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Identification and characterisation of a regulatory region in the *Toxoplasma gondii* hsp70 genomic locus[☆]

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Abstract

Toxoplasma gondii is an important human and veterinary pathogen. The induction of bradyzoite development in vitro has been linked to temperature, pH, mitochondrial inhibitors, sodium arsenite and many of the other stressors associated with heat shock protein induction. Heat shock or stress induced activation of a set of heat shock protein genes, is characteristic of almost all eukaryotic and prokaryotic cells. Studies in other organisms indicate that heat shock proteins are developmentally regulated. We have established that increases in the expression of bag1/hsp30 and hsp70 are associated with bradyzoite development. The *T. gondii* hsp70 gene locus was cloned and sequenced. The regulatory regions of this gene were analysed by deletion analysis using β -galactosidase expression vectors transiently transfected into RH strain *T. gondii*. Expression was measured at pH 7.1 and 8.1 (i.e. pH shock) and compared to the expression obtained with similar constructs using *BAG1* and *SAG1* promoters. A pH-regulated region of the Tg-hsp70 gene locus was identified which has some similarities to heat shock elements described in other eukaryotic systems. Green fluorescent protein expression vectors driven by the Tg-hsp70 regulatory region were constructed and stably transfected into *T. gondii*. Expression of green fluorescent protein in these parasites was induced by pH shock in those lines carrying the Tg-hsp70 regulatory constructs. Gel shift analysis was carried out using oligomers corresponding to the pH-regulated region and a putative DNA binding protein was identified. These data support the identification of a pH responsive *cis*-regulatory element in the *T. gondii* hsp70 gene locus. A model of the interaction of hsp70 and small heat shock proteins (e.g. BAG1) in development is presented.

Keywords

Toxoplasma gondii; hsp70; Gene regulation; Differentiation; Transfection; Apicomplexa; Heat shock response; Heat shock element; Transcription factors

1. Introduction

Toxoplasma gondii is a ubiquitous apicomplexan parasite of mammals and birds. It has long been recognised as an important opportunistic pathogen of immunocompromised hosts and has emerged as a major opportunistic pathogen of the AIDS epidemic (Luft and Remington, 1992; Wong and Remington, 1993). Although overwhelming disseminated toxoplasmosis has been reported, the predilection of this parasite for the central nervous system (CNS) causing necrotising encephalitis constitutes its major threat to patients with HIV infection (AIDS). The development of *Toxoplasma* encephalitis is believed due to the transition of the resting or latent bradyzoite stage to the active rapidly replicating form, the tachyzoite stage (Frenkel and Escajadillo, 1987; Weiss and Kim, 2000). It is likely that in chronic toxoplasmosis tissue cysts (bradyzoites) regularly transform to tachyzoites and that these active forms are removed or sequestered by the immune system. Such a dynamic equilibrium between encysted and replicating forms would lead to recurrent antigenic stimulation, possibly accounting for the life-long persistence of antibody titers found in chronically infected hosts (Weiss et al., 1988).

It is now recognised that stress conditions are associated with the induction of bradyzoite development, i.e. there are more bradyzoites under these conditions than would be expected from a simple inhibition of tachyzoite replication (Bohne et al., 1999; Weiss and Kim, 2000; Lyons et al., 2002). Temperature stress (43 °C (Soete et al., 1994)), pH stress (pH 6.8 or 8.2 (Soete et al., 1994; Weiss et al., 1995)) or chemical stress (sodium arsenite, nitric oxide, sodium nitroprusside (Bohne et al., 1993; Soete et al., 1994; Weiss et al., 1995; Soete and Dubremetz, 1996)) result in an increase in bradyzoite antigen expression and the development of cyst-like structures of *T. gondii* in vitro. In bone marrow derived murine macrophage lines, interferon gamma (IFN- γ) increased bradyzoite antigen expression and this appeared to be related to nitric oxide (NO) production (Bohne et al., 1993). These stressors probably function by slowing replication allowing differentiation to occur at a particular point in the *T. gondii* cell cycle (Bohne et al., 1994; Radke et al., 2003). Bradyzoite differentiation probably has features in common with other stress induced differentiation systems such as glucose starvation and hyphae formation in fungi or spore formation in *Dictyostelium* (Soderbom and Loomis, 1998; Thomason et al., 1999; Estruch, 2000). These systems have demonstrated unique proteins related to specific differentiation structures in each organism as well as the utilisation of phylogenetically conserved pathways. Many of these signalling pathways involve cyclic nucleotides and kinases as part of the regulatory system in differentiation. Heat shock proteins are also involved in these pathways as chaperones for both regulatory and stage-specific proteins involved in differentiation.

The heat shock proteins fall into several subfamilies, namely, the low molecular weight (16–35 kDa) or small heat shock proteins (smHSP), the hsp60 family, the hsp70 family (68–78 kDa), and the high molecular weight (89–110 kDa) families (hsp90 and hsp100) (Morimot et al., 1994). These proteins interact in the regulation of many host cell responses and act as chaperones. The various heat shock proteins chaperone different sets of proteins. For example, hsp90 is known to interact with proteins involved in many of the signalling pathways and smHSPs have been associated with cytoskeletal proteins as well as with proteins induced during developmental processes (Morimot et al., 1990; Noyer, 1991; Morimot et al., 1994). Heat exposure, chemical agents (sodium arsenite), mitochondrial inhibition (2,4 dinitrophenol, sodium azide and other uncouplers of oxidative phosphorylation), transition series metals, hydrogen peroxide and anaerobic conditions are all associated with the induction of heat shock proteins (Morimot et al., 1994). Many of these agents are associated with bradyzoite induction in vitro (Soete et al., 1994; Tomavo and Boothroyd, 1995). In addition to affecting gene expression, heat shock can also change

cellular metabolism. For example, in *Xenopus* heat shock results in an interruption of oxidative phosphorylation leading to anaerobic glycolysis (Nickells and Browder, 1985; Heikkila, 1993a, b). A change in metabolic pathway utilisation is believed to occur during bradyzoite differentiation with the expression of stage-specific glycolytic enzymes (Weiss and Kim, 2000; Tomavo, 2001).

It has been previously determined that a *T. gondii* hsp70 homologue (AF045559, U85649, U85648) is induced during bradyzoite differentiation (Lyons and Johnson, 1995, 1998; Weiss et al., 1998; Miller et al., 1999). In *T. gondii* exposed to pH 8.1 compared to pH 7.1 media there is a 4-fold induction of hsp70 (Weiss et al., 1996, 1998). This 3- to 4-fold change in hsp70 in *T. gondii* is similar to the magnitude of the hsp70 response seen in *Trypanosoma cruzi*, *Theileria annulata* and *Plasmodium falciparum* during stress or differentiation (Shiels et al., 1997; del Cacho et al., 2001). The current study was performed to identify a regulatory region of the *T. gondii* hsp70 gene that is associated with stress induced transcription, and that may be involved in the differentiation program in this organism. This is the first demonstration in *T. gondii*, by gel shift assay, of a putative regulatory protein binding to a *cis*-regulatory element.

2. Materials and methods

2.1. Identification of *T. gondii* hsp70 genomic clones

Primers hsp70-158f 5'-CCIGCITA(T/C) TT(T/C)AA (T/C)GA-3', hsp70-385R 5'-GCIACIGC(T/C)TC (A/G)-TCIGG-3' were used to amplify a 680 bp amplicon from a *T. gondii* hsp70 cDNA clone and the amplicon was cloned into a TA PCRII vector (Invitrogen-Gibco-BRL, Gaithersburg, MD), taking advantage of the 5' a overhang generated by Taq polymerase. The plasmid, Tg-hspB3, containing the insert was subsequently purified using Wizard Plus (Promega, Madison, WI).

Dilutions of a RH *T. gondii* genomicDNA library in λ DASHII (NIH reagent 2862) were incubated with the XL1-Blue MRF' *Escherichia coli*, plated in Top Agar (0.8% agar in LB broth) on LB plates and incubated for 42 °C for 18 h. Plates containing plaques were overlaid with a nitrocellulose membrane, the membrane lifted after 5 min, membrane bound DNA was then denatured with 0.5 N NaOH/1.5 M NaCl and neutralised with 1 M Tris-HCl (pH 7.5)/1.5 M NaCl treatment followed by UV cross-linked using 0.600 J/cm² in an ISS UV Translink (Natick, MA). Nitrocellulose membranes were then prehybridised in Dig Easy Hyb (Boehringer Mannheim, Indianapolis, IN) for 3 h at 42 °C followed by hybridisation in Dig Easy Hyb containing 7.5 ng/ml of heat denatured digoxigenin random primer labeled Tg-hspB3 insert [High Prime Digoxigen DNA Labeling Kit (Boehringer Mannheim)] for 6 h. The membranes were then washed twice for 5 min at 25 °C with 2 × SSC/0.1% SDS and for 15 min once at 68 °C with 0.1 × SSC/0.1% SDS. Membranes were then incubated for 5 min in maleic acid buffer (100 mM maleic acid/150 mM NaCl, pH 7.5) containing 3% (v/v) Tween 20 and blocked for 2 h in maleic acid buffer containing 1% blocking reagent (Boehringer Mannheim). Membranes were then incubated with a 1:5000 dilution of alkaline phosphatase conjugated anti-DIG antibody (Boehringer Mannheim) in the blocking solution for 30 min, washed twice for 15 min in maleic acid buffer containing 3% (v/v) Tween 20 and reactive plaques detected with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) in 100 mM Tris-HCl/100 mM NaCl (pH 9.5) over 2 h. Eight clones were identified (G1-1, G1-2, G2-1, G2-2, G3-1, G3-2, G3-3, G3-4) and subsequently plaque purified. Clones were excised out of the λ -ZAP phage as an ampicillin-resistant pBS phagemid vector using the ExAssist interference-resistant helper phage and XL0LR bacterial strain of *E. coli* [Lambda ZAP cloning Kit (Stratagene, La Jolla, CA)]. Plasmid DNA was subsequently purified using Wizard Plus (Promega).

Clone G3-1 containing an insert of about 15,500 bp was mapped by restriction digestion using *Eco* RI, *Pst* I, *Bam* HI, *Eco* RV, *Hind* III, *Sac* I, *Apa* I, *Xha* I, *Sma* I, *Sac* I, *Kpn* I or *Xba* I followed by agarose gel electrophoresis and Southern blotting using Tg-hspB3 as a probe. A 1700 bp fragment that hybridised to Tg-hspB3 was subcloned into pBluescript SK[−] and sequenced on an ABI Prism Model 377 DNA Sequencer (Perkin Elmer Corporation, Foster City, CA) using T7 and T3 oligomers flanking the insertion site. Additional sets of 18–20 bp oligomers were synthesised based on observed sequence data in order to sequence the clones in both directions. This sequence demonstrated that this clone Tg-hsp70-1700 contained about 1000 bp that were upstream of the initial ATG codon of hsp70 and 700 bp of the hsp70 coding region. In addition, these primers were used to obtain sequence data from the hsp70G 3–2 clone and primers were used to verify the sequence of the hsp70 locus in this genomic clone. Sequencing oligomers were selected using Oligo Primer Analysis Software (NBI, Plymouth, MN). Sequence data sets were assembled using the SeqMan program of the LaserGene Software Package (Dnastar, Madison, WI) (Burland, 2000).

2.2. Isolation of RNA and identification of transcription start of Tg-hsp70

High quality RNA was isolated from RH strain *T. gondii*, purified from human fibroblast culture by 3 mm nucleopore filtration, using TRIZOL reagent system (Invitrogen-Gibco-BRL) which is modified guanidine isothiocyanate/acid phenol method. A 5' rapid amplification of cDNA ends (RACE) technique (Invitrogen-Gibco-BRL) was used to obtain the transcription start site. First stand cDNA synthesis was performed for 50 min at 42 °C with 5 µg *T. gondii* RNA, 100 nM primer Tg-hsp70GSP1: 5'GCTGA-GGCCGGCAATGGT3' and 200 U SuperScript II reverse transcriptase in reaction buffer (20 mM Tris-HCl (pH 8.4), 50 mM KCL, 2.5 mM MgCl₂, 10 mM DTT, 400 µM dATP, 400 µM dCTP, 400 µM dGTP, and 400 µM dTTP). To produce 5'dC tailed cDNA, 10 µl of GlassMAX spin cartridge purified cDNA was incubated with 200 µM dCTP and 1 µl of terminal deoxynucleotidyl transferase in tailing buffer (10 mM Tris-HCl (pH 8.4), 25 mM KCL, 1.5 mM MgCl₂). dC tailed cDNA was then amplified by PCR using the primer set Tg-hsp70GSP2: 5'CGACCGCGATTTC CTTCATTTTG3' and 5' RACE Abridged Anchor Primer 5'GGCCACGCGTCTGACTAGTACGGGIIGGGIIGGGII-IIG3' in PCR buffer (20 mM Tris-HCl (pH 8.4), 50 mM KCL, 1.5 mM MgCl₂, 200 µM dATP, 200 µM dCTP, 200 µM dTTP, 200 µM dGTP, 2.5 U Taq polymerase). Amplification was for 35 cycles (94 °C, 1 min; 55 °C, 1 min; 72 °C, 1 min) with a terminal 72 °C extension for 5 min. The amplicon was purified using a GlassMax spin column and *Not* I and *Acc* I sites were generated using the exonuclease activity of T4 DNA polymerase by incubating the amplicon in the presence of dTTP, dATP and T4 DNA polymerase in Tris acetate buffer. The amplicon was then ligated into *Not* I-*Acc* I cut pBlueScript KS[−]. Three clones were sequenced on a ABI Prism Model 377 DNA Sequencer (Perkin Elmer Corporation, Foster City, CA) using T3, M13 reverse and T7 primers.

2.3. Northern blot of Tg-hsp70

Total RNA (20 µg), isolated from *T. gondii* ME49 and RH strain and was electrophoresed on 1% agarose/formaldehyde gels and capillary blotted to nylon membranes using standard procedures. Nylon membranes were then prehybridised in Dig Easy Hyb (Boehringer Mannheim) for 3 h at 42 °C followed by hybridisation in Dig Easy Hyb containing 7.5 ng/ml of heat denatured digoxigenin random primer labeled Tg-hspB3 insert [High Prime Digoxigen DNA Labeling Kit (Boehringer Mannheim)] overnight at 42 °C. The membranes were then washed twice for 20 min at 25 °C with 2 × SSC/0.1% SDS and for 20 min once at 65 °C with 0.1 × SSC/0.1% SDS. Membranes were then incubated for 5 min in maleic acid buffer (100 mM maleic acid/150 mM NaCl (pH 7.5)) containing 3% (v/v) Tween 20 and blocked for 2 h in maleic acid buffer containing 1% blocking reagent (Boehringer Mannheim). Membranes were then incubated with a 1:5000 dilution of alkaline phosphatase

conjugated anti-DIG antibody (Boehringer Mannheim) in the blocking solution for 30 min, washed twice for 15 min in maleic acid buffer containing 3% (v/v) Tween 20. Detection of bound antibody was performed using the Western Light Kit (Tropix Inc., Bedford, MA) employing chemiluminescence using CSPD[®]. Gel analysis and densitometry was performed on bands using Sigma Gel (version 1.05) (Jandel Scientific Software, San Rafael, CA).

2.4. Construction of promoter expression plasmids Tg-hsp70/ β -gal and Tg-hsp70/GFP

In order to study the promoter activity, a 951 kb fragment of 5' upstream region of Tg-hsp70 was derived by digesting Tg-hsp1700 with *Kpn* I and *Nco* I. This fragment was then ligated into the SAG1/ β -gal plasmid that had been digested with *Kpn* I and *Nco* I. SAG1/ β -gal is a plasmid that has the 5' upstream region of SAG1 and the 3'UTR of SAG1 flanking a β -galactosidase gene (McFadden et al., 1997). This plasmid can be used to study promoter activity as measured by the induction of β -galactosidase in transfected *T. gondii*. The ligation reaction resulted in the 951 bp fragment being placed upstream of β -galactosidase (β -gal) gene replacing the SAG promoter with the Tg-hsp70 promoter region. Previously, we had determined in cDNA clones that the poly (A) tail of *hsp70* mRNA starts 230 bp downstream of the *hsp70* stop codon, therefore, this 3' flanking region was used to replace the 3'UTR of SAG1 in the construction of the *hsp70* promoter plasmid. The 230 bp fragment was amplified by using a Tg-hsp70 cDNA clone that we had previously subcloned in pBluescript as a template and primers, 3'Tg-hsp70 PacIF: 5'CCTTAATTAAGTGTG-AAGCGGAAAG3' and 3'Tg-hsp70 NotIR: 5'ATAGTTTA-GCGGCCGCGAGAAAGTTGATGC3', that added a *Pac* I site at the 5' and a *Not* I site at the 3' end of the amplicon. The Tg-hsp70/ β -gal construct was digested with *Pac* I and *Not* I to remove the 3'UTR of SAG1 and the 230 bp fragment digested with *Pac* I and *Not* I was ligated into the Tg-hsp70/ β -gal plasmid at the 3' end of β -galactosidase gene. This construct, Tg-hsp70P, was used to test the promoter activity of the 951 bp region in driving the β -gal gene expression.

The following deletion constructs were made in order to locate the promoter region in the *hsp70P* construct. A 641 bp fragment was generated by digesting the *hsp70P* plasmid with *Kpn* I and *Ehe* I to remove 310 bp followed by ligation of the plasmid. This plasmid was designated *hsp70-640*. A 410 bp fragment was obtained by digesting the *hsp70/ β -gal* plasmid with *Kpn* I and *Nsi* I to remove 541 bp followed by ligation of the plasmid. This plasmid was designated *hsp70-410*. Another fragment containing -1 to -200 upstream of the start site of *hsp70P* was generated by PCR technique by amplifying *hsp70P* primer: Tg-*hsp70-200F* 5'GGGGTACCGTGTGCTGTCCGGTACGAG3' containing a *Kpn* I and Tg-*hsp70-200R* 5'CATGCCA-TGGCATGTTGTCTTCTGCAGG3' containing a *Nco* I site. Subsequently, this amplicon was ligated into the *Kpn* I/*Nco* I cut SAG1/ β -gal plasmid. This plasmid was designated *hsp70-200*. Inverse PCR was used in order to delete a 79 bp region within *hsp70P*: the *hsp70P* plasmid was amplified with primers containing *Spe* I restriction sites, Tg-*hsp70* DelF: 5'GACTAGTGGCTTGAACAGCGA-GATTTGCAC3' and Tg-*hsp70* DelR: 5'ACTAGTCTAC-ACTCCTTTGCGCTCCTTAC3'. The amplicon which lacked the 79 bp region -340 to -429 was restricted with *Spe* I and then ligated to produce the intact plasmid. This construct was designated 429 Δ 340. All of the constructs were sequenced to confirm the correct orientation and confirm deletions.

For the construction of a Tg-hsp70 promoter GFP vector the 951 kb fragment derived by digesting Tg-hsp1700 with *Kpn* I and *Nco* I was ligated into a *Kpn* I and *Nco* I digested GRA1/GFP/GRA2-SK vector (Striepen et al., 1998; Kim et al., 2001) replacing the GRA1 promoter in this vector with the Tg-hsp70 promoter region. This *hsp70P/GFP/GRA2-SK* construct was used to test the promoter activity for driving GFP expression in transient transfection assays and to produce stable transformants in *T. gondii* PLK strain. Inverse PCR was used, as described above, to produce a 79 bp internal deletion in the *hsp70P/GFP/*

GRA2-SK construct. This construct was designated *429Δ340/GFP/GRA2-SK*. All of the constructs were sequenced to confirm the correct orientation and confirm deletions.

2.5. Construction of *BAG1/β-gal* plasmid

We had previously identified a 16 kb genomic clone containing the *BAG1* locus from a PLK strain *T. gondii* genomic library in λDASHII (NIH reagent depository catalogue number 2863) and subcloned this clone into a pBluescript KS + plasmid which we designated Bg2 (Zhang et al., 1999b). A restriction map of this clone had been previously obtained in our development of a *T. gondii* *BAG1* knockout (Zhang et al., 1999b). The Bg2 plasmid was therefore digested with *Sac* I to yield a fragment containing the 5' upstream region and exon 1 of the *BAG1* gene. This band was subcloned into pBluescript KS + and the presence of the 5' upstream region and *BAG1* exon1 confirmed by sequencing with T7 and T3. The 5' upstream region of *TgBAG1* starting at the initial ATG was then cloned from this plasmid by PCR using T3: 5'TTAATTGGGAGTGATTCCCC3' and *BAG1* NR: 5'Nsi-CATCTTTTTTGAATATCATACG3'. The amplicon was digested with *Hind*III and *Nsi* I and this fragment was ligated into a *GRA1/β-gal* plasmid (gift of Dr David Sibley) that had been digested with *Sac* I and *Nsi* I to produce the *BAG1/β-gal* plasmid.

2.6. *T. gondii* isolation and culture

RH (a type I strain), ME49 (a type II strain) and PLK (a type II strain that is a clonal isolate of ME49) *T. gondii* were maintained by twice weekly passage of the parasites into confluent flasks of human fibroblasts [ATCC CRL 1475 (CCD-27SK)] (Weiss et al., 1995). Dulbecco's modified Eagle's Medium (DME) supplemented with 10% foetal calf serum (Invitrogen-Gibco-BRL) and 1% Penicillin–Streptomycin was replaced weekly. Fibroblasts were subcultured weekly using 0.25% trypsin/0.03% EDTA at a subcultivation ratio of 1:4 and used between passage 6 and 30. To produce pH buffered DME 10 mM HEPES was added and the pH was adjusted to 7.1 or 8.1.

2.7. Chemicals

Indomethacin (Sigma, St Louis, MO) was dissolved in ethanol at 25 mg/ml. Ethanol was added in similar concentrations to controls. Dimethyl sulfoxide (DMSO) and ethanol had no effect on bradyzoite formation in the concentrations used. Sodium nitroprusside was used as a nitric oxide (NO) donor, a stock solution of 10 mg/ml in distilled water was diluted in media prior to use.

2.8. Transient and stable transfection of *T. gondii*

Electroporation of parasites was performed using the methods we have previously published for the disruption of the *BAG1* gene (Zhang et al., 1999b) employing 10⁷ freshly harvested parasites for each transfection of 50 µg of circular plasmid in a volume of 800 µl of cytomix. RH strain *T. gondii* was used for transient transfection of the β-galactosidase and GFP plasmid constructs. The PLK (ME49) strain was used for stable transfection of GFP plasmid constructs. All β-galactosidase constructs were co-transfected with 25 µg of circular pT230/CAT plasmid containing a TUB1 promoter upstream of chloramphenicol acetyltransferase (*cat*), e.g. a ratio of 2:1 for β-galactosidase to *cat* plasmid constructs. pT230/CAT provided an independent standard for the efficiency of transfection. All plasmids were dissolved in cytomix prior to being added to parasites in cytomix in the electroporation cuvette. Parasites were then transfected by electroporation with a single pulse using a BTX Electro Cell Manipulator 600, charging voltage 2.0 kV and a resistance of 48 Ω. After transfection parasites were transferred into T25 flasks with human fibroblast cells. In order to evaluate the effect of pH on promoter activity parasites were cultured at both pH 7.1 and pH 8.1 media for 24 h post-transfection.

In order to select stable transformants chloramphenicol was added into the media of GFP transfected PLK strain parasites at a final concentration of 40 μ M, a concentration that allowed no detectable growth of wild type parasites. After 3 weeks under chloramphenicol selection, parasites were aliquoted into 96 well plates at 1:10, 1:100 and 1:1000 dilutions and cultured for 1 week. Wells containing single plaque colonies were selected and subcloned. Expression of GFP was detected by fluorescence microscopy using a Nikon Diaphot inverted microscope with a UV-A filter cube as well as by immunoblot employing an anti-GFP monoclonal antibody (Clontech, Palo Alto, CA).

2.9. Immunoblot

Organisms were purified from human fibroblasts by rupture with a 27 gauge needle followed by filtration through a 3.0 μ m nucleopore filter and equal numbers of organisms were dissolved in gel sample buffer (Weiss et al., 1988). Equal amounts of organism (by counting of extracellular organisms in a hemocytometer) were loaded onto SDS-PAGE gels, electrophoresed and transferred to nitrocellulose as previously described (Weiss et al., 1988). The amount of GFP expression was ascertained by immunoblotting using a 1:1000 dilution of an anti-GFP monoclonal antibody (Clontech, Palo Alto, CA) followed by detection using the Western Light Kit (Tropix Inc., Bedford, MA) employing chemiluminescence with CSPD and an alkaline phosphatase labeled secondary anti-mouse IgG antibody (1:10,000 dilution). Gel analysis and densitometry was performed on bands using Sigma Gel (version 1.05) (Jandel Scientific Software, SanRafael, CA).

2.10. Chloramphenicol acetyltransferase and β -galactosidase activity assays

T. gondii infected human fibroblast cell monolayers were rinsed three times with PBS (pH 7.2), scraped off and resuspended in 110 μ l of 250 mM Tris (pH 7.5). After three cycles of freezing/thawing, an aliquot of the cell lysate was used to determine both chloramphenicol acetyltransferase (CAT) and β -galactosidase activity. Protein concentrations of parasite lysates were determined by Bio-Rad assay. CAT assays were performed using a non-radioactive FAST CAT (deoxy) Green Chloramphenicol Acetyltransferase Assay Kit (Molecular Probes, Eugene, OR). CAT activity was measured using thin liquid chromatography (TLC) as the percentage BODIPY 1-deoxychloramphenicol (BDC) that was acetylated in the presence of *T. gondii* extract and acetyl CoA. TLC plates were analysed using a Storm[®] (Amersham Biosciences Corp., Arlington Heights, IL) imaging system for autoradiographic and non-radioactive (fluorescent and chemifluorescent) detection and quantitation of images. Percent conversion (PC) of the substrate was calculated as the measured fluorescent intensity of acetylated BDC/(acetylatedBDC plus BDC). The usual PC was 50% and the transfection efficiency correction factor (TCF) was therefore set up as TCF = PC/50. The β -galactosidase activity assay was performed using 4-methylumbelliferyl- β -D-galactopyranoside (MUG) 0.1 mg/ml as a substrate and fluorescence at 460 nm was measured in a Hoefer TKO100 Mini-Fluorometer (excitation 365 nm). Diluted samples (1:100 and 1:1000) were used for replicate measurements of β -galactosidase activity to obtain fluorescence intensity (FI) values in the range of 0–500 and these values were then standardised to the reflect the FI resulting from 50 μ g of *T. gondii* lysate. The β -galactosidase FI was then corrected (FIc = FI/TCF) for the measured CAT activity in order to correct the FI values for any differences in the efficiency of transfection that occurred between experiments. All experiments were performed in triplicate and the FIc values are used for analysis in the figures.

2.11. Electrophoretic mobility shift assay

The following oligonucleotides and their complementary oligomers were synthesised: Tg-hsp70-REG: 5'TGCAAGAAAGAAAGGCTTTC GGAAAGGAAACCG3', Tg-hsp70-CTL: 5' TGCAATAAATAAAGGATTCGTAAAGGA AACCG3', BAG1P 5'TAGAGA

AAGGCAGAAGGCCGGAG CGTTTTCTCAG3'. Oligonucleotides were synthesised with and without a biotin tag on the 5' end and the complementary primer pairs annealed to produce double stranded labeled oligomers. Unlabeled double stranded oligomers were produced by annealing complementary oligomers that were synthesised without a biotin tag at the 5' end of the oligomer. Electrophoretic mobility shift assay (EMSA) was performed by using either the LightShift™ Chemiluminescent EMSA Kit (Pierce, Rockford, IL) following the manufacturer's instructions or a ³²P end labeled oligomer gel shift assay system (Promega) (Huang et al., 1999).

Protein lysate was prepared from 3.0 µm nucleopore filtration purified ME49 strain *T. gondii* (Weiss et al., 1988; Zhang et al., 1999a). By microscopy no host cells were seen in the purified parasite preparations. Parasites were sonicated in buffer B [20 mM Hepes (pH 8.0), 0.1 KCL, 0.5 mM DTT, 0.2 mM EDTA, 20% glycerol and one protease inhibitor cocktail tablet (Roche, Germany) per 10 ml of solution]. The whole parasite lysate was centrifuged at 3000 × g in order to remove insoluble materials. The protein concentration of the lysate was measured and 50 µg aliquots were stored at -80 °C.

For the LightShift™ Chemiluminescent EMSA binding assay 15 µg of lysate was incubated with biotin labeled oligonucleotides for 20 min at room temperature in 10 mM Tris (pH 7.5), 50 mM KCL, 1 mM DTT, 5 mM MgCl₂, 2.5% glycerol, 50 ng/µl poly (dI-dC). For competition assay, excess amounts of unlabeled oligonucleotides (10 ×, 20 × and 50 ×) were added to the reaction before the biotin labeled oligonucleotides was added. The reaction was added to loading buffer and electrophoresis was performed at 100 V for 3 h using a 5% native polyacrylamide gel and 0.5 × TBE buffer. The gel was then transferred overnight onto a nylon membrane using a capillary transfer method employing 20 × SSC as the transfer buffer. The membrane was cross-linked at 120 mJ/cm² using a UV cross-linker (254 nm bulbs), blocked for 15 min in LightShift blocking buffer and incubated with a 1:300 dilution of LightShift™ stabilised streptavidin horseradish peroxidase conjugate. The membrane was then washed four times in 1 × LightShift™ wash buffer, incubated for 5 min in Light-Shift™ substrate equilibration buffer and then LightShift™ luminol/enhancer with peroxide was placed on the membrane and it was wrapped in plastic wrap. X-ray film was then exposed to the membrane for 2–5 min in the dark and developed.

For the ³²P EMSA assay oligomers were labeled using T4 polynucleotide kinase and γ-³²P ATP (3000 Ci/mmol; 10 mCi/ml)(Amersham Biosciences Corp.) and the assay performed according to the manufacturers protocol (Promega) as previously published (Huang et al., 1999). *T. gondii* lysate (10 µg) was incubated with labeled oligonucleotides for 20 min at room temperature in 10 mM Tris (pH 7.5), 50 mM KCL, 1 mM DTT, 5 mM MgCl₂, 2.5% glycerol, 50 ng/ml poly (dI-dC). For cold competition excess amounts (50 ×) of unlabeled oligonucleotides were added to the reaction before the ³²P-labeled oligonucleotides were added. The reaction was added to loading buffer and electrophoresis was performed at 100 V for 3 h using a 5% native polyacrylamide gel and 0.5 × TBE buffer. The gels were dried and subjected to autoradiographic exposure for 12–48 h.

3. Results

3.1. Characterisation of the Tg-hsp70 gene locus

We had previously cloned the *hsp70* gene (AF045559) of *T. gondii* (Weiss et al., 1998). Northern blotting demonstrated that the mRNA for Tg-hsp70 was 2.6 kb (Fig. 1). A similar size mRNA was seen in both RH and ME49 strains and the size did not change with pH shock (data not shown). The transcription start of the *Tg-hsp70* gene determined using 5'RACE PCR was at position -153 from the initial ATG of the coding sequence (Fig. 2).

Analysis of the region of -1 to -951 (Genbank accession number AY481576) from the initial ATG of *Tg-hsp-70* (designated *Tg-hsp70P*) revealed that the sequence of this upstream region cloned from the RH strain of *T. gondii* was identical to that of the upstream region of the *Tg-hsp70* (sequence ID: TGG_1932) of the ME49 strain being used for the *Toxoplasma* genome project (<http://www.ToxoDB.org/ToxoDB.shtml>). At -650 bp from the initial ATG the sequence AGAGACG is present (Fig. 2, double underline), which has been described as a *cis*-acting element that acts as an enhancer in the transcription of several *T. gondii* genes (Mercier et al., 1996). As illustrated in Fig. 2 there is a series of nGAAn repeats -385 from the initial *Tg-hsp70* ATG or -232 from the transcription start site, which have similarity to the heat shock element (HSE) described in other eukaryotes (Morimot et al., 1994). An element CCGGGG (Fig. 2, underline) located right next to the putative HSE in the *Tg-hsp70* is predicted as an Sp1 site using GenQuest (Lasergene, DNA STAR) and is similar to the sp1-hsp70 site in the human hsp70 promoter (Morgan, 1989). Finally, there are several AGGGG or CCCCT regions (Fig. 2, underline), which are similar to the core region of the stress response element (STRE) described in many eukaryotic genes (Estruch, 2000). None of these STRE regions are present, however, in the area mapped as the location of the pH responsive region of the *Tg-hsp70* promoter (Fig. 3).

3.2. Characterisation of the *Tg-hsp70* promoter

The region of -1 to -951 from the initial ATG of *Tg-hsp70* was cloned into the SAG1- β Gal vector replacing the SAG1 promoter region with that of *Tg-hsp70*. The 3' region from the cDNA *Tg-hsp70* clone was substituted for the 3' region of SAG1 in the SAG1- β Gal vector to complete construction of the *Tg-hsp70P* vector. A BAG1- β Gal construct was used as a control. *BAG1*, a marker for bradyzoite development, is known to be expressed within 24 h of the exposure of cultures to conditions that result in bradyzoite differentiation.

As demonstrated in Table 1, the expression of *BAG1*, *SAG1* and *Tg-hsp70P*- β Gal constructs correlated with the observed levels of BAG1, SAG1, and hsp70 proteins seen during bradyzoite differentiation in response to pH 7.1 and pH 8.1 media (Bohne et al., 1997, 1999; Weiss et al., 1998; Weiss and Kim, 2000). Expression of β -galactosidase, as measured by MUG, increased 1.8 fold if regulated by the promoter for *BAG1* or *Tg-hsp70P* and decreased 1.9 fold if under the control of the *SAG1* promoter at pH 8.1. As nitric oxide (NO) is known to increase bradyzoite differentiation, the effect of sodium nitropruside (SNP) on the induction of *BAG1*- β -Gal vector was examined. SNP increased *BAG1* driven β -galactosidase expression. Previous observations using RT-PCR as well as immunoblot analysis indicated that *Tg-hsp70* expression was increased in the presence of indomethacin. A similar phenomenon was seen with the *Tg-hsp70* promoter construct. Indomethacin increased the expression of β -galactosidase at both pH 7.1 and pH 8.1 in the *Tg-hsp70* promoter vector.

3.3. Deletion analysis of the *Tg-hsp70* promoter

A series of deletions were made in the upstream region of *Tg-hsp70* were made: yielding vectors containing 720 bp (*hsp70-720*), 410 bp (*hsp70-410*) and 230 bp (*hsp70-200*) of the upstream region. The map at the top of Fig. 3 represents the cloned 951 bp region prior to the initial ATG of the *Tg-hsp70* gene with the transcription starting point indicated by an arrow. All constructs had comparable activity at pH 7.1 (Fig. 3). Comparison of β -galactosidase activity of these constructs (see graph in Fig. 3) demonstrated that the putative stress sensitive *cis*-element was located between -200 and -410 from the initial ATG codon. The fold stimulation of gene expression (adjusted for CAT activity) of pH 8.1/pH 7.1 is illustrated at the right of each *hsp70* construct. Although basal activity remains, the pH 8.1 inducible β -galactosidase activity is clearly lost when the region between -200 and -410 bp is deleted. A vector *hsp70P-429* Δ *340* having a deletion of the 79-bp region that contains the putative *cis*-element from the full length hsp70 promoter construct was constructed using an

inverse PCR technique. The construct, *hsp70P-429Δ340*, did not have any pH inducibility of β -galactosidase activity at pH 8.1 (fold stimulation 1.0). The β -galactosidase activity at pH 8.1 or pH 7.1 of *hsp70P-429Δ340* was similar to that of *hsp70-200*. Thus, deletion of this 70 bp region was sufficient to ablate pH 8.1 inducibility, consistent with this region containing a *cis*-acting regulatory region for *Tg-hsp70*.

Results with *Tg-hsp70P* and *Tg-hsp70P-429Δ340* β -gal constructs were confirmed with transient transfection in RH strain and stable transfection in PLK strain *T. gondii* of GFP reporter constructs. As demonstrated in Fig. 4A, pH 8.1 shifts leads to an increased expression of GFP in the *Tg-hsp70P* construct in RH strain *T. gondii* (transient transfection). Almost no GFP expression was seen at pH 8.1 or pH 7.1 with the *Tg-hsp70P-429Δ340* construct. By immunoblot an increase in expression at pH 8.1 (4-fold increase) can be seen in PLK strain *T. gondii* (Fig. 4B) that have been stably transfected with *Tg-hsp70P*, but almost no expression is seen with the *Tg-hsp70P-429Δ340* construct at pH 8.1. Prolonged exposure does demonstrate basal expression of *hsp70* in all constructs at pH 7.1 and pH 8.1 that is not affected by the 79 bp deletion (data not shown).

3.4. *T. gondii* proteins bind to the putative *hsp70* *cis*-acting element

Analysis of the 79 bp deletion region of *Tg-hsp70* revealed regions with some similarity to HSEs (repeating nGAAn motifs). Therefore, two double stranded consensus sequences were constructed for the shift analysis: *Tg-hsp70*-REG:

5'TGCAAGAAAGAAAGGCTTTCGGAAA GGAAACCG3' control *Tg-hsp70*-CTL:

5'TGCAATAAAATAAGGATTTTCGTAAA GGAAACCG3' (and its complement; bp changes indicated in bold, nGAAn motifs underlined). These were used for gel shift analysis (EMSA) using a radiometric method previously used for the analysis of NF- κ B during *T. cruzi* infection (Huang et al., 1999) and a non-radiometric method. Specificity studies were performed with a 50 and 100-fold molar excess of unlabeled oligonucleotide. *Tg-hsp70*-REG associates with a protein in *T. gondii* lysates resulting in a positive EMSA (Fig. 5A, lane 1). This binding could be competed by cold unlabeled probe *Tg-hsp70*-REG (Fig. 5A, lanes 2–4), but not by the negative control probe *Tg-hsp70*-CTL (Fig. 5B, lane 4). The *BAG1* regulatory region had been previously mapped to a location about 400 bp upstream from the initial ATG of this gene (Bohne et al., 1997). In this region, we also identified an area with a nGAAn motif (underlined), although this was not identical to *Tg-hsp70*-REG. Oligonucleotides made to this region of *BAG1*,

5'TAGAGAAAGGCAGAAGGCCGAGCGTTTTCTCA-G3', did not bind protein in a GSA assay and could not compete with the *Tg-hsp70P* oligomers (Fig. 5B, lane 5).

4. Discussion

We have previously determined that a *T. gondii* *hsp70* homologue (AF045559) is induced during bradyzoite differentiation (Weiss et al., 1998). The current paper defines a new *cis*-regulatory element that is associated with the response of *hsp70* to pH stress. This element appears to be present in virulent type I strains such as RH as well as in avirulent type II strains such as PLK (ME49). This element is independent of those required for basal transcription of *Tg-hsp70*. Previously, some *cis*-regulatory elements have been identified in *T. gondii* in the characterisation of SAG1, GRAs and NTPase, but these do not appear to be upregulated with stress (Soldati and Boothroyd, 1995; Mercier et al., 1996; Nakaar et al., 1998).

The magnitude of the response we observed with the identified *hsp70* *cis*-regulatory element is similar to that seen in other *hsp70* genes. Extracellular *T. gondii* treated with a 1-h exposure to pH 8.1, which lead to bradyzoite development, expressed a 72 kDa inducible *hsp70* (detected with mAb C92F3A-5; Stressgen) (Weiss et al., 1998). Human fibroblasts

infected with *T. gondii* exposed to pH 8.1 media demonstrated a 4-fold induction of the hsp70 levels compared to *T. gondii* grown in pH 7.1 media (Weiss et al., 1996, 1998). The three to 4-fold change demonstrated in *T. gondii* with stress as well as differentiation is comparable with the magnitude of the hsp70 response demonstrated in *T. cruzi*, *T. annulata* and *P. falciparum* (Shiels et al., 1997; del Cacho et al., 2001). Similar results were obtained with in vivo cysts during reactivation in a murine model induced by anti- γ -interferon (Silva et al., 1998), suggesting that hsp70 may be important in both tachyzoite to bradyzoite and bradyzoite to tachyzoite differentiation. Quercetin, a known inhibitor of heat shock protein synthesis, was able to suppress hsp70 levels and indomethacin, a known inducer of heat shock transcription, was able to increase hsp70 levels (Weiss et al., 1996, 1998).

It has been reported that hsp70 may also act as a virulence factor and that higher basal levels of hsp70 are seen in vivo in RH strain (Lyons and Johnson, 1995, 1998; Miller et al., 1999, 2000). Analysis of 900 bp of the upstream region of hsp70 in both RH (a type I) and ME49 (a type II) strain demonstrates that they are identical, so the mechanism of regulation of hsp70 as a virulence factor does not appear to depend on unique upstream elements in this region of the gene.

Heat shock- or stress induced activation of a set of heat shock protein genes, is a characteristic of eukaryotic and prokaryotic cells. These genes act as chaperones for protein folding and transport (Noyer, 1991; Morimot et al., 1994). In many eukaryotes, heat shock-induced transcription of heat shock proteins is under the control of heat shock factor (HSF) which is activated post-translationally and binds specifically to the heat shock element (HSE) in the upstream promoter region of heat shock protein genes (Noyer, 1991; Morimot et al., 1994). It is notable that HSEs mediate not only the response to heat and other forms of stress but have been demonstrated to respond during differentiation.

Analysis of other eukaryotic heat shock genes has resulted in the definition of a HSE as a repeating array of the 5-bp sequence 5'-nGAAn-3' ($\rightarrow$), where repeats are usually inverted relative to the immediately adjacent repeat (Abravaya et al., 1991; Cunniff and Morgan, 1993; Morimot et al., 1994). In the consensus sequence nGAAn the 'G' at position 2 is absolutely conserved, with base substitutions here abolishing heat-induced expression, whereas the 'A' at position 3 and 4 are less conserved since base substitutions at these sites occur. In the first position 'A' is preferred, but is not essential. Heat shock promoters from different heat shock genes in diverse organisms, have the same 5-bp building block. The number of 5-bp units in a functional HSE can vary but usually ranges from 3 to 6. For example, the promoter region of the *Drosophila* hsp70 gene has four HSEs (Morimot et al., 1994). In addition an HSE can tolerate inserts (of up to 10 bp) between the repeating units provided the phase of the repeat is maintained. In yeast (*Saccharomyces cerevisiae*) the hsp90 HSE has the structure GAA3nTTC7nTTC3nTTC3nGAA6nGAA3nTTC6nTTC2nAGA7nGAA. Similar variant yeast HSEs have been reported for SSA, STI1 hsp26, and phosphoglycerate kinase (Morimot et al., 1994). The location of HSEs is variable and ranges from about 40 to 300 bp upstream of the transcriptional start site. HSEs have not been described in other protozoan heat shock genes that have been examined (Lee and Van der Ploeg, 1990; Bock and Langer, 1993; Biswas and Sharma, 1994; Dunn et al., 1995; Andersen et al., 1996; Adamson et al., 2001; del Cacho et al., 2001; Zilka et al., 2001; Banumathy et al., 2003).

The regulatory region of Tg-hsp70 has a series of nGAAn repeats -385 from the initial Tg-hsp70 ATG or -232 from the transcription start site (Fig. 2). A repeating motif of nGAAn ($\rightarrow$) and nCTTn ($\leftarrow$), similar to a single HSE, is indicated with two flanking nGAAn sequences. HSFs display a remarkable flexibility in their ability to interact with HSEs containing different numbers and arrangements of 5-bp units. The smallest array that

demonstrates binding of purified *Drosophila* HSF in vitro contains two 5 bp units either head to head (nGAAnnTTCn; → ←) or tail to tail (nTTCnnGAAn; → ←) (Morimot et al., 1994).

The gel shift assay data demonstrates that the putative Tg-hsp70 *cis*-regulatory element 5'TGCAAGAAAGAAAGGCTTTCGGAAAGGAAACCG3' can bind to a protein in *T. gondii* lysate that is a presumptive transcription factor. This protein is not bound by the Tg-BAG1 oligomer that displays a similar nGAAn motif located in the region of the mapped BAG1 promoter. Examination of the *Plasmodium* genome database version 4.1 (<http://PlasmoDB.org>) and the *Toxoplasma* genome database version 2.3 (<http://ToxoDB.org>) does not reveal any genes homologous to known HSFs. The described *T. gondii* binding site is not a classical HSE and the protein binding this site may not be related to known HSFs. Examination of hsp90 and hsp60 loci in the *T. gondii* genome did not demonstrate the presence of the *cis*-element we identified in Tg-hsp70. This regulatory region may be unique to Tg-hsp70 and not present in other *T. gondii* heat shock proteins.

There is a significant body of evidence relating heat shock proteins with differentiation in various phyla (Heikkila, 1993a,b). In *Drosophila* hsp70 and hsp68 are associated with the blastoderm stage (Arrigo and Tanguay, 1991). In *Xenopus* hsp70 expression coincides with activation of the zygote genome (Nickells and Browder, 1985). In K652, a human cell line, induction of differentiation to synthesise haemoglobin is associated with activation of hsp70 gene expression (Heikkila, 1993b). The heat shock response of fungi such as *S. cerevisiae* and *Neurospora crassa* varies with development (Kurtz and Lindquist, 1984; Plesofsky-Vig and Brambl, 1985). For example, in *Blatocladia emersonii* hsp70 is induced during sporulation and hsp70 is associated with hyphal branching and secretion in response to steroids in *Achlya ambisexualis* (Heikkila, 1993a). In *Histoplasma capsulatum*, mitochondrial ATPase activity and hsp70 induction are correlated with the transition from mycelium to yeast phase (Patriarca et al., 1992). Similar heat shock protein associations with development and intracellular survival have been noted in protozoa. For example, in *Leishmania chagasi* heat shock proteins are associated with the capacity to survive an oxidant stress and play a role in the promastigote to amastigote transition (Wilson et al., 1994). Post-translational modifications of heat shock proteins have also been described to occur in a tissue specific pattern during development and to vary during differentiation events (Fink and Goto, 1998; Welsh and Gaestel, 1998).

Small heat shock proteins have also been observed to be increased in many organisms undergoing differentiation, have been noted to be expressed in tissue specific fashions during development and can account for over 1% of the total protein of a cell during differentiation (Welsh and Gaestel, 1998). Small heat shock proteins are a diverse group of proteins that display less conservation than other heat shock proteins. In *Drosophila* hsp 27, hsp26, hsp 23 and hsp 22 are expressed in a tissue specific manner during development and appear to have key functions in development (Arrigo and Tanguay, 1991). Knockout of small heat shock proteins has been demonstrated to interfere with differentiation events (Wehmeyer et al., 1996; Moerman and Klein, 1997; Unno et al., 1998; Yuan et al., 1998). Inhibition of hsp27 interferes with the granulocyte differentiation of human promyelocytic HL-60 cells and the differentiation of murine embryonic stem cells (Welsh and Gaestel, 1998). The ability of *Mycobacterium tuberculosis* to survive in macrophages as a latent organism (i.e. to differentiate to its latent stage) was impaired by knockout of Acr (a 16-kDa smHsp) (Yuan et al., 1998).

BAG1 (a *T. gondii* smHsp) is expressed during bradyzoite differentiation (Bohne et al., 1995; Parmley et al., 1995) and we have shown that knockout of BAG1 affects this development (Zhang et al., 1999b). Small heat shock proteins are characterised by the

presence of an α -crystalline domain at the carboxy-terminus, including the characteristic GVL motif seen in BAG1, that is often involved in their oligomerisation (Morimot et al., 1994; Fink and Goto, 1998; Welsh and Gaestel, 1998). Small heat shock protein may be involved in preventing the aggregation of newly synthesised or denatured proteins formed during differentiation and the final folding of such proteins would depend on the involvement of hsp70. Fig. 6 provides a model based on the functions ascribed to heat shock proteins in other eukaryotic systems for the involvement of hsp70 and small heat shock proteins in differentiation.

In summary, the current report provides further evidence of a pH stress regulated element in the *T. gondii* hsp70 locus (Weiss et al., 1998). This pH regulated *cis*-element was mapped to the region -340 to -420 from the initial ATG of the hsp70 gene. Within this area is an element containing several nGAAn repeats and a region similar to the sp1-hsp70 element described in other eukaryotes. The nGAAn repeat region was demonstrated to bind a putative transcription protein. The upstream region of the hsp70 in both RH (a type I) and ME49 (a type II) strain is identical and may reflect the presence of important regulatory elements in this critical protein that appears to be involved in both virulence (Miller et al., 1999; Dobbin et al., 2002) and developmental stage transitions (Silva et al., 1998; Weiss et al., 1998). It is clear that hsp70 has both constitutive promoters as well as stress regulated promoter elements. While the identified pH regulated element has similarities to HSEs that have been described in other eukaryotes analysis of the *T. gondii* genome suggests it is unlikely that *T. gondii* has traditional HSFs and the mechanism(s) of the regulation of stress responses in this organism remain to be elucidated.

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Fig. 1.

Northern blot analysis of *Toxoplasma gondii* hsp70. Total RNA (20 µg), isolated from *T. gondii* ME49 was electrophoresed on a 1% agarose/formaldehyde gels, capillary blotted to a nylon membrane and probed with a digoxigenin labeled Tg-hsp70 probe. Lane 1: Digoxigenin labeled RNA Standard (Boehringer Mannheim), Lane 2: 20 µg RNA from ME49 *T. gondii*. The hsp70 mRNA band is at approximately 2600 bp.

GGTACCCCCTTCTGCGACTCTTTACAAACGCGGAATCCAACAGTTAAGCACCCGCCTAACATGAACTTCACTGGCCCT
 CCTAGTGCTTCTTTTCTTTGTTTATTGCGGTTCCGGTAGTAATATTTGAAGCACCCGAGAAGCTATCCCACTTTCTG
 TGAGGGGCGCGTTCTAGATATTGTCCGTAACGCGTTGTGACTGGCTATTGCCAGTGGAAACGTCCGGCGCCGTGCGCA
 -650
 ACCCCCCCCCCCTCCCCCGACGGGGACAACACAACAGCCGTTATTTGGCAGCTGTCAGGGAGAGACGCAGGACATGCAGT
 GGACCGTGCAAGCAGCGTCAGCCTCTTGCGGGGAGTGCTGCTTTTTTGACCTCGTTGAGTAAGCTCCCGCCCCCCCC
 ACCCGAAGACTTGCGTCATCCTGCTCGTCACCTCCCCCCCCCTCCCCCACGAGCGACGCCGGTACTCATCAGAGGA
 -420
 ACCTTTGTGCTGTCCGTGTCGCAAGCGTCAGTATGGAGCGCAAAGGAGTGTAAGATGCATTGTTCCGGTCAAGTATC
 -340
 TGCAAGAAAGAAAGGCTTTCGGAAGGAAACCGGGGCTCTGCAAGAAATCGCCAGTGTGTTTCGTCTTTTGGCTTG
 AACAGCGAGATTGCACCGTGTGCTGCCTCCGCAGCACGGACAAGTTGCTCCAGCACCTACGTCCGAGTACATACCA
 CACACCGCCCTCTTCGTCCCTCATCATCCCCCTGCTGTGCGCGGGTGTGCTGTCCGGTACGAGTCATAGGGTGTGTCC
 -153
 GGCACCTCGCCCCGcatttcacatttttaggagagtgcggttccgtgcggtgcagctcaactaaggacggatcgaaggtc
 agtggttttttccttccaaccatttttcctttccactccccggttctttttgtgtcgcggtgcagttggttgccct
 +1
 gcagaagacaacATG

Fig. 2. *Toxoplasma gondii* hsp70 promoter region. The sequence of the 5' upstream region of the *T. gondii* hsp70 gene locus (951 bp) is shown (Genbank accession number AY481576). The transcription start site identified by 5' RACE is at -153 from initial ATG which is highlighted. Lowercase letters represent the 5' UTR region of the gene. '⏏' indicates locations of restriction sites and primers used to produce deletion constructs (see Section 2). '→' indicates location of nGAAn or nTTCn motifs. An Sp1-hsp70 motif is underlined (Morgan, 1989). The double underlined element at -650 has been described as an enhancer in other *T. gondii* genes (Soldati and Boothroyd, 1995; Mercier et al., 1996; Nakaar et al., 1998). The dotted underlined text indicates sequence identical to the core region (AGGGG or CCCCT) of stress related elements (STRE) described in other eukaryotic systems (Estruch, 2000).

Fig. 3.

Deletion analysis of the *Toxoplasma gondii* hsp70 promoter region. The top of this figure shows a map of the upstream genomic locus of the *T. gondii* hsp70 gene indicating the location of restriction sites and primers used for creation of the deletion constructs. The transcription start site is indicated by an arrow and the start of the gene by an ATG. The position of deletion constructs is illustrated below the genomic map. The vector hsp70P-429Δ340 has a deletion of a 79-bp region from -340 to -429 (thin dotted gray line) from the initial ATG. To the right of each construct is the adjusted fold stimulation for parasites maintained at pH 8.1 (bradyzoite induction) versus those at pH 7.1 (tachyzoites) for each construct (see Section 2 for calculation). The regulatory region for pH stress-induction maps to the region indicated by the box. The chart below indicates the β -galactosidase activity (normalised for *cat* activity, see Section 2 for calculation) of each construct at pH 8.1 and pH 7.1 during transient transfection in the RH strain of *T. gondii*.

Fig. 4.

Tg-hsp70P-GFP expression is induced by pH shock in *Toxoplasma gondii*. (A) Representative microscopic fields of the transient expression of Tg-hsp70P-GFP in RH strain *T. gondii* at pH 7.1 and pH 8.1. GFP driven by the 5' upstream region of Tg-hsp70 is induced by pH stress. By microscopy approximately 5% of *T. gondii* are GFP positive at pH 7.1 versus 25% at pH 8.1. (B) Immunoblot of GFP constructs under the control of different promoters stably expressed in PLK strain *T. gondii*. A 1:1000 dilution of an anti-GFP monoclonal antibody (Clontech) was used to detect GFP expression. Equal amounts of *T. gondii* (as determined by counting parasites) were loaded in each lane. Lane 1: GRA1-GFP (pH 7.1), Lane 2: Tg-hsp70P-GFP (pH 7.1), Lane 3: Tg-hsp70P-GFP (pH 8.1), Lane 4: BAG1-GFP (pH 7.1), Lane 5: BAG1-GFP (pH 8.1), Lane 6: Tg-hsp70P-429Δ340-GFP (pH 7.1), Lane 7: Tg-hsp70P-429Δ340-GFP (pH 8.1). BAG1 and hsp70P driven expression are upregulated in response to pH 8.1 and that the activity of this regulatory region is decreased by deletion of the -340 to -429 region. At pH 7.1 the Tg-hsp70P-429Δ340-GFP construct also has a decrease in its expression of GFP compared to the Tg-hsp70P-GFP construct. This is consistent with a loss of inducible expression with this deletion as PLK, a type II *T. gondii* strain, often has bradyzoite development observed at pH 7.1 in tissue culture.

Fig. 5.

Gel shift assay. (A) electrophoretic mobility shift assay (EMSA) (using biotin labeled oligonucleotides) demonstrating binding of a putative regulatory protein (arrowhead) to Tg-hsp70-REG. “*” indicates the location of unbound (free) probe. *Toxoplasma gondii* ME49 strain protein lysate was prepared by sonication of purified parasites in 20 mM Hepes (pH 8.0), 0.1 KCL, 0.5 mM DTT 0.2 mM EDTA 20% glycerol containing a protease inhibitor cocktail tablet (Roche) followed by centrifugation at $3000 \times g$ to remove insoluble materials. Lane 1: Biotin labeled Tg-hsp70-REG, Lane 2: 10 $\times$ unlabeled Tg-hsp70-REG added to reaction, Lane 3: 20 $\times$ unlabeled Tg-hsp70-REG added to reaction, Lane 4: 50 $\times$ unlabeled Tg-hsp70-REG added to reaction, Lane 5: no oligomers added to the reaction (negative control). As can be seen cold unlabeled Tg-hsp70-REG competes with the binding of this protein to the biotin labeled Tg-hsp70-REG. (B) EMSA (using ^{32}P labeled

oligonucleotides). Lane 1: ^{32}P labeled Tg-hsp70-REG demonstrating binding to putative regulatory protein (arrowhead), Lane 2: $50 \times$ unlabeled Tg-hsp70-REG added to the reaction, Lane 3: ^{32}P labeled Tg-hsp70-REG, Lane 4: $50 \times$ unlabeled Tg-hsp70-CTL added to ^{32}P labeled Tg-hsp70-REG, Lane 5: $50 \times$ unlabeled BAG1 added to ^{32}P labeled Tg-hsp70-REG. ‘*’ indicates the location of unbound (free) probe.

Fig. 6.

A model of the interactions of small heat shock proteins and hsp70 in differentiation in *Toxoplasma gondii*. The size of the gray arrows indicates relative degree of upregulation observed during differentiation. The oligomerisation of small heat shock proteins, such as BAG1, has been described in other eukaryotic systems. Such oligomerisation, [smHsp]_n formation, is associated with the formation of heat shock granules. In other eukaryotic systems hsp70 is known to interact and bind ATP. Sequence analysis suggests that this may also occur with *T. gondii* hsp70. During differentiation small heat shock proteins and hsp70 work together in response to stress and protein translation and transport.

Table 1
Tg-hsp70 β -galactosidase expression is enhanced by bradyzoite induction conditions

Promoter	pH 7.1	pH 8.1
SAG1	256	132
BAG1	128	200
BAG1 + SNP ^a	290	nd ^b
hsp70P	116	219
hsp70P + IND ^c	356	490

β -Galactosidase values are expressed as arbitrary MUG fluorescence units normalised to co-transfected CAT activity as described in Section 2. Experiments were performed in triplicate. Mean values of these replicates of MUG fluorescence are given. The SEM was $\leq 10\%$ for these values.

^a SNP, sodium nitroprusside (an NO donor)

^b nd, not determined

^c IND, indomethacin