CHAPTER 3

Molecular Tools for Genetic Dissection of the Protozoan Parasite *Toxoplasma gondii*

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=========== **I. Introductory Overview**

A. Genetics of *Toxoplasma gondii*

Despite the importance of protozoan infections in human and veterinary disease worldwide, it has been difficult to unravel the mechanisms of pathogenesis for many of these organisms—particularly intracellular parasites. In part, this problem stems from the difficulty of culturing many such pathogens, and from the lack of suitable genetic systems. The Apicomplexan parasite *Toxoplasma gondii* provides a promising exception to this rule. *T. gondii* is convenient and safe to grow *in vitro* using standard cell culture techniques; the tachyzoite (merozoite) form is haploid and capable of indefinite asexual replication in culture. Beginning in the 1970s, pioneering work by Dr. E. R. Pfefferkorn and colleagues established the potential of *T. gondii* for genetic studies (Pfefferkorn, 1988). Parasites are readily mutagenized (Pfefferkorn and Pfefferkorn, 1979) and the clonal progeny of individual mutants can be plaque purified (Foley and Remington, 1969; Pfefferkorn and Pfefferkorn, 1976). In the wild, *T. gondii* is an unusually promiscuous parasite, infecting virtually any vertebrate tissue (Frenkel, 1973). *In vitro,* this wide host range facilitates experiments conceptually similar to somatic cell genetic studies, where existing mammalian cell mutants are used to determine what the parasite can do for itself and what it requires from its host (Pfefferkorn, 1981).

Within cells of the cat intestinal epithelium (but, alas, not yet in culture), *T. gondii* parasites are also capable of sexual differentiation and mating (Frenkel, 1973), permitting classical genetic crosses (Pfefferkorn and Pfefferkorn, 1980). The resultant progeny—8 haploid sporozoites within a single oocyst—are readily purified, and individual sporozoites can be isolated by micromanipulation (D.S. Roos and E. R. Pfefferkorn, unpublished). *Toxoplasma* exhibits normal Mendelian linkage patterns and chromosomal reassortment through the sexual cycle, with a level of reciprocal recombination suitable for genome mapping (Sibley *et al.,* 1992).

The molecular genetics of protozoan parasites have proved nearly as intractable as classical genetic approaches. Transformation of the Kinetoplastida (*Trypanosoma, Leishmania,* etc.) has only become possible within the past few years (Bellofatto and Cross, 1989; Laban *et al.,* 1990; Cruz and Beverley, 1990; Lee and van der Ploeg, 1990; ten Asbroek *et al.,* 1993), and there is still no practical system for transformation of such important pathogens as *Plasmodium* sp. (malaria) (Miles, 1988; Wellems, 1991). In contrast to many protists, the genetic structure of *Toxoplasma* is notable chiefly for being relatively conventional, i.e., similar to that of its mammalian host cells with respect to gene organization, codon usage, and nucleotide bias (Roos, 1993; Ellis *et al.,* 1993). These observations have led several investigators to examine the feasibility of molecular transformation in this parasite, and such studies have proved very successful (Soldati and Boothroyd, 1993; Donald and Roos, 1993; 1994; Kim *et al.,* 1993;

Sibley *et al.*, 1994). This review outlines the use of several of the molecular genetic tools that have recently been developed for the *T. gondii* system. Because the availability of these tools has resulted in a significant increase in the number of investigators interested in the basic biology of *Toxoplasma*, a brief introduction to parasite culture techniques is also provided.

B. The Parasite Life Cycle

The life cycle of *T. gondii* can be thought of as two independent cycles, intersecting in the form of the intracellular tachyzoite (Fig. 1). Asexual replication can occur in virtually any nucleated animal cell, and consists of the following steps (for reviews, see Joiner and Dubremetz, 1993; Sibley, 1993):

1. Attachment to the host cell. A variety of ligands, including laminins, may be involved.
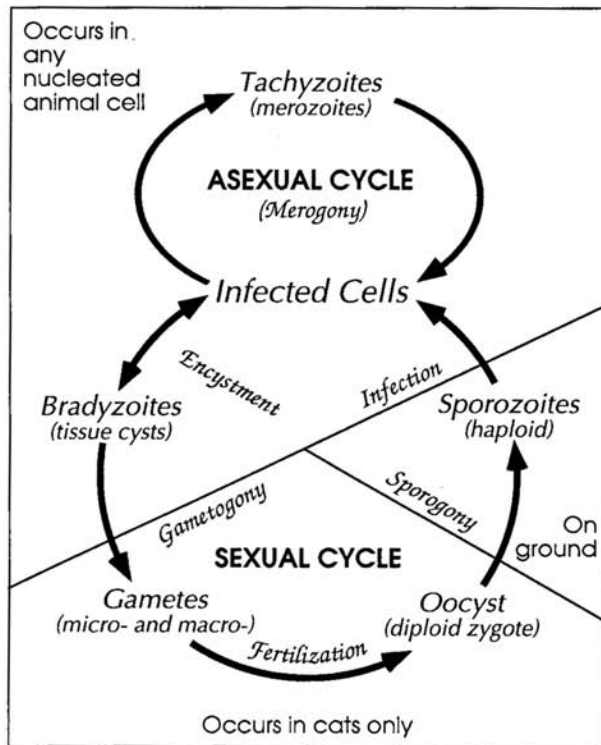

Fig. 1 Asexual and sexual cycles of *Toxoplasma gondii* replication. Asexual replication of *T. gondii* "tachyzoites" (merozoites) can be maintained indefinitely *in vitro*, in virtually any nucleated animal cell. Sexual differentiation occurs only within feline intestinal epithelium. See text for details on the genetic possibilities provided by this parasite.

2. Invasion into a specialized parasitophorous vacuole. The mechanism of invasion and precise origin of the vacuole is unknown, but this process is usually associated with clearing of one or more of the rhoptries—specialized organelles located at the apical end of the parasite. Secretion from dense granules is thought to play a role in establishing and/or maintaining vacuolar membrane function.

3. Multiple rounds of replication within the parasitophorous vacuole. *Toxoplasma* replicates by endodyogeny, producing two daughter parasites in each division. In contrast, many Apicomplexan parasites replicate multiple times before assembly of the mature daughter cells (merogony). Because all parasites within a given vacuole divide synchronously, the number of tachyzoites in any vacuole is invariably a power of two (Fig. 2).

4. Host cell lysis and parasite exit, facilitated by a twisting motility of the parasites. The motility of parasites during escape contrasts with their quiescence during replication, and is probably induced by the ionic changes that occur as the host cell membrane begins to give way under mechanical strain from the growing parasitophorous vacuole (Endo *et al.*, 1987; Endo and Yagita, 1990).

Within animal tissues, tachyzoites are also capable of differentiating into a more slowly replicating form—termed bradyzoites—which develop a periodic acid/Schiff-positive wall and may persist within the tissues for months or years, reemerging periodically to provide a natural boost to host immunity (McLeod and Remington, 1987). Given the high frequency of latent infection (10–90% in populations worldwide), bradyzoites represent a serious danger to immuno-compromised individuals (Luft and Remington, 1992).

In contrast to the broad host range of asexual parasite forms, sexual differentiation is known to occur only in feline species (Frenkel, 1973). When bradyzoites infect intestinal epithelial cells (following ingestion of an infected mouse, for example), the parasites can differentiate into macro- or microgametes. The latter rupture out of infected cells and fuse with macrogametes to produce diploid oocysts, which develop a thick, impermeable wall and are shed with the feces. Upon exposure to oxygen and ambient temperature, the oocyst undergoes sporogony: two meiotic divisions and a single mitotic division produce eight haploid sporozoites, divided into two sporocysts within the oocyst wall. Sexual crosses can be carried out in cats under laboratory conditions (Pfefferkorn and Pfefferkorn, 1980), but although bradyzoite cysts have been observed in culture (Lindsay *et al.*, 1991; Bohne *et al.*, 1993; Soete *et al.*, 1993; and D. S. Roos, unpublished observations), it has not yet proved possible to reproduce the complete *Toxoplasma* sexual cycle *in vitro*. For further information on the biology of *T. gondii* infections see Frenkel (1973).
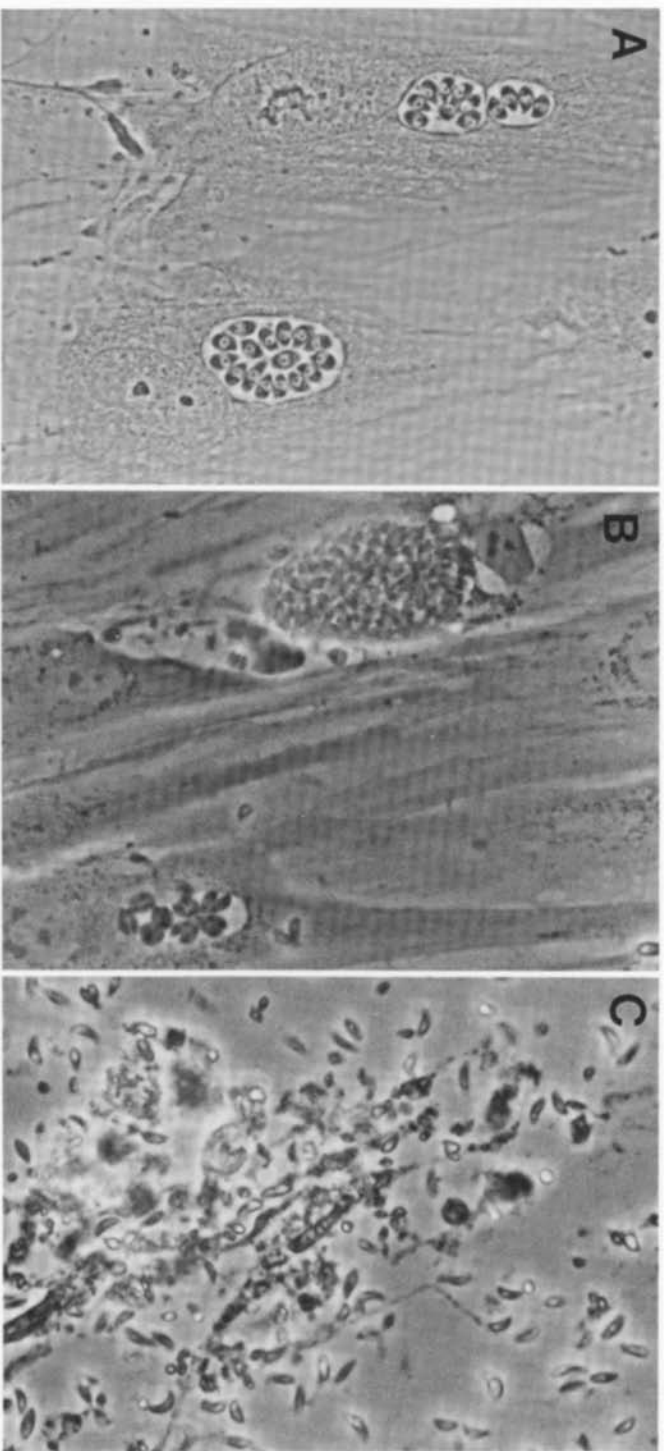
Fig. 2 *In vitro* culture of *T. gondii* in HFF cells. Tachyzoites replicate synchronously (by endodyogeny) within intracellular parasitophorous vacuoles. These vacuoles therefore always contain $2n$ parasites, where n indicates the number of divisions that have taken place since invasion. (A) Vacuoles at early stages of infection contain "rosettes" of 4, 8, or 16 parasites (fixed and stained with Giemsa). (B) Parasites continue to replicate within the parasitophorous vacuole, grossly distending the cell membrane. The large vacuole extends far out of the plane of focus in this phase-contrast micrograph. (C) The entire host cell monolayer is eventually destroyed, releasing highly refractile extracellular tachyzoites.

II. *In Vitro* Culture of *T. gondii* Tachyzoites

A. Growth of Parasites

1. Parasite Strains

Many strains of *T. gondii* have been studied in different labs, and although these differ in virulence and other respects, all strains are thought to constitute a single species (the only species within the genus *Toxoplasma*) (Levine, 1988). The virulent RH strain (Sabin, 1941) is commonly used because of its rapid replication rate, its high productivity, and its highly efficient lysis of host cells—facilitating the isolation of large numbers of tachyzoites relatively uncontaminated by host cell material. It should be noted that virulent strains identified as RH in several laboratories around the world appear to differ (Howe and Sibley, 1994), although it is not known whether these differences reflect heterogeneity within the original RH isolate, independent mutations over the past 50 years in culture, or laboratory contamination. To ensure strain homogeneity, it is advisable to work with strains of known provenance which have recently been clonally rederived (see below).

Toxoplasma RH forms few if any tissue cysts in mice—perhaps due to its virulence, or perhaps because of genetic alterations which prevent bradyzoite differentiation. RH strain *T. gondii* also fails to produce oocysts in cats. The ME49 strain has often been employed for studies on latent infection and sexual differentiation, and clonal isolates of this strain are available (Ware and Kasper, 1987).

Tachyzoites from virulent stains may be passaged either *in vitro* or by intraperitoneal infection of mice. Avirulent strains may be maintained in mice as latent tissue cysts, or in culture as tachyzoites. Only *in vitro* techniques are discussed below; for a discussion of *Toxoplasma* culture in animals, see Boothroyd *et al.,* (1994).

2. Host Cells

In clinical infections, *T. gondii* parasites are often found within macrophages and in the central nervous system (Frenkel, 1973), but these observations probably reflect the frequency with which macrophages encounter pathogenic organisms *in vivo*, and the immunologically privileged nature of the central nervous system, rather than any strong tissue tropism on the part of the parasite itself. Tachyzoites—the proliferative asexual form of *Toxoplasma*—may be maintained in virtually any mammalian cell type, including transformed cell lines (CHO, HeLa, LM, MDBK, Vero, 3T3, etc.). Parasites infect monolayer cultures more efficiently than suspension cultures, presumably because contact and invasion is easier to accomplish.

For routine cell culture, *T. gondii* tachyzoites are often grown in primary human foreskin fibroblasts (HFF cells), which offer several of advantages:

(i) The large, flat morphology of HFF cells provides an extensive plasma membrane, permitting multiple cycles of parasite replication within each cell before lysis. HFF cells therefore yield high parasite titers with minimal cell debris. (ii) HFF cells are strongly contact inhibited, permitting confluent monolayers to be prepared many weeks in advance and maintained until needed. (iii) Confluent HFF monolayers are highly resistant to many metabolic inhibitors (as a consequence of their strong contact inhibition), facilitating drug selection of parasites. (iv) As primary human cells, HFF cells provide a host comparable in certain respects to a clinical infection. Disadvantages of HFF cells include their limited replicative lifespan, slow growth rate (especially at high passage number), differences between HFF cultures available in different labs, and the possibility of contamination with latent human viruses.

HFF cells are maintained according to standard tissue culture techniques for monolayer cells and are released for passage using trypsin digestion. Care must be taken not to overtrypsinize HFF cells, as they are readily damaged. HFF cells are inoculated at moderate density (>2000 cells/cm^2) into tissue culture flasks (or plates, or wells) in modified Eagle's medium (MEM; ~ 0.3–0.4 ml/cm^2) containing 10% heat-inactivated newborn bovine serum (*HFF medium*), and incubated at 37°C in a humidified CO_2 incubator. Cells typically reach confluence ($\sim 3 \times 10^4$ cells/cm^2) in ~ 5–10 days, depending on passage number. Acid production is sufficiently low in HFF cells that flasks may be sealed after CO_2 levels have equilibrated (typically overnight), to minimize culture contamination. Confluent monolayers may be maintained for several weeks without replacing the original medium, but cultures differing in passage number, age, density, etc. may exhibit significant differences in their ability to support parasite growth. It is therefore advisable to use sister flasks of HFF cells set up in parallel whenever results from parallel parasite infections are to be compared.

3. Routine Parasite Culture

Because tachyzoite replication takes place intracellularly, serum growth factors are necessary primarily to satisfy the host monolayer rather than the *T. gondii* parasites themselves. (For labeling tachyzoite proteins with [^{35}S]-methionine, intracellular parasite replication appears to proceed normally for at least 12 hr in serum-free, methionine-free medium.) To minimize the possibility of exposing parasites to serum antibodies and complement, we typically replace the HFF medium with 1% heat-inactivated fetal bovine serum (FBS) in MEM (*Infection medium*) prior to inoculation with parasites. Certain transformed cell lines require higher serum content for maintenance in culture, but 1% FBS is sufficient for HFF cells once the cultures have reached confluence. For drug studies, dialyzed FBS may be employed.

T. gondii tachyzoites can be maintained indefinitely by serial passage, al-

though continued *in vitro* culture may affect the ability of parasites to traverse the sexual cycle in cats or form tissue cysts in animals (as is apparently the case for the RH strain). Complete lysis of a 25-cm^2 T-flask (T25) containing confluent HFF cells typically produces $\sim 5 \times 10^7$ RH-strain tachyzoites. Because tachyzoites die with a half-life of ~ 10 hr unless they invade a new host, it is important that extracellular parasites be used shortly after lysis of the cell monolayer. Plaquing efficiencies for freshly "lysed-out" RH-strain parasites are typically ~ 20–40%, but other strains may exhibit lower viability, even if released from the host cells by syringe passage (see below).

Passage for routine parasite maintenance

1. Aspirate HFF medium from a T25 flask containing confluent HFF cells. [Transformed cell lines (CHO, J774, LM, Vero, etc.) may also be employed for parasite growth, but infection must be coordinated with cell growth so that parasite lysis peaks close to the time of cell confluence.]

2. Add 9 ml fresh Infection medium.

3. Infect with freshly lysed-out tachyzoites: a 1-ml inoculum of RH-strain parasites ($\sim 5 \times 10^6$ tachyzoites) will completely destroy the host cell monolayer in 2 days; a 0.1-ml inoculum will lyse out in 3–4 days.

4. Incubate at 37°C in a humidified CO_2 incubator. Once pH equilibrium has been attained (typically overnight, depending on the rate of gas exchange) flasks may be sealed to minimize the risk of contamination.

5. Monitor at least daily for parasite growth, using an inverted microscope equipped with phase-contrast optics. Intracellular parasites are visible as banana-shaped organisms within the relatively transparent, intracellular parasitophorous vacuole. As replicate replication proceeds, vacuoles containing 2, 4, 8, etc., parasites become apparent, often organized as "rosettes" with the parasites' apical ends directed outward (Fig. 2). As vesicles swell still further through continued parasite replication, cells become grossly distended and sausage-like. After cell lysis, emerging parasites often squirm vigorously. Extracellular parasites are markedly more refractile ("phase-bright") than intracellular forms; "phase-dull" extracellular parasites are lysed, and fail to exclude trypan blue and other vital stains.

6. When the host cell monolayer is destroyed, this entire procedure may be repeated from step 1, above. All waste material should be decontaminated with bleach, alcohol, or by autoclaving before disposal.

Once established within the intracellular parasitophorous vacuole, tachyzoite replication proceeds with a doubling time of ~ 7.5 hr (RH-strain). The time between infection and lysis of the host cell is variable, and probably depends chiefly on space constraints: vacuoles in smaller cells (or within narrow extensions of large cells) typically rupture after 2–4 parasite doublings (≤ 16 tachyzoites), whereas vacuoles within the body of larger cells (e.g., HFF) may

contain as many as 256 (2^8) tachyzoites before lysing out of the host cell. No specific trigger for host cell lysis has been identified, and it is likely that lysis is simply a function of the cell's inability to contain an exponentially increasing parasite load. As noted above, emergence from the host cell is associated with a dramatic induction of parasite motility. Parasites may be at any stage in the replicative cycle when released from the host cell, and it is not uncommon to observe 'Siamese twins' afloat in the supernatant medium due to host cell lysis prior to the completion of endodyogeny (cytokinesis).

4. Purification of Tachyzoites

T. gondii tachyzoites may be purified by various means, although purification is not generally necessary for routine passage. The banana-shaped tachyzoites are ~6 μm in length, but only ~2 μm in diameter, and parasites may therefore be purified as a single cell suspension by filtration through 3-μm-pore-size polycarbonate filters (Nuclepore). Aggregated parasites and most host-cell debris remain behind. Filters and filter holders are available in a variety of sizes; a single 47-mm-diameter filter will generally suffice for filter purification of parasites from two 175-cm^2 T-flasks (T175s) of HFF cells. Small fragments of host cell debris remain in the supernatant following centrifugation for 20 min at 1500 × g. Tachyzoites may also be purified by filtration over glass fiber or cellulose columns (Bodmer *et al.*, 1972). Parasite density is determined using a standard hemocytometer.

Many parasite strains fail to emerge from the host cell monolayer as cleanly as the virulent RH laboratory strain. For such strains, tachyzoite yields may be improved by scraping the infected monolayer with a rubber policeman and forcing this material through a 27 gauge needle. Forcibly released parasites can be filtered as above.

5. Optimizing Production of Viable Parasites

Although tightly synchronous infection with *Toxoplasma* cannot be achieved at present (as noted above, the time between infection and cell lysis is variable, and host cells may lyse and release parasites at any stage during their replicative cycle), it is nevertheless possible to observe fluctuations in parasite titer in the supernatant medium, caused by alternating cycles of intracellular replication followed by parasite emergence. To maximize the production of viable parasites, it is necessary to consider the inoculating titer relative to total host cell number. If the penultimate cycle of infection results in lysing 50% of the available host cells, for example, the total yield of viable parasites will be suboptimal—residual extacellular tachyzoites from that round of infection will lose viability while parasites are replicating in the remaining host cells. For large-scale culture, it is therefore advisable to inoculate many flasks at high density, rather than inoculating at low density and waiting for the eventual destruction of the host

cell monolayer. To favor the relatively synchronous emergence of parasites grown in multiple flasks, it is helpful to use host cell monolayers that are set up and maintained in parallel.

Large-scale culture protocol

1. Infect T25 flask as described above.

2. When parasites emerge, divide the entire 10-ml culture ($\sim$2–6 $\times$ 10^7 tachyzoites) between two T175s, each containing a total of 50 ml Infection medium.

3. When these parasites, in turn, 'lyse out' of the host cells ($\sim$2 days), the emerging parasites are used to infect 26 $\times$ T175s.

4. Follow cultures by inspection under an inverted microscope (at least twice daily), and when the host monolayer reaches >80% lysis filter supernatant medium through 3-μm polycarbonate membrane (see above) into 6 x 250-ml conical centrifuge bottles.

5. Vigorously knock flasks against palm of hand to dislodge residual parasites, rinse the residual monolayer with 5 ml phosphate-buffered saline (PBS), filter as above, and combine with the original filtrate. Repeat this procedure.

6. Remove a small aliquot from each centrifuge bottle for quantitation using a hemocytometer.

7. Pellet parasites for 20 min at 1500 $\times$ *g* in a refrigerated centrifuge maintained at 4°C. Yields are improved by slow deceleration.

8. Remove supernatant medium, resuspend pellets in a small volume of PBS, combine in a 15-ml conical centrifuge tube, and repellet as above. Yield for RH-strain: 5–10 $\times$ 10^9 tachyzoites ($\sim$0.1–0.2 ml packed cell volume); ME49 strain, 2–6 $\times$ 10^9. When working with highly concentrated parasites, it is particularly important to guard against possible infection through splashes to the eyes; see safety precautions discussed below.

It is also possible to scale-up parasite production using roller culture (Leriche and Dubremetz, 1991), although continuous agitation may inhibit effective invasion and therefore increase the percentage of dead extracellular tachyzoites harvested from the supernatant medium.

6. Cloning by Limiting Dilution or Growth under Agar

Both because extracellular tachyzoites may aggregate and because the residue of lysed host cells often contains many parasites enmeshed in cell debris, it is essential that tachyzoites be purified by filtration prior to cloning. Individual parasite clones may be isolated by limiting dilution in microtiter plates containing confluent host cells. Assuming 20–40% viability of the extracellular tachyzoites, inoculation of 96-well plates with 0.25 tachyzoites per well yields a predicted frequency of 5–9 wells containing a single parasite clone, and <0.5 wells containing more than one parasite. Wells containing single parasite plaques

can be identified easily using an inverted microscope, and parasite clones removed to T-flasks for expansion. Depending on the rate of parasite growth it is generally possible to identify clones within 7–10 days of plating, and expand these to $>5 \times 10^7$ tachyzoites (i.e., the yield from one T25) in a total of 2–3 weeks.

As an alternative to cloning by limiting dilution, parasite clones may also be isolated by plaque purification. Serial dilutions of parasites are inoculated into confluent HFF monolayers grown in 6-well plates (or larger dishes), and incubated at 37°C for 8–16 hr to permit infection. The supernatant medium is then replaced with a 1:1 mixture of $2\times$ Infection medium and 1.8% BactoAgar (agar melted at 56°C and cooled to <41°C before addition). After the agar has solidified at room temperature, infected dishes are incubated at 37°C until plaques are visible under the microscope—typically 7–10 days for RH-strain *T. gondii*. After marking the position of plaques under the microscope, plugs containing individual parasite plaques are removed using a sterile, plugged Pasteur pipette and inoculated directly into T25s containing confluent HFF cells.

7. Long-Term Storage

Toxoplasma tachyzoites may be frozen in liquid nitrogen using DMSO as a cryoprotectant. As noted above, parasites die rapidly in the extracellular environment, but intracellular tachyzoites fare very poorly during freezing. It is therefore preferable to prepare parasites for freezing by high-titer infection of HFF host cells, and to freeze large numbers of freshly lysed-out extracellular parasites (concentrated by centrifugation) in a small volume of cryoprotectant medium.

The protocol outlined below has been optimized with respect to time of parasite harvest (intracellular vs extracellular tachyzoites; parasites in the midst of host cell lysis vs completely lysed-out cultures), cryoprotectants (DMSO vs glycerol), cryoprotectant concentration, and serum concentration. Prolonged exposure to DMSO reduces viability, and it is therefore important that parasites be frozen as soon as possible after addition of the cryoprotectant. In contrast to others' experience, we have not found that more gradual addition of cryoprotectant improves parasite viability. In our hands, slow freezing protocols fail to improve upon simply placing of parasites in a precooled freezer box at −80°C, but direct freezing in either liquid nitrogen or a dry ice/ethanol bath is detrimental to parasite survival.

Freezing protocol

1. Infect a confluent T175 containing HFF cells with the entire contents of a freshly lysed-out T25 in a total of 50 ml Infection medium and incubate overnight at 37°C.

2. Observe the culture to ensure that virtually all cells are infected and follow until ~80% of the host cell monolayer has lysed.

3. Transfer the supernatant medium to a 50-ml conical centrifuge tube (filtration is not required), remove an aliquot for quantitation, and concentrate the parasites by centrifugation for 15 min at $1500 \times g$.

4. Aspirate the supernatant medium and resuspend parasites to a concentration of at least 10^8/ml in ice-cold Infection medium.

5. Add an equal volume of ice-cold Infection medium previously supplemented with 20% heat-activated FBS and 25% DMSO and mix by gently inverting (final concentrations: 10% FBS, 12.5% DMSO).

6. Pipette 0.5-ml aliquots (larger volumes do not thaw well) into 1.8-ml capacity round-bottom cryotubes and freeze at $-80°C$ in a precooled freezer box.

7. After checking viability by thawing one vial for each sample frozen, the remaining vials may be transferred to liquid nitrogen. (Viability at $-80°C$ is variable, probably because of temperature fluctuations introduced through use of most laboratory freezers; in liquid nitrogen, parasites remain viable for at least 15 years).

It is always advisable to confirm the viability of a frozen sample prior to discarding the parental parasite culture. As for mammalian cells, speed is essential in thawing parasite samples. The round-bottom cryotubes recommended above are preferable to tubes with a molded base because of their more rapid thawing rate; glass vials are still more effective vessels for parasite storage provided that an oxyacetylene torch is available for sealing.

Thawing protocol

1. Remove a frozen vial from liquid nitrogen (or $-80°C$ freezer) directly into a beaker of water at 37°C and agitate continuously until thawed (~40 sec).

2. Decontaminate the vial surface in 70% ethanol and wipe completely dry.

3. Transfer the thawed parasite sample directly to a 15-ml conical centrifuge tube containing 10 ml Infection medium and mix gently by inverting.

4. Pellet parasites for 15 min at $1500 \times g$.

5. Aspirate supernatant medium, gently resuspend parasite pellet in 10 ml fresh Infection medium, and add to a confluent monolayer of HFF cells in a T25 flask.

6. Observe cultures to verify that infection has been established and that no contamination is present.

Some investigators report improved parasite survival when the frozen aliquot is gradually diluted into Infection medium over the course of 15 min. Alternatively, thawed parasites may be directly inoculated into T25 flasks, and DMSO removed by replacing the medium after parasites have infected the monolayer. (It is not clear whether the presence of low concentrations of DMSO during the early stages of infection and replication is more detrimental to tachyzoites than centrifugation in the presence of the cryoprotectant.)

Using wild-type RH-strain tachyzoites it is commonly possible to rescue >50% of the parasite titer present before freezing (determined by plaque assay). Other strains and parasite mutants may freeze less successfully.

B. Replication Assays

Several techniques are available for following the survival of parasites under various conditions. As shown in Fig. 3A, all four of the assays described below yield comparable results in measuring the effects of pyrimethamine, an antifolate commonly used for inhibition of *T. gondii* both in culture and in the clinic.

1. Plaque Assays

Parasite viability may conveniently be assessed by plaque assay on HFF cell monolayers. Other cell types may also be used, but the slow replication rate of HFF cells (especially in the low serum Infection medium) permits plaque assays to be carried out even under conditions where parasite replication is greatly diminished. Although plaque assays may be performed in petri dishes or multiwell plates, plaque morphology is improved when the assays can be shielded from excess vibration (such as frequent opening and closing of incubator doors); T25 flasks offer the advantage of being easily sealed, after which they may be transferred to a standard (i.e., non-CO_2, nonhumidified) incubator or warm room for the duration of the assay.

Plaque assay protocol (adapted from Foley and Remington, 1969; Pfefferkorn and Pfefferkorn, 1976)

1. Inoculate T25 flasks, 35-mm dishes, or 6- or 24-well plates containing confluent HFF cells with serial 10-fold dilutions of freshly lysed-out tachyzoites in fresh Infection medium. For T25 flasks, up to ~200 plaques per flask can reliably be scored, so inocula of 200, 2000, 2×10^4, etc., are usually prepared (depending on the expected kill curves). For 35-mm dishes or 6-well plates, a maximum of ~50 plaques can be scored, vs ~15 per well in 24-well plates. Because of the narrower range of acceptable inocula using smaller plates or wells, statistical concerns mandate shallower dilutions (e.g., threefold) and inoculation of replicate wells.

2. In addition to the above, inoculate one or two control flasks/dishes/wells with a dilution of untreated parasites calculated to produce a reasonable number of plaques (e.g., 50 plaques per T25).

3. After pH equilibrium has been established but before any host cell lysis occurs (~8–24 hr), T25 flasks may be sealed and transferred to another incubator or warm room, if desired.

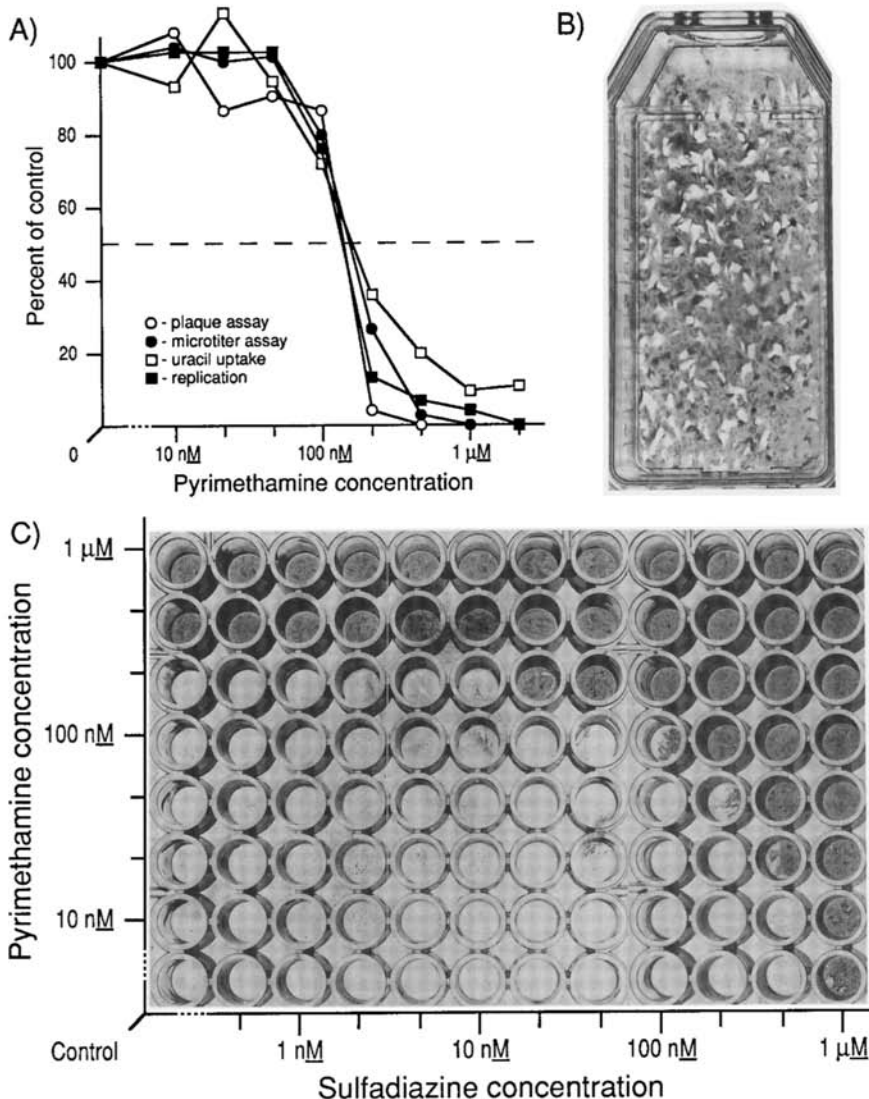4. Incubate undisturbed for at least 6 days.

Fig. 3 *In vitro* assays. (A) Four independent assay techniques yield similar 50% inhibitory concentrations (IC$_{50}$) for pyrimethamine against RH-strain *T. gondii*. (○) Plaque assay (% control plaques); (●) microtiter assay (100—% maximum density); (□) uracil uptake assay (% control incorporation, 24–26 hr postdrug addition); (■) intracellular replication assay (% control doublings, 24–48 hr postdrug addition). See text for details on assay procedures. (B) Plaque assay. Eight days postinfection, the culture was fixed in methanol and stained with crystal violet to reveal clear, irregular parasite plaques against the mottled purple background typical of HFF cells. (C) Microtiter assay. HFF cells were infected with *T. gondii* tachyzoites in the presence of pyrimethamine and/ or sulfadiazine at the indicated concentrations. This highly virulent strain originally isolated from a patient with AIDS-toxoplasmosis is approximately twofold less sensitive to pyrimethamine than the RH-strain shown in (A) (parasites kindly provided by Drs. J. S. Remington and B. Danneman). As indicated in the text, such assays readily lend themselves to the analysis of multidrug interactions, such as the demonstration of approximately fivefold synergy between these two inhibitors of the folate pathway (note log–log scale).

5. Without disturbing experimental samples, remove one control flask or plate to assess the extent of plaque growth. Such controls can be examined for a maximum of 2–3 days after first being moved before satellite plaques begin to appear.

6. When controls indicate that plaques are of adequate size for visualization (usually ~9 days postinfection), experimental samples should be aspirated, rinsed in PBS, fixed in for 5 min in methanol, stained in crystal violet (5X stock: dissolve 25g crystal violet in 250 ml ethanol and add to 1000 ml 1% ammonium oxalate), and air-dried. Parasite plaques appear as irregular clear areas against the mottled violet background produced by confluent HFF cells (Fig. 3B).

To prevent formation of secondary plaques, especially if it is difficult to establish an undisturbed location for parasite incubation, medium may be aspirated 16–24 hr postinfection (i.e., after invasion has occurred, but before lysis of the host cells begins) and replaced with medium containing 1.8% BactoAgar, as described above. After plaques appear, the remaining host cells may be stained overnight by the addition of medium containing 0.01% neutral red dye on top of the agar. Parasites grown under agar typically take 1–2 days longer to form plaques than when grown in liquid culture.

2. Monolayer Disruption Assays

Parasite viability may also be determined by microtiter assays that assess the ability of parasites to lyse the host cell monolayer. These assays are particularly valuable for generating kill-curves following drug (or other) treatment. Although less quantitative than plaque assays, this approach is more rapid (results can often be obtained in 4–5 days), less dependent on precise estimation of inoculum titer, produces less plastic waste (and is commensurately less expensive), and is readily automated both for set-up and analysis. Microtiter assays also lend themselves to two-dimensional analysis (e.g., for drug interaction/synergy studies; Fig. 3C).

Microtiter assay protocol (one-dimensional assay)

1. 96-well microtiter plates containing HFF cells may be set up several weeks prior to use (typically inoculated with 10^3 cells per well in 250 μl HFF medium) and maintained until needed, but note that microtiter plates are unusually susceptible to desiccation—particularly at the edges and corners—and must be kept well humidified.

2. Aspirate medium from a 96-well microtiter plate(s) and replace with 200 μl Infection medium per well. (Aspirator manifolds and multiple-pipettors are helpful in working with microtiter plates.)

3. To the first well in each row, add 173 μl Infection medium containing 2.70X the top drug concentration desired.

4. Serially dilute 173 μl medium from one well to the next along the row. To provide an untreated control, do not add diluted drug to the last well (discard the 173-μl aliquot removed from penultimate well).

5. Dilute freshly lysed-out, filter-purified parasites to 2×10^4/ml in Infection medium and add 50 μl to each well of microtiter plate (final inoculum = 1000 parasites per well).

6. Incubate at 37°C in humidified environment containing 5% CO_2.

7. Observe daily and record cytopathic effect.

8. When the cell monolayer is destroyed in the control well (or later, to follow the progress in intermediate wells), aspirate and decontaminate medium, rinse in PBS, fix in methanol, and stain using crystal violet (see above). Quantitate monolayer destruction by densitometry, if desired.

As noted above, these assays readily lend themselves to two-dimensional analysis, producing a matrix of drug interactions (cf. Figure 3C). In setting up such assays, both drugs must be added prior to parasite addition. To ensure that drugs added in the second dimension do not affect the concentration of the first drug, increase the concentration of drug added to the first well in the first row *only* by 1.87-fold (beyond the 2.7-fold noted above; 5.03-fold above the desired final concentration). Addition of the second drug to this row (the first well in each column) will dilute the first drug to the proper final concentration.

3. Incorporation of [³H]-Uracil

Unlike its mammalian host cells, *T. gondii* tachyzoites are capable of taking up and utilizing uracil directly for pyrimidine salvage (Schwartzman and Pfefferkorn, 1981; Iltzsch, 1993). [³H]-Uracil therefore serves as a parasite-specific metabolic label for measuring viability (Pfefferkorn and Pfefferkorn, 1977). As a metabolic assay, uracil uptake does not require multiple cycles of parasite replication, and is therefore even more rapid than microtiter assays. Twenty-four-well plates provide a convenient level of labelling, but the protocol outlined below can readily be adapted for other configurations.

Uracil uptake assay protocol (adapted from Pfefferkorn and Guyre, 1984)

1. Inoculate confluent monolayers of HFF cells grown in 24-well plates with freshly harvested, filtered *T. gondii* parasites in 1 ml Infection medium and incubate under desired conditions.

2. Add 5 μCi [5,6-³H]-uracil (30–60 Ci/mmol) and incubate for 2 hr at 37°C. (Note: [³H]-uracil degrades within days in aqueous solution at 4°C and is stable for no more than a few weeks at -80°C. Aliquots may be stored for a period of many months in 70% ethanol at -80°C, however, and lyophilized and resuspended in culture medium immediately prior to use.)

3. Chill the tray(s) at -20°C for 2 min, add 1 ml ice-cold 0.6 *N* trichloroacetic acid (TCA) to existing medium, and incubate on ice for 1 hr to fix the monolayer.

4. Remove TCA solution to radioactive waste container, rinse, and immerse the plate(s) in running water bath overnight (or at least 4 hr).

5. Shake the plate(s) dry and add 0.5 ml 0.1 N NaOH per well. Incubate at 37°C for 1 hr to dissolve the TCA precipitate.

6. Count 0.25 ml from each well in scintillation fluid suitably acidified to neutralize NaOH (3.6 ml glacial acetic acid per gallon; use 3 ml scintillant per sample). Inoculation of plates with 10^4 RH-strain tachyzoites 24 hr prior to labeling typically results in incorporation of 10^4 cpm during the course of a 2-hr incubation (background from uninfected host cells is typically ~300 cpm).

Although uracil incorporated into extracellular parasites is precipitated as readily as for intracellular parasites, the TCA treatment does not necessarily fix extracellular parasites to the cell monolayer. If a substantial number of extracellular parasites are present, incorporation in the TCA supernatant may be collected by filtration through glass-fiber (Whatman GF/C) filters (Pfefferkorn and Pfefferkorn, 1977).

4. Direct Measurement of Doubling Time

As a more direct means of assessing the effect of various treatments, parasite replication rates may be determined by following the number of cell doublings as a function of time. Although somewhat laborious, these assays provide data at the level of individual parasites rather than a population average, without requiring the multiple cycles of infection necessary for plaque production.

Taking advantage of the fact that all parasites within a single vacuole replicate synchronously, it is possible to determine the number of mitotic divisions since infection as $\log_2$(parasite number). Although it is not currently possible to synchronize precisely parasite replication between different vacuoles (see above), a reasonable degree of synchronization can be established by inoculating cells in a small volume of medium and replacing the medium after ~4 hr—rinsing away parasites that failed to infect during the 4-hr window. The average number of cell divisions in the population (e.g., average of 100 randomly selected vacuoles) is scored at 4 to 8-hr intervals.

Alternatively, the replication rate of individual vacuoles may followed by video microscopy or scored by marking individual vacuoles and following the replication rate by visual inspection of the same exact vacuoles at 4-hr intervals.

C. Safety Issues

Thirty percent of the U. S. population is estimated to become infected with *T. gondii* in the course of their life (risk factors include gardening or farming, preference for rare meat, and cat ownership; Frenkel, 1973; McLeod and Remington, 1987). Infection in an otherwise healthly adult is normally asymptomatic (McLeod and Remington, 1987). Moreover, as opposed to the bradyzoite (tissue cyst) and oocyst forms, the tachyzoite parasite form discussed in this review

is only infectious by direct introduction into the bloodstream or heavy inocula-
tion of the mucous membranes (e.g., through an eye-splash). Nevertherless,
in vitro culture probably selects for increased virulence, and regardless of
the worker's antitoxoplasma serum titer, any needle stick or other potentially
infectious exposure should be considered a presumptive case of toxoplasmosis
and treated promptly with pyrimethamine–sulfadiazine accompanied by leuko-
vorin rescue (Brooks *et al.*, 1987).

All work with live organisms should be carried out in disposable tissue culture
ware using class-2 biosafety hoods. Eye protection should be worn when work-
ing outside of laminar flow hoods, particularly when using highly concentrated
parasite cultures. The use of sharp implements (needles, Pasteur pipettes, etc.)
should be limited, and flasks, pipettes, etc., that contact live parasites should
be immediately decontaminated with alcohol, bleach, or acid, or by autoclaving
or boiling. All laboratory personnel must be informed of the potential danger
of *Toxoplasma* as a human pathogen and alerted to symptoms indicating possible
parasite infection. Additional considerations apply to work with drug-resistant
organisms (see below). Because of the special danger of toxoplasmosis for
pregnant women and HIV-positive individuals, such individuals should not
work with live parasites.

III. Molecular Transformation Systems for *Toxoplasma*

A. Vectors

A series of transformation vectors has been developed for *T. gondii*, based on
regulatory and coding sequences derived from the parasite's fused dihydrofolate
reductase–thymidylate synthase gene (DHFR-TS; Donald and Roos, 1993;
1994a). Figure 4 illustrates the basic structure of several constructs employed
in the transformation schemes described below; DHFR-TS genomic and cDNA
sequences are available from GenBank, with the Accession No. L08489. All
plasmids are based on Bluescript pKS plasmid (Strategene), with polylinker
cloning sites indicated. For transient transformation, a chloramphenicol acetyl-
transferase (CAT) reporter gene was engineered for expression under control
of genomic DHFR-TS 5' and 3' noncoding sequences (p*dhfr*CAT*dhfr*). Stable
transformation vectors employ these regulatory regions linked to DHFR-TS
coding sequences, either as a cDNA-derived 'minigene' (pDHFR-TSc3) or in
the native genomic configuration (pDHFR-TSg8). Selection is facilitated by the
introduction of mutations in the DHFR domain predicted—by analogy with
drug-resistant *Plasmodium falciparum* isolates (Hyde, 1990; Wellems,
1991)—to provide resistance to pyrimethamine (indicated by asterisks in Fig.
4). To test the expression of foreign genes linked to the DHFR-TS selectable
marker, a CAT reporter construct under control of 5' and 3' noncoding se-
quences derived from the *T. gondii* major surface antigen P30 (SAG$^{1/2}$CAT

Soldati and Boothroyd, 1993) was introduced upstream of the DHFR-TS se-lectable marker (p*sag*CAT::DHFR-TSc3). Further details of vector construc-tion have been reported elsewhere (Donald and Roos, 1993, 1994a).

B. Transient Expression

Flanking sequences from a variety of *T. gondii* genes have been successfully used to drive expression of CAT reporter plasmids introduced into parasites by electroporation (Soldati and Boothroyd, 1993; Donald and Roos, 1993). As shown in Fig. 5A, promoter sequences are essential for expression, whereas 3′ flanking sequences provide only modest enhancement of the signal (downstream sequences may be more important for other *Toxoplasma* promoters; Soldati and Boothroyd, 1993). Electroporation of HFF cells with *T. gondii* CAT constructs yields no detectable expression.

Basic electroporation protocol (Soldati and Boothroyd, 1993; Donald and Roos, 1993)

1. Pellet freshly harvested, filter-purified tachyzoites by centrifugation at $1500 \times g$ for 15 min and resuspend in filter-sterilized "intracellular" electropora-tion buffer (van den Hoff *et al.*, 1992) (*electroporation buffer:* 120 mM KCl, 0.15 mM CaCl$_2$, 10 mM K$_2$HPO$_4$/KH$_2$PO$_4$, pH 7.6, 25 mM HEPES, pH 7.6, 2 mM EDTA, 5 mM MgCl$_2$ 2 mM ATP, 5 mM glutathione). A sterile solution containing all salts and buffers may be prepared in advance and kept indefinitely at room temperature, to be supplemented with fresh ATP and glutathione (GSH) and resterilized by filtration through a 0.22-μm filter immediately prior to use.

2. Repellet parasites, resuspend in electroporation buffer to a concentration of 3.3×10^7/ml, and transfer 300 μl parasites (10^7) to a sterile 2-mm-gap electroporation cuvette.
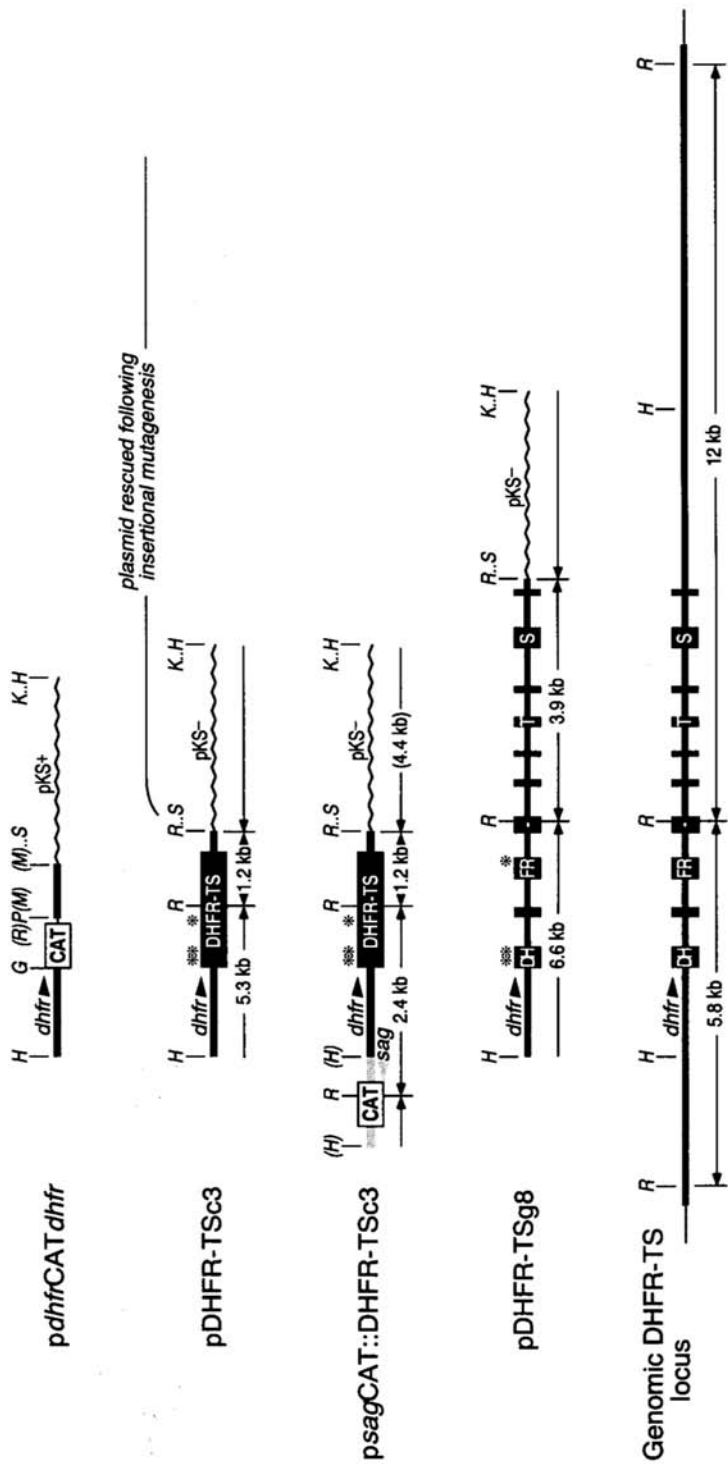
3. Add 50 μg plasmid DNA resuspended in 100 μl electroporation buffer and mix gently by pipetting.

4. Electroporate parasites using a single 1.5-kEV pulse at a resistance setting of 24 Ω (pulse time ~0.25 msec; conditions optimized using a BTX model 600 Electro Cell Manipulator).

5. Leave cuvette undisturbed for 15 min at room temperature.

6. Inoculate parasites into a T25 flask containing a confluent monolayer of HFF cells in 10 ml infection medium and incubate at 37°C. If direct comparisons are desired, transfer only 0.3 ml (75%) of the electroporation mix, as it is difficult qantitatively to remove the entire sample from the cuvette.

Electroporation under these conditions diminishes parasite viability by ~25% (by plaque assay; but note that only 20–40% of extracellular parasites are viable at the time of harvest, as discussed above). CAT activity can be detected beginning ~8 hr posttransfection and continues to increase for at least 24 hr. (As

pdhfrCATdhfr

pDHFR-TSc3

psagCAT::DHFR-TSc3

pDHFR-TSg8

Genomic DHFR-TS locus

plasmid rescued following insertional mutagenesis

CAT is an unusually stable enzyme, these levels probably reflect accumulated product; the time of peak CAT transcription has not been determined.) Under these conditions, at least 50% of all viable parasites express CAT protein detectable by immunofluorescence (Fig. 5B).

Although the above conditions have been optimized for expression of p*dhfr*CAT*dhfr* in RH-strain tachyzoites, they are probably generally applicable, as acceptable electroporation parameters are rather broad: charging voltages from 0.25 to 1.5 kEV/mm and resistance settings from 0 to 64 Ω produce significant CAT activity. CAT activity can be readily detected using ≤ 1 μg plasmid and 10^6 parasites, and total expression increases as an approximately linear function of parasite number and plasmid concentration up to 10^7 parasites and 50 μg plasmid. Further increases (up to 5 x 10^7 parasites and 200 μg plasmid) enhance the CAT signal somewhat, but with decreased efficiency on a per parasite or per microgram basis. Linearized and intact circular plasmid DNA are equally effective in transient transformation assays.

Interestingly, of commonly used reporter genes, only CAT appears to function effectively in *Toxoplasma*. We have observed only trace expression from luciferase constructs and no expression whatsoever of β-glucuronidase. The possibility of transient expression is not limited to CAT, however—pyrimethamine-

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Fig. 4   Transformation vectors for *T. gondii*. The DHFR-TS genomic map is shown at bottom; solid boxes indicate coding sequence—fragmented by 9 introns. The 5' end of mature mRNAs lies ~350 nt upstream of the translational start; polyadenylation can occur at >10 sites from ~250 to ~900 nt downstream of the stop codon. Genomic *Eco*RI sites (R) and *Hind*III sites (H) are indicated, along with the size of expected *Eco*RI restriction fragments.

All plasmid vectors are shown in linearized form p*dhfr*CAT*dhfr* contains 1.4 kb of 5' and 0.8 kb of 3' genomic sequence from the parasite's DHFR-TS gene fused to a bacterial CAT reporter cassette using synthetic *Bgl*II sites (G) introduced 7–12 nt upstream of the ATG codon in both CAT and the 5' DHFR-TS untranslated region. Wavy lines indicates pKS vector sequences; most of the additional restriction sites shown derive from the multiple cloning site: H, *Hind*III (ligated to genomic *Hind*III site); R, *Eco*RI; P, *Pst*I; M, *Sma*I (contains blunt-ligated DHFR-TS 3' end); S, *Sac*I; K, *Kpn*I. Restriction sites destroyed in the course of vector construction are indicated by parentheses. ".." denotes additional sequence derived from the pKS polylinker, which may be useful for vector manipulation. pDHFR-TSc3 is a "minigene" in which genomic 5' flanking sequences were fused to a full length cDNA clone. Restriction sites are abbreviated as above; internal *Eco*RI site corresponds to genomic site shown. The DHFR-TS 3' end terminates at an *Eco*RI site introduced during cDNA cloning. *Eco*RI restriction fragments expected for digestion of circular plasmid are indicated. Asterisks represent point mutations introduced to confer resistance to pyrimethamine (see text and Donald and Roos, 1993). Bracket shows plasmid sequence rescued along with flanking genomic DNA in insertional mutagenesis protocol (see text). p*sag*CAT::DHFR-TSc3 is identical to the above, except for the insertion of a CAT reporter gene in opposite orientation, under control of the SAG1 promoter (vector SAG ½ CAT kindly provided by Drs. D. Soldati and J. C. Boothroyd). The 4.4-kb *Eco*RI fragment does not hybridize with the DHFR-TS probe used in Fig. 6. Both the *dhfr* and the *sag* promoters are shown by arrows representing directionality. pDHFR-TSg8 is a genomic clone spanning the DHFR-TS gene from the endogenous *Hind*III site to a synthetic *Eco*RI site in the 3' flanking region.
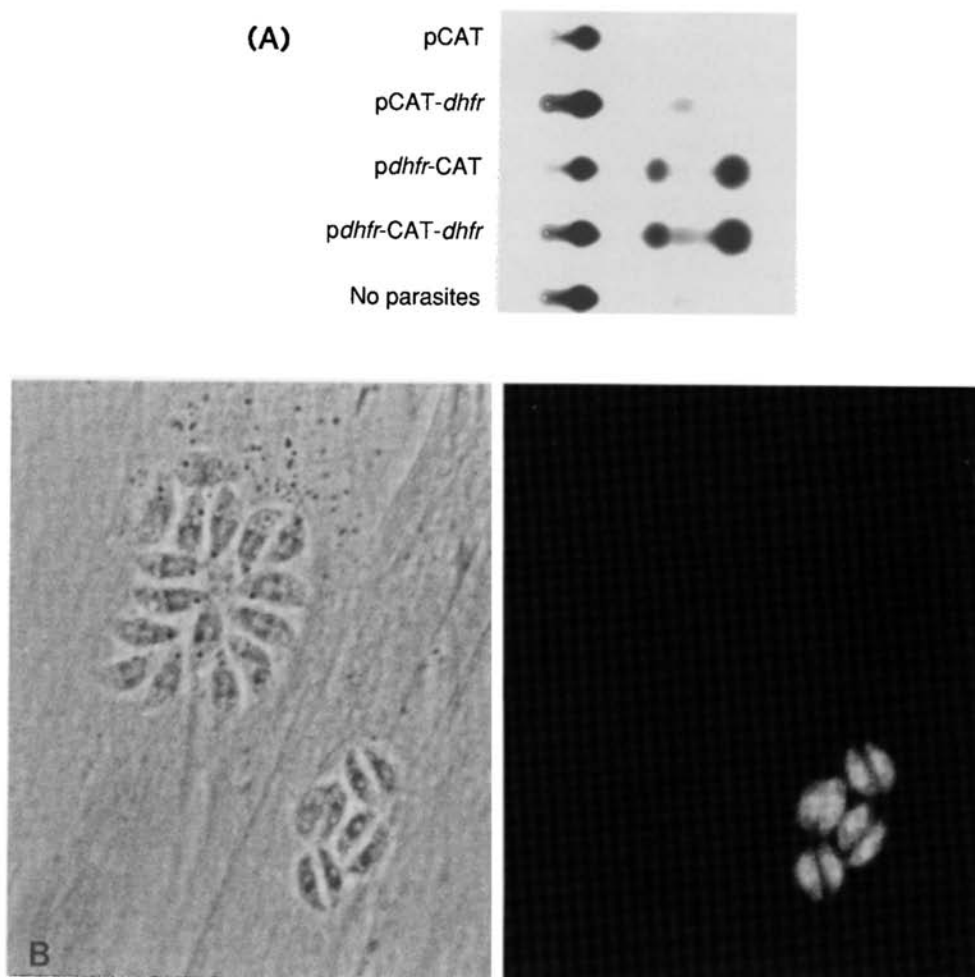
Fig. 5 Transient expression of recombinant CAT in electroporated *T. gondii*. (A) Extracellular parasites were transfected with a reporter cassette flanked by 5′ and/or 3′ sequences derived from the DHFR-TS gene, inoculated into HFF cells, and assayed for CAT activity 24 hr later. The 5′ DHFR-TS domain confers high levels of CAT expression; the 3′ domain is helpful but not essential. (B) 24 hr after transfection with p*dhfr*–CAT–*dhfr*, 55% of the parasitophorous vacuoles in infected HFF cells express CAT by immunofluorescence (right panel; compare with phase-contrast image at left). Each vacuole contains the progeny of a single parasite.

resistant DHFR-TS genes can also be detected in ~50% of viable electroporated parasites (Donald and Roos, 1993), and other groups have explored additional markers (unpublished).

Detection of CAT activity in transfected Toxoplasma tachyzoites

1. Harvest parasite-infected host cell monolayers ~24 hr postinfection using a rubber policeman, wash monolayer with PBS, and pellet combined mixture at $1500 \times g$ for 15 min.

2. Resuspend in 0.5 ml ice-cold electroporation buffer (without ATP or GSH) supplemented with 15% glycerol and 10 mM β-mercaptoethanol and transfer to a microfuge tube. Samples may be stored frozen at $-80°C$ at this point if desired.

3. On ice, add phenylmethylsulfonyl fluoride (PMSF) to 1 mM and sonicate 2×20 sec at ~55 W using a microtip (e.g., setting 4 on Branson Model 250 Sonifier). (PMSF may be prepared in advance as a 100 mM stock and stored in ethanol at $-20°C$. Thaw on ice immediately before use.)

4. Clarify by centrifugation for 10 min at 4°C in a microcentrifuge (at top speed) and transfer supernatant to fresh microfuge tube.

5. Pipette 50 μl of the sonic extract directly into the bottom of a microfuge tube and place the open tube(s) in the microfuge. The remaining sonic extract may be stored at $-80°C$ for further use (e.g., for protein assay).

6. Carefully pipette a 5 μl drop of 40 mM acetyl coenzyme A and 3.5 μl [^{14}C]-chloramphenicol (30–60 Ci/mmol) on opposite walls of each tube. Spin briefly to initiate reactions synchronously.

7. Gently tap tubes to mix thoroughly and incubate for 30 min at 37°C.

8. Stop reactions by the addition of 300 μl ethyl acetate, vortex, and centrifuge 10 min in microfuge.

9. Remove the organic (upper) phase to fresh microfuge tube, lyophilize, resuspend in 10 μl ethyl acetate, and spot samples on glass thin-layer chromatography (TLC) plate (TLC plate should be dried for ~2 hr at >70°C before spotting).

10. Separate mono- and di-acetylated chloramphenicol from unacetylated substrate by ascending chromatography in an equilibrated chamber containing chloroform/methanol (95:5)

11. After plate has dried, cover with single layer of plastic wrap and expose to X-ray film or phosphor-imager cassette.

The relative strength of the various available *Toxoplasma* promoters remains unexplained. Surprisingly, the SAG1 promoter (from the P30 major surface antigen) appears to be the weakest in transient transformation assays, whereas promoters derived from DHFR-TS, ROP1 (a rhoptry protein), and TUB1 (β-tubulin) are approximately 5- to 10-fold more powerful. TUB1 is probably the strongest *Toxoplasma* promoter yet identified (Soldati and Boothroyd, 1993). In addition to the full-length 1.4-kb DHFR-TS promoter illustrated, a variety of comparably effective truncated promoters have also been engineered, includ-

ing a fully active PCR cassette and *Nsi*I constructs spanning the ATG initiation codon.

C. Stable Transgene Expression and Overexpression

To develop a marker for selection of stable transformants, DHFR-TS minigenes lacking introns were constructed containing 5' and 3' genomic flanking sequences fused to a cDNA-derived coding region (pDHFR-TSc3 in Fig. 4). Although DHFR-TS gene structure and expression appears to be unchanged in pyrimethamine-resistant mutants selected *in vitro*, several point mutations were introduced into the coding sequence by analogy with drug-resistant *P. falciparum*.

Mutation T83N (Thr^{83}→ Asn, formerly designated M3 in Donald and Roos, 1993; 1994) is analogous to Ser^{108}→ Asn in *Plasmodium*, which confers moderate pyrimethamine resistance.

F245S (Phe^{245}→ Ser, formerly M4) confers low-level pyrimethamine resistance to *P. falciparum* (Phe^{223}→ Ser) (known only from *in vitro* studies; Tanaka *et al.*, 1990).

S36R (Ser ^{36}→ Arg, formerly M2; analogous to Cys^{59}→ Arg in *P. falciparum*) confers no phenotype in isolation, but dramatically enhances resistance when combined with Ser^{108}→ Asn in *Plasmodium* or either T83N or F245S in *Toxoplasma*.

For further discussion of these mutants, see Donald and Roos (1993); for reviews of DHFR-TS mutations in pyrimethamine-resistant malaria, see Hyde (1990) and Wellems (1991).

Upon transformation of pDHFR-TSc3/T83N or pDHFR-TSc3/F245S into *T. gondii* tachyzoites and selection in 1 μM pyrimethamine (approximately sixfold above the IC$_{50}$; see Fig. 2), transient replication above background levels was seen in ~50% of transfected parasites (Donald and Roos, 1993). Maintaining drug selection allowed the isolation of pyrimethamine-resistant parasites at a frequency of ~10^{-6}. These mutants remain drug-resistant even in the absence of continued selection. Hybridization with genomic DNA isolated from cloned, stably resistant mutants (Fig. 6) reveals the presence of multiple copies of transgenic DNA integrated into the parasite genome either as a tandem array (lane 2) or replicating as a stable extrachromosomal episome (lane 3). Selection in 600 nM pyrimethamine permitted isolation of drug resistant clones at somewhat higher frequency, and these clones harbor commensurately fewer DHFR-TS transgenes (lane 1).

DHFR-TS vectors containing double mutations T83N + S36R or F245S + S36R produced stable transformants at much higher frequency: ~5% of all viable transformants develop resistance to 1 μM pyrimethamine, even in the absence of drug selection (Donald and Roos, 1993; 1994). The majority
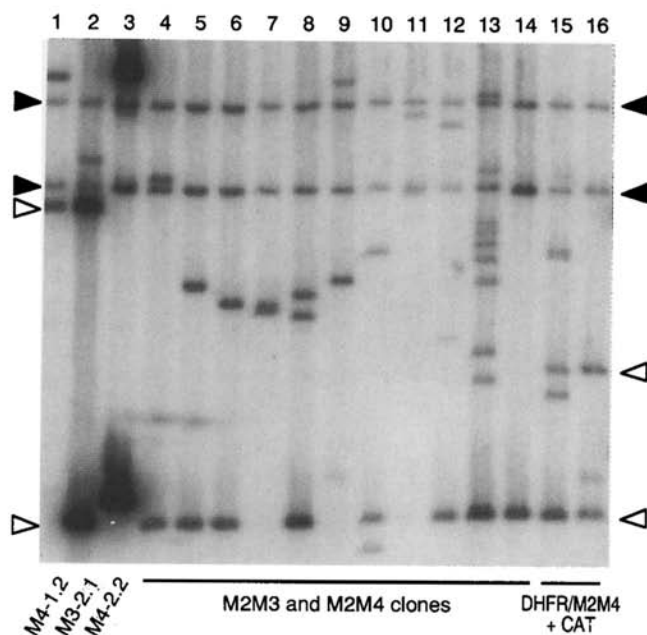
Fig. 6 Nonhomologous integration of stable DHFR-TS transgenes. DNA from drug-resistant parasite clones was digested with *Eco*RI and probed with DHFR-TS coding sequences. Each lane represents an independently cloned parasite line transfected with supercoiled circular plasmid pDHFR-TSc3 (see Fig. 4). Lanes 1–3 were isolated from clones transfected with single DHFR mutations M3 (T83N) or M4 (F245S); 4–14 were transfected with mutants M2M3 (T83N + S36R) or M2M4 (F245S + S36R). The endogenous 12- and 5.8-kb genomic bands are indicated by solid arrowheads, and were unaltered in any of these clones. Plasmid-derived bands are indicated by open arrowheads (5.3 and 1.2 kb for lanes 1–14). Most parasites transfected with the double mutants contain one plasmid band and one novel band, indicating that a single plasmid integrated by nonhomologous recombination. Resistance to 1 μM pyrimethamine is conferred by the M3 or M4 mutations only when present in high copy number (lanes 2 and 3). M4-1.2 was selected in 600 nM drug. Clones in lanes 15 and 16 were transfected with p*sag*CAT::DHFR-TSc3. These parasites stably express both pyrimethamine-resistant DHFR-TS and CAT. For further discussion see text. Reprinted from Donald and Roos (1993), with permission.

of these mutants contain single-copy insertions of the transfected plasmid, integrated into parasite DNA by nonhomologous recombination at sites dispersed throughout the genome (lanes 4–14).

Protocol for stable transformation (Donald and Roos, 1993)

1–5. Follow basic electroporation protocol, above.

6. Inoculate parasites into a T175 flask containing confluent HFF cells in 50 ml Infection medium *prepared with dialyzed serum* and supplemented with

1 μM pyrimethamine and incubate at 37°C. If multiple independent clones are desired, up to 10 T25 flasks may be inoculated. Pyrimethamine is stored at 4°C as a 10 mM stock in ethanol, and diluted in culture medium before use. As pyrimethamine is a "static" rather than "cidal" inhibitor, drug selection may be applied to parasites immediately after electroporation, or added up to 24 hr post-transfection.

7. When the host cell monolayer nears complete destruction, filter-purify extracellular tachyzoites and inoculate 96-well plates for cloning by limiting dilution as described above (maintaining drug selection, in medium prepared with dialyzed serum). The frequency of stable resistance among parasites that emerge from a first cycle of infection depends on the particular drug resistance allele employed and the precise conditions of selection. It is therefore advisable to inoculate microtiter plates at several dilutions. Alternatively, parasites may be passaged for another cycle in T-flasks prior to cloning.

Linear and circular DHFR-TS plasmids are equally effective at stable transformation. In most cases, integration of circular molecules occurs without significant loss or rearrangement of either host or plasmid genetic material—consistant with a model involving reciprocal crossing over at a single site (see below). Linear molecules also generally integrate at a single site without rearrangement, but are usually accompanied by the loss of a few (1–30) terminal nucleotides, and are sometimes ligated to short fragments derived from transfected plasmid DNA.

Selection of the particular pyrimethamine resistance allele to be used for transformation depends on the application at hand. Lanes 15 and 16 in Fig. 6 demonstrate that a foreign reporter gene (e.g., CAT) can be successfully integrated into the parasite genome in parallel with the DHFR-TS marker. These genes are stably maintained and expressed in the absence of specific selection. Insertion of the reporter gene upstream of DHFR-TS and in the opposite orientation (psagCAT::DHFR-TSc3 in Fig.4) results in higher CAT expression than introduction in the same orientation as DHFR-TS. It is probable (but yet untested) that transfections using low-level pyrimethamine-resistance alleles coupled with a foreign gene would result in overexpression of both DHFR-TS and the gene of interest. By analogy with other systems (Kaufman, 1990), it is also likely that such mutants isolated (at higher frequency) in lower pyrimethamine concentrations could be "stepped-up" to higher drug levels with concomitant coamplification of the desired gene.

The surprisingly high frequency of stable integration using high-level pyrimethamine-resistance alleles permits many exciting genetic approaches to the study of *T. gondii*. It should be noted, however, that the traditional reliance on antifolates for clinical management of acute toxoplasmosis means that such mutants pose an unusual hazard (see discussion of special considerations for working with pyrimethamine-resistant organisms, below). Unfortunately, although other selectable markers are available for *Toxoplasma* (Kim *et al.*,

1993; Sibley *et al.,* 1994), the frequencies of resistance provided by these systems are ~100-fold lower than for the DHFR-TS double mutants, precluding some of the more promising applications described below.

D. Nonhomologous Recombination, Insertional Mutagenesis, and Marker Rescue

The high frequency of nonhomologous chromosomal insertion observed using cDNA-derived DHFR-TS minigenes suggested that it might be possible to knock out gene function efficiently by insertional mutagenesis, tagging the locus in the process. This approach has revolutionized the identification of important genes in other genetic systems (Berg and Spradling, 1991; Feldmann, 1991). Given that 5% of viable electroporated parasites stably integrate recombinant transgenes (Donald and Roos, 1993; 1994a), transfection of 10^7 parasites with a survival rate of 20% should yield ~10^5 independent random insertion events. Assuming completely random insertion (an unlikely prospect) this predicts an average of more than one hit in any 1-kb segment of the 8×10^7 base pair *Toxoplasma* genome.

The ability to identify insertional mutants of interest depends on a practical screen or selection strategy. Negative selectable markers such as uracil phosphoribosyltransferase (UPRT), hypoxanthine/guanine phosphoribosyltransferase (HGPRT), or adenosine kinase provide a suitable test (Donald and Roos, 1994b).

UPRT is a nonessential single-copy gene, whose loss confers resistance to fluorideoxyuridine (FUDR; Pfefferkorn, 1977). Eight transfections of RH-strain tachyzoites according to the procedure presented below yielded at least four independent FUDR-resistant clones. To ensure that mutants were independent, only one clone was isolated from each transfection, but the estimated frequency of >3 $\times$ 10^{-7} is within one order of magnitude of the theoretical value for truly random insertions. FUDR-resistant clones were unable to incorporate [^{3}H]-uracil and lack UPRT activity. Flanking genomic DNA was rescued along with the pKS vector by bacterial transformation and used to demonstrate that all of the FUDR-resistant mutants had integrated transfected plasmid into nearby (but distinct) sites within the parasite genome. Corresponding cDNA and genomic clones were isolated to provide the complete sequence of the *T. gondii* UPRT gene. To confirm the function of the putative UPRT locus and provide a mutant incapable of reversion to wild-type, homologous recombination technology (see below) was employed to replace the endogenous locus in wild-type parasites with a genomic clone from which essential coding sequences were deleted. Transfection of both FUDR-resistant insertional mutants and the UPRT "knock-out" clones with either reconstructed minigenes or the wild-type genomic locus restored UPRT activity.

Similar experiments have permitted identification of the *T. gondii* HXGPRT gene, based on selection for resistance to 6-thioxanthine. (Because the host

cell enzyme exhibits no XPRT activity, thioxanthine is parasite specific; we are indebted to Dr. E. R. Pfefferkorn for suggesting this ingenious selection.) In addition to their value as a test for insertional mutagenesis, the UPRT and HXGPRT genes provide negative selectable markers for the development of positive–negative selection systems in *Toxoplasma* and candidates for alternative positive selection vectors (see below).

For reference in the insertional mutagenesis and marker rescue protocols outlined below, the linearized depiction of plasmid pDHFR-TSc3 shown in Fig. 4 can be considered to have integrated so as to disrupt the target gene of interest. The bracket indicates the rescued plasmid, including the bacterial vector and flanking genomic sequence from the target gene.

Protocol for insertional mutagenesis

1–6. Follow stable transformation protocol, above, using high-level pyrimethamine resistance vectors pDHFR-TSc3/T83N + S36R or pDHFR-TSc3/F245S + S36R *linearized* by restriction at the unique *Hind*III site. Although both linear and circular DNA produce insertional mutants, using linear molecules facilitates marker rescue and mapping of the resultant integrants (see below).

7. When the host cell monolayer nears complete destruction, filter-purify the pyrimethamine-resistant tachyzoites and apply desired secondary screen or selection (maintaining pyrimethamine selection as well). For identification of UPRT mutants, T175 flasks were inoculated with 10^7 tachyzoites in 5 μM FUDR. In some cases it may be desirable to force parasites out of the host cells by syringe-passage, to avoid selection against mutations that retard growth.

8. Isolate positive clones by plaque purification or limiting dilution and confirm phenotype.

Marker rescue protocol, for identification of the mutated genomic locus

1. Isolate parasite genomic DNA from putative insertional mutants.

2. Map the nature of transgene insertion by Southern analysis. In the case of a single insertion, restriction with polylinker enzymes that cut once in plasmid pDHFR-TSc3 will reveal a single-copy fragment extending into flanking genomic DNA (or two bands, depending on the location of the restriction site relative to the probe employed). Multiple independent insertions will produce multiple novel bands. Insertion may also occur as a tandem array, in which case multiple copies of the complete 6.5-kb vector will be observed, in addition to the single-copy flanking band(s). Head-to-head or tail-to-tail insertions have also been observed, producing more complicated (but predictable) restriction patterns.

As unique cutters that separate the selectable DHFR-TS marker from bacterial vector sequences, *Spe*I and *Xba*I are particularly useful for mapping. These enzymes may be used to map flanking restriction sites of suitable size for marker rescue. Additional enzymes that may be useful for marker rescue include *Eco*RI,

*Pst*I, *Xma*I, *Eag*I, *Sac*II, and *Sac*I. Note: restriction with enzymes expected to cleave close to the ends of the introduced plasmid (e.g., *Hind*III, *Cla*I) may yield confusing results due to deletion of these sites by intracellular exonucleases prior to integration, as noted above.

3. Restrict genomic DNA with an enzyme(s) predicted to generate fragments of suitable size for bacterial replication as a plasmid (up to ~20 kb, including the entire 3-kb pKS vector). Note: if mapping reveals multiple tandem insertions, double digestion may facilitate plasmid rescue from the tandem array. For example, *Spe*I/*Sph*I may be employed if the flanking *Spe*I site is of suitable size and is proximal to the flanking *Sph*I site; 4.6-kb fragments derived from the tandem array will have incompatible ends.

4. Ligate 500 ng restricted genomic DNA overnight in dilute solution ($<1\mu g/$ ml), to promote intramolecular ligation.

5. Transfect competent *Escherichia coli* with 50 ng of the ligated genomic DNA, select on ampicillin plates, and isolate resistance plasmids. It is essential to use bacteria of high transformation competency for this procedure ("library"-competent or electrocompetent cells).

6. Confirm recovery of the expected size plasmid. Genomic DNA flanking the insertion point may be sequenced using standard pKS sequencing primers.

7. Probe Southern blots of DNA from wild-type parasites and mutant clones with flanking sequence fragment, to confirm that independent mutants all harbor insertions in the same region of genomic DNA.

8. Isolate cDNA clones and genomic clones corresponding to the tagged sequence. (Genomic and cDNA libraries for *T. gondii* are available from several sources, including the AIDS Research and Reference Reagent Program of the NIH.) Genomic DNA fragments or reconstructed minigenes may be introduced into mutant parasites according to the above transformation protocols, to confirm restoration of wild-type phenotype.

Because integration of electroporated plasmid DNA does not depend on pyrimethamine treatment, it should not be necessary to employ DHFR-TS vectors if direct selection for the desired mutants is sufficiently powerful—a decided advantage in terms of laboratory safety (see below). In other cases, pyrimethamine resistance is used to select for transgenic parasites, which can then be further screened by procedures appropriate to the gene of interest. As *Toxoplasma* tachyzoites are haploid, insertional mutagenesis of essential genes is lethal, but it should be possible to use the above procedure to tag and clone any nonessential gene for which a suitable selection or screen is available.

E. Homologous Recombination: Pseudo–diploids, Gene Knock-outs, and Perfect Gene Replacement

Although the cDNA-derived vectors discussed above appear to integrate into the *Toxoplasma* genome at random, vectors containing more extensive regions
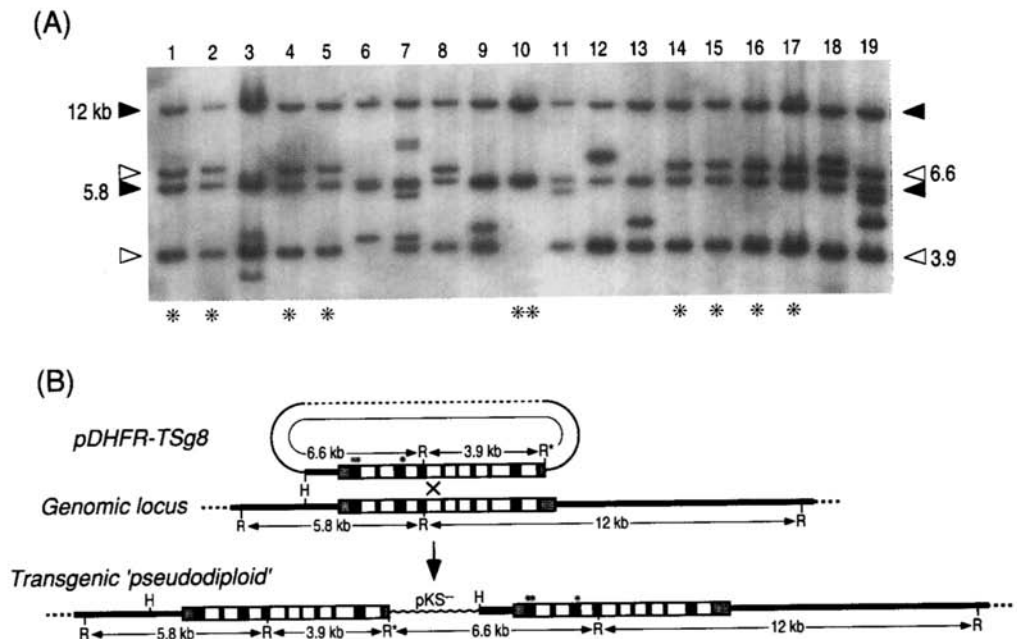
(A)

(B)

Fig. 7 Homologous recombination in *Toxoplasma*. As indicated by single asterisks, many drug resistant parasites transfected with alleles derived from the complete genomic DHFR-TS locus (plasmid pDHFR-TSg8 in Fig. 4) contain only endogenous and plasmid-derived bands of the expected sizes (solid and open arrows, respectively). As no free plasmid remains in these parasites, integration must have occurred by reciprocal crossing-over, as indicated in (B) and described in the text. Lane 10 (**) shows a parasite clone with no detectable plasmid sequences. Direct sequencing indicates that this clone is a perfect gene replacement, harboring the mutant DHFR-TS allele at its proper locus and no trace of the original wild-type allele. For further discussion, see Donald and Roos (1994) (reprinted with permission).

of continuous sequence homology (cf. pDHFR-TSg8 in Fig. 4) preferentially integrate by homologous recombination, at the same high frequency (Donald and Roos, 1994a). As shown in Fig. 7, when an 8-kb genomic clone spanning the DHFR-TS locus was transfected as a circular plasmid (according to the stable transformation protocol outlined above), approximately half of the drug-resistant parasites harbored transgenes integrated by homologous recombination. When a 16-kb genomic clone was employed, >80% homologous recombination was observed (Donald and Roos, 1994a).

Two forms of homologous recombination have been identified in *Toxoplasma*. Integration by reciprocal crossing-over at a single site produces a "pseudodiploid" duplication of the locus, as diagrammed in the bottom panel of Fig. 7. Integration by gene replacement results in loss of the wild-type allele by the mutant gene (cf. lane 10 in Fig. 7). Functional replacement could occur by several different mechanisms—double crossing-over, gene conversion, or re-

combination between the duplicated loci in a pseudodiploid (removing either the wild-type or the mutant gene copy, depending on the site of recombination). Unlike most eukaryotic systems (Orr-Weaver *et al.*, 1981; Kucherlapati and Smith, 1988; Cruz and Beverley, 1990; Hasty *et al.*, 1991; Finbarr-Tobin and Wirth, 1992) homologous recombination in *T. gondii* is far more common using circular plasmids than linearized DNA, suggesting that the "pseudodiploid intermediate" model is probably dominant (pseudodiploidy has never been observed in tachyzoites transfected with linear DNA). Depending on the nature of the mutant allele, gene replacement results in either allelic exchange or a functional gene knockout.

Protocol for pseudo-diploid formation or gene replacement

1–6. Follow stable transformation protocol, above, using plasmid DNA containing as large a segment of the targetted locus as feasible. See notes below for further considerations in plasmid design, the use of circular vs linear DNA, and selection schemes.

7. Isolate parasite clones and screen for presence of the mutant and wild-type alleles. In DNA hybridizations or PCR reactions, the pseudodiploid configuration results in the presence of both genomic and plasmid bands (and no novel bands); gene replacement yields only the genomic fragments (or fragments expected for the mutant allele, if different).

Linking a cDNA-derived pyrimethamine-resistant DHFR-TS gene to any target sequence of interest should permit pseudodiploid formation when *circular* plasmid DNA is transfected and parasites selected for pyrimethamine resistance. Pending the development of coupled positive–negative selection vectors such as those used in animal systems (Mortensen, 1993), production of gene knock-outs in *Toxoplasma* requires either selection for resistance encoded within the locus itself or extensive screening. Where negative selection is available, as for the loss of UPRT activity in FUDR-resistant mutants, nonfunctional genes can be transfected in either linear or circular form, and mutants screened for gene replacement. Deletion of a small internal fragment (for the production of irreversible knock-outs) does not appear to affect significantly the frequency of homologous vs nonhomologous recombination. Where no direct selection is available, gene knock-outs can be produced by inserting a selectable marker (e.g., CAT or a cDNA-derived mutant DHFR-TS allele) into the middle of the target gene), transfecting linear DNA to bias against pseudodiploid formation, and screening for loss of the targetted gene against the background of nonhomologous recombinants (Kim *et al.*, 1993). Alternatively, it may be possible to transfect circular plasmids containing both a positive selectable marker and the mutant gene of interest, and to screen pseudodiploids for subsequent loss of the wild-type gene once removed from selection. For this strategy it is essential to provide comparable lengths of genomic sequence on both sides of the intro-

duced mutation, to maximize the frequency of pseudodiploid resolution into the mutant form.

F. Cloning by Complementation

The high frequency of stable transformation suggests that it should be feasible to clone *T. gondii* genes by complementation (as is now possible for *Leishmania;* Ryan *et al.,* 1993). To test this possibility, total genomic DNA was prepared from parasites harboring a drug-resistant DHFR-TS gene (lane 10 in Fig. 7), digested with various restriction enzymes, and electroporated into wild-type tachyzoites. Transfection of parasites with 50 μg of total DNA according to the procedures described above produced pyrimethamine-resistant plaques at a frequency of $\sim2.5 \times 10^{-6}$, regardless of the size of the genomic fragment spanning the DHFR-TS locus (9 to ~35 kb) or the position of the DHFR-TS gene within that fragment. Taking into account the $\sim10^4$-fold difference in size between the 8×10^7 bp *T. gondii* genome and the plasmid vectors employed ($6-19 \times 10^3$ bp), this frequency is surprisingly close to the 5% transformation rate measured for purified plasmid (Donald and Roos, 1993; 1994a). No plaques were observed when wild-type parasite DNA was used for transformation. These results demonstrate that molecular cloning by complementation is indeed feasible in *Toxoplasma*. Identification of unknown mutations would presumably require a *T. gondii* expression library in an appropriate bacterial shuttle vector. Alternatively, it may be possible to tag transfected genomic DNA by cotransfection with a bacterial plasmid (Kaufman, 1990).

G. Special Considerations for Working with Pyrimethamine–Resistant Organisms

Because classical therapy for acute toxoplasmosis involves treatment with antifolates, the use of pyrimethamine-resistant parasites poses a special hazard. Mice infected with transgenic RH-strain parasites harboring the high level pyrimethamine-resistance alleles T83N+S36R or F245S+S36R are completely refractory to pyrimethamine treatment (B. J. Luft, personal communication). Even very high inocula of these pyrimethamine-resistant parasites remain sensitive to azithromycin, clindamycin, atovaquone and sulfonamides, however. (Pyrimethamine-resistant parasites are actually hypersensitive to sulfonamides both *in vitro* and *in vivo*.) Any possible infectious contact with these mutants should be treated with clindamycin+sulfa or macrolide+sulfa.

As the tachyzoite form of *T. gondii* is infectious only by direct bloodstream introduction, release of drug resistant organisms into general circulation—even in the event of an accidental laboratory infection—is only possible if the subject is eaten by a cat. To preclude the production of infectious oocysts, pyrimethamine resistant parasites should not be passed through a feline host.

Development of new selectable markers that permit mutant selection at frequencies comparable to high-level pyrimethamine resistance DHFR-TS alleles via a folate-independent pathway is an important goal for the future.

===== **IV. Summary and Outlook**

A. Genetic Tools for the Future

Through the efforts of several laboratories, the development of molecular transformation techniques and strategies for *T. gondii* has proceeded with remarkable rapidity. In brief, recombinant molecules can be expressed either transiently or as stable transformants, as episomes or integrated into the parasite genome, and as single copy or multicopy transgenes. Stable integration can be produced by random nonhomologous recombination, single-site homologous recombination (producing a duplication at the transfected locus), or perfect gene replacement. Many of these outcomes can now be selected specifically, by the use of appropriate vectors and transformation conditions. The extraordinarily high frequencies of stable transformation observed permit cloning by complementation, insertional mutagenesis/marker rescue, gene knock-outs, and allelic replacement. In combination with available classical (Pfefferkorn, 1988; Pfefferkorn and Pfefferkorn, 1980) and "cell-genetic" (Pfefferkorn, 1981; 1988) possibilities and physical and genetic mapping strategies (Sibley *et al.*, 1992; Sibley and Boothoyd, 1992), these tools provide a powerful arsenal for investigations into the biology of intracellular parasitism.

Additional molecular tools would enhance genetic manipulation of *T. gondii* still further. The availability of negative selectable markers (e.g., UPRT, HGPRT) should readily permit development of positive–negative selection vectors for forced gene replacement (Mortensen, 1993). Episomal transformation vectors would be useful for the study of essential genes (and a variety of other applications) and for the production of bacterial shuttle vectors. The ability to establish pyrimethamine-resistant DHFR-TS transgenes in episomal form (cf. Fig. 6, lane 3) argues that such an approach is possible. As previously noted, cDNA libraries for transgenic expression in *T. gondii* would be useful for gene cloning by complementation.

Finally, the potential danger of pyrimethamine-resistant parasites in an accidental laboratory infection argues for use of alternative selectable markers. Three such markers have been developed to date, conferring resistance to chloramphenicol (Kim *et al.*, 1993), phleomycin (Perez *et al.*, 1989), or an ingenious complementation of tryptophan auxotrophy (Sibley *et al.*, 1994). Unfortunately, thus far none of these systems appears to provide transformation frequencies comparable to those observed using DHFR-based vectors, but the search for a suitable replacement remains a high priority. Identification of the *T. gondii* UPRT and, especially, HXGPRT genes (as noted above) provide possible positive—as well as negative—selection systems.

B. Outstanding Problems in the Cell Biology and Pathogenesis of *T. gondii*

Now that many of the necessary molecular genetic tools are available for studying *Toxoplasma*, the challenge for the future is to apply these techniques

to the analysis of important biological problems. Among the many interesting questions of cell biological interest, we may wish to consider the following:

Lacking flagella or cilia, what structures and mechanisms provide motility to parasite tachyzoites (gliding motility during the ''search'' for host cells and twisting motility during cell invasion and exit)?

What structures and molecules are necessary for host cell invasion? What site(s) of attachment is involved?

How is the parasitophorous vacuole membrane established and maintained? What is the membrane composed of and what does it do?

How is the highly polarized parasite structure established and maintained? How are the components of these organelles targeted to the proper destinations?

What regulates the strictly synchronous division of parasites within the vacuole?

What is needed from the host cell; i.e., why is *Toxoplasma* an *obligate* intracellular parasite?

Are there specific signals for interconversion between bradyzoites and tachyzoites, and what regulatory and biochemical changes are involved?

Why does sexual differentiation occur only in feline intestinal epithelium, and what signals and controls are involved in this developmental pathway?

What essential features of the *T. gondii* are recognized by the immune system of the parasitized organism, and how might this information assist in the treatment of congenital infections and toxoplasmosis in AIDS?

Can novel metabolic pathways or structural elements be identified in *Toxoplasma* as potential targets for drug development, and how do such novelties broaden our appreciation of eukaryotic diversity?

What aspects of cell biology and biochemistry are conserved across the vast evolutionary gulf separating *T. gondii* from more familiar animal, plant, and fungal systems, and what do these similarities tell us about the essential elements of eukaryotic design?

Acknowledgments

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