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Transient and stable transfection in the protozoan parasite *Entamoeba invadens*

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Abstract

Entamoeba histolytica is an important human pathogen and a major health problem worldwide. Many aspects of parasite biology can be studied with the exception of stage conversion, which cannot be reproduced adequately in *E. histolytica*. The reptile parasite *Entamoeba invadens* is a vital model system for studying stage conversion since it can be induced to undergo both encystation and excystation with high efficiency *in vitro*. However, functional studies using *E. invadens* have been limited by the lack of genetic tools in this species. Here, we report a new method for both transient and stable transfection of *E. invadens*. These new tools will greatly enhance research into *Entamoeba* development.

Keywords

Entamoeba; electroporation; development; stage conversion

Amebiasis, caused by the protozoan parasite *Entamoeba histolytica*, is a major health problem in humans, affecting 50 million people a year and causing an estimated 100,000 deaths [1]. The life cycle involves two stages: the invasive trophozoite form and the environmentally resistant cyst form. Spread of the parasite to new hosts requires conversion from the disease-causing trophozoite form to the infectious cyst form; thus, stage conversion is essential for disease transmission [2]. Some information regarding the developmental cascade has been identified using clinical *E. histolytica* isolates in which cysts are present, including identification of developmentally regulated transcripts and a stage-specific transcription factor which is important in regulating encystation [3, 4]. Although *E. histolytica* recent clinical isolates have cysts, regulated, high-level encystation has not been possible in this species despite significant efforts [2, 5]. Thus, research into this vital aspect of parasite biology has been hampered by the lack of a good *in vitro* model for *E. histolytica* encystation.

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In the absence of such a model, researchers have utilized a related species, *Entamoeba invadens*, which infects reptiles and causes invasive disease similar to *E. histolytica* infection in humans [6]. In *E. invadens*, both encystation and excystation can be performed *in vitro* with high efficiency [7]. Encystation is triggered by glucose starvation and osmotic shock, and excystation is initiated by incubation in rich media with the addition of bile salts, which mimics the environment in the small intestine [8-10]. However, the utility of this model system is compromised by a dearth of genetic tools to manipulate gene expression. Here, we report development of a method for transfection of *E. invadens*, which can be used both for transient expression of genes of interest and to generate stable transfectant parasite lines. These advances will allow analyses of genes in *E. invadens* at the molecular level and facilitate genetic dissection of the developmental cascade.

To create the transient transfection expression vector, we wanted to use promoters of genes that have high expression in both trophozoites and cysts. The *E. invadens* enolase gene (EIN_093390) was chosen and RT-PCR confirmed high gene expression in *E. invadens* trophozoites, and continued expression during encystation up to 48 hours (data not shown). To generate the vector (pEi-eno-Luc), the 5' and 3' flanking regions of the *E. invadens* enolase gene were amplified from genomic DNA by PCR and cloned into pBluescript SK- (Agilent). For the 5' promoter region, a 480bp segment (including a 12bp portion of the enolase coding region) was amplified, and cloned into *Bam*HI/*Hind*III sites. For the 3' downstream regulatory region, a 484bp fragment was used and cloned using *Xho*I/*Kpn*I. Finally, firefly luciferase was amplified from the TP4i vector [11] and inserted between the enolase flanking sequences using the *Xho*I and *Hind*III sites (Figure 1A). For use in stable transfection, the pEi-eno-Neo construct was generated by replacing the luciferase coding region with the coding region for neomycin phosphotransferase, which had been PCR amplified from pKT-3M (Figure 1B). Sequences for all cloning primers are listed in Table 1.

E. invadens strain IP-1 was maintained in LYI-S-2 [12] at 25°C [13]. In order to determine optimal conditions for electroporation, transient transfection assays were performed with pEi-eno-Luc. We first attempted transfection following the previously published protocols for *E. histolytica* [14], however, we did not achieve significant levels of luciferase expression with this method. Hence, we adapted the electroporation protocol from a previously published method for transfection of *T. brucei* [15], a parasite which, like *E. invadens*, grows at 25°C. For each trial, one 25cm² flask of log-phase trophozoites was iced, harvested, and resuspended in 0.4ml ZM buffer (132mM NaCl, 8mMKCl, 8mM Na₂PO₄, 1.5mM KH₂PO₄, 0.5mM Magnesium acetate, 0.09mM CaCl₂) at RT, and transferred to a 2mm gap cuvette. Parasites were electroporated with the pEi-eno-luc vector using 1.5kV and 25μF, giving an average time constant of ~0.2ms. After transfection, the trophozoites were transferred to LYI-S-2 media in 15ml glass tubes, and left to recover at 25°C. Transfection efficiency was assayed by measuring luciferase activity with the Promega Luciferase Assay kit following the manufacturers instructions. A number of different electroporation parameters were attempted, including differing amounts of DNA (30μg and 60μg) and numbers of pulses (one versus two pulses). Luciferase activity was measured at 17 and 24 hours post electroporation, with the highest activity at 17h post transfection, with considerable loss of activity by 24h. As in *E. histolytica*, increasing the amount of DNA and using 2 pulses increased the transfection efficiency (data not shown) [14]. The highest luciferase activity detected (using 60μg DNA, 2 pulses, and measured at 17h post transfection) was >3000 fold over lysate from untransfected cells, indicating that transfection was successful and allowing us to proceed with attempts at generating stable transfectants.

Before attempting to make stable transgenic lines, we first tested the growth of *E. invadens* IP-1 in neomycin (G418) and hygromycin in order to determine selection conditions. We

performed killing curves with neomycin (from 0.1 µg/ml to 100 µg/ml). Trophozoites were grown under standard culture conditions and drug was added to log phase parasites. At 24-hour intervals, tubes were examined for living, adherent parasites. *E. histolytica* HM-1:IMSS parasites were used as a control and were susceptible to 1 µg/ml G418. In contrast, *E. invadens* IP-1 was much more resistant to neomycin, killing 100% of parasites only at 50 µg/ml G418. This strain was even more resistant to hygromycin, requiring up to 100 µg/ml for complete killing (data not shown).

Stable transfectants were first generated using the pEi-eno-Neo construct. Electroporation was performed with 50 µg plasmid following the protocol outlined above with 2 pulses of 1.5 kV. Drug selection was begun at 24 h post transfection. Interestingly, despite our finding that 50 µg/ml G418 was required for killing untransfected amoeba, multiple attempts to select at that concentration did not succeed, with 100% lethality of the parasites. We hypothesized that this was possibly due to increased sensitivity of the parasites after electroporation and thus attempted selecting for stable transfectants in a graded manner. We were successful in selecting for stable transgenic parasites by culturing in 20 µg/ml G418, beginning at 24 h after electroporation and continuing until >90% of parasites had died off (approximately 1 week). This was followed by reduction of drug concentration to 5 µg/ml for ~2 weeks, until cells had gone through two passages, after which drug concentration was slowly increased to 60 µg/ml. Mock-transfected parasites (electroporated with no DNA) subjected to the identical treatment all died and did not recover despite the graded drug selection, indicating that parasites which survived in the experimental sample were not drug resistant mutants but had maintained the plasmid and Neo cassette. To confirm that we had true transgenic strains, we performed a Northern blot for expression of Neo (Figure 2A). RNA was isolated from untransfected *E. invadens* IP-1 trophozoites, and from pEi-eno-Neo transfectants growing at 30 µg/ml and 60 µg/ml G418, using Trizol reagent (Invitrogen). Purified RNA (10 µg) was run on a 1% agarose gel, transferred to nitrocellulose, and hybridized with a [α -³²P] labeled PCR probe generated against Neo. Expression of Neo was seen at both 30 µg/ml and 60 µg/ml G418, although with higher expression at the higher drug concentration, indicating a higher copy number consistent with episomal maintenance of the plasmid, as has been seen previously with the stable transfection of *E. histolytica* [14], and in a recently published method for *E. invadens* transfection [16]. As expected, no Neo expression was seen in untransfected trophozoites. Expression of the endogenous enolase gene was probed as a loading control and demonstrated equivalent levels of RNA in all lanes (Figure 2A). Sequences for primers used for probe amplification are listed in Table 1.

In order to overexpress genes in *E. invadens*, we developed the construct pEi-ck-Myc (Figure 1C). This plasmid was derived from pEi-eno-Neo by introduction of the flanking regions from the casein kinase II (ckII) gene (EIN_083650). This gene, like enolase, has high expression in both trophozoites and cysts, as demonstrated by RT-PCR (data not shown). A 489 bp segment from the ckII 5' regulatory region (including 15 bp of coding region) was amplified from genomic DNA and cloned into pEi-eno-Neo using *NotI/XbaI*. A 445 bp segment of the ckII 3' flanking regulatory region was cloned into the vector with *SacI/SacII*. A sequence coding for a 3xMyc tag was amplified by PCR from the plasmid pKT-3M [17], and added by cloning into the *NotI* and *SacII* sites. This Myc-tag region includes the additional cloning sites *NheI* and *AvrII*, for C-terminal and N-terminal tagging, respectively (Figure 1C). Finally, as the addition of these sequences resulted in an in-frame stop codon, the plasmid was modified by site-directed mutagenesis using the QuikChangeII kit (Agilent) to remove the stop codon and allow expression of the gene of interest.

A Myb domain containing protein (EIN_241140) was chosen for overexpression studies and cloned into this construct. This gene was chosen based on homology to a gene previously identified as upregulated in *E. histolytica* cysts and which serves as a potential regulator of

stage conversion [3, 4]. In addition, this gene has low expression in *E. invadens* trophozoites and thus would allow us to determine whether our construct facilitated its overexpression (data not shown). The full-length coding region was amplified by PCR from *E. invadens* genomic DNA, and cloned into the *AvrII* site of pEi-ck-Myc, which should result in an N-terminal fusion of the 3xMyc tag.

Stable transfectants were generated from this construct, using the protocol outlined above. When the ck-Myb parasites were growing to confluence at 40μg/ml G418, RNA was extracted with Trizol reagent, and used to test for gene expression. This RNA, as well as RNA extracted from untransfected trophozoites, was used to generate cDNA using previously published methods [3]. RT-PCR was performed using a forward primer that hybridizes to the Myc tag, and a reverse primer that hybridizes to the EiMyb coding region. This primer pair should only amplify transcript from transfected cells, and should not detect the endogenous Myb transcript. We identified a PCR product in the cDNA from the ck-Myb overexpressing cells and not from the untransfected controls (Figure 2B). Actin (EIN_093130) was used as a loading control, and showed equivalent signal from both samples. Control samples (with no reverse transcriptase) as expected gave no PCR product. In addition, we performed a Northern blot using 20μg of RNA following the procedures outlined above. An oligonucleotide probe directed at the Myc tag, plus an additional 12bp targeted to hybridize with the portion of ckII coding region that was included as a fusion, was labeled with [γ -³²P] ATP and hybridized overnight. We detected signal at the appropriate size in RNA from the ck-Myb parasites but not for the control parasites (Figure 2C). Enolase was used as a loading control. Sequences for primers used are listed in Table 1.

Our establishment of a system for transient and stable transfection in *E. invadens* is an important step in developing tools that will facilitate studying the genetic and molecular basis of stage conversion in *Entamoeba*. A similar method was recently developed by another group, which also used electroporation and graded selection for stable transfectants [16, 18]. Some differences in the approaches include our use of electroporation conditions for *Trypanosomes* whereas the group of Bhattacharya used conditions more similar to those used for *E. histolytica*, with some modifications including a higher V/cm. However, many similar findings emerge from both studies, including the apparent episomal nature of the plasmid (similar also to what is noted in *E. histolytica*), the higher resistance of *E. invadens* to neomycin, and the relatively slow selection process obtaining stable transfectants (a time frame that is significantly longer than that in *E. histolytica*). These advances in genetic manipulation of *E. invadens* will enable molecular characterization of proteins that are potential regulators of encystation and will be an important use of the available genome sequence information. Many studies, including analysis of promoter activity, protein localization, effect of gene overexpression and genetic manipulation (such as regulated expression, dominant-negative approaches, or antisense) can now be attempted. In addition, the ability to generate stably transfected parasites lays the foundation for the development of RNAi-based techniques for gene knockdown, which have been used successfully in *E. histolytica* [19, 20], and which will contribute greatly to our understanding of the role targeted genes play in amebic development. The ability to genetically manipulate *E. invadens* will greatly enhance the study of *Entamoeba* development.

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***Entamoeba invadens* transfection Ehrenkaufer and Singh**

- *E. invadens* is a model system for studying *Entamoeba* stage conversion
- Lack of genetic tools has rendered this system less useful for molecular studies
- We have developed methods for transient and stable transfection of *E. invadens*
- These will be valuable tools for studying molecular basis of parasite development

Figure 1. Schematic of constructs used

(A) pEi-eno-Luc contains the firefly luciferase gene flanked by 5' and 3' regions of enolase in a pBS backbone and was used for optimization of transfection conditions. (B) pEi-eno-Neo contains the gene for Neomycin resistance flanked by 5' and 3' regions of enolase in a pBS background and was used to generate stable transgenic parasites and as the backbone for pEi-ck-Myc. (C) pEi-ck-Myc contains the eno-Neo cassette with the addition of flanking promoter sequences from the ckII gene, which was used to drive gene expression. A 3xMyc tag and *NheI* and *AvrII* sites have been introduced for inserting the gene of interest.

Figure 2. Overexpression of Neomycin and Myb in stably transfected *E. invadens* IP-1 parasites
(A) Northern blot analysis of the Neomycin resistance gene expressed in trophozoites stably transfected with the pEi-eno-Neo construct. RNA was extracted from parasites growing at 30 μ g/ml and 60 μ g/ml G418. Note the increased expression of Neo in the 60 μ g/ml sample and lack of signal in the untransfected WT control. The endogenous enolase transcript shows equal loading for all samples. **(B)** RT-PCR from cDNA from wild-type untransfected (WT) and Myb overexpressing transgenic parasites (Myb OX) was tested for expression of the Myb transcript using primers to the Myc tag and the Myb transcript. For all samples, a control reaction without reverse transcriptase was performed and was negative. Actin (EIN_093130) was used as a loading control. **(C)** Northern blot analysis showing Myb transcript in untransfected wild-type trophozoites (WT) and those stably transfected with the pEi-ck-Myb construct (Myb OX). RNA was extracted from parasites growing at 40 μ g/ml G418. The enolase transcript shows equal loading for all samples.

Table 1
Oligonucleotide sequences of the primers used

Sequences for all cloning, Northern blot and PCR primers are listed along with the ID for each *E. invadens* gene targeted.

Cloning primers	Sequence (5' -> 3')	Ei gene targeted
enolase 5' BamHI	GGATCCCAATAAAATTAACCTTCATCAAACATAACAC	EIN_093390
enolase 5' HindIII	AAGCTTAATG GTAGACATCTCTGTTTTTGC	EIN_093390
enolase 3' XhoI	CTCGAGAGTTAATTTAAACCTTTCGTTGGTC	EIN_093390
enolase 3' KpnI	GGTACCAAAAGCAGTCTTGTTCAGATT	EIN_093390
Luciferase HindIII	AAGCTTATGGAAGACGCCAAAAACATAAAG	
Luciferase XhoI	CTCGAGTTACAATTTGGACTTTCGCCCC	
Neo HindIII	AAGCTTATGATTGAACAAGATGGATTGC	
Neo XhoI	CTCGAGTCAGAAGAACTCGTCAAGAAGG	
ckll 5' XbaI	TCTAGATCAGTTGGTATTTGAGGTTTCAA	EIN_083650
ckll 5' NotI	GCGGCCGCAGGACGAGAATCATGGAAATATTTTTT	EIN_083650
ckll 3' SacII	CCGCGGCTGCTTTTGTTACGTCTAGTTTA	EIN_083650
ckll 3' SacI	GAGCTCATTCGTGTTTTGGGTTTGA	EIN_083650
Myb for AvrII	ACCTAGGGGAGGAGAATTGATAACTGCAGAAATGGAAG	EIN_241140
Myb rev AvrII	ACCTAGGTCATATTTCCGAATAATGATGCG	EIN_241140
mutagenesis for	TCTCGTCTGCGGCCGCAATGGCTAGCGAA	
mutagenesis rev	TTCGCTAGCCATTGCGGCCGAGGACGAGA	
Northern blot primers		
Neo PCR probe for	CAGACAATCGGCTGCTCTGATG	
Neo PCR probe rev	GATACTTTCTCGGCAGGAGCAAG	
enolase PCR probe for	ATTGCCAAGGCTGGTTACAC	EIN_093390
enolase PCR probe rev	GCAAGCCTTCTTCTCAATGG	EIN_093390
Myc oligo probe	AAGATCTTCTTCTGAAATAAGTTTTTGTTCGCTAGCCATTGCGGCCGAGGACGAGAACT	
RT-PCR primers		
Myc RT for	TGGCTAGCGAACAACAAAACTT	EIN_241140
Myb RT rev	GGTTGCATTTAGCCATGTGA	EIN_241140
Actin RT for	GCTGCAATGAAAGCAAAACA	EIN_093130
Act in RT rev	GTTGTGCTCCAAGTCCCAAT	EIN_093130