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# Influence of 5′ sequences on expression of the *Tet* repressor in *Giardia lamblia*

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**Abstract**

Gene expression is poorly understood in *Giardia lamblia*. Previously we utilized the *Escherichia coli* tetracycline regulatory elements to develop a giardial-inducible gene expression system. In this study, we tested the hypothesis that regions flanking the *tet* repressor (tet R) may influence its expression and affect inducibility of the regulatory system. We found that addition of a 6-His tag or nuclear localization signal (NLS) at the N- but not C-terminus of tet R, increased the induction ratios >100-fold. A non-specific sequence also increased the induction ratio. Fusing NLS at the N-terminus, also led to exclusively nuclear tet R localization. Changing the promoter from *gdh* or α-giardin to α2-tubulin increased the induction ratio slightly. Tet R expression at both RNA and protein levels correlated with repression efficiency, indicating that coding sequences of the 6-His tag or NLS may contribute to transcriptional activation of the exotic *tet R* gene in *Giardia*. In addition, we found that the tet R system mediated gene repression and induction during encystation. Previous studies used an artificial reporter gene. In this study, we were able to induce overexpression of epitope-tagged cyst wall protein 1 (CWP1) in vegetatively growing *Giardia* trophozoites. Moreover, we could repress or induce expression of exogenous CWP1 in encysting cells. Taken together, our data suggest that expression of tet R in *Giardia* is complex and can be strongly influenced by additional sequences, especially at its N-terminus. This system provides insights into expression of an alien gene and can be exploited to regulate gene expression and study important functions in *G. lamblia*.


*Keywords:* Inducible; Tetracycline; Repressor; *Giardia*

## 1. Introduction

*Giardia lamblia*, a major cause of waterborne diarrheal disease, is also of basic biological interest as an early diverging eukaryote [1]. Like *Entamoeba* and *Cryptosporidium*, it undergoes differentiation from a pathogenic trophozoite form into a resistant infectious cyst form. *Giardia* is a valuable model for differentiation of such parasites, as its life cycle can be reproduced in vitro [2–4]. Moreover, the *Giardia* Genome Project is very advanced and SAGE analysis of the transcriptome over the life cycle is in progress [5]. However, >60% of the predicted genes have no known function (http://gmod.mbl.edu/perl/site/giardia?page=intro) and regulation of differentiation is poorly understood [6]. Gene knockout in *Giardia* is not feasible because of its 4N ploidy [7,8]. The ability to repress and induce expression of a transgene may be crucial, especially in the case of a dominant negative mutant or a protein that may be toxic. *Tet*-inducible systems have proven valuable in many parasites [9,10]. Therefore, we developed an inducible gene expression system in *G. lamblia* by introducing the *Escherichia coli tet* repressor-operator system into the stable DNA transfection system we previously developed [11]. In that system, two copies of the *tet* operator were inserted downstream of the minimal *ran* promoter,

*Abbreviations:* NLS, nuclear localization signal; tet R, tet repressor; CWP1, cyst wall protein 1

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which had been shown to confer transcription initiation and promoter activity in *G. lamblia* [12]. The gene encoding the *tet* repressor protein (tet R) was fused with a 6-His tag to permit its localization and expressed under the control of a 52-bp α-giardin promoter. This system allows the level of expression of a target gene to be regulated in a reversible manner as it produced a 50-fold induction of luciferase activity upon the addition of tetracycline to inactivate the tet R.

The tet R mediates repression of the basal level of the reporter gene expression as well as induction in response to tetracycline [13,14]. A tight repression could result from sufficiently high amounts of the tet R expressed from a strong promoter [14]. In this study, we tested the hypothesis that regions flanking the tet R coding region might affect its expression and could lead to higher induction ratios. Our original constructs contained an N-terminal 6-His tag in order to facilitate immunolocalization and purification. Since tet R did not target exclusively to the nucleus, we also appended a nuclear localization signal (NLS), which was previously shown to function in *Giardia*. Since neither the NLS nor the 6-His tage is a normal giardial sequence, we asked whether these foreign sequences might influence giardial expression or nuclear targeting of tet R. We also asked if the *tet*-inducible system could be exploited to regulate expression of a giardial encystation specific gene during vegetative growth and differentiation.

## 2. Materials and methods

### 2.1. Giardia culture

Trophozoites of *G. lamblia* isolate WB (ATCC 30957), clone C6, were cultured in modified TYI-S33 medium [15]. Encystation was induced according to our published procedure [16].

### 2.2. RNA extraction, Northern blot analysis, and nuclear run on assays

Total RNA was extracted from *G. lamblia* wild type or indicated transfectants using TRIzol reagent (Life Technologies). Standard procedures were used in electrophoresis, blotting and hybridization of total RNA [17]. Total RNA (10 μg) was fractionated and transferred to Zeta-Probe blotting membrane (Bio-Rad). Strand-specific probes were labeled using the Prime-It II kit (Stratagene). The membranes were hybridized and washed as described [18]. Hybridization signals were imaged and quantified using a Storm system (Molecular Dynamics). Nuclear run on assays were performed as previously described [19]. RNA was subsequently purified by using TRIzol reagent (Life Technologies). The synthesized RNA was hybridized to a nylon membrane which contained *tet R* or *neo* gene coding region. The *tet R* gene coding region was amplified by PCR using primers TF (TCTAGATTAGATAAAAGTAAA) and TR (TTAAGACCCACTTTCACATTT) and pUHD15-1 [20] template. The *neo* gene coding region was amplified by PCR using primers NEOF (ATGATTGAACAAGATGGATTGCAC) and NEOR (TCAGAAGAACTCGTCAAGAAGGCG) and pRANneo [21] template.

### 2.3. Plasmid construction

The pNLop2-GtetR, pPop2 and pNLop2-GItetR have been described before [11,16]. All constructs were verified by DNA sequencing with BigDye Terminator 3.1 DNA Sequencing kit and the reaction products were analysed on an ABI 3100 DNA Analyser (Applied Biosystems). The coding sequences of NLS of SV40 large T antigen (KKKRKV; 5′-AAGAAGAAGCGCAAGGTG-3′) and 6-His tag (HHHHHH; 5′-CATCACCATCACCATCAC-3′) were added to specific constructs by PCR using specific primers. For constructing pNLop2-GtetRX, pNLop2-GtetRCH, pNLop2-GtetRNNL, the *tet R* gene was amplified by PCR on pUHD15-1 [20] template using primer pairs TXF (GGCGCCATGGATTCTAGATTAGATAAAAGTAAA) and TXR (CGGACTCGAGTTAAGACCCACTTTCACATTT), TXF and TCR (CGGACTCGAGTTAGTGATGGTGATGGTGATGAGACCCACTTTCACATTT), TNLF (GGCGTCATGAAGAAGAAGCGCAAGGTGTCTAGATTAGATAAAAGTAAAG) and TXR, respectively. The resulting fragments were digested with *Nco*I (*Bsp*HI for TNLF, TXR PCR product) and *Xho*I, and ligated in place of *Nco*I/*Xho*I-excised *tet R* in pGtetR [11]. The *tet R* expression cassettes in the resulting constructs pGtetRX, pGtetRCH, or pGtetRNNL were amplified by PCR using primers GS (CGATGTCGACGACCACAAATAACGCCTTTA) and GC (AATAATCGATGGTACCAGCTGATCGGCGCC). Each of the PCR products was digested by *Sal*I and *Cla*I, and cloned into *Kpn*I/*Cla*I-digested pRANneo together with *Kpn*I/*Xho*I-digested pLop2. This entailed ligating three fragments together. For constructing pNLop2-GtetRCNL, oligonucleotides (NL2F, TAACCCCAAAAAAGAAGAGAAAGGTCGAAA; NL2R, TAATTCGACCTTTCTCTTCTTTTTTGGGGT) containing the coding sequence of the NLS were phosphorylated, annealed, and cloned into the *Nde*I-digested and phosphatase-treated pNLop2-GtetRX. The C-terminal insertion of the NLS resulted in a disruption of Ile194 in α helix 10 of the tet R [14]. For constructing pN-TtetR, pNLop2-TtetR [11] was digested with *Kpn*I and self-ligated. For constructing pNRLop2-GItetR, the neomycin phosphotransferase (*neo*) expression cassette in pRANneo was amplified by PCR using NXF (GGCCTCTAGAAATGGGACAGGATCTAAC) and NHR (GGCCAAGCTTATCGATGTAACGAACCGCTAGAAG), digested with *Xba*I and *Hin*dIII, and cloned into pUC18 vector. The *neo* expression cassette in the resulting construct was digested with *Kpn*I, and ligated in place of the *Kpn*I-excised *neo* expression cassette in pNLop2GItetR. The clone with the *neo* expression cassette in the same orientation with the *tet R* expression cassette was picked.