

# Transcriptional regulation of two stage-specifically expressed genes in the protozoan parasite *Toxoplasma gondii*

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Received February 9, 2005; Revised and Accepted March 2, 2005

DDBJ/EMBL/GenBank accession no. AY155668

## ABSTRACT

The protozoan parasite *Toxoplasma gondii* differentially expresses two distinct enolase isoenzymes known as ENO1 and ENO2, respectively. To understand differential gene expression during tachyzoite to bradyzoite conversion, we have characterized the two *T.gondii* enolase promoters. No homology could be found between these sequences and no TATA or CCAAT boxes were evident. The differential activation of the *ENO1* and *ENO2* promoters during tachyzoite to bradyzoite differentiation was investigated by deletion analysis of 5'-flanking regions fused to the chloramphenicol acetyltransferase reporter followed by transient transfection. Our data indicate that in proliferating tachyzoites, the repression of *ENO1* involves a negative distal regulatory region (nucleotides –1245 to –625) in the promoter whereas a proximal regulatory region in the *ENO2* promoter directs expression at a low level. In contrast, the promoter activity of *ENO1* is highly induced following the conversion of tachyzoites into resting bradyzoites. The *ENO2* promoter analysis in bradyzoites showed that there are two upstream repression sites (nucleotides –1929 to –1067 and –456 to –222). Furthermore, electrophoresis mobility shift assays demonstrated the presence of DNA-binding proteins in tachyzoite and bradyzoite nuclear lysates that bound to stress response elements (STRE), heat shock-like elements (HSE) and other *cis*-regulatory elements in the upstream regulatory regions of *ENO1* and *ENO2*. Mutation of the consensus AGGGG sequence,

completely abolished protein binding to an oligonucleotide containing this element. This study defines the first characterization of *cis*-regulatory elements and putative transcription factors involved in gene regulation of the important pathogen *T.gondii*.

## INTRODUCTION

*Toxoplasma gondii* is an obligate intracellular protozoan parasite that is capable of infecting most nucleated mammalian cells. It is an important opportunistic pathogen that is becoming a major threat to immunocompromised hosts, such as patients with the acquired human immunodeficiency virus (AIDS) infection (1,2). The parasite can also cause severe congenital disease to the growing foetus during pregnancy (3). Cats and felines in general are the final hosts for the parasite, but man and a wide range of other animals may serve as intermediate hosts. The parasite is transmitted through consumption of raw or undercooked tissue cyst infected meat or by oocyst ingestion produced by the final hosts. The parasite has a complex life cycle involving asexual and sexual stages in the infected hosts. This includes a rapidly growing stage known as the tachyzoite and a slowly dividing stage called the bradyzoite. The bradyzoite is also a cyst forming stage. Encysted bradyzoites escape the immune system and therefore remain in the host during their lifetime. If allowed to do so, in immunocompromised hosts and AIDS patients, they will convert back to the tachyzoite form and reactivate toxoplasmosis. This reactivation defines the cause of fatal encephalitis in immunocompromised *Toxoplasma* infected patients (2). The factors that trigger *Toxoplasma* differentiation are not very well known, and a better understanding of these factors will help in the control and treatment of toxoplasmosis.

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The establishment of methods for stable and transient transfection in *T.gondii* (4,5) has allowed the generation of mutants that has led some insights into the molecular processes of parasite differentiation and pathogenesis (6,7). *In vitro* culture studies have also been valuable in understanding the possible factors involved in *Toxoplasma* differentiation. *In vitro* studies of tachyzoite to bradyzoite conversion suggested that this process was linked to a number of stress factors. These included an increase in culture temperature or a change in pH of the culture medium (8). Similarly, agents inducing nitric oxide production (9) or inhibitors of mitochondrial function (10) were also demonstrated to induce bradyzoite formation. Several morphological and metabolic changes accompany *Toxoplasma* differentiation. This is evident from stage-specific expression of genes that are probably involved in the differentiation process. Some cell surface parasite molecules as well as key glycolytic isoenzymes were found to be stage specific (11–14).

Enolase (2 phospho-D-glycerate hydrolase, EC 4.2.1.11) is a glycolytic metal-activated metalloenzyme belonging to the enolase superfamily (15). The enzyme catalyses the Mg<sup>2+</sup>-dependent reversible dehydration of 2-phospho-D-glycerate to phosphoenolpyruvate during both glycolysis and gluconeogenesis. The enzyme from a large variety of organisms is conserved and biologically active as a dimer with subunits of ~45–48 kDa. The protein appears to serve other functions in some organisms. It is a major lens protein in some vertebrates (16). In yeast, it was found to be identical to the heat shock protein HSP 48 and is involved in thermal tolerance and growth control in this organism (17). In *T.gondii*, enolase was found to be differentially expressed (12,13). The parasite expresses two plant-like enolases (ENO1 and ENO2) with distinct enzymatic and antigenic properties (13). The two enolases were found to be encoded by single copy genes while the deduced amino acid sequences showed 73.65% identity (13). Semi-quantitative reverse transcriptase PCR analysis demonstrated that *ENO1* mRNA was only detected in the bradyzoites isolated from chronically infected mice while *ENO2* mRNA was exclusively found in the tachyzoites (12,13). The absence of *ENO1* mRNA in the tachyzoite stage suggested repression of *ENO1* transcription during bradyzoite to tachyzoite conversion.

The transcription of protein coding genes by RNA polymerase II in eukaryotes is directed by specific sequence elements that bind transcription factors in the promoters of these genes (18). Other upstream or downstream regulatory sequences may regulate gene transcription as well. In *T.gondii*, the gene-regulatory processes governing development are poorly understood, and of particular interest are the molecular mechanisms underlying the tachyzoite to bradyzoite stage conversion. It should be mentioned that, despite the isolation and characterization of numerous genes, no functional TATA and CCAAT boxes have yet been described in *T.gondii*. In our efforts to understand the stage-specific transcription of enolase isoenzymes, we have cloned and sequenced the promoters of the two *T.gondii* enolase genes. Transient transfection of promoter constructs allowed the identification of regions necessary for the transcription of both genes. Sequence-specific promoter regions that bind to putative transcription factors were mapped precisely.

## MATERIALS AND METHODS

### Cultivation of parasites

*T.gondii* strain 76K tachyzoites were propagated *in vitro* in human foreskin fibroblasts (HFF) using DMEM (Bio Whittaker) supplemented with 10% fetal calf serum (Dutscher), 2 mM glutamine and 0.05% gentamycin. Bradyzoite differentiation was induced *in vitro* by culturing tachyzoite infected cells in absence of CO<sub>2</sub> (19) and encysted bradyzoites freed by pepsin digestion (0.05 mg/ml pepsin in 170 mM NaCl, 60 mM HCl). Tachyzoites were allowed to grow until they lysed HFF cells spontaneously and were harvested by filtration through a glass wool column and through a 3 µm pore filter.

### Cloning of *ENO1* and *ENO2* flanking sequences

A PCR DNA fragment was generated from the *ENO2* open reading frame (ORF) using the sense 5'-CTACCGTTACTCACTTCCAAC-3' and antisense 5'-CCAATATTATCAACGAAACTGC-3' oligonucleotide primers. The 1.6 kb PCR product was labelled using a non-radioactive labelling kit (Boehringer) according to the manufacturer's instructions. The probe was used to screen a *T.gondii* strain 76K genomic library made in the bacteriophage lambda DashII vector (Stratagene). Briefly, *E.coli* XL1 blue P2 cells (Stratagene) were infected with the recombinant phages containing insert size of 10–20 kb and about 500 000 phage plaques were lifted on to hybrid N+ membranes (Amersham). Plaque lifts were treated as previously described (20), and then hybridized with the digoxigenin-11-dUTP labelled *ENO2* probe. Hybridization was done overnight at 42°C in hybridization buffer (2% blocking reagent, 5× SSC, 0.1% N-lauroyl-sarcosine, 0.02% SDS and 50% formamide). Filters were washed at room temperature with 2× SSC, 0.1% SDS and at higher stringency (0.1× SSC, 0.1% SDS at 68°C). Filters were then washed for 5 min in maleic acid washing buffer (0.1 M maleic acid pH 7.5, 0.15 M NaCl, 0.3% Tween-20) and incubated for 30 min in blocking buffer (1% blocking reagent in 0.1 M maleic acid pH 7.5, 0.15 M NaCl). An anti-digoxigenin alkaline phosphatase-conjugated antibody was diluted (1:10 000) in blocking buffer and incubated with the filters for 30 min. Filters were washed twice in washing buffer. The hybridizing plaques were detected by chemiluminescence using CDP-Star substrate (Boehringer) diluted in detection buffer (0.1 M Tris-HCl pH 9.5, 0.1 M NaCl). After the primary screen, positive plaques were rescreened with the same probe and the purified plaques used to make phage DNA (20). Phage DNA was digested with the appropriate restriction enzymes and sub-cloned into pBluescript plasmid vector (Stratagene). Plasmid DNA was sequenced by the dideoxy chain termination method using ALF express Autoread or Thermo-sequenase dye terminator sequencing kits (Amersham). Sequence reactions were run on an ALF express sequencer (Amersham), and data analysis and sequence alignments were performed using software programs available from the Swiss Institute of Bioinformatics ExpASy Molecular Biology Server or from the resources centre INFOBIOGEN.

### Construction of *ENO1* and *ENO2* reporter plasmids

Plasmid constructs for transfection were made from PCR-amplified DNA fragments using pairs of forward and reverse primers. For *ENO1* constructs, the following forward primers

**E1F:** AGCCCCCATGGCACCTCCAGTCA, **E1F1:** TGGA-CAGTCTGGCATGTGAGTTGC, **E1F2:** TATCACATGTGAGATGTGAC, **E1F3:** GCAACAAGCTGCTGTGTGC-CACGC, **E1F4:** CTGATAATCAAGCTTTGGCTGG were combined with **ENO1rev:** CATCGTGACAGGCGCGTAG-ATGAG. For ENO2 constructs, each forward primer **E2F:** TATCGTCGCATCGGAAATGCG, **E2F1:** TTCTGATTTA-CCGATTGTGTAAGG, **E2F2:** CAGCCTGTGTTCTGC-TGGATGTGA, **E2F3:** CGCGGATCCGCACACGTACAA-GCG, **E2F4:** GCGGAACGGCGGTCGCATGTCTCAT, **E2F5:** CGTCCCATTACGTTTTTCTTTTTGC was combined with **ENO2rev:** CATTTTGTGGAAGTTGAGTAACGGT. The sense primer contained a ClaI restriction site and the antisense primer an NsiI site. The PCR was done using 20 ng of DNA template and 50 pmol of each primer. The reactions were cycled for 35 times by heating at 95°C for 1 min, annealing at 57°C for 1 min, elongation at 72°C for 2 min and a final elongation step for 10 min at the same temperature. Buffers and Taq DNA polymerase used for PCR were from Promega. PCR-amplified DNA was digested with ClaI and NsiI restriction enzymes, gel purified using a gene clean spin kit (Qbiogene) and cloned into the reporter vector. All constructs were derived from the basic pTUB5CAT (5) by removing the TUB5 promoter using ClaI and NsiI restriction enzymes and replacing it with the enolase promoter constructs. Thus, these constructs contained the 3'-untranslated region (3'-UTR) of the major surface antigen 1 gene (SAG1) of the original pTUB5/CAT (5). To generate constructs with ENO1 and ENO2 3'-flanking sequences, the 3' SAG1 sequence was then removed from pTUB5CAT or from ENO1 or ENO2 promoter constructs by digesting the plasmids with PacI and NotI restriction enzymes. Oligonucleotide primers containing PacI and NotI restriction enzyme sites were used to amplify 3'-UTRs of the ENO1 and ENO2 genes. The amplified DNA was cut with the same restriction enzymes and cloned into the vectors.

### Transfection and CAT assays

*T. gondii* (76K strain) tachyzoites were grown and purified as described above. Cells ( $1.0 \times 10^7$ ) were washed once in PBS and resuspended in 800  $\mu$ l of cytomix buffer (120 mM KCl, 0.15 mM CaCl<sub>2</sub>, 10 mM K<sub>2</sub>HPO<sub>4</sub>/KH<sub>2</sub>PO<sub>4</sub> pH 7.6, 25 mM HEPES pH 7.6, 2 mM EGTA, 5 mM MgCl<sub>2</sub>) containing 2 mM ATP pH 7.6, 5 mM glutathione and 50  $\mu$ g of plasmid construct and 20  $\mu$ g of control plasmid *GRA1/LacZ* for  $\beta$ -galactosidase activity. Electroporation was done in a 4 mm gap cuvette using a BTX electro cell manipulator 600 (BTX, San Diego) set at a voltage of 2.5 kV cm<sup>-1</sup>, 25  $\mu$ F capacitance and 48  $\Omega$  serial resistance (5). Electroporated cells were immediately allowed to infect HFF monolayer cells for about 6 h after which fresh medium was added. Cells were grown for 48 h and then harvested for CAT assays. For CAT assays, HFF-infected monolayers were harvested, centrifuged at 800 g and the cell pellet washed once in PBS pH 7.5. The cells were then resuspended in 100  $\mu$ l of 0.25 M Tris-HCl pH 7.8 and a lysate prepared by four cycles of freezing in liquid nitrogen and thawing at 37°C. The lysate was centrifuged at 14 000 g and the supernatant used immediately for CAT assays or stored at -20°C until used. CAT and  $\beta$ -galactosidase assays were performed as previously described (5,21).

### RNA isolation

Total RNA was isolated from tachyzoites and from *in vitro*-differentiated bradyzoites. RNA was isolated from the parasites by a hot phenol method. Briefly, parasites were lysed in 200  $\mu$ l lysis buffer (1% SDS, 50 mM NaC<sub>2</sub>H<sub>2</sub>O<sub>2</sub> and 10 mM EDTA). An equal volume of phenol (pH 5.2) was added and the mixture vortexed and incubated at 65°C for 15 min. After centrifugation at 14 000 g for 10 min, the upper aqueous phase was removed and the phenol phase extracted again with 100  $\mu$ l of lysis buffer as before. The aqueous phases were combined and extracted twice with an equal volume of phenol/chloroform, once with an equal volume of chloroform alone and the RNA precipitated with ethanol. The RNA was treated with RNase-free DNase I (1 U/ $\mu$ g RNA) re-extracted as above and precipitated with ethanol. The RNA was used immediately for primer extension analysis or stored in ethanol at -80°C.

### Primer extension analysis

Oligonucleotide primers (10 pmol) were end-labeled at 37°C with [ $\gamma$ -<sup>32</sup>P]ATP (Amersham) using 10 U of polynucleotide kinase (New England Biolabs). The labelled oligonucleotides were then passed through sephadex G-50 quick spin columns (Roche) and precipitated together with 10  $\mu$ g of total RNA. The sequences of the antisense oligonucleotides were 5'-CCAAAATCTCGCGTGCAACGATGTC-3' for ENO1 and 5'-CGAGGATCTGACGAGCAGTGATGTC-3' for ENO2. After precipitation and washing in 80% ethanol, the pellet was dried and resuspended in RNase-free water containing 1 $\times$  first strand cDNA buffer (50 mM Tris-HCl, 6 mM MgCl and 75 mM KCl). The primers were annealed to the RNA by heating at 60°C for 1 h and cooling slowly at room temperature. After this, 40 U of rRNasin (Promega), 150  $\mu$ M dNTPs and 100 U of Powerscript reverse transcriptase (Stratagene) were added and the reaction incubated at 42°C for 1.5 h. The reaction (50  $\mu$ l) was stopped by adding 1  $\mu$ l of 0.5 M EDTA (pH 8.0) and DNase-free RNase A was added to a final concentration of 5  $\mu$ g/ml, and the reaction incubated at 37°C for 30 min. Three volumes of TE pH 7.5 (10 mM Tris-HCl and 1 mM EDTA) containing 100 mM NaCl were added and the reaction extracted with an equal volume of phenol/chloroform. After centrifugation at 12 000 g, the aqueous phase containing the primer extension products was precipitated with 2.5 volumes of ethanol and the pellet washed, dried and resuspended in 4  $\mu$ l of TE. Then, 6  $\mu$ l of formamide loading buffer was added and the extension products boiled for 2 min at 95°C, chilled immediately on ice and then loaded on a 6% polyacrylamide gel. A sequence reaction with the same primer prepared with the Sequenase<sup>TM</sup> Quick-Denature Plasmid Sequencing Kit (United States Biochemicals) was run next to the primer extension product to act as a marker.

### Nuclear extracts

Nuclear extracts for band shift assays were obtained from about  $5 \times 10^9$  tachyzoites or  $2 \times 10^9$  bradyzoites obtained *in vitro* (19). The parasites were harvested as described above and washed once in cold PBS and then resuspended in 4 ml of ice-cold buffer A (10 mM HEPES pH 7.9, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.5 mM DTT, 0.1 mM EDTA, 0.65% NP40 and 0.5 mM PMSF). The parasite lysate was incubated for 10 min

on ice and then centrifuged at 1500 *g* for 10 min at 4°C. The supernatant was discarded and the nuclei pellet was lysed by resuspending in 400 µl of Buffer B (20 mM HEPES pH 7.9, 1.5 mM MgCl<sub>2</sub>, 420 mM NaCl, 0.2 mM EDTA, 0.5 mM DTT, 25% glycerol and 0.2 mM PMSF). After incubation on ice for 15 min, nuclear extracts were centrifuged at 14 000 *g* for 10 min at 4°C. The supernatant was aliquoted in 30 µl volumes and stored at -80°C. The protein concentration in the nuclear lysate was determined by the Bradford method.

### Electrophoresis mobility shift assays

Electrophoresis mobility shift assays (EMSA) were performed using a band shift assay kit (Amersham) following the manufacturer's instructions. Briefly, DNA fragments (50–300 bp) were generated by PCR using primers originally designed for sequencing the promoters of *ENO1* and *ENO2* genes. The smaller fragments, whose sizes ranged from ~40 to 50 bp were also generated by PCR and the products resolved on 1.5–2% agarose gels. In our experience, fragments above 50 bp could be purified using the gene clean spin kit (Qbiogene). However, fragments smaller than this were purified by electrophoresis in dialysis membranes. About 100 ng of the purified DNA was dephosphorylated with 20 U of calf intestine alkaline phosphatase (Roche) in dephosphorylation buffer (50 mM Tris-HCl and 0.1 mM EDTA). The enzyme was heat-inactivated at 65°C for 10 min and an equal volume of TE (50 µl) added to the reaction which was then extracted once with an equal volume of phenol/chloroform. Another extraction was done with an equal volume of chloroform and the DNA precipitated with ethanol at -80°C for at least 30 min and then centrifuged at 14 000 *g* for 20 min. The DNA pellet was air-dried and then end-labelled with [ $\gamma$ -<sup>32</sup>P]ATP (Amersham) using 10 U of polynucleotide kinase. To label oligonucleotides, single-stranded oligonucleotides were annealed in buffer (10 mM Tris-HCl, pH 7.5 and 50 mM NaCl) by heating at 95°C for 5 min, 65°C for 15 min, 55°C for 15 min and 37°C for 30 min. The annealed oligonucleotides (500 fmol) were labelled by a fill-in-reaction with [ $\alpha$ -<sup>32</sup>P]dATP (Amersham) using 5 U of Klenow DNA polymerase I (New England Biolabs). Alternatively, they were end-labelled with [ $\gamma$ -<sup>32</sup>P]ATP using 10 U of polynucleotide kinase. The labelled DNA fragments or oligonucleotides were purified by passage through a Sephadex G-50 column. The probes were used immediately or stored at -20°C and used within one week. For gel shift assays, labelled DNA fragments (2–5 ng), or double-stranded oligonucleotides (20 fmol) were incubated for 20 min at room temperature with 5 µg of nuclear extracts in binding buffer (10 mM Tris-HCl at pH 7.5, 50 mM NaCl and 0.5 mM DTT) containing 1 µg Poly(dI-dC):Poly(dI-dC) (Amersham), 0.01% NP40 and 10% glycerol. For competition experiments, 10- to 50-fold excess of self or unrelated unlabelled DNA fragments or 50- to 300-fold excess of unlabelled double-stranded oligonucleotides were added to the binding reaction together with the nuclear extracts before addition of the labelled probe. To the bound and unbound DNA-protein complexes, loading dye (25 mM Tris-HCl at pH 7.5, 0.02% bromophenol blue, 0.02% xylene and 4% glycerol) was added and the complexes separated on 6% non-denaturing polyacrylamide gels in running buffer (7 mM Tris-HCl at pH 7.5, 3 mM NaC<sub>2</sub>H<sub>2</sub>O<sub>2</sub> and 1 mM EDTA).

### Nucleotide sequence accession number of *ENO1* and *ENO2* genes

Sequence data have been submitted to the GenBank database under accession no. AY155668.

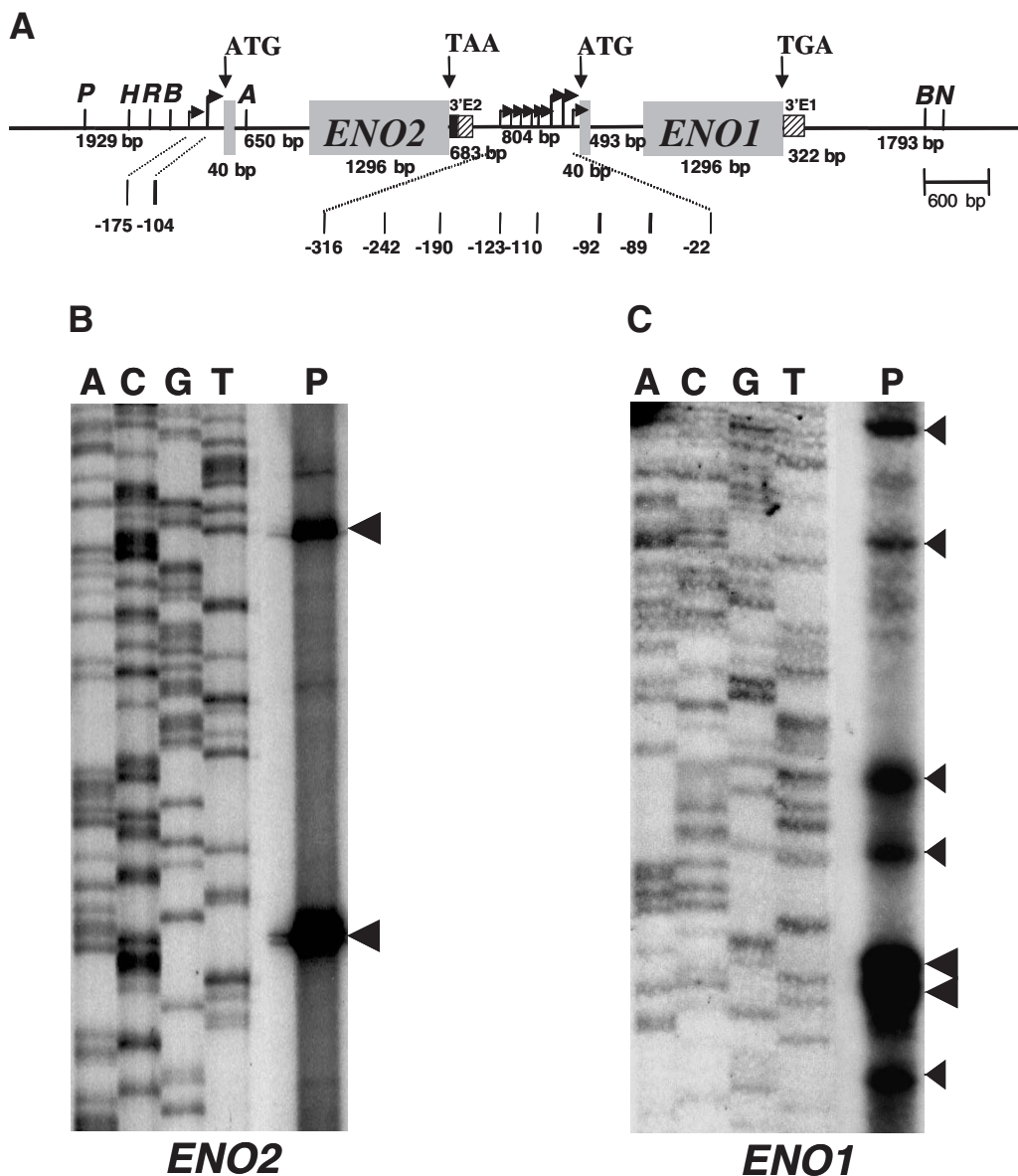
## RESULTS

### Structure of the *ENO1* and *ENO2* upstream and downstream regions

A *T.gondii* genomic library constructed in a lambda Dash II vector was screened with the 1.6 kb *ENO2* probe. A genomic DNA fragment hybridizing to the probe was subcloned and sequenced. The information derived from the sequence indicated that both *ENO1* and *ENO2* genes were located in this fragment. The whole sequence obtained from the enolase locus was ~9.3 kb and contained sequences from the ORFs of *ENO1* and *ENO2*, putative promoter sequences of both genes and ~2 kb sequence downstream of the *ENO1* gene (Figure 1A). A comparison of the genomic sequence with the cDNA sequences of *ENO1* and *ENO2* (12,13) showed that both genes contained single introns located exactly at the same position (Figure 1A). The sizes of the introns in both genes were different but both started 40 bp downstream of their ATG initiation codons. The intron in *ENO1* genomic DNA was 493 bp while that of *ENO2* was 650 bp. The consensus splicing donor and acceptor sequences were conserved and similar to those of other eukaryote genes with a GT at the 5' end and an AG at the 3' end of the intron. The sequences of the introns were completely different and had no homologous regions in their entire sequences. The sequences of the two putative promoters of *T.gondii* enolases were also completely different. The A/TGAGACG repeats identified in other *T.gondii* promoters and shown to be critical for the transcription of these genes (22,23) were not evident. Similarly, TATA and CCAAT boxes often found within 100 bases upstream of the transcription start sites and common in many eukaryotic genes could not be identified. The initiator element with the consensus sequence TCAGTTT which was identified in the nucleoside triphosphate hydrolase genes of *T.gondii* (24) was not found in either promoter.

### Determination of the transcription initiation sites of the *ENO1* and *ENO2* genes

As an initial step in the characterization of the *ENO1* and *ENO2* promoters, we mapped the transcription start sites of the respective genes using primer extension. When mRNA was isolated from tachyzoites, *ENO2* gene was shown to contain two transcription start sites located at -104 and -175 upstream of the ATG codon (Figure 1B) while no signal was detected with the *ENO1* probes (data not shown), confirming the transcriptional repression or the absence of *ENO1* messenger in the tachyzoites. The *ENO1* transcription start sites were only detected using mRNA isolated from *in vitro* induced bradyzoites. In this case, the bradyzoite *ENO1* extension products revealed multiple initiation sites with two major sites at -89 and -92 upstream of the ATG codon (Figure 1C). Several other minor initiation sites were also evident upstream and downstream of the two major initiation sites of *ENO1* at -22, -110, -123, -190, -242 and -316 position relative to



**Figure 1.** (A) Tandemly arrayed organization of the two stage-specific *T. gondii* enolase genes. Approximately 9.3 kb of the whole sequence of both enolase loci is drawn to scale. Both *ENO1* and *ENO2* genes contained single dissimilar introns located 40 bp downstream of their ATG initiation. An intron of 493 bp is present in *ENO1* genomic DNA while *ENO2* contains an intron of 650 bp. The transcription start sites of *ENO1* and *ENO2* are indicated by vertical arrows and the numbers above correspond to their nucleotide positions relative to the ATG codon. 3'E2 indicates the 242 bp of the 3'-UTR of *ENO2* (up to the polyadenylation signal) plus the 441 bp of the adjacent 5'-flanking region of *ENO1* (distal promoter). 3'E1 corresponds to the 3'-UTR of *ENO1*. Some restriction enzyme sites *Apa*I (A), *Eco*RI (R), *Bam*HI (B), *Hind*III (H), *Pst*I (P) and *Not*I (N) are indicated. (B and C) Mapping of the transcription start sites of *ENO2* and *ENO1* by primer extension using RNA from tachyzoites and bradyzoites, respectively. Note that one major transcription start site and a minor one were obtained for *ENO2* (arrows) whereas *ENO1* gave two major, and at least five minor transcription start sites (arrows). The letters A, C, G and T indicate the products of a sequencing reaction that was run along the primer extension fragments for size determination.

the ATG. The nucleotide positions of these transcription start sites are also shown in Figure 1A. The presence of multiple transcription start sites in the *ENO1* gene was also confirmed by 5'RACE. After nucleotide sequencing of the 5'RACE products, the existence of several 5' DNA fragments with different nucleotide lengths confirms the primer extension data (data not shown). We could not rule out the possibility that some of the minor transcription initiation sites and 5'RACE fragments observed in *ENO1* can be associated with the degradation of the primary transcript. Nevertheless, our data remain in good

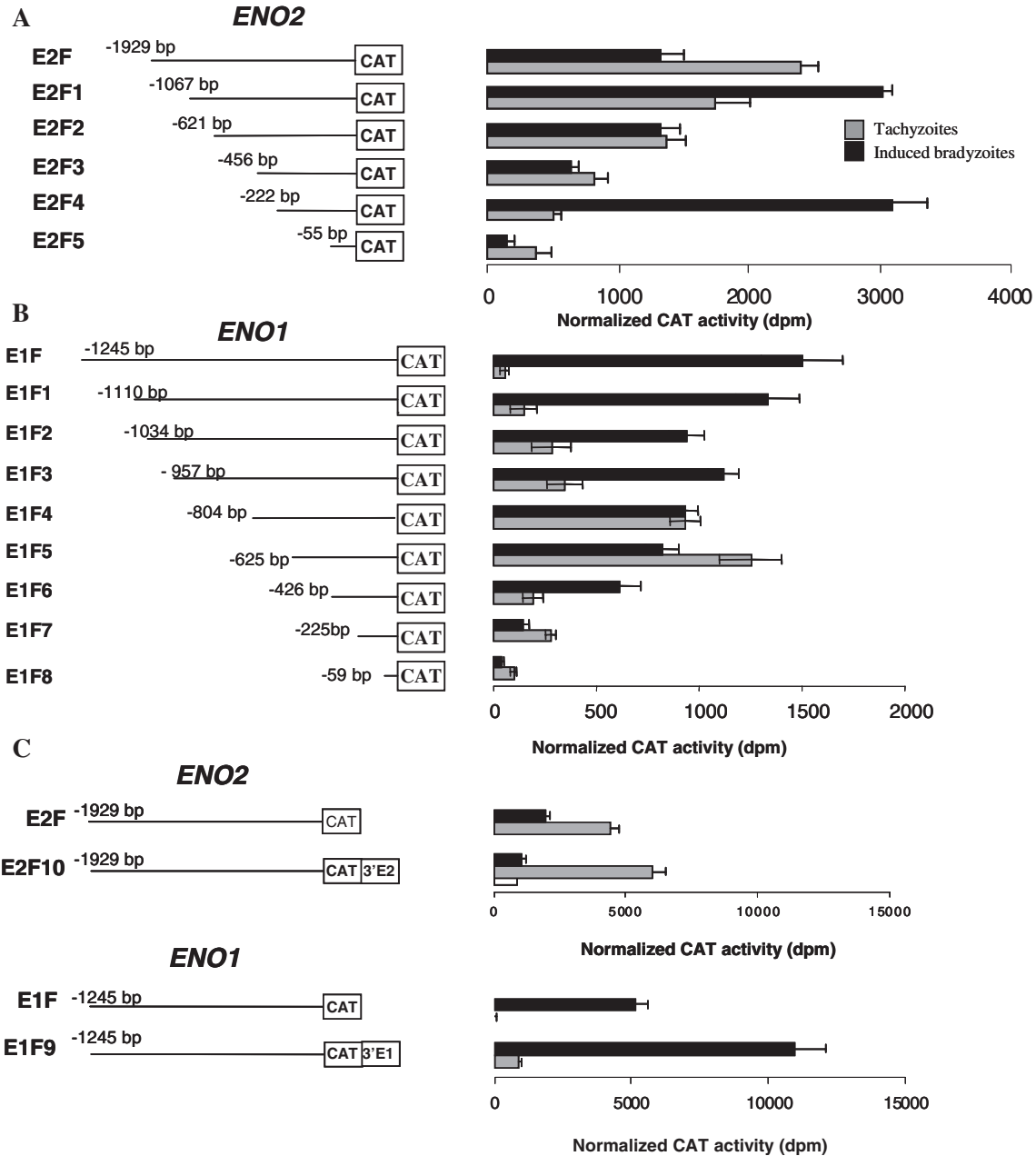
agreement with the presence of multiple transcription start sites that are indeed frequently seen in higher eukaryotic promoters lacking TATA and CCAAT boxes (25–27).

#### Functional mapping of the *ENO2* and *ENO1* promoters

To gain insight into the DNA elements involved in the expression of the *ENO1* and *ENO2* genes, a series of reporter plasmids containing various lengths of the *ENO1* (from nucleotides –1245 to –59) and *ENO2* (from nucleotides –1929

to -55) 5'-flanking regions upstream of the CAT gene were transfected into the tachyzoites. Transient transfections of these various deletion constructs gave rise to low CAT activities for both *ENO1* and *ENO2*, suggesting that *T.gondii* enolase genes exhibited weak promoter activity similar to that found with the surface antigen gene 1 (*SAG1*) (23) and lactate dehydrogenase (*LDH*) promoters (14). Serial deletion of the

*ENO2* promoter leads to consistent and progressive decrease of CAT activity, suggesting that the construct E2F4 (deletions up to -222 bp upstream of the ATG codon) may contain all the *cis*-regulatory elements necessary for the expression of *ENO2* gene (Figure 2A). In contrast, when CAT-chimeric constructs of the *ENO1* promoter were transfected into the tachyzoites, the region with the first 620 bp downstream of the *ENO2*



**Figure 2.** Identification of transcriptional regulatory elements in the *ENO1* and *ENO2* genes by transient CAT assays. Transfections of tachyzoites were performed with *ENO2* (A) and *ENO1* (B) promoter-reporter constructs that are schematically depicted on the left of the panels. The transfected tachyzoites were also subjected to stress thereby converting to bradyzoites (see Materials and Methods). (C) Activities of the *ENO1* and *ENO2* promoters during tachyzoite to bradyzoite conversion. The plasmids E2F or E1F contain the 3'-UTR of *SAG1* gene while E2F10 and E1F9 contain the 3'-UTR of *ENO2* and *ENO1*, respectively. The CAT-constructs depicted on the left-hand side (E2F versus E2F10 and E1F versus E1F9) were transfected into tachyzoites and these organisms were divided equally into two flasks of human foreskin fibroblasts. After 6 h of invasion, one flask was subjected to experimental stress conditions to induce bradyzoite conversion and the second flask was kept in tachyzoite growth conditions (see Materials and Methods). Levels of CAT signal in tachyzoites and bradyzoites were determined after removing the promoterless CAT vector activities and adjustments for  $\beta$ -galactosidase activity used as internal control. The data are averages of four independent experiments. Error bars represent the mean and SD values of four independent experiments.

polyadenylation site (E1F construct) contain sequences that downregulate the expression of the bradyzoite-specific *ENO1* in the tachyzoite stage (Figure 2B). As judged from the CAT reporter gene activity, deletion of this region (constructs E1F1 to E1F5) showed a steady-level increase in promoter activity in the tachyzoite. The deletion of this 620 bp region in E1F5 construct resulted in 20-fold increase of CAT activity (Figure 2B). Further deletions of *ENO1* promoter to give constructs E1F6 to E1F8 produced a further reduction in CAT activity until the background.

When these series of *ENO2* constructs (E2F–E2F5) were transfected in tachyzoites which were then subjected to stress conditions inducing bradyzoites (see Materials and Methods), the *ENO2* promoter analysis revealed two upstream repression sites (nucleotides –1929 to –1067 and –456 to –222) which were not previously detected in tachyzoites (Figure 2A), suggesting that the transcriptional activity of *ENO2* promoter in bradyzoites relies on different components. In contrast, the promoter activity of the largest construct E1F which contains negative regulatory elements (nucleotides –1245 and –625) as evidenced in tachyzoites, increased ~30-fold following bradyzoite differentiation (Figure 2B). The other smaller constructs tested (from E1F1 to E1F8) in bradyzoites gave a progressive decrease of CAT activity down to the basal promoter activity reached in E1F8. In order to test the effect of the first exon and the intron on CAT activity, other CAT-chimeric constructs containing either the first exon or both exon and intron (see the map shown in Figure 1A) were generated from *ENO1* and *ENO2* promoters with their respective exon and intron sequences. Transfection of these constructs did not appear to produce any significant changes in CAT activity when compared with that of the *ENO1* and *ENO2* promoter alone. This suggested that the first exon and the intron probably did not contain any sequences regulating the transcription of the *ENO1* and *ENO2* genes.

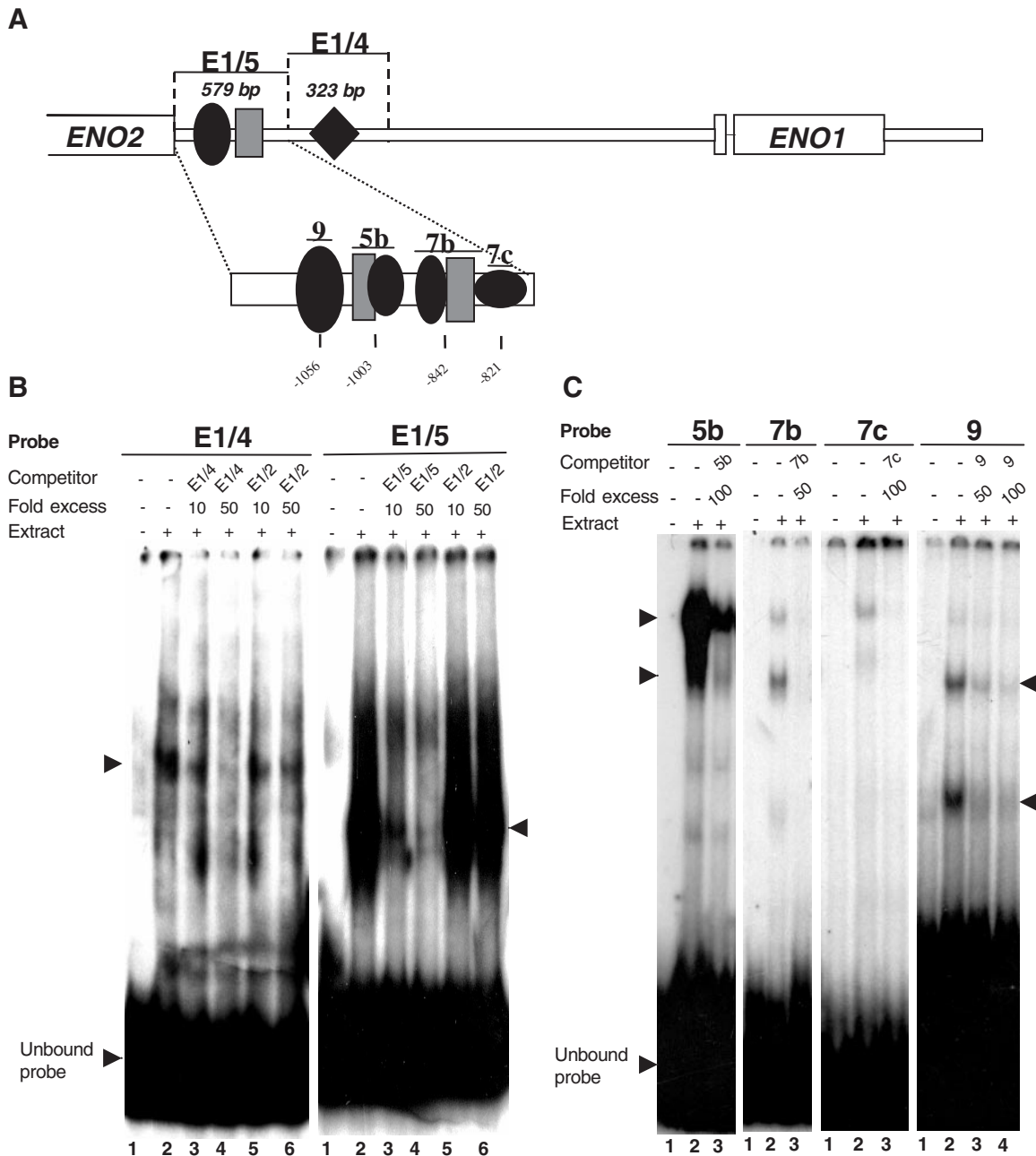
### The *ENO1* and *ENO2* upstream regions contain differentially regulated promoters

When the 3'-UTR of *SAG1* was replaced by the *ENO2* or *ENO1* 3'-UTRs to generate respectively the E2F10 and E1F9 constructs, a significant increase of CAT activity was observed as compared to E2F and E1F (Figure 2C). The repression of the *ENO1* promoter activity seen in the distal DNA element (fragment nucleotides from –1245 to –625) was still present and, this region appears to have no influence on the *ENO2* promoter activity when it is placed downstream of the CAT gene in the E2F10 construct (Figure 2C). The generation of these two constructs allowed us to investigate the transcriptional regulation occurring at both downstream and upstream regions of *ENO1* and *ENO2* during tachyzoite to bradyzoite conversion. To do this, we compared the CAT activity of promoter constructs with or without their respective 3'-UTRs during tachyzoite to bradyzoite conversion. The expression of each CAT construct was affected differently by incubation of transfected tachyzoites in low CO<sub>2</sub> growth conditions that induce conversion to the bradyzoite stage (19). While CAT expression from E2F and E2F10 was reduced to 2- to 6-fold in induced bradyzoites, that of E1F and E1F9 was induced to 10- to 25-fold (Figure 2C). On the other hand, the expression of the CAT-chimeric constructs driven by the

promoter and/or the 3'-UTR of the tachyzoite-specific *ENO2* gene was reduced 2- to 6-fold (50–85% reduction compared to CAT expression in tachyzoite) when the tachyzoites convert to bradyzoites while the constructs from the bradyzoite-specific *ENO1* gene were upregulated to 10- to 25-fold in the induced bradyzoite. The reduction or increase of CAT expression are consistently higher in E2F10 and E1F9 than E2F and E1F, confirming that these CAT-chimeric constructs with the respective 3'-UTR of *ENO1* and *ENO2* gave more pronounced CAT activity than constructs with the heterologous *SAG1* 3'-UTR.

### Evidence for specific protein–DNA interactions within the promoter of *ENO1*

To characterize the *cis*-regulatory elements and *trans*-acting factors that play a role in the transcriptional regulation of the *ENO1* gene in tachyzoites, we performed a series of EMSAs. DNA fragments from the intergenic region between the ATG initiation codon of the *ENO1* gene and the polyadenylation site of *ENO2* were generated by PCR and tested for protein binding in EMSA. We tested protein binding with a nuclear lysate made from purified tachyzoites. Out of the five PCR fragments from the promoter of *ENO1*, only two fragments located at the distal regulatory region of *ENO1* promoter bound proteins in the tachyzoite lysate. The fragments, referred to as E1/4 and E1/5 were 323 and 579 bp, respectively (Figure 3A). The larger DNA fragment bound several proteins strongly while the smaller fragment bound weakly to one protein (Figure 3B, lanes 2). The binding of proteins to fragment E1/4 and E1/5 was specific since competition with 10- to 50-fold excess of the same unlabelled fragment prevented binding to the labelled fragment (Figure 3B, lanes 3 and 4). Competition with similar concentrations (10- to 50-fold excess) of unlabelled non-specific fragment E1/2 had no effect on protein binding (Figure 3B, lanes 5 and 6, arrowheads). The specific regions that bind proteins in the 579 bp fragment were then determined with smaller overlapping PCR fragments. The fragment was divided into two overlapping fragments of 424 and 161 bp, respectively. The 424 bp fragment was further divided into seven fragments of decreasing sizes of 349, 296, 234, 76, 75, 63 and 49 bp, respectively. On the other hand, the 161 bp fragment was divided into two overlapping fragments of 90 and 93 bp, respectively. Two smaller overlapping fragments of 57 and 52 bp were obtained from the 93 bp fragment. All the fragments were labelled and tested for protein binding by EMSA. From these, only four fragments referred to as fragments 5b (63 bp), 7b (57 bp), 7c (52 bp) and 9 (49 bp) bound proteins in EMSA (Figure 3C). Double-stranded oligonucleotides were then designed with the information obtained from the EMSA studies and tested for protein binding. These oligonucleotides also bound proteins in EMSA with the same gel shift patterns, confirming the data obtained with the PCR fragments. Competition with specific oligonucleotides demonstrated that the oligonucleotides were binding proteins specifically. The sequences in the oligonucleotides were checked for homology to transcription factor binding elements in the TRANSFAC and TFSEARCH databases (28). The putative *cis*-regulatory elements identified in the distal regulatory elements of *ENO1* promoter and the consensus sequences for these elements are summarized in Figure 8C.



**Figure 3.** Evidence for specific protein–DNA interactions within the promoter of *ENO1*. (A) A schematic diagram showing the intergenic region between *ENO1* and the 3' end of *ENO2* which was used to generate DNA fragments for proteins binding in EMSA. Their relative positions upstream of the ATG codon are indicated. The only two fragments E1/4 and E1/5 binding specifically to proteins in EMSA with tachyzoite nuclear extracts are indicated. The numbers 9, 5b, 7b and 7c represent the probes/competitors described in (C). (B) Electrophoretic band shift assay showing DNA–protein complexes with DNA fragments E1/4 and E1/5 located at the distal promoter region of *ENO1*. Lane 1, free probe; lane 2, no competitor; lane 3, specific competitor (10-fold excess of the homologous cold fragment); lane 4, specific competitor (50-fold excess); lane 5, unspecific competitor (10-fold excess of *ENO1* fragment 2 or E1/2); and lane 6, same unspecific competitor (50-fold excess). The DNA–protein complexes are shown by arrowheads. (C) Gel shift binding assay with fragment 5b at the promoter of *ENO1* and the nuclear extract from tachyzoites. Lane 1, free probe; lane 2, no competitor; and lane 3, specific competitor (100-fold excess). Arrowheads show the DNA–protein complexes. Gel shift binding assays with fragments 7b and 7c. Lane 1, free probe; lane 2, no competitor; and lane 3, specific competitor (50-fold or 100-fold excess). DNA–protein complexes with fragment 9. Lane 1, free probe; lane 2, no competitor; lane 3, specific competitor (50-fold excess); and lane 4, specific competitor oligonucleotide (100-fold excess).

**Mutations in the stress response element (STRE) present in the *ENO1* promoter abolish protein binding in EMSA**

Oligonucleotide 5b1 (45 bp) was generated from part of the 63 bp PCR fragment 5b that gave the most intense signal in

EMSA. This strong signal corresponds to a complex of proteins with at least one intensely binding protein and at least two minor ones (Figure 3C, left panel). One of the protein binding *cis*-regulatory sequences in oligonucleotide 5b1 was found to be similar to the yeast stress response element (STRE) (29–31). The putative STRE element located within



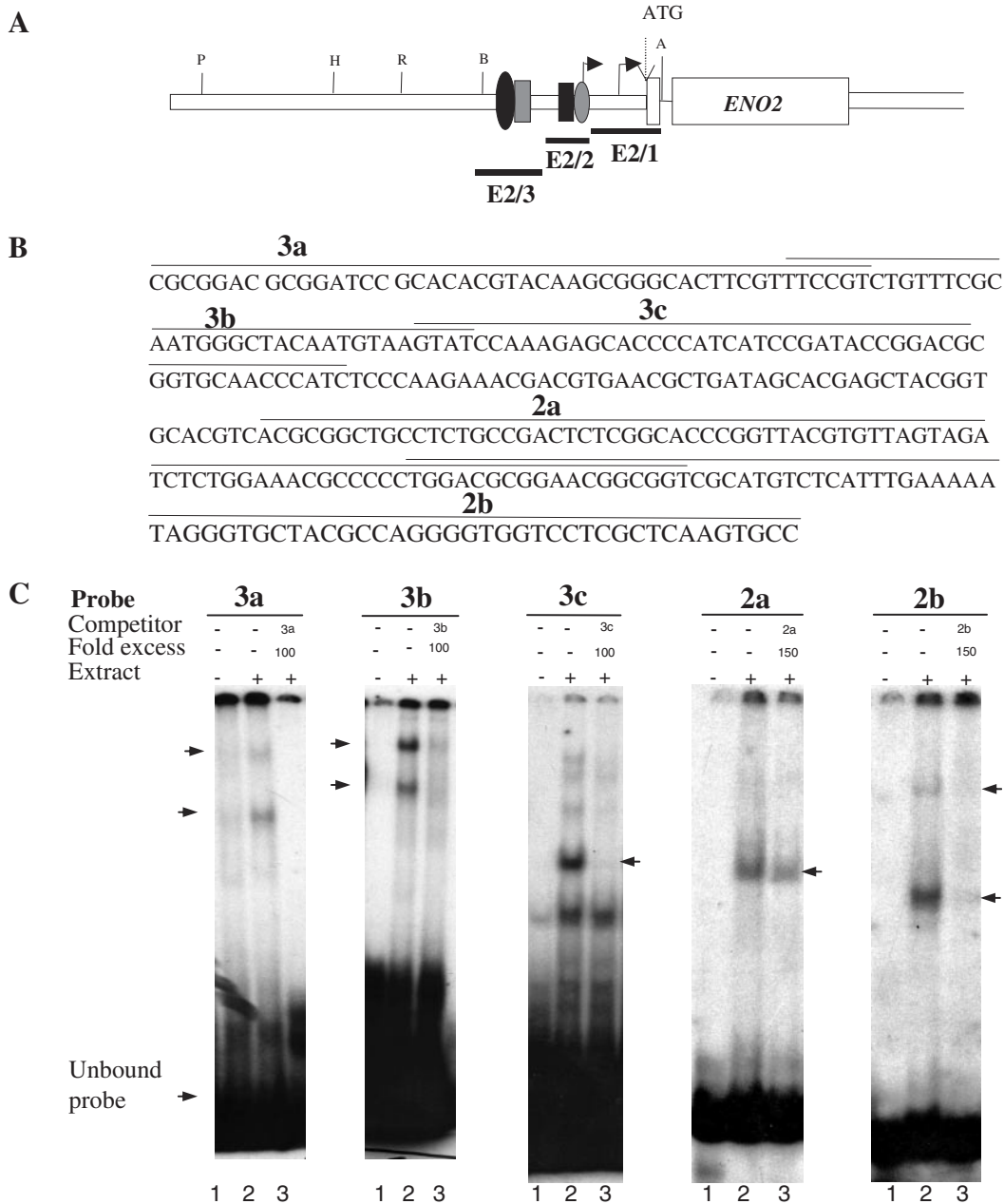


factors also bind to the oligonucleotide 5b1 in addition to STRE. This conclusion was strongly supported by the ability of oligonucleotide 5b1 to completely disrupt binding of *T.gondii* nuclear extract to the yeast canonical STRE (Figure 4B, lane 15). In addition, these results demonstrate that a STRE-like factor that is capable of specifically binding to yeast STRE is present in *T.gondii* nuclear lysate (Figure 4B, lane 13). Taken together, these data indicate that the STRE may play a central role in assembling the transcription complex that binds to oligonucleotide 5b1. It is possible that a

complex of two or more proteins is formed by protein–DNA and protein–protein interaction.

**Specific proteins binding to the promoter of ENO2**

From the 5' promoter region of *ENO2*, four fragments were generated by PCR and an additional four were generated by using restriction enzymes *EcoRI*, *BamHI*, *PstI* and *HindIII* (Figure 5A). Two other PCR fragments were generated from the *ENO2* intron. Fragment E2/1 was 191 bp and included

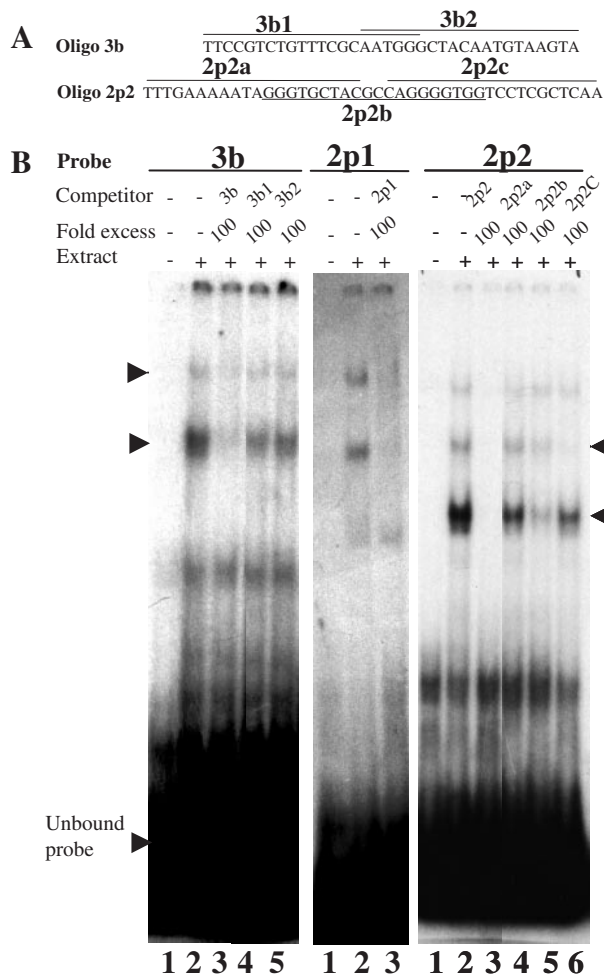


**Figure 5.** The proximal regulatory elements of *ENO2* promoter is a target for DNA-binding proteins. (A) A schematic representation showing the 5'-flanking region of *ENO2* that was used to generate DNA fragments for proteins binding in EMSA. The two fragments E2/2 and E2/3 binding specifically to proteins in EMSA using the tachyzoite nuclear extracts are indicated. (B) The nucleotide sequence of the contiguous E2/2 and E2/3 fragments is depicted with the oligonucleotides further designed for protein binding in EMSA. (C) Band shift assay with oligonucleotides from E2/2 and E2/3. Oligonucleotides 3a, 3b and 3c: lane 1, free probe; lane 2, no competitor; lane 3, specific competitor (50-fold or 100-fold excess); and oligonucleotides 2a and 2b: lane 1, free probe; lane 2, no competitor; lane 3, specific competitor (150-fold excess). The arrowheads show the DNA–protein complexes.

sequences of the first exon. Fragment E2/2 was 140 bp and extended up to  $-291$  bases upstream of the ATG codon. Fragment E2/3 was 162 bases and extended a few bases upstream of the BamHI site (Figure 5A). When these fragments were tested for protein binding in EMSA, only fragments E2/2 and E2/3 were found to specifically bind several proteins (data not shown). The nucleotide sequence of fragments E2/2 and E2/3 is shown in Figure 5B. To better define the interaction of the several proteins that bind to fragment E2/2 and E2/3, we have generated five PCR fragments from this region. Two fragments 2a and 2b of 82 and 74 bp were generated from fragment E2/2 (Figure 5B). On the other hand, fragment E2/3 was divided into three smaller overlapping fragments referred to as fragments 3a, 3b and 3c whose sizes ranged from  $\sim 40$  to 50 bp (Figure 5B). Gel shift assays using the labelled fragments showed that the fragments bound a particular set of proteins (Figure 5C, lanes 2). Using the information from EMSA analysis, double-stranded oligonucleotides were designed and these were tested for protein binding in EMSA. In all, three double-stranded oligonucleotides referred to as oligo 2p1, oligo 2p2 and oligo 3b were synthesized from the promoter of *ENO2*. The sizes of the oligonucleotides were 36, 42 and 35 bp, respectively. When these were tested on EMSA, it was apparent that some of the oligonucleotides bound more than one protein (Figure 6B, lanes 2). Oligonucleotide 2p1 bound two proteins whereas oligonucleotide 2p2 bound three major proteins and few minor ones revealed upon longer exposures (Figure 6B, left panel). The specificity of the oligonucleotides binding to the proteins was tested in assays where the corresponding unlabelled oligonucleotides were included in the binding reaction before the addition of the lysates. As can be seen from Figure 6B (lanes 3), 100 $\times$  molar excess of the unlabelled oligonucleotides was able to disrupt the specific binding. Competition experiments with two overlapping oligonucleotides of 3b1 and 3b2 (Figure 6A, upper sequence) showed that these two oligonucleotides 3b1 and 3b2 could not compete efficiently for protein binding with the labelled 3b oligonucleotide (Figure 6B, lanes 4 and 5 compare to lane 3). In contrast, it was found that oligonucleotide 2p2b and 2p2c could each compete for a single protein binding to oligonucleotide 2p2, the 42 bp oligonucleotide that bound to three or more different proteins (Figure 6B, right panel, lane 2 compared with lanes 5 and 6). To identify DNA regulatory elements that probably bind transcription factors, the oligonucleotide sequences were checked for homology to sequences in the TRANSFAC and TFSEARCH databases (Figure 8C).

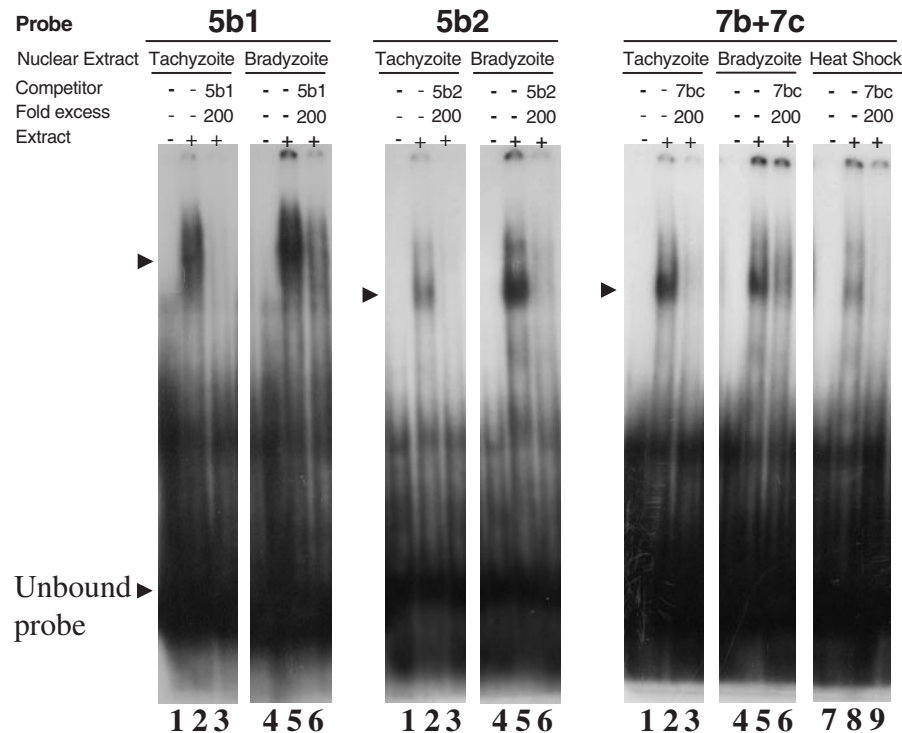
### The *ENO1* promoter activity and STRE binding are increased under stress-induced bradyzoite differentiation

In order to test the DNA–protein interactions under the stress conditions tested above, nuclear extracts were prepared from stress-induced bradyzoites ( $\text{CO}_2$  starvation method) and used for EMSAs. The specific DNA-binding activities in EMSAs described above have been obtained by comparing equal amount of nuclear extracts from non-stressed tachyzoites and stress-induced bradyzoites (Figure 7). In comparison to the tachyzoite EMSAs signals (Figure 7, lanes 2), these specific DNA-binding activities were significantly increased when the STREs (oligonucleotides 5b1 and 5b2 of *ENO1*



**Figure 6.** Localization of protein binding within the 35 and 42 bp DNA of oligonucleotides 3b and 2p2. (A) The sequence of oligonucleotide 3b and the extent of the oligonucleotides 3b1 and 3b2 used in the competition experiments using the tachyzoite nuclear extracts are shown. (B) Right panel: lane 1, free probe; lane 2, no competitor; lane 3, specific competitor (100-fold excess); and lanes 5 and 6 show competition with oligonucleotides 3b1 and 3b2, respectively (100-fold excess each). Arrowheads show the oligonucleotide–protein complexes. Band shift assay with oligonucleotides 2p1 and 2p2. The sequence of oligonucleotide 2p2 and the extent of the oligonucleotides 2p2a, 2p2b and 2p2c used in the competition experiments are shown in (A). Lane 1: free probe; lane 2, no competitor; and lane 3, specific competitor (100-fold excess). Lanes 4–6 show competition of labelled oligonucleotide 2p2 with the short overlapping self oligonucleotides shown in (A). Note the disappearance of the oligonucleotide protein complexes in lanes 5 and 6 (arrowed).

promoter) were incubated with nuclear extract from stress-induced bradyzoites (Figure 7, lanes 5 compare to lanes 2). In contrast, no marked difference was seen when the nuclear extract from stress-induced bradyzoites were used for DNA-binding activities with the oligonucleotides 7b+7c and 9 (data not shown) containing the putative heat shock elements (HSEs) of *T.gondii*. Importantly, neither the HSE-containing oligonucleotide 7b+7c (lane 8 compare to lane 2) nor the oligonucleotides 5b1 and 5b2 (data not shown) displayed an increased DNA-binding activities using nuclear extract from tachyzoites subjected to heat shock treatment. This suggests that the HSE-like motifs in the *ENO1* promoter may not be functional as heat shock-inducible elements under our experimental conditions.



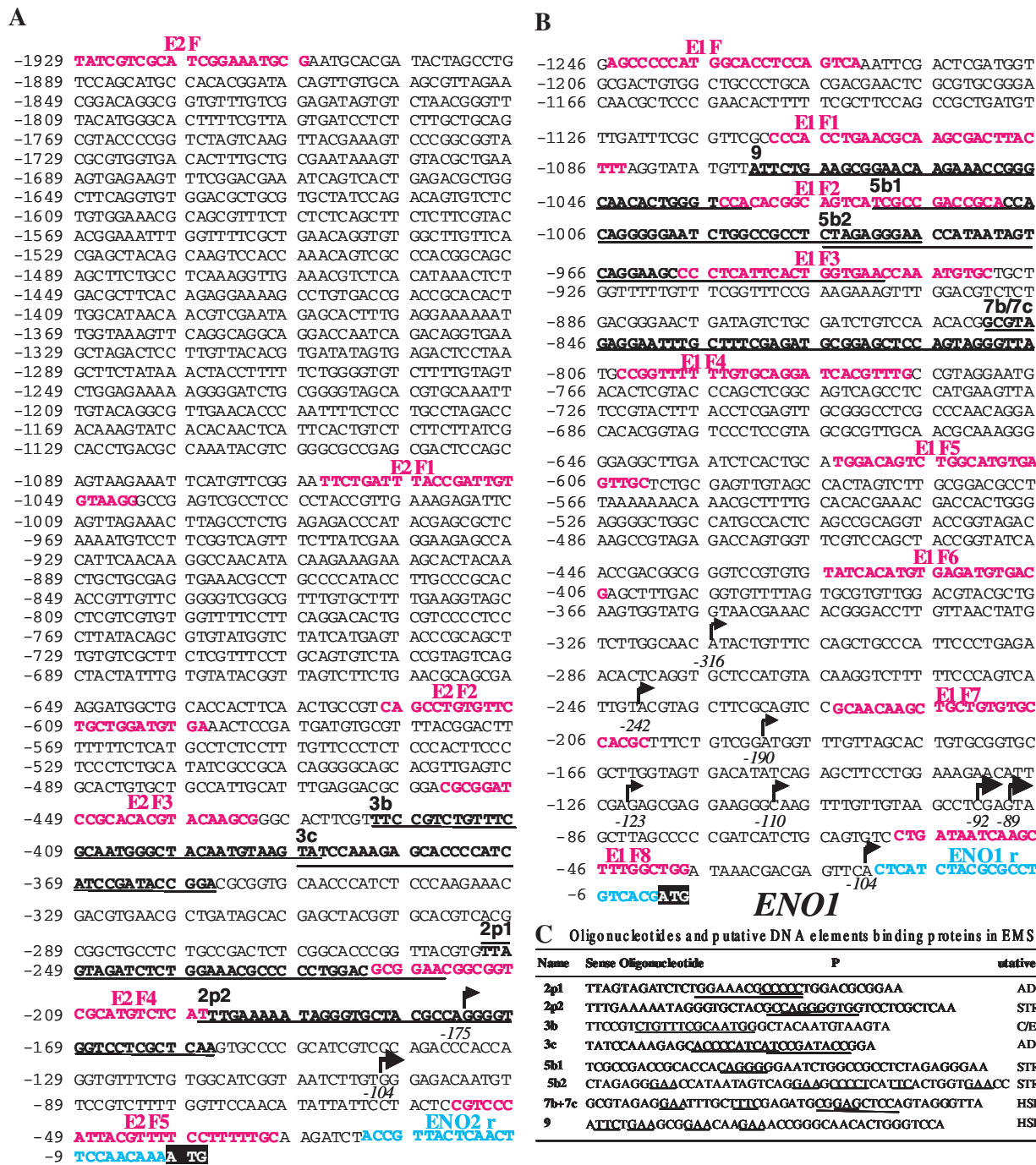
**Figure 7.** Increased DNA–protein interactions with nuclear extract from stress-induced bradyzoites. Electrophoretic band shift assays showing DNA–protein complexes with *ENO1* promoter DNA oligonucleotides containing STRE motifs (5b1 and 5b2) or HSE-like motifs (7b+7c) and nuclear extract from non-stressed tachyzoites (lanes 1–3), nuclear extract from stress-induced bradyzoites (lanes 4–6) or nuclear extract from heat shock treated tachyzoites (oligo 7b+7c, lanes 7–9). Equal amount of nuclear extracts have been used. Lane 1, free probe; lane 2, probe with tachyzoite nuclear extract and no competitor; lane 3, probe with tachyzoite nuclear extract and specific competitor (200-fold excess of the homologous cold fragment); lane 4, free probe; lane 5, probe with stress-induced bradyzoite nuclear extract and no competitor; lane 6, probe with stress-induced bradyzoite nuclear extract and specific competitor (200-fold excess); lane 7, free probe; lane 8, probe with nuclear extract from tachyzoite heat shock treated and no competitor; and lane 9, probe with tachyzoite nuclear extract and specific competitor (200-fold excess). Arrowheads show the DNA–protein complexes.

## DISCUSSION

To understand stage-specific gene transcription in the protozoan parasite *T.gondii*, it is necessary to identify regulatory DNA elements and the proteins binding to these elements. The available information on the DNA elements that bind to transcription factors in the *T.gondii* genome is sketchy. Only few elements have so far been characterized and shown to be critical for transcription of *T.gondii* genes (22–24). The current study was performed to identify the regulatory regions of *T.gondii* enolase genes that bind putative transcription factors. We summarize in Figure 8A–C, the deletion constructs for promoter activity, the transcriptional *cis*-regulatory elements and the putative *trans*-acting factors identified in the *ENO1* and *ENO2* genes. This is the first detailed demonstration by gel shift assay and mutagenesis of *cis*-regulatory elements in *T.gondii* promoters that bind to putative regulatory factors. The biochemical isolation and further characterization of these transcription factors are underway.

*T.gondii* enolase is a differentially expressed glycolytic enzyme (12,13). The two distinct enolases (*ENO1* and *ENO2*) are each expressed at only one stage *in vivo*. *ENO1* is expressed only in the bradyzoite stage and *ENO2* only in tachyzoites (13). Like *T.gondii* enolase, differential expression of other enolase genes is linked to stress factors, disease state or developmental transition. In mammals, enolase is a dimeric enzyme which exhibit cell type specific isoforms and is differentially

expressed in degenerating and regenerating skeletal muscle myofibres (32,33). *S.cerevisiae* like *T.gondii*, has two enolase genes designated *ENO1* and *ENO2* which are differentially expressed. *ENO2* is induced more than 20-fold in cells grown on glucose while *ENO1* is not (34,35). The expression of *ENO1* was found to be similar in cells grown on glucose and in cells grown on gluconeogenic carbon sources (35). The *ENO2* promoter was found to contain a *cis*-acting regulatory region mediating glucose-dependent induction of gene expression (34). Taken together, these studies show that stress induces transcription of enolase genes in *S.cerevisiae*, and *T.gondii* enolases may also be regulated by a similar mechanism. Differential gene expression in protozoan parasites probably serves as a strategy for these organisms to adapt to different environmental conditions. Environmental, oxidative, heat and nutrient limitation stresses are some of the stressful conditions that protozoan parasites encounter within or outside their hosts. Therefore, these parasites have to keep on changing the expression patterns of their genes in order to adapt to their current environment. The STRE-binding activity detected in nuclear extract from stress-induced bradyzoites was significantly higher than that from non-stressed tachyzoites. Because it has been documented that tachyzoite to bradyzoite conversion are induced by different stress conditions (8–10,36), it is tempting to speculate that the presence of stress responsive elements in the promoters of the *T.gondii* enolase genes may



## ENO2

**Figure 8.** (A and B) Overview of the results obtained by promoter analysis and by EMSAs. The promoter regions of *ENO1* and *ENO2* are shown. The forward primers used for CAT expressing constructs are indicated in red while the common reverse primers are shown in blue. The name of these constructs are displayed in red on top of the forward primers. The DNA fragments used in EMSAs are indicated by bold bars and underlined. The transcription start sites are shown by black arrows. The *cis*-regulatory elements identified are displayed as boldface, black, underlined sequences on top and at the beginning of the EMSA fragments. (C) The nature of *cis*-regulatory elements and the corresponding putative *trans*-acting or transcription factors are indicated in the table. The oligonucleotide sequences were checked for homology to sequences in the TRANSFAC and TFSEARCH databases.

be indicative of the responsiveness of these genes to a variety of stress conditions. Similarly, in *S.cerevisiae*, the multistress response element mediates induction of transcription by a variety of stresses including oxidative stress, nitrogen starvation,

glucose starvation and approach-to-stationary phase (29–31). The zinc finger transcription factors Msn2p and Msn4p are required for transcription induction through the STRE and disruption of the two genes results in sensitivity to a variety of

stresses (29). It is interesting to note that the *T.gondii* HSE without the canonical motif, such as yeast HSE, is also found in the *T.gondii* enolase promoters. In yeast, the single essential HSF binds constitutively to DNA and allows a wide range of distinct interactions of DNA binding domains within HSEs. Therefore, yeast cells utilize this single HSF to activate the expression of a wide variety of genes in response to heat and other stress, and to also coordinate the expression of genes required for growth under normal physiological conditions (37). Although the presence of STRE and HSE elements in the promoter does not imply functionality of these elements *in vivo*, it is interesting to note that both STRE and HSE elements have also been recently identified in *T.gondii* Hsp 70 genes (38). Examination of the *Toxoplasma* genome database (<http://toxodb.org>) does not reveal any obvious candidates homologous to known HSFs or STRE, suggesting that *T.gondii* transcriptional factors binding to these *cis*-regulatory sites may either be unrelated or distantly related to the classical HSF and STRE of yeast or higher eukaryotes. Interestingly, the generation of progressive deletions in the -1245 to -804 bp region of *ENO1* that removed the HSE and STRE motifs led to a significant increase of *ENO1* promoter activity in the tachyzoites whereas the presence of these *cis*-acting elements gave no obvious influence on promoter activity in the bradyzoites (Figure 2). This suggests that the region between -1245 and -804 bp contains *cis*-acting elements that are bound by negative regulators in the tachyzoites but not in the bradyzoites. In addition, further increase in *ENO1* promoter activity was observed in a construct lacking an additional DNA fragment of 179 bp, suggesting that the whole 620 bp region seems to participate in the stage-specific expression control. This also suggests that in addition to both STRE and HSE, other sequences may be involved in the stage expression control of promoter activity, indicating that there is an additional layer of complexity due to the involvement of other sequences.

Recent studies show that enolase has a multifunctional role depending on its subcellular localization. In *Arabidopsis thaliana*, enolase is required for cold acclimation and chilling resistance and its mutation impairs cold responsive gene transcription (39). In animal cells, an alternative transcription product of enolase referred to as MBP1 is a DNA binding transcription factor (40). MBP1 represses the expression of c-Myc proto-oncogene by binding to the TATA box, and thus preventing the TATA-binding protein's binding to the promoter (40). Interestingly, our recent localization of *T.gondii* enolases in the parasite nucleus suggests other possible function for this protein (41).

## ACKNOWLEDGEMENTS

We would like to thank Drs Martine Duterque-Coquillaud and Steven Ball for critically reading the manuscript. This work was supported by the Centre National de la Recherche Scientifique (CNRS) through the 'Action Thématique Incitative sur Programme et Equipe' (ATIPE). Michael Kibe and Alexandra Coppin were supported by fellowships from the CNRS and the Agence National de la recherche sur le Sida (ANRS), respectively. Funding to pay the Open Access publication charges for this article was provided by Centre National de la Recherche Scientifique (CNRS).

*Conflict of interest statement.* None declared.

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