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SUMMARY

Toxoplasma gondii has recently come under intense study as a model for intracellular parasitism because it has a number of properties that facilitate experimental manipulation. Attention is now being turned towards understanding the developmental biology of this complex parasite. The differentiation between the two asexual stages, the rapidly growing tachyzoites and the more slowly dividing, encysted bradyzoites, is of particular interest. Progression from the former to the latter is influenced by the host's immune response. This paper describes current progress on a number of research fronts, all aimed at understanding the triggers that push the tachyzoite–bradyzoite equilibrium in one or other direction and the changes that occur in gene expression (and ultimately metabolism and function). Chief among the techniques used for these studies are genetics and molecular genetics. Recent progress in these areas is described.

1. INTRODUCTION

Toxoplasma gondii is an obligate intracellular parasite in the family Apicomplexa (for an excellent collection of recent reviews, see the book by Gross (1996)). It is one of the most common parasites of animals, being found worldwide in a large range of warm-blooded verterbrates and at a very high prevalence. In France, for example, *T. gondii* infection is detectable in up to 85% of otherwise healthy adults (Desmonts & Couvreur 1974). The initial infection is characterized by a disease of variable severity but typically giving influenza-like symptoms and lymphadenopathy (Luft & Remington 1992). How the disease progresses depends largely on the immune status of the individual infected. Traditionally, this parasite had been well known as a cause of severe foetal infection in cases where a mother acquires her first infection during pregnancy. More recently, however, it has also emerged as an important opportunistic pathogen of immunocompromised persons, especially AIDS patients, in whom a potentially fatal pneumonia and toxoplasmic encephalitis can ensue.

The parasite has many natural properties that have attracted a growing number of investigators to its study. Immunologists have been attracted by the efficient immune response it elicits, which yields protection against significant disease except in the two scenarios given above. Cell biologists have been attracted by its elegant ultrastructure, which includes a number of organelles dedicated to the intracellular lifestyle (i.e. attachment, invasion and intracellular growth and egress). More recently, developmental biologists have become interested in this protozoan because, as with many parasites, *T. gondii* has a complex life cycle, which includes a number of discrete developmental stages. In figure 1, the life cycle is depicted in a somewhat unconventional way as consisting of two potentially independent cycles, one asexual and one sexual. This emphasizes that the parasite appears fully capable of propagating itself in nature through either cycle. Indeed, recent evidence suggests that different strains rely on the two cycles to different degrees and, in the virulent lines such as RH, perhaps only the asexual mode is used (Sibley & Boothroyd 1992; Darde *et al*. 1992). In practical terms, this uncertainty means that it is not clear what fraction of human infection is a result of ingesting meat containing tissue cysts (arrow 3) rather than a result of accidental ingestion of oocysts (arrow 7).

The sexual cycle is a classical coccidian one and involves gametogenesis, zygote formation and development into an oocyst that is shed in the faeces of the cat (the only known animal in which the sexual cycle can occur). Following a short time in the environment (*ca.* 2 d), the oocyst matures and becomes fully infectious. In terms of genetics, this sexual cycle shows classical mendelian properties as described in detail in Pfefferkorn & Pfefferkorn (1980).

The asexual cycle, which can occur in almost any warm-blooded animal, is simpler. It consists of two stages, the rapidly dividing tachyzoite (Greek *tachy*, fast)

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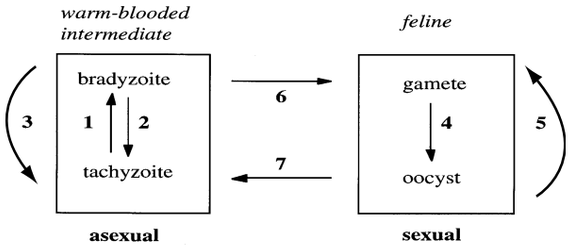

Figure 1. The life cycle of *T. gondii*. The asexual cycle can occur in a large number of warm-blooded animals and is shown on the left. It involves an equilibrium between the rapidly dividing tachyzoite and the more slowly dividing bradyzoite (arrows 1 and 2). The balance in this equilibrium is believed to be determined by environmental factors, especially stress provided by the host's immune response; encystment results from a strong immune response whereas reactivation occurs when the pressure is relaxed. To what degree the process is inductive, rather than selective, is not yet clear. Transmission through the asexual cycle is by ingestion of (undercooked) meat and other organs containing the infectious, encysted bradyzoites (arrow 3; e.g. rat to pig, or, pig to human). Scavenging allows the parasite to cycle through the food chain (e.g. pig to rat). The sexual cycle is shown on the right and involves schizogony, gametogenesis and fertilization (arrow 4) in the gut epithelium of felines. Cat-to-cat transmission through the sexual cycle is by ingestion of oocysts in faecal contamination (arrow 5). Crossover between the two cycles is represented by arrows 6 and 7 but, as mentioned in the text, the relative extent of this in nature is not known for all strains.

and the more slowly growing, encysted bradyzoite (*brady*, slow). In an infected animal there is an equilibrium between these two stages so that early in the infection tachyzoites predominate but as the host's immune response engages the parasite there is a shift toward the encysted bradyzoite. Both forms are capable of appearing in almost any tissue in the animal.

2. MATERIALS AND METHODS

Except as noted below, all materials and methods used standard reagents and protocols as recommended by the supplier and/or generally available laboratory manuals (see, for example, Harlow & Lane 1988; Ausubel *et al*. 1996).

(*a*) *Parasite strain*

As with any project where mutants are to be studied, the choice of parental strain is critical to be sure that the desired phenotype can be obtained and further studied. For this study, a recently cloned derivative of the ME49 strain of *T. gondii*, which has become the 'workhorse' of many laboratories interested in bradyzoite development (the origin of this strain is described by Boothroyd *et al*. 1995). Detailed restriction fragment length polymorphism (RFLP) analysis of ME49 and a cloned derivative therefrom showed no differences; this result suggested that the ME49 strain was already effectively clonal at the time when the subclones were derived (Sibley *et al*. 1992). This is not surprising given that the strain was originally isolated from a piece of infected lamb, which was unlikely to have carried a large number of tissue cysts, and that it has been passed extensively in animals since then.

The clone used for the bulk of the work described in this paper has been designated PDS and was generated from ME49 oocysts produced by Elmer Pfefferkorn. Note that the derivative was cloned out of oocysts to ensure that the line would be fully competent for all stages of the life cycle. This is especially important should a cross in cats ever be attempted: the ability to productively infect these animals (i.e. to produce oocysts) can be lost after continuous passage as tachyzoites (E. R. Pfefferkorn, personal communication). Thus, low-passage-number cultures were used for all mutant and genetic analyses.

The advantages of the ME49 (PDS) strain are as follows.

1. It is a representative of one of the most commonly seen genotypes in infected people (Sibley & Boothroyd 1992; Darde *et al*. 1992; Howe & Sibley 1995).
2. It is not highly virulent, and thus meaningful measurements of changes in LD_{50}, for example, can be obtained (compared with RH, which has an LD_{50} in mice of less than 10).
3. It grows sufficiently well *in vitro* to be able to prepare reasonable amounts of material.
4. It readily differentiates to bradyzoites *in vitro* under appropriate stimuli (see below).
5. It is readily transfected (Kim & Boothroyd 1995).
6. There are many reagents available for this strain, including a genetic map (Sibley *et al*. 1992), cDNA and genomic DNA libraries, and specially engineered strains and mutants (e.g. those with atovaquone resistance (Tomavo & Boothroyd 1995) or those that are hypoxanthine/xanthine/guanine phosphoribosyl-transferase (HXGPRT)-deficient (Donald *et al*. 1996)).

(*b*) *Protocol for differentiation of tachyzoites to bradyzoites* in vitro

To induce tachyzoites to differentiate to bradyzoites *in vitro*, the high-pH method of Soete *et al*. (1993) was chosen. This method has the advantage of being cheap, simple, and easily monitored (through pH indicators). It has also proven the most efficient.

The protocol is as follows. A confluent monolayer of human foreskin fibroblasts (HFF) is infected with tachyzoites from a recently lysed culture at a multiplicity of infection of *ca*. 0.1. These are allowed to grow in standard tachyzoite conditions for four hours (i.e. in Dulbecco's modified Eagle's medium (DMEM) with 10% Nu-serum at pH 7.2, under 5% CO_2) to allow invasion and initial growth. After this, the medium is removed and replaced with inducing medium (RPMI/HEPES, pH 8.1, 5% fetal bovine serum) and the culture placed in a 37 °C air incubator. The inducing medium is replaced every 2 d. In longer incubations, the pH can vary and is readjusted by regulating exposure to air and/or by adding 1–2 drops of base (NaOH). By about 2 d, the vacuoles show distinct signs of

becoming cysts (rounding up and showing stacked parasites, compared with the flattened rosettes of the tachyzoite vacuoles) and parasite division is reduced. By about 6 d, clear cyst-like structures are apparent, containing 50–100 parasites.

3. RESULTS

A number of approaches to studying the developmental biology of *T. gondii* have been taken, focusing on biochemical, genetic and molecular genetic strategies. As yet, attention has been restricted to the asexual development (tachy–brady switch) for simple logistical reasons: the sexual cycle occurs only in cats and has not yet been reproduced in culture.

(*a*) *The tissue-cyst wall contains GlcNAc, probably in chitin*

In recent years, a number of groups have found conditions that cause tachyzoites growing *in vitro* to differentiate into bradyzoites (Soete *et al*. 1993; Bohne *et al*. 1993, 1994; Weiss *et al*. 1995). Although these may not be identical to fully 'mature' bradyzoites found *in vivo*, they are unquestionably well along the pathway leading to mature cysts. This conclusion is based on the facts that (a) they show strong expression of several genes whose expression is normally absolutely restricted to bradyzoites, and (b) they are resistant to pepsin digestion in acid, a biologically relevant hallmark of the natural cyst (this property allows survival through the stomach and release in the gut). Under the high-pH conditions developed by Soete *et al*. (1993), the ability of the cysts to bind a number of lectins *in vitro* was tested. Of the twelve tested, only two, *Dolichos biflorus* seed lectin and succinylated wheat-germ agglutinin (S-WGA), showed specific binding (figure 2). This was confirmed by specific inhibition of binding with their sugar haptens, *N*-acetylgalactosamine (GalNAc) and *N*-acetylglucosamine (GlcNAc), respectively.

To directly assess the nature of the S-WGA-binding material and because the (β-1,4-linked polymer of GlcNAc (chitin) serves as the major cell-wall component in other systems such as yeast, *in vitro*-derived cysts were treated with chitinase; their ability to bind S-WGA was then assessed. The result was a loss in S-WGA binding and a disruption in the cyst wall, leading to release of some of the bradyzoites within. These results suggest that chitin may represent a significant part of the cyst wall composition.

(*b*) *Expressed sequence tags from bradyzoites* in vitro

One way to gain some idea of the differences in gene expression in two developmental forms is to look at the differences in their mRNA expression. This can be done in a number of ways, including differential display (Liang & Pardee 1992). In the present study, efforts and projects were combined to generate a large number of expressed sequence tags (ESTs) from the tachyzoite and bradyzoite stages. Although it is not particularly elegant, this strategy has the advantage

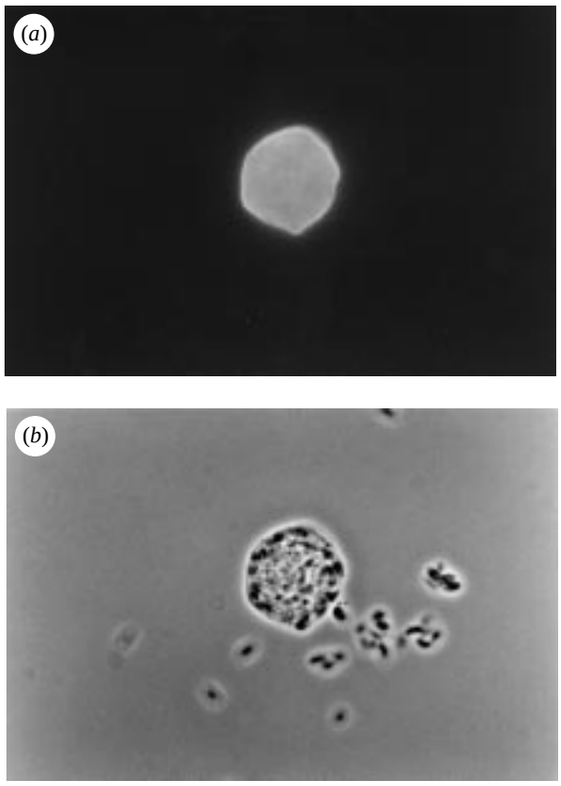

Figure 2. The tissue-cyst wall contains GlcNAc, probably in chitin. *In vitro* tissue cysts were generated by growth in medium at pH 8.1 and then stained with (biotinylated) succinylated wheat-germ agglutinin (*a*). Detection was with streptavidin conjugated to texas red. (*b*) A phase micrograph of the same field. Note that the lectin staining is specific to the cyst wall and not to the free parasites.

that stage-specific ESTs can be gleaned from a scan of the database and any promising candidates are easy to pursue because a partial sequence and cDNA clone are available. Additionally, the choice of which to pursue can be made with some knowledge of probable coding function. A cDNA library has been generated from the ME49 strain (PDS clone), starting with either *in vitro* tachyzoites or *in vitro* bradyzoite mRNA. The latter material was from cultures infected with tachyzoites but then switched to high pH, as described above. In this case, the parasites were harvested 6 d after inducing the switch, by which time they are expressing many bradyzoite-specific markers, including the cyst wall. The hope is that this population includes parasites that are at different stages in the switching pathway and thus the ESTs that will be obtained will include genes that are turned on early and late in the process. Sequencing of the tachyzoite library has been completed (Ajioka *et al*. 1998). This augments another completed effort on cDNA from tachyzoites of the RH strain (Wan *et al*. 1996; Ajioka *et al*. 1998). Sequencing of the *in vitro* bradyzoite library has been completed (J.B., A.H., L. Hillier, I.M., M. Marra, L. D. Sibley and R. Waterston, unpublished results). Results from this multicentre collaborative effort are most easily accessed through the very useful website set up by the

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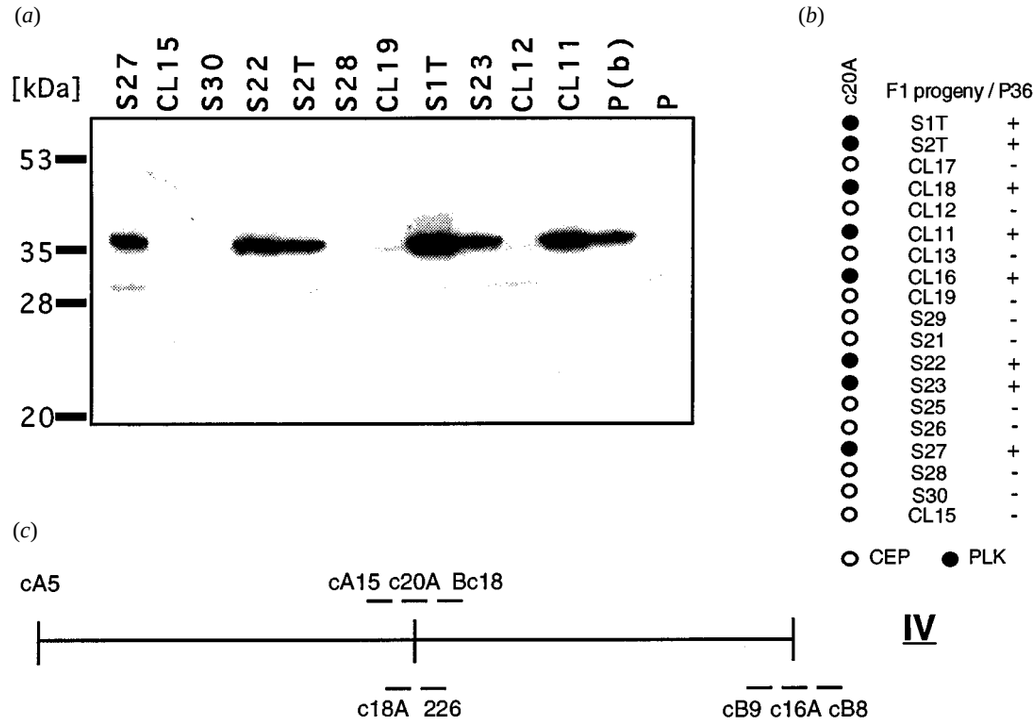

Figure 3. Genetic mapping of a natural P36 polymorphism. Two strains and their F1 progeny have been examined for the ability to turn on P36 expression upon placement in bradyzoite conditions. (*a*) Lysates of one parental and 11 progeny strains are shown probed with a monoclonal antibody to P36. Lane P is the PDS clone (asexually derived from the parental ME49 strain) grown in tachyzoite conditions. Lane P(b) is the PDS strain grown under bradyzoite conditions. The other lanes are 11 of the F_1 progeny of the cross (Sibley *et al*. 1992). (*b*) The P36 reactivity cosegregates with marker c20A and adjacent markers on chromosome IV in all 19 progeny examined. (*c*) The rudimentary map of chromosome IV showing the position of the c20A locus.

Cambridge group (`http://www.ebi.ac.uk/parasites/toxo/toxpage.html`).

(*c*) *Genetic mapping of a bradyzoite-specific gene, P36*

Another way to examine the differentiation process is to exploit the ability to perform genetic crosses with this parasite and map natural (or selected) variants in the process. It had previously been noted that the CEP strain did not show expression of a bradyzoite-specific surface antigen (P36) when exposed to switching conditions, at least as judged by Western blot analysis and immunofluorescence assay with a monoclonal antibody (mAb) for this protein (Tg4A12) (Tomavo *et al*. 1991). To map this polymorphism, we examined the previously characterized progeny of a cross between the CEP and ME49 strains (Sibley *et al*. 1992) for reactivity with this monoclonal antibody. Figure 3(*a*) shows a Western blot in which lysates from 11 of the F_1 progeny of this cross are probed with the anti-P36 mAb. When this was compared with the previously determined segregation pattern for over 60 RFLP markers (Sibley *et al*. 1992), there was perfect cosegregation of the P36 reactivity and the c20A RFLP marker in all 19 strains examined, indicating tight linkage (figure 3*b*). Figure 3(*c*) shows the map position indicated by this result.

The basis for this polymorphism is currently under investigation. It could result from a difference in the P36 structure such that the epitope seen by the mAb is not present in the CEP allele. Alternatively, it could indicate the total absence of the P36 protein from the CEP strain or a failure to turn on bradyzoite-specific genes in response to the high-pH conditions. Use of other bradyzoite-specific antibodies will help to resolve this issue.

(*d*) *Complementation protocols*

Regardless of the nature of the P36 polymorphism, it should be possible to complement the CEP strain with a library from ME49 (i.e. to restore anti-P36 reactivity). Currently, there are a large number of techniques available for high-efficiency transformation of *Toxoplasma* (reviewed in Roos *et al*. 1994; Boothroyd *et al*. 1995). Although these could allow the simple complementation envisaged here, an episomal vector potentially has several other advantages, including even higher efficiency and making recovery of the complementing gene trivial. A genomic sequence of 500 bp that confers replicative stability on a pBluescript backbone has been selected. The resulting plasmid has been used

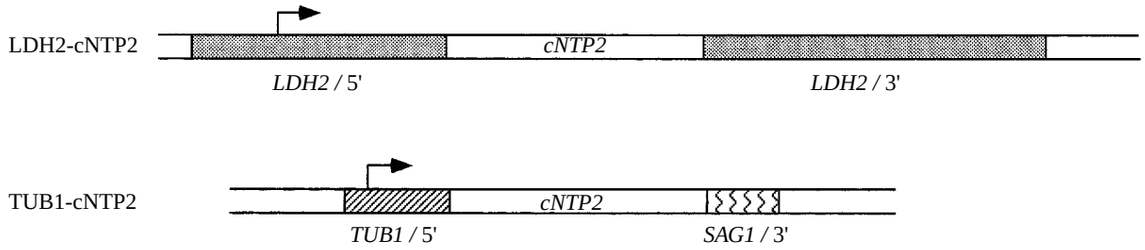

Figure 4. Use of cytosolic NTPase as a negatively selectable marker. Schematic diagram showing the cytosolic *NTP2* gene inserted downstream of either the bradyzoite-specific *LDH2* or the constitutive *TUB1* (encoding (α-tubulin) promoter to yield *LDH2c–NTP2* or *TUB1–cNTP2*, respectively.

for constuction of a genomic library and this has successfully been used to complement an Hpt$^-$ mutant, yielding the wild-type *HPT* gene in the recovered episome, as expected. A similar library will be used to complement the CEP strain for anti-P36-reactivity. It will also be used for complementation of the differentiation mutants whose generation is described below.

(*e*) *Use of an engineered NTPase for negative selection*

As with the natural 'mutant' just described (i.e. the CEP strain), it should be possible to identify genes critical for the differentiation process by generating mutants in the laboratory. To do this, a strategy is needed that will select for differentiation mutants over the wild type. The approach in the present study has been to introduce a negative selectable marker under control of a bradyzoite-specific promoter such that when wild-type parasites switch from tachyzoite to bradyzoites, they will die. Upon mutagenesis (e.g. with ethyl nitrosourea) it should then be possible to select for mutants that are unable to switch.

Two negative selectable markers have been chosen: hypoxanthine–xanthine–guanine phosphoribosyl transferase (HPT (Donald *et al*. 1996); see below) and an engineered form of the potent *Toxoplasma* nucleotidase (NTPase (Asai *et al*. 1983, 1995; Bermudes et al. 1994)). This latter enzyme is normally synthesized as a secreted protein, which is stored in the dense granules before release. Upon invasion into a host cell, the contents of the dense granules are delivered into the developing parasitophorous vacuole where the NTPase serves to convert host NTPs (which diffuse into the vacuole through pores) into NMPs that are presumably acted on by a phosphatase to produce nucleosides for uptake (Joiner *et al*. 1994). The strategy in this study has been to engineer the NTPase gene (specifically *NTP2* by Asai's nomenclature) such that it lacks the region encoding the N-terminal signal peptide that normally serves to target the NTPase to the secretory pathway and then place this under control of a bradyzoite-specific promoter. The expectation is that, on differentiation to the bradyzoite form, such an enzyme will be synthesized and retained within the cytosol, where it will prove highly toxic because it will degrade the parasite's own NTP stores. This cytosolic form of the enzyme is termed cNTP.

To test this concept, a plasmid with the *LDH2* gene, including flanking sequence, was obtained from Dr S. Parmley (Palo Alto Medical Foundation). This gene was isolated in a screen for bradyzoite-specific cDNAs and encodes a form of lactate dehyrogenase apparently found only in bradyzoites, as no mRNA for this gene is detectable in tachyzoites (Yang & Parmley 1995). The region extending from *ca*. 720 bp upstream of the transcription start site (as mapped by Dr Parmley) through to the start codon, *ca*. 270 bp downstream of this site (F. Seeber, S. Parmley and J.C.B., unpublished data), has been subcloned. This was placed upstream of a form of the *NTP2* gene lacking the signal peptide (generated by polymerase chain reaction (PCR)). Downstream of this coding region, *ca*. 1750 bp of 3′-LDH2 gene sequence was inserted.

The resulting construct (*LDH2–cNTP2*) (figure 4) was cotransfected into wild-type PDS parasites along with the *cat* selectable marker and chloramphenicol selection was applied (Kim *et al*. 1993). As a control, parasites were transfected with a constitutively expressing version (under control of the *TUB1* promoter (Soldati & Boothroyd 1993)). The results are summarized in table 1. They show that 20% of the parasites transfected with the *cat* and *LDH2–cNTP2* constructs and selected for chloramphenicol resistance had both plasmids whereas parallel experiments with *cat* and the *TUB1–cNTP2* yielded no cotransfectants. Thus, *LDH2–cNTP2* can be tolerated in tachyzoites although some integration events may have led to loss of regulation and subsequent toxicity in the tachyzoites. The constitutively expressing *TUB1–cNTP2* plasmid appears to be highly deleterious to tachyzoites, as expected: no cotransfectants were obtained.

Next, the effect of switching the parasites to bradyzoites *in vitro* was examined for one of the parasites successfully transfected with the *LDH2–cNTP2* construct. The results (table 1) showed that *LDH2–cNTP2* is indeed turned on and toxic in the bradyzoites: the parasite vacuoles swelled and growth generally stopped at the 2–4-cell stage with no further cyst development. When the cultures were treated with pepsin to digest the cyst (Freyre 1995) and tachyzoite cultures were initiated, no parasites were recovered. Parasites with only the *cat* construct differentiated efficiently and were fully viable after pepsin digestion and recovery as tachyzoites. This strategy is now being further refined by using the episomal vector described in the previous section. Ultimately, it should allow for generation of

Table 1. *Efficiency of cotransfection and effect of LDH2-driven cNTP2 expression on differentiating bradyzoites*

(NA, not applicable; ND, not done.)

Transfecting plasmids	co-transfection[a] (%)	differentiation *in vitro* (Tz to Bz)[b] (%)	recovery (Bz to Tz)[c] (%)
cat	NA	100	100
cat + *SAG1–βgal*	100	ND	ND
cat + *LDH2–cNTP2*	20	<1	0
cat + *TUB1–cNTP2*	0	NA	NA

[a]Percentage of parasites resistant to chloramphenicol that also showed uptake of the cotransfecting plasmid (assessed by enzymatic activity for β-galactosidase and by Southern blotting for the *cNTP2* constructs).

[b]Efficiency of cyst development in response to conditions of pH 8 *in vitro* relative to strains that received the *cat* plasmid only. One of the lines that received *cat* + *LDH2–cNTP2* was expanded as tachyzoites and then switched to bradyzoite conditions. Of over 100 vacuoles examined in this strain, none showed other than aborted development whereas the line that received *cat*-only control showed the usual 90–95% successful differentiation. (Note that it was not possible to do the analysis with the *TUB1–cNTP2* construct as no stable transformants harbouring this gene were obtained.)

[c]Relative viability of bradyzoites *in vitro*. After the induction of cysts *in vitro*, cultures were treated with pepsin at low pH to digest the cysts (Freyre *et al*. 1995), and then passed to fresh cultures under tachyzoite conditions to measure the viability of the organisms by plaque assay.

mutants that are deficient in their ability to differentiate to the bradyzoite stage in response to the high-pH conditions.

(*f*) *Promoter trap with HPT*

A second selectable marker, HPT, which has the major advantage of being usable in both positive and negative selection strategies, has also been pursued. Expression of HPT in the presence of 6-thioxanthine (6-TX) is growth-inhibitory to the parasite: HPT converts the 6-TX to 6-thioxanthineribose monophosphate, which can inhibit guanosine monophosphate (GMP) synthetase and/or be further phosphorylated and then misincorporated into RNA. Conversely, growth in the presence of mycophenolic acid (MPA) and xanthine will select for the presence of HPT, because MPA will inhibit the only alternative route to xanthosine monophosphate (XMP) (inosine monophosphate (IMP) dehydrogenase), making the parasites fully dependent on HPT for growth. The use of this selection strategy was developed for *Toxoplasma* by Drs E. Pfefferkorn, B. Ullman, D. Roos and colleagues (Donald *et al*. 1996); protocols and reagents were kindly provided to us by Dr Roos and/or obtained from the Office of AIDS Research Repository.

The selection strategy requires starting with a strain of parasite that is deficient in HPT. To create such a strain, Dr Roos' laboratory used a cloned derivative of wild-type ME49 (in this case PLK) and inactivated the endogenous *HPT* gene by targeted insertional mutagenesis. Such a strain has been found to be completely resistant to up to 400 μg ml^{-1} 6-TX (over this concentration the host cells begin to be affected). Growth of wild-type PLK, in contrast, is completly eliminated by concentrations as low as 40 μg ml^{-1} 6-TX.

Once this Hpt^- strain had been obtained, it was necessary to engineer it such that it carried the *HPT* gene under control of a bradyzoite-specific promoter. To do this, a promoterless copy of the *HPT* gene was inserted into a plasmid to yield the construct shown in figure 5(*a*). This *HPT* cassette includes 30 bp of buffer sequence (with no start or stop codons) upstream of the HPT coding region so that if any chew-back occurs on integration the *HPT* will not be affected. The resulting plasmid was linearized immediately upstream of this cassette and electroporated into the Hpt^- PLK strain by using restriction-enzyme-mediated integration (REMI) (Black *et al*. 1995)). It was found that REMI gave a considerable enhancement of stable transformation frequency in *Toxoplasma*. In this particular experiment, DpnII was used as the added enzyme; DpnII will not digest the bacterially grown plasmid but will damage the parasite DNA and thus induce the repair machinery that is thought to mediate the integration.

Stable transformants were selected for chloramphenicol resistance (Kim *et al*. 1993). Because an attempt was being made to select bradyzoite-specific genes, the resulting population was grown as tachyzoites in the presence of either 40 or 400 μg ml^{-1} 6-TX to select for parasites with variable *HPT* expression. Both concentrations will stop the growth of all parasites that have the promoterless *HPT* integrated downstream of a promoter that is on in tachyzoites. The population that survived this treatment was then induced to differentiate to bradyzoites and subjected to a positive selection by growing the parasites in medium containing 50 μg ml^{-1} xanthine (X) as a substrate for HPT and either 100 or 200 μg ml^{-1} MPA. In this way, parasites that have turned on the *HPT* gene will survive whereas those that had the plasmid integrated into transcriptionally silent regions (or inserted in any way that did not give active expression) will die.

Several lines have been generated by using this or simlar protocols. The growth of one of these strains under bradyzoite conditions was virtually unaffected by the presence of 200 μg ml^{-1} MPA+X. For convenience, this line will be referred to as the BH1 line (for bradyzoite control of HPT expression). Most critically, the killing of BH1 when grown as bradyzoites in the presence of 6-TX was examined. To do this, duplicate cultures were infected with 7×10^5 tachyzoites of the parental (Hpt^-) or BH1 lines. The medium was changed after 4 h to bradyzoite-inducing conditions and incubation was continued at 37 °C in air. Approximately 14 h after this, 6-TX was added to one of each pair of flasks to a concentration of 300 μg ml^{-1} and incubation was continued for another 32 h. The parasites were harvested and syringed and dilutions were used to infect fresh monolayers under tachyzoite-culturing conditions, again in the presence or absence of the drug, and incubated at 37 °C in 5% CO_2. Fourteen hours after reinfection, the medium (without 6-TX)

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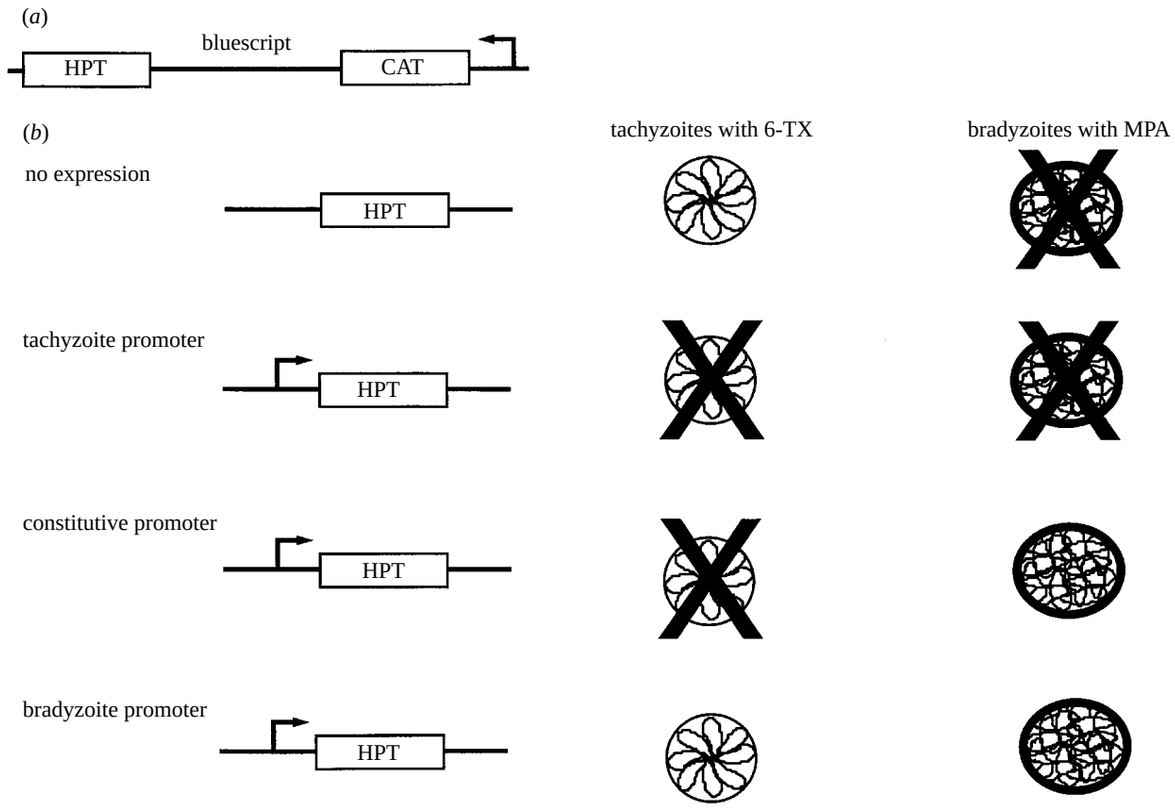

Figure 5. Promoter trap for identification of bradyzoite-specific genes. (*a*) Promoterless constructs used for insertion. Note that the promoterless HPT is in the opposite orientation to the selectable CAT gene. (*b*) Strategy for selection of bradyzoite-specific expression of the promotorless HPT inserts. Four scenarios are shown (note that 'no expression' could be due to insertion into a transcriptionally silent region, insertion in the wrong orientation, or insertion into the wrong frame or in any other way that fails to yield expression). The outcome of selection with 6-TX under tachyzoite growth conditions and MPA under bradyzoite conditions are shown (the large X indicates a block in growth; see text for explanation).

was replaced in all flasks. The flasks were incubated for 7 d, after which plaque numbers were determined by standard methods. The results showed that BH1 had only about 4% parasite survival, compared with growth in the absence of drug. The parental control, on the other hand, showed the expected *ca.* 95% survival. Further examination of BH1, however, showed that it was also expresed at the tachyzoite stage, albeit minimally; it is therefore not strictly a bradyzoite-specific gene. By increasing the stringency of the 6-TX and/or the incubation times, it has been possible to remove constitutive expressers from the pool of mutants and select genes whose expression appears to be restricted to the bradyzoite stage. These new strains are currently being tested for ability to survive 6-TX in bradyzoite conditions. These should ultimately provide the necessary genetic background to allow the selection of mutants based on an inability to respond to the stimuli for differentiation *in vitro* and thereby survive treatment with 6-TX. Work is now in progress to select such mutants and identify the genes that may be crucial to the differentiation process.

4. CONCLUSIONS

A major effort to understand the intricate biology and biochemistry of development in *T. gondii* has begun. Thanks to the efforts of many laboratories over the past five years or so, many of the tools and reagents essential to this undertaking are now available. In the coming five years, we can look forward to major advances in the understanding of the developmental biology of this important parasite. Translating these to the benefit of the animals and people affected will be the next challenge. For example, such knowledge could aid in treatment of the infection through enabling researchers to 'push' the parasites in the direction of the less virulent bradyzoite or 'pull' them back to the more drug-accessible tachyzoite stage.

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