoQ column. Lane 1 shows the column output material, lanes 5 and 6 contain the bulk of the EGFP, the position of which is indicated.

Table 1

Quantification of EGFP in lysates of transfected cell lines

Cell Line	µg EGFP	µg Total Protein	µg EGFP/mg Total Protein
WT	0	2160	0
pGAPDHEGFP; 10 µg Neo/ml.	250	4080	61.3
PGAPDHEGFP 50 µg Neo/ml.	55	4320	12.7
PTPBFEGFP 10 µg Neo/ml.	20	4920	4.1
PTPBFEGFP 50 µg Neo/ml.	22	3960	5.6