Received 21 November; accepted 17 December 2001.

1. Nossal, G. J. V. Negative selection of lymphocytes. *Cell* **76,** 229–239 (1994).
2. Swat, W., Ignatowicz, L., von Boehmer, H. & Kisielow, P. Clonal deletion of immature $CD4^+$ $CD8^+$ thymocytes in suspension culture by extrathymic antigen-presenting cells. *Nature* **351,** 150–153 (1991).
3. Surh, C. D. & Sprent, J. T-cell apoptosis detected *in situ* during positive and negative selection in the thymus. *Nature* **372,** 100–103 (1994).
4. Clayton, L. K. *et al.* T-cell receptor ligation by peptide/MHC induces activation of a caspase in immature thymocytes: the molecular basis of negative selection. *EMBO J.* **16,** 2282–2293 (1997).
5. O'Connor, L. *et al.* Bim: a novel member of the Bcl-2 family that promotes apoptosis. *EMBO J.* **17,** 384–395 (1998).
6. Bouillet, P. *et al.* Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity. *Science* **286,** 1735–1738 (1999).
7. Gross, A., McDonnell, J. M. & Korsmeyer, S. J. Bcl-2 family members and the mitochondria in apoptosis. *Genes Dev.* **13,** 1899–1911 (1999).
8. Strasser, A., O'Connor, L. & Dixit, V. M. Apoptosis signaling. *Annu. Rev. Biochem.* **69,** 217–245 (2000).
9. Smith, K. G. C., Strasser, A. & Vaux, D. L. CrmA expression in T lymphocytes of transgenic mice inhibits CD95 (Fas/APO-1)-transduced apoptosis, but does not cause lymphadenopathy or autoimmune disease. *EMBO J.* **15,** 5167–5176 (1996).
10. Newton, K., Harris, A. W., Bath, M. L., Smith, K. G. C. & Strasser, A. A dominant interfering mutant of FADD/Mort1 enhances deletion of autoreactive thymocytes and inhibits proliferation of mature T lymphocytes. *EMBO J.* **17,** 706–718 (1998).
11. Walsh, C. M. *et al.* A role for FADD in T cell activation and development. *Immunity* **8,** 439–449 (1998).
12. Sentman, C. L., Shutter, J. R., Hockenbery, D., Kanagawa, O. & Korsmeyer, S. J. *bcl-2* inhibits multiple forms of apoptosis but not negative selection in thymocytes. *Cell* **67,** 879–888 (1991).
13. Strasser, A., Harris, A. W. & Cory, S. *Bcl-2* transgene inhibits T cell death and perturbs thymic self-censorship. *Cell* **67,** 889–899 (1991).
14. Huang, D. C. S. & Strasser, A. BH3-only proteins—essential initiators of apoptotic cell death. *Cell* **103,** 839–842 (2000).
15. Zong, W. X., Lindsten, T., Ross, A. J., MacGregor, G. R. & Thompson, C. B. BH3-only proteins that bind pro-survival Bcl-2 family members fail to induce apoptosis in the absence of Bax and Bak. *Genes Dev.* **15,** 1481–1486 (2001).
16. Nakayama, T. *et al. In vivo* calcium elevations in thymocytes with T cell receptors that are specific for self ligands. *Science* **257,** 96–99 (1992).
17. Martin, S. & Bevan, M. J. Antigen-specific and nonspecific deletion of immature cortical thymocytes caused by antigen injection. *Eur. J. Immunol.* **27,** 2726–2736 (1997).
18. Jenkinson, E. J., Kingston, R. & Owen, J. J. Newly generated thymocytes are not refractory to deletion when the α/β component of the T cell receptor is engaged by the superantigen staphylococcal enterotoxin B. *Eur. J. Immunol.* **20,** 2517–2520 (1990).
19. Barnden, M. J., Allison, J., Heath, W. R. & Carbone, F. R. Defective TCR expression in transgenic mice constructed using cDNA-based α- and β-chain genes under the control of heterologous regulatory elements. *Immunol. Cell Biol.* **76,** 34–40 (1998).
20. Bouillet, P., Cory, S., Zhang, L.-C., Strasser, A. & Adams, J. M. Degenerative disorders caused by Bcl-2 deficiency are prevented by loss of its BH3-only antagonist Bim. *Dev. Cell* **1,** 645–653 (2001).
21. von Boehmer, H. Developmental biology of T cells in T cell-receptor transgenic mice. *Annu. Rev. Immunol.* **8,** 531–556 (1990).
22. Strasser, A., Harris, A. W., Von Boehmer, H. & Cory, S. Positive and negative selection of T cells in T cell receptor transgenic mice expressing a *bcl-2* transgene. *Proc. Natl Acad. Sci. USA* **91,** 1376–1380 (1994).
23. Puthalakath, H., Huang, D. C. S., O'Reilly, L. A., King, S. M. & Strasser, A. The pro-apoptotic activity of the Bcl-2 family member Bim is regulated by interaction with the dynein motor complex. *Mol. Cell* **3,** 287–296 (1999).
24. Ma, A. *et al.* Bclx regulates the survival of double-positive thymocytes. *Proc. Natl Acad. Sci. USA* **92,** 4763–4767 (1995).
25. Ogilvy, S. *et al.* Constitutive bcl-2 expression throughout the hematopoietic compartment affects multiple lineages and enhances progenitor cell survival. *Proc. Natl Acad. Sci. USA* **96,** 14943–14948 (1999).
26. Grillot, D. A. M., Merino, R. & Nuñez, G. Bcl-x_L displays restricted distribution during T cell development and inhibits multiple forms of apoptosis but not clonal deletion in transgenic mice. *J. Exp. Med.* **182,** 1973–1983 (1995).
27. Calnan, B. J., Szychowski, S., Chan, F. K., Cado, D. & Winoto, A. A role for the orphan steroid receptor Nur77 in apoptosis accompanying antigen-induced negative selection. *Immunity* **3,** 273–282 (1995).
28. Zhou, T. *et al.* Inhibition of Nur77/Nurr1 leads to inefficient clonal deletion of self-reactive T cells. *J. Exp. Med.* **183,** 1879–1892 (1996).
29. Suzuki, A. *et al.* T cell-specific loss of Pten leads to defects in central and peripheral tolerance. *Immunity* **14,** 523–534 (2001).
30. Dijkers, P. F., Medemadagger, R. H., Lammers, J. J., Koenderman, L. & Coffer, P. J. Expression of the pro-apoptotic Bcl-2 family member Bim is regulated by the forkhead transcription factor FKHR-L1. *Curr. Biol.* **10,** 1201–1204 (2000).

Acknowledgements

We are grateful to H. von Boehmer and W. Heath for HY-TCR and OT-II TCR transgenic mice. We thank A. Naughton and C. Tilbrook for animal care, A. Harris, J. Miller, D. Huang and L. O'Reilly for discussions and critical comments on the manuscript, and G. Smyth and R. Thomson for statistical advice. This work was supported by fellowships and grants from the National Health and Medical Research Council (Canberra), the Dr Josef Steiner Cancer Research Foundation (Bern), the Leukemia and Lymphoma Society of America, the Anti-Cancer Council of Victoria and the NIH.

Competing interests statement

The authors declare that they have no competing financial interests.

Correspondence and requests for materials should be addressed to A.S. (e-mail: strasser@wehi.edu.au).

De novo pyrimidine biosynthesis is required for virulence of *Toxoplasma gondii*

Barbara A. Fox & David J. Bzik

Department of Microbiology and Immunology, Dartmouth Medical School, Lebanon, New Hampshire 03756, USA

Toxoplasma gondii **is a ubiquitous protozoan parasite that is responsible for severe congenital birth defects and fatal toxoplasmic encephalitis in immunocompromized people[1]. Fundamental aspects of obligate intracellular replication and pathogenesis are only now beginning to emerge for protozoan parasites. *T. gondii* has a fragmented pathway for salvaging pyrimidine nucleobases derived from the parasite or host cell, and this limited pyrimidine salvage capacity is funnelled exclusively through uracil phosphoribosyltransferase[2,3]. Disrupting the function of this enzyme does not affect the growth of *T. gondii* tachyzoites[4], which suggests that the *de novo* pyrimidine biosynthesis pathway may be necessary for growth. We have examined the virulence of *T. gondii* mutants that lack carbamoyl phosphate synthetase II (uracil auxotrophs) to determine whether *de novo* pyrimidine biosynthesis is required *in vivo*. Here we show that *T. gondii* uracil auxotrophs are completely avirulent not only in immune-competent BALB/c mice but also in mice that lack interferon-γ. A single injection of the uracil auxotroph into BALB/c mice induces long-term protective immunity to toxoplasmosis. Our findings indicate the significance of the *de novo* pyrimidine biosynthesis pathway for the virulence of parasitic protozoa, and suggest routes for developing vaccines and chemotherapy.**

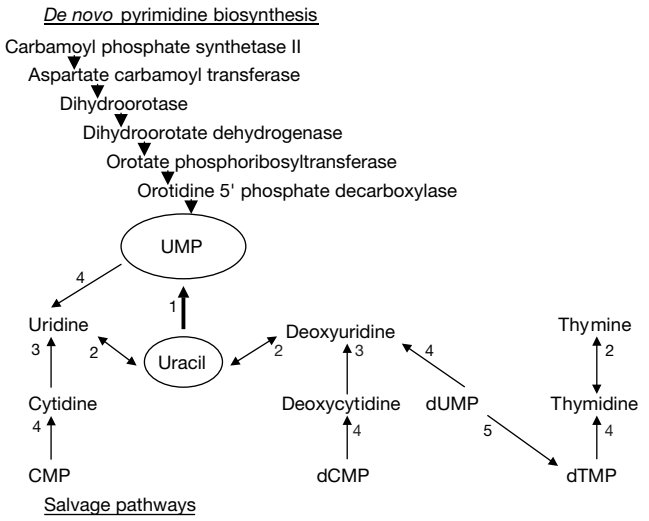

Figure 1 Pyrimidine salvage and *de novo* pyrimidine biosynthesis pathways in *T. gondii*. Pathways that produce UMP, the precursor of all pyrimidines used by *T. gondii*, are shown. The six steps of the *de novo* pyrimidine biosynthesis pathway and their corresponding enzymes are shown. Salvage pathway[2,3] steps that have been detected in *T. gondii* are shown in solid lines. Double arrowheads indicate that the activity is capable of both conversions. Enzyme activities: uracil phosphoribosyltransferase (1); nucleoside phosphorylase (2); nucleoside deaminase (3); nucleoside 5′-monophosphate phosphohydrolase (4); and thymidylate synthase (5; part of the bifunctional DHFR-TS[6,19]). Thymidylate synthase is not considered a salvage enzyme; it is a key enzyme in the interconversion of pyrimidine nucleotides. Many potential salvage enzyme activities have not been detected in *T. gondii*[3].

Because of its genetic accessibility[5–7] and natural virulence in mice, *T. gondii* is an excellent model for the discovery and evaluation of auxotrophic mutants that capitalize on differences in metabolism between protozoan parasites and their hosts. Like its host, *T. gondii* has an intact pathway for *de novo* pyrimidine biosynthesis, but differs in that it has only a limited pyrimidine salvage pathway (Fig. 1). In *T. gondii*, as in other parasites of the phylum Apicomplexa, the key regulatory enzyme of *de novo* pyrimidine biosynthesis is carbamoyl phosphate synthetase II (CPSII), a parasite enzyme that has distinctive properties compared with the CPSII activity of the mammalian host cell[8]. In addition, apicomplexan parasites have a monofunctional CPSII domain fused onto the same polypeptide as the glutamine amidotransferase domain, which produces an enzyme architecture that is not observed in bacteria, fungi or mammals[9,10]. These unique features of *T. gondii* CPSII make this activity an attractive target to disrupt to obtain null mutants.

We cloned a 6.6-kilobase (kb) *Hin*dIII *CPSII* genomic DNA fragment from strain RH that contains exons with significant similarity to CPSII from fungi, plants and mammals (Supplementary Information). The single chromosomal copy of this 6.6-kb *Hin*dIII fragment was disrupted to obtain uracil auxotroph strains *cps1-1* and *cps2-1*. Neither strain had detectable CPSII activity compared with the activity measured in the wild-type RH strain (57 nmol h^{-1} mg^{-1}). Without uracil supplementation the parasite invaded normally, but it failed to replicate once intracellular. Essentially, no growth was observed at uracil concentrations lower than 20 μM (Fig. 2a). Normal parasite growth was seen between about 0.2 and 0.6 mM uracil; however, growth was suppressed in uracil concentrations higher than 0.8 mM, which indicates that normal regulation of pyrimidine pools or salvage mechanisms may be disrupted in the auxotrophic mutants. The ability of added uracil to rescue this nonreplicating uracil auxotroph declined with time (Fig. 2b).

We verified that disruption of the *CPSII* gene was the genetic lesion responsible for the uracil auxotrophy of *cps1-1* and *cps2-1*. Transfection of *cps1-1* and *cps2-1* with a linearized 6.6-kb *CPSII Hin*dIII fragment from the wild-type strain RH rescued significant numbers of parasites (scored as plaques), which then grew normally in the absence of uracil (Table 1). Rescued isolates (*cps1-1rs*) all had the wild-type RH CPSII genotype and phenotype on the basis of Southern blot (data not shown), CPSII activity (61 nmol h^{-1} mg^{-1} protein), and growth responses in uracil (Fig. 2a). In contrast, transfection of *cps1-1* or *cps2-1* with only plasmid DNA, or with the Δ6.6-kb *CPSII Hin*dIII plasmid containing an internal 1.1-kb *Bam*HI-generated deletion failed to rescue any additional plaques in the absence of uracil (Table 1).

We tested the virulence of the *T. gondii CPSII* gene knockout mutants in a BALB/c mouse model of lethal toxoplasmosis. The parental RH strain is hypervirulent in all mice strains, with an estimated 100% lethal dose of fewer than 10 parasites[11]. Mice injected intraperitoneally with only a low dose of the parental wild-type RH strain succumb rapidly to the infection (median survival of 9 d). In contrast, mice injected with the *cps1-1* or *cps2-1* mutant survived the infection at inoculating doses of 10^3, 10^4, 10^5, 10^6 (data not shown) and 10^7 tachyzoites (Fig. 3a). Parasites could not be recovered from the intraperitoneal cavity of mice at 3 weeks after inoculation. Mice inoculated with the uracil auxotroph mutants survived longer than 12 months with no evidence of any phenotype of parasite persistence (tachyzoites or brain cysts). In contrast, parasites such as *cps1-1rs*, which were rescued by transfection of the uracil auxotroph mutants with the wild-type 6.6-kb *CPSII Hin*dIII fragment (Table 1), were highly virulent in BALB/c mice (Fig. 3a).

We examined the virulence of *cps1-1* in homozygous interferon-γ (IFN-γ) knockout (*gko*) mice on a BALB/c background. Because the cytokine IFN-γ is necessary for host control of *T. gondii* infection, IFN-γ knockout mice rapidly succumb to toxoplasmosis even after infection with normally avirulent strains[12]. As expected, *gko* mice rapidly succumbed to the RH strain (median survival of 9 d; Fig. 3b). Unexpectedly, *gko* mice injected with the *cps1-1* mutant survived the

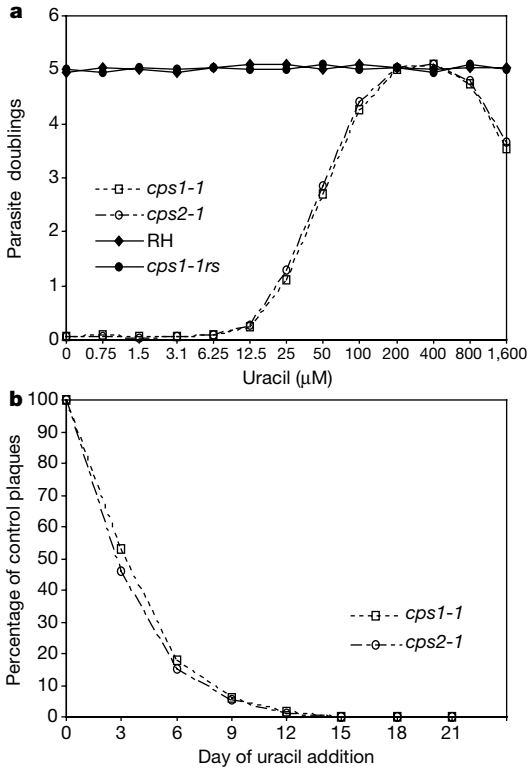

Figure 2 Growth and viability of uracil auxotrophs of *T. gondii*. **a**, Growth rate of uracil auxotroph mutants was measured as a function of the uracil concentration of the infection medium. Parasites were allowed to invade HFFs for 3 h, the cells were then washed and incubated with infection medium containing various concentrations of uracil. Host cells were microscopically inspected 36 h later to identify individual vacuoles containing parasites. The average number of tachyzoites per vacuole was determined by scoring 50 independent vacuoles for each data point. The average number of parasites per vacuole was converted into 'parasite doublings' (for example, 1 parasite doubling = 2 parasites per vacuole, and 5 parasite doublings = 32 parasites per vacuole). Growth responses for auxotrophic mutants *cps1-1* and *cps2-1* are compared with that of the RH parent and a representative clone (*cps1-1rs)* of a rescued parasite obtained after transfection of *cps1-1* with the wild-type 6.6-kb *CPSII Hin*dIII fragment (see Table 1). **b**, Ability of added uracil to rescue the nonreplicating intracellular parasites was determined by a plaque assay (Methods). For uracil addition on day 15, 18 and 21, plaques from the auxotrophic mutants (*cps1-1* and *cps2-1*) were reduced to about 0.2%, 0.02% and 0.003%, respectively, of the control p.f.u.

Table 1 Frequency of rescue of uracil auxotroph mutants after transfection of plasmid DNA

Plasmid DNA	Parasite	p.f.u. rescued ($\times 10^{-7}$)
6.6-kb *CPSII Hin*dIII	*cps1-1*	24
6.6-kb *CPSII Hin*dIII	*cps2-1*	21
Δ6.6-kb *CPSII Hin*dIII	*cps1-1*	< 1
Δ6.6-kb *CPSII Hin*dIII	*cps2-1*	< 1
pBluescript	*cps1-1*	< 1
pBluescript	*cps2-1*	< 1
Mock	*cps1-1*	< 1
Mock	*cps2-1*	< 1

Auxotrophic mutants were transfected with plasmid DNA and plaques (p.f.u.) were scored in uracil-free medium (Methods). Either *Hin*dIII-digested plasmid DNA (20 μg) was transfected, or no plasmid was transfected (mock). Parasites (1.2×10^7) were transfected as indicated and were cultured in a single large HFF flask. Plaques were scored 8 d after transfection by visual and microscopic inspection of plaques. Data shown are the mean from three independent experiments. In other experiments, the spontaneous *in vitro* reversion frequency of *cps1-1* and *cps2-1* was determined to be about 2.8×10^{-8}.

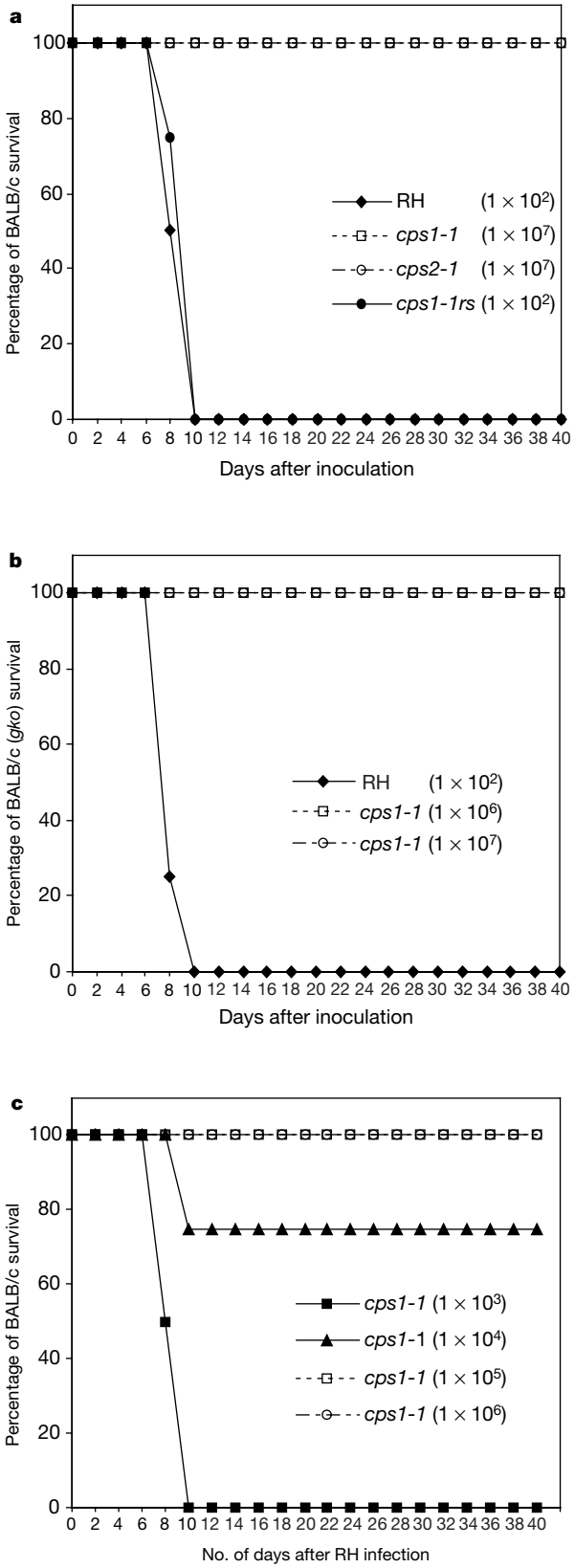

Figure 3 Uracil auxotroph mutants are avirulent in mice and confer long-term protective immunity. **a**, Tachyzoites of the indicated strains were injected i.p. into BALB/c mice ($n = 4$) and monitored for more than 40 d (Methods). The inoculation doses are shown. **b**, Tachyzoites of the indicated strains were injected i.p. into homozygous IFN-γ knockout (BALB/c background) mice ($n = 4$) and monitored for more than 40 d. The inoculation doses are shown. **c**, Various doses of tachyzoites of *cps1-1* were injected i.p. into BALB/c mice ($n = 4$). Mice were challenged 40 days later with 200 p.f.u. of wild-type strain RH tachyzoites administered by the i.p. route.

infection at inoculating doses of 10^3, 10^4, 10^5 (data not shown), 10^6 and 10^7 tachyzoites (Fig. 3b). The *cps1-1*-inoculated *gko* mice showed long-term survival (> 6 months), with no apparent phenotype of parasite persistence.

We examined whether the avirulent uracil auxotrophic mutants could protect mice from a lethal toxoplasma challenge infection. Groups of BALB/c mice were injected once with various doses of *cps1-1* tachyzoites. These mice were injected 40 d later with a lethal challenge dose of strain RH. Parasite doses greater than 10^4 *cps1-1* tachyzoites were highly effective in inducing a long-term protective immunity in BALB/c mice (Fig. 3c).

Our data indicate that the obligate intracellular *T. gondii* parasite may have evolved to have a strict reliance on its own *de novo* pyrimidine biosynthesis pathway *in vivo*. The parasite's pyrimidine salvage pathway cannot apparently salvage significant amounts of pyrimidines from its host cell. This adaptation of the protozoan parasite may have arisen as a consequence of the apparent low availability of pyrimidines in animal tissues[13,14]. Notably, the pyrimidine starvation phenotype of the *cps1-1* and *cps2-1* mutants results in a complete block of parasite replication *in vitro* (Fig. 2) and *in vivo* (Fig. 3). Identifying compounds that limit the *de novo* biosynthesis of pyrimidines in *T. gondii* might be a route for antiparasite drug design. The auxotrophic mutants described here are remarkably avirulent and can also induce long-term protective immunity to toxoplasmosis. To our knowledge, these are the first reported mutants of *T. gondii* that do not kill *gko* mice[15]. *T. gondii* elicits a strong cell-mediated immune response that controls its own growth and that can stimulate nonspecific resistance to unrelated pathogens and tumours[16,17]. Consequently, these auxotrophic mutants may offer even greater promise as a strategy for vaccine development. Developing pyrimidine auxotrophs in other protozoan parasites might verify drug targets in *de novo* pyrimidine biosynthesis and might also provide a broad-ranging approach to obtaining protozoan parasite mutants that are severely attenuated in their virulence. □

Methods

Parasite culture and phenotypic analysis

Human foreskin fibroblasts (HFFs) were cultured in EMEM medium in 10% fetal bovine serum (FBS) at 37 °C in 95% air/5% CO_2. We cultured parasites in EMEM in 1% FBS at 37 °C in 95% air/5% CO_2. Plaques were visualized by fixing HFF monolayers in 50% (v/v) methanol and 7% (v/v) glacial acetic acid, and staining with saturated Coomassie blue dissolved in fixative. The CPSII enzyme assays were done as described[8]. We isolated parasite DNA and carried out Southern analysis as described[18].

To determine whether added uracil could rescue intracellular mutants, replicate sets of flasks of confluent HFF cells were inoculated with 2×10^5, 2×10^4, 2×10^3 or 2×10^2 plaque forming units (p.f.u.) of uracil auxotroph tachyzoites. After a 3-h incubation at 37 °C in medium lacking uracil for attachment and invasion, the remaining extracellular parasites were removed ($t = 0$) by washing the monolayer with PBS, and cultures were incubated in medium lacking uracil. At various times, medium was removed from duplicate sets of the inoculated flasks and was replaced with medium containing 0.2 mM uracil. We stained monolayers and scored plaques by visual inspection 8 d after uracil addition.

Mutant construction and analysis

A genomic DNA fragment of the *T. gondii CPSII* gene (strain RH) was cloned using a polymerase chain reaction (PCR) signature homology strategy. A *CPSII* PCR DNA fragment of the expected size of 450 base pairs (bp) was isolated and subcloned into pBluescript. We examined 30 individual clones, each of which had an identical sequence with a high amino-acid similarity to other CPSII species. We used the 450-bp *CPSII* fragment to identify a single-copy 6.6-kb *Hin*dIII fragment of genomic DNA by Southern blot analysis and to screen a *T. gondii Hin*dIII plasmid library constructed in pBluescript to obtain the 6.6-kb *CPSII Hin*dIII clone. The Δ6.6-kb *CPSII Hin*dIII plasmid was constructed by creating a 1.1-kb *Bam*HI deletion (deleting bp 2,261 to 3,347) in the central part of the 6.6-kb *CPSII Hin*dIII clone.

We made the targeting plasmid by inserting a dihydrofolate reductase/herpes simplex virus thymidine kinase/thymidylate synthase positive/negative selection marker[18] adjacent to the 3′ *CPSII* sequences in the Δ6.6-kb *CPSII Hin*dIII plasmid. This plasmid was integrated into the *CPSII* locus by transfection[18] of strain RH and selection in pyrimethamine with uracil supplementation. We screened clones derived from this selection for ones that grew normally in medium with 0.2 mM uracil, but had disrupted CPSII activity and could not plaque in medium lacking uracil. These *cps1* and *cps2* mutants were then subjected to negative selection in ganciclovir in the presence of uracil. This strategy

selected parasites that lost expression of the integrated thymidine kinase marker[18]. We subcloned ganciclovir-resistant and pyrimethamine-sensitive parasites from this selection to produce *T. gondii* strains, *cps1-1* and *cps2-1*. Uracil auxotrophs *cps1-1* and *cps2-1* have two tandem copies of the targeting plasmid integrated into the *CPSII* locus. Only the *CPSII* locus was disrupted, and integration was achieved by homologous recombination in *CPSII* sequences on the 5′ side of the *Bam*HI sites of the 6.6-kb *CPSII Hin*dIII fragment (data not shown). We maintained the uracil auxotrophs in culture in medium supplemented with 0.2 mM uracil.

Murine virulence assay

We obtained tachyzoites by allowing infected HFF monolayers to lyse completely. Tachyzoites were purified by filtration through sterile 3-μm nuclepore membranes, washed in PBS and collected by centrifugation. We resuspended tachyzoites pellets in PBS and counted them under the microscope. Tachyzoites were injected intraperitoneally (i.p.) in 0.2 ml into mice aged 6–8 weeks. The actual p.f.u. in the inoculum was determined by plaque assay. In all of the mouse injection experiments and for all of the parasite strains tested, the p.f.u. to parasite ratio was between 0.4 and 0.6. We used four mice per parasite dose with each strain. Experiments with groups of mice were repeated twice. Mice were monitored for 40 d, and in some experiments for more than 1 year. We cared for mice according to NIH guidelines.

Received 15 August; accepted 22 November 2001.

1. Luft, B. J. & Remington, J. S. Toxoplasmic encephalitis in AIDS. *Clin. Inf. Dis.* **15,** 211–222 (1992).
2. Pfefferkorn, E. R. *Toxoplasma gondii*: the enzymic defect of a mutant resistant to 5-fluorodeoxyuridine. *Exp. Parasitol.* **44,** 26–35 (1978).
3. Iltzsch, M. H. Pyrimidine salvage pathways in *Toxoplasma gondii*. *J. Euk. Micro.* **40,** 24–28 (1993).
4. Donald, R. G. & Roos, D. S. Insertional mutagenesis and marker rescue in a protozoan parasite: cloning of the uracil phosphoribosyltransferase locus from *Toxoplasma gondii*. *Proc. Natl Acad. Sci. USA* **92,** 5749–5753 (1995).
5. Soldati, D. & Boothroyd, J. C. Transient transfection and expression in the obligate intracellular parasite *Toxoplasma gondii*. *Science* **260,** 349–352 (1993).
6. Donald, R. G. & Roos, D. S. Stable molecular transformation of *Toxoplasma gondii*: a selectable dihydrofolate reductase-thymidylate synthase marker based on drug-resistance mutations in malaria. *Proc. Natl Acad. Sci. USA* **90,** 11703–11707 (1993).
7. Sibley, L. D., Messina, M. & Niesman, I. R. Stable DNA transformation in the obligate intracellular parasite *Toxoplasma gondii* by complementation of tryptophan auxotrophy. *Proc. Natl Acad. Sci. USA* **91,** 5508–5512 (1994).
8. Asai, T. *et al.* Enzymes of the *de novo* pyrimidine biosynthetic pathway in *Toxoplasma gondii*. *Mol. Biochem. Parasitol.* **7,** 89–100 (1983).
9. Flores, M. V., O'Sullivan, W. J. & Stewart, T. S. Characterisation of the carbamoyl phosphate synthetase gene from *Plasmodium falciparum*. *Mol. Biochem. Parasitol.* **68,** 315–318 (1994).
10. Chansiri, K. & Bagnara, A. S. The structural gene for carbamoyl phosphate synthetase from the protozoan parasite *Babesia bovis*. *Mol. Biochem. Parasitol.* **74,** 239–243 (1995).
11. Pfefferkorn, E. R. & Pfefferkorn, L. C. *Toxoplasma gondii*: isolation and preliminary characterization of temperature-sensitive mutants. *Exp. Parasitol.* **39,** 365–376 (1976).
12. Suzuki, Y., Orellana, M. A., Schreiber, R. D. & Remington, J. S. Interferon-γ: the major mediator of resistance against *Toxoplasma gondii*. *Science* **240,** 516–518 (1988).
13. Fields, P. I., Swanson, R. V., Haidaris, C. G. & Heffron, F. Mutants of *Salmonella typhimurium* that cannot survive within the macrophage are avirulent. *Proc. Natl Acad. Sci. USA* **83,** 5189–5193 (1986).
14. Mahan, M. J., Slauch, J. M. & Mekalanos, J. J. Selection of bacterial virulence genes that are specifically induced in host tissues. *Science* **259,** 686–688 (1993).
15. Scharton-Kersten, T. M. *et al.* In the absence of endogenous IFN-γ, mice develop unimpaired IL-12 responses to *Toxoplasma gondii* while failing to control acute infection. *J. Immunol.* **157,** 4045–4054 (1996).
16. Mahmoud, A. A., Warren, K. S. & Strickland, G. T. Acquired resistance to infection with *Schistosoma mansoni* induced by *Toxoplasma gondii*. *Nature* **263,** 56–57 (1976).
17. Charest, H. *et al.* Recombinant attenuated *Toxoplasma gondii* expressing the *Plasmodium yoelii* circumsporozoite protein provides highly effective priming for $CD8^+$ T cell-dependent protective immunity against malaria. *J. Immunol.* **165,** 2084–2092 (2000).
18. Fox, B. A., Belperron, A. A. & Bzik, D. J. Negative selection of herpes simplex virus thymidine kinase in *Toxoplasma gondii*. *Mol. Biochem. Parasitol.* **116,** 85–88 (2001).
19. Bzik, D. J., Li, W. B., Horii, T. & Inselburg, J. Molecular cloning and sequence analysis of the *Plasmodium falciparum* dihydrofolate reductase-thymidylate synthase gene. *Proc. Natl Acad. Sci. USA* **84,** 8360–8364 (1987).

Supplementary Information accompanies the paper on *Nature*'s website (http://www.nature.com).

Acknowledgements

We thank members of the Bzik lab, and D. S. Roos, S. N. Fiering and E. R. Pfefferkorn for discussions. This work was supported by grants from the National Institutes of Health and the US Department of Defense.

Competing interests statement

The authors declare that they have no competing financial interests.

Correspondence and requests for materials should be addressed to D.J.B. (e-mail: David.J.Bzik@Dartmouth.edu). The sequence of the 6.6-kb *CPSII Hin*dIII fragment has been deposited with GenBank (accession code AY059630).

A Rad26–Def1 complex coordinates repair and RNA pol II proteolysis in response to DNA damage

Elies C. Woudstra*, Chris Gilbert*, Jane Fellows*, Lars Jansen†, Jaap Brouwer†, Hediye Erdjument-Bromage‡, Paul Tempst‡ & Jesper Q. Svejstrup*

* *Mechanisms of Gene Transcription Laboratory, Cancer Research UK, Clare Hall Laboratories, Blanche Lane, South Mimms, Hertfordshire EN6 3LD, UK*

† *MGC Department of Molecular Genetics, Leiden Institute of Chemistry, Leiden University, 2300 RA Leiden, The Netherlands*

‡ *Molecular Biology Programme, Memorial Sload-Kettering Cancer Center, York Avenue 1275, New York 10021, USA*

Eukaryotic cells use multiple, highly conserved mechanisms to contend with ultraviolet-light-induced DNA damage[1]. One important response mechanism is transcription-coupled repair (TCR), during which DNA lesions in the transcribed strand of an active gene are repaired much faster than in the genome overall[2]. In mammalian cells, defective TCR gives rise to the severe human disorder Cockayne's syndrome (CS)[3]. The best-studied CS gene, CSB, codes for a Swi/Snf-like DNA-dependent ATPase, whose yeast homologue is called Rad26 (ref. 4). Here we identify a yeast protein, termed Def1, which forms a complex with Rad26 in chromatin. The phenotypes of cells lacking *DEF1* are consistent with a role for this factor in the DNA damage response, but Def1 is not required for TCR. Rather, *def1* cells are compromised for transcript elongation, and are unable to degrade RNA polymerase II (RNAPII) in response to DNA damage. Our data suggest that RNAPII stalled at a DNA lesion triggers a coordinated rescue mechanism that requires the Rad26–Def1 complex, and that Def1 enables ubiquitination and proteolysis of RNAPII when the lesion cannot be rapidly removed by Rad26-promoted DNA repair.

In order to purify Rad26, yeast whole-cell extract derived from a strain expressing Myc-decahistidine tagged Rad26 (MHRad26) was fractionated into a DNA-free 'soluble' fraction and a salt-stable chromatin fraction as previously described[5]. MHRad26 partitioned approximately equally between these fractions and was purified to homogeneity by a combination of conventional and affinity chromatography. As can be seen in Fig. 1a, there was a noticeable difference between the polypeptide composition of Rad26 isolated from the soluble fraction and that isolated from chromatin after high salt extraction. Silver staining of MHRad26 isolated from the soluble fraction showed a single protein band (MHRad26) (Fig. 1a, lane 1), whereas MHRad26 extracted and purified from the salt-stable chromatin fraction appeared to be a doublet (lane 3). Both fractions were analysed by western blot analysis, and the proteins from the doublet were identified by a combination of peptide mass fingerprinting using matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometry (MS), and mass spectrometric sequencing using NanoES triple-quadrupole MS/MS (ref. 6). We found that the slower-migrating protein purified from chromatin was Rad26, whereas the faster-migrating protein was the product of the YKL054C ORF on budding yeast chromosome XI (predicted M_r 83,900). We named this gene *DEF1* (RNAPII degradation factor 1).

In order to investigate the biochemical behaviour of Def1 and to confirm its association with Rad26, decahistidine-haemagglutinin (HA) tagged Def1 (Def1HH) was immuno-purified from extracts derived from *MHRAD26 DEF1HH* cells. The majority of Def1 was found in the soluble (DNA-free) fraction, but only very small amounts of Rad26 could be immunoprecipitated with it from this fraction (data not shown). As in the case of Rad26, SDS–poly-