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International Journal for Parasitology 36 (2006) 443–452

www.elsevier.com/locate/ijpara

Tight control of transcription in *Toxoplasma gondii* using an alternative tet repressor

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Received 8 November 2005; received in revised form 28 December 2005; accepted 5 January 2006

Abstract

Fusion of yellow fluorescent protein (YFP) to the N-terminus of the *Escherichia coli* Tn10 tet repressor (TetR) created a functional YFP–TetR repressor with the capacity of 88-fold repression of transcription when expressed in *Toxoplasma gondii*. As a test promoter we used the *T. gondii* ribosomal protein RPS13 promoter for which we provide experimental evidence of having a single major transcriptional start site, a condition favourable to the design of inducible expression systems. Integration of four tet operator (tetO) elements, 23–43 bp upstream of the RPS13 transcriptional start site, resulted in maximal repression of transcription (88-fold). Moreover, integration of these four tetO elements reduced the promoter activity only 20% in comparison with the wildtype promoter. Regulation was six-fold higher compared with an inducible expression system employing wildtype TetR. Importantly, only 0.1 μg/ml tetracycline was required for maximal induction demonstrating a higher affinity of tetracycline for YFP–TetR than for wildtype TetR which required 1 μg/ml tetracycline for maximal induction. The use of 0.1 μg/ml tetracycline allows prolonged continuous culturing of *T. gondii* for which levels of 1 μg/ml tetracycline are toxic. Our results show that YFP–TetR is superior to TetR for transcriptional regulation in *T. gondii* and we expect that its improved characteristics will be exploitable in other parasites or higher eukaryotes.

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Keywords: *Toxoplasma gondii*; Inducible expression; Transcriptional regulation; Tet repressor; Tetracycline; Ribosomal protein; YFP–TetR

1. Introduction

Tetracycline-inducible expression systems are widely used for exogenous control of gene expression in eukaryotes. These systems are based on two regulatory elements derived from the tetracycline resistance operon of the *Escherichia coli* Tn10 transposon, being a tet repressor (TetR) protein and the tet operator (tetO) DNA sequence. Interaction of dimeric TetR with tetO elements, which are placed in the vicinity of the transcriptional start site will sterically interfere with binding of RNA polymerase or auxiliary transcription factors and block transcription of a gene (Berens and Hillen, 2003). Addition of tetracycline causes conformational changes and dissociation of TetR from tetO elements, thereby allowing gene transcription. This system enables inducible expression of heterologous genes as well as functional studies of endogenous genes. Currently, a tetracycline-inducible system is established in many eukaryotes including plants (Gatz and Quail, 1988), yeast (Dingermann et al., 1992; Faryar and Gatz, 1992) and a number of protozoan parasites such as *Trypanosoma brucei* (Wirtz and Clayton, 1995), *Entamoeba histolytica* (Hamann et al., 1997; Ramakrishnan et al., 1997) and *Giardia lamblia* (Sun and Tai, 2000). Recently, a tetracycline-inducible system was described for *Toxoplasma gondii*, where a codon optimised tet repressor (named $TetR^S$) was expressed, which allowed a 15-fold regulation of transcription by an artifical promoter containing four tetO elements (Meissner et al., 2001). However, integration of multiple tetO elements caused a drastic reduction in promoter activity, which limits its use for functional studies. Most likely, integration of tetO elements then interferes with essential promoter sequences. In general, protozoan tetracycline-inducible expression systems need 1–10 μg/ml tetracycline for complete induction (Hamann et al., 1997; Ramakrishnan et al., 1997; Sun and Tai, 2000; Yan et al., 2001; Inoue et al., 2002), which are levels that are toxic for *T. gondii*. Therefore, a less toxic tetracycline derivative had to be used (Meissner et al., 2001).

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0020-7519/$30.00 © 2006 Australian Society for Parasitology Inc. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.ijpara.2006.01.005

In the present study, alternative tet repressors have been constructed, of which a TetR fusion with yellow fluorescent protein (named YFP–TetR) showed improved regulatory properties. Furthermore, tetO integration sites were identified for the strong *T. gondii* ribosomal protein RPS13 promoter, which were not detrimental for promoter activity, not even upon integration of four tetO elements. In combination with YFP–TetR, these tetO-containing promoters allowed high expression of genes and high levels of transcriptional regulation at reduced non-toxic tetracycline concentrations in *T. gondii*.

2. Materials and methods

2.1. Parasite strains and culturing

The *T. gondii* tachyzoites were maintained in culture at 5% CO_2 and 37 °C by serial passage in Vero cells or human foreskin fibroblasts (HFF) and grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% (v/v) heat-inactivated foetal calf serum and 2 mM L-glutamine. With the exception of RHΔHXGPRT (Donald et al., 1996) and Rep1.2 (expressing $TetR^S$, Meissner et al., 2001), cultures of stable transfectants were also supplemented with 20 μM chloramphenicol (water-soluble; Sigma).

2.2. Tet repressor constructs

Three different TetR constructs were made based on the p*CAT-GFP* plasmid (Striepen et al., 1998). This construct contains a fusion of chloramphenicol acetyltransferase (CAT) to green fluorescent protein (GFP), which is driven by the dihydrofolate reductase (DHFR) promoter. First, the DHFR promoter was replaced by the *T. gondii* alpha-tubulin (TUB) promoter, which was amplified by PCR from the ptub*YFP-YFP*/sag*CAT* plasmid (Gubbels et al., 2003) using the following primers (restriction sites are underlined): TUB-FW (*Hin*d III); 5′-CGATAAGCTTCGAATCTCTGAACG GATGTGT-3′ and TUB-RV (*Bgl* II); 5′-CGAGATCTGG GAATTCAAGAAAAAATGCCAACG-3′. The PCR product was inserted in p*CAT-GFP* with *Hin*d III/*Bgl* II, resulting in *p*TUB*CAT-GFP*. Subsequently, three different TetR constructs were made in which expression of the repressor gene was driven by the TUB promoter.

In the following, underlined residues are restriction sites, and start- and stopcodons are given in bold. (i) *TetR*, which contained the wildtype *E. coli* Tn10 tet repressor, was PCR amplified with the primers TetR-*Bgl* II FW; 5′-CGATAGATC T*AAA***ATG**TCTAGATTAGATAAAAG-3′ and TetR-*Pst* I RV; 5′-CGTCTGCAG**TTA**AGACCCACTTTCACATT TAA G-3′, digested with *Bgl* II/*Pst* I and inserted in *p*TUB*CAT-GFP* by replacing the *CAT-GFP* sequence. The resulting construct was named TetR. To increase translation initiation a triple adenine (italics) was inserted immediately upstream of the *TetR* coding sequence (Seeber, 1997).

(ii) *AdaptTetR*, which contained *T. gondii* optimised codon usage (www.kazusa.or.jp) for the first 20 amino acids of the *E. coli* Tn10 *TetR*, was PCR amplified with the primers adaptTetR-*Bgl* II FW; 5′- CGATAGATCT*AAA***ATG**TC GCGCCTCGACAAGAGCAAGGTCATCAACTCGGCGC TGGAGCTCCTGAACGAAGTCGGCATCGAAGGTTTAA CAACCCG-3′ and TetR-*Pst* I RV. The PCR product was digested with *Bgl* II/*Pst* I and inserted in *p*TUB*CAT-GFP* similar to *TetR* resulting in the construct adaptTetR.

(iii) *YFP-TetR*, which contained a fusion of the *YFP* gene to the *E. coli* wildtype *TetR* gene, was made in two steps. First, *TetR* was PCR amplified with the primers TetR-*Avr* II FW; 5′-CGATCCTAGG**ATG**TCTAGATTAGATAAAAG-3′ and TetR-*Pst* I RV, digested with *Avr* II/*Pst* I and inserted in *p*TUB*CAT-GFP* by replacing the *GFP* sequence. Subsequently, *YFP* was obtained from ptub*YFP-YFP*/sag*CAT* by *Bgl* II/*Avr* II digestion after which it was used to replace the *CAT* sequence, resulting in YFP–TetR.

Finally, a chloramphenicol resistance cassette containing the *CAT* gene under the control of the *T. gondii* SAG promoter was inserted in all TetR constructs (Kim et al., 1993). The CAT cassette was PCR amplified from ptub*YFP-YFP*/sag*CAT* using the primers T3; 5′-ATTAACCCTCAC TAAAG-3′ and CAT-RV (*Hin*d III); 5′-CGATAAGCTT TCGGGGGGGCAAGAATTGTGT-3′, digested with *Hin*d III and inserted at the *Hin*d III site immediately upstream of the TUB promoter in all constructs (in the same orientation as the *TetR* genes).

2.3. Stable transfectants and Western blotting

Stable transfectants (expressing the different TetR constructs) were obtained by transfecting freshly harvested *T. gondii* RHΔHXGPRT tachyzoites (10^7) with 25 μg of either circular or linearised (x *Not* I) sterile plasmid DNA as described previously (Kim et al., 1993) or by the restriction enzyme-mediated integration method (Black et al., 1995). Parasites and DNA were mixed in a total volume of 400 μl electroporation buffer, which was composed of 120 mM KCl, 0.15 mM $CaCl_2$, 10 mM K_2HPO_4/KH_2PO_4 pH 7.6, 25 mM HEPES, 2 mM EDTA and 5 mM $MgCl_2$. Immediately prior to use, fresh ATP and glutathione were supplemented to the electroporation buffer to a final concentration of, respectively 2 and 5 mM. Samples were placed in a 2 mm gap cuvette, after which electroporation was performed using the settings 1.8 kV, 100 Ω, 25 μF (BTX electroporator). Transfectants were added to confluent Vero cell monolayers (T25 flask) and 1 day p.i. chloramphenicol (30 μM) was added. Limiting dilution was performed at 20 μM chloramphenicol, after which individual clones were picked and tested for stability by a CAT ELISA (Roche) according to the supplier's manual.

Lysates of transfectants stably expressing the TetR constructs were analysed on a 4–20% NuPAGE Bis/Tris gel (Invitrogen). The gel was Western blotted and TetR was detected using an anti-tet repressor antibody (monoclonal mouse IgG1 mix, Mobitec; 1:500). As secondary antibody a goat antibody specific to mouse IgG was used which was conjugated with alkaline phosphatase (Zymed; 1:1000).

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