We have exploited a variety of molecular genetic, biochemical, and genomic techniques to investigate the roles of purine salvage enzymes in the protozoan parasite *Toxoplasma gondii*. The ability to generate defined genetic knockouts and target transgenes to specific loci demonstrates that *T. gondii* uses two (and only two) pathways for purine salvage, defined by the enzymes hypoxanthine-xanthine-guanine phosphoribosyltransferase (HXGPRT) and adenosine kinase (AK). Both HXGPRT and AK are single-copy genes, and either one can be deleted, indicating that either one of these pathways is sufficient to meet parasite purine requirements. Fitness defects suggest both pathways are important for the parasite, however, and that the salvage of adenosine is more important than salvage of hypoxanthine and other purine nucleobases. HXGPRT and AK cannot be deleted simultaneously unless one of these enzymes is provided in *trans*, indicating that alternative routes of functionally significant purine salvage are lacking. Despite previous reports to the contrary, we found no evidence of adenine phosphoribosyltransferase (APRT) activity when parasites were propagated in APRT-deficient host cells, and no APRT ortholog is evident in the *T. gondii* genome. Expression of *Leishmania donovani* APRT in transgenic *T. gondii* parasites yielded low levels of activity but did not permit genetic deletion of both HXGPRT and AK. A detailed comparative genomic study of the purine salvage pathway in various apicomplexan species highlights important differences among these parasites.

Like all parasitic protozoa, the obligate intracellular parasite *Toxoplasma gondii* lacks the ability to synthesize the purine ring *de novo*, and thus relies entirely on the salvage of purines from the host cell to meet its nutritional needs (1–3). This requirement, coupled with the shortcomings of conventional therapies for treating congenital toxoplasmosis and opportunistic infections associated with AIDS and other immunosuppressive conditions (4–8), makes purine salvage an attractive target for chemotherapy.

The purine metabolism of *T. gondii* has previously been examined biochemically, resulting in the identification of various activities capable of assimilating nucleosides and nucleobases from the host cell into the purine nucleotide pools of the parasite (2, 3). (See “Discussion” for a model of the purine salvage pathway in *Toxoplasma* and other apicomplexan parasites.) Reported salvage activities include the phosphoribosylation of adenine, guanine, hypoxanthine, and xanthine, and the phosphorylation of adenosine. The latter seems to contribute most significantly to parasite purine economy, as adenosine is incorporated into nucleotide pools at a considerably higher rate than any purine nucleobase (2, 3).

Most of the reported salvage activities can be accounted for by two enzymes: hypoxanthine-xanthine-guanine phosphoribosyltransferase (HXGPRT) and adenosine kinase (AK). The genes for both have been cloned and expressed in bacterial systems, and the purified proteins have been examined biochemically and structurally (9–13). Genetic studies indicate that neither enzyme is essential for parasite viability, suggesting that the purine salvage pathways of the parasite are functionally redundant. The incorporation of labeled inosine and hypoxanthine into adenosine nucleotides, and of labeled adenosine into IMP, indicates that AMP and IMP are interconvertible. The conversion of AMP to IMP (via AMP deaminase), and of IMP to AMP (via adenylosuccinase synthetase/lyase) probably predominate, although adenine and adenosine deaminase activities (3) may also play a role. Despite the previously measured incorporation of adenine by *Toxoplasma*, no adenine phosphoribosyltransferase gene has been identified in this or any other apicomplexan parasite.

In the present study, we have integrated genetic, biochemical, and genomic approaches to explore the contributions of HXGPRT and AK in maintaining a robust purine salvage pathway. The ability of *T. gondii* to grow in virtually any nucleated mammalian cell (14), combined with the availability of various mammalian somatic cell mutants and the ability to genetically manipulate the parasite (15), has allowed for a comprehensive analysis of the role played by individual parasite enzymes in overall purine nutrition. These studies provide formal proof that all purine salvage in *T. gondii* proceeds via HXGPRT or AK (there is no functional APRT activity), and fitness assays support the suggestion that AK is metabolically more important than HXGPRT. We have also

1 The abbreviations used are: HXGPRT, hypoxanthine-xanthine-guanine phosphoribosyltransferase; AK, adenosine kinase; AMP, adenosine 5’-monophosphates; APRT, adenine phosphoribosyltransferase; 6TX, 6-thioxanthine; 8-azaA, 8-azaadenine; 2-FA, 2-fluoroadenine.
exploited the large-scale genomic datasets now available for multiple apicomplexan species to create detailed maps of purine salvage pathways for several of these parasites, an important first step toward identifying potential drug targets for broad-spectrum anti-parasitic chemotherapy.

MATERIALS AND METHODS

Parasites, Cells, Chemicals, and Reagents—T. gondii strain RH (available through Ogden Bioservices Corporation, Rockville, MD) or mutants derived from this strain were used for all in vitro tissue culture experiments and were cultivated in primary human foreskin fibroblasts (HFF cells) or other fibroblast cells, as described previously (15, 16). [14C]Adenine (56 Ci mol−1), [14C]xanthine (57 Ci mol−1), [3H]Hudarcic acid (48 Ci mmol−1), and [3H]uracil (20 Ci mmol−1) were purchased from Moravek Biochemicals (Brea, CA). All unlabeled substrates of purine salvage enzymes were purchased from Sigma. DNA modifying enzymes were acquired from New England Biolabs (Beverly, MA). DE81 anion-exchange filters were obtained from Whatman (Hillsboro, OR).

Molecular Genetic Manipulations—All molecular manipulations were performed according to standard protocols (15). Isolation of parasite genomic DNA was carried out by digestion with proteinase K (200 μg/ml) in the presence of 1% sodium dodecyl sulfate, followed by phenol/chloroform extraction and ethanol precipitation. Digested genomic DNA was blotted to Nytran filter paper and processed as described. The AK probe consisted of the open reading frame of the AK cDNA excised from the pBAe6AK expression vector (11) by digestion with NotI and XbaI. The HXGPRT probe was isolated from a similar expression vector (9) by digestion with NdeI and XbaI. Probes were purified from agarose gels by Qia-gen gel purification kit and [32P] labeled utilizing Stratagene Prime-It II random primer labeling kit.

Generation of Defined Knockouts—Plasmids for generating defined knockouts at the HXGPRT or AK loci (constructs HXGPRT-KO and AK-KO) have been described previously, as have the methods employed to isolate mutant parasites (9, 10). The HXGPRT::AK knockout construct was constructed by inserting an AK cDNA driven by the T. gondii dihydrofolate reductase promoter into the HXGPRT-KO vector. The construct pminAK (10) was digested with HindIII and NotI to remove the AK expression cassette (AK cDNA flanked by dihydrofolate reductase 5′ and 3′ sequences), and the resulting fragment was blunted and gel-purified using the Qiagen gel purification kit and [32P] labeled utilizing Stratagene Prime-It II random primer labeling kit.

Enzyme Assays—After lysis of the host cell monolayer, parasites were isolated from residual host cell debris by filtration through 3-μm Nucleopore filters. Parasites were pelletted by centrifugation at 2500 rpm for 20 min at 4 °C and washed twice in phosphate-buffered saline before resuspending them at 105 parasites/ml in TMD buffer (100 mM Tris, pH 7.5, 5 mM MgCl2, 2 mM dithiothreitol) and lysis by sonication on ice or the addition of 1% Triton X-100. Protein concentrations were determined by using Bio-Rad protein assay and normalized for all enzymatic determinations. All enzyme assays were conducted according to the radiometric methods described previously (17, 18). AK reactions were performed in a 50-μl volume at 37 °C in TMD buffer with 1 mM ATP, 5 mM NaF (to inhibit phosphatase activity), 25 μM [14C] xanthine and appropriate volumes of crude parasite lysate. Reactions were terminated by spotting 10 μl of the reaction mix onto DEAE anion-exchange filters. Filters were washed 3× in water and once in ethanol, dried completely at 60 °C; radioactivity was assessed by liquid scintillation counting. Measurements were taken over a 120-min time course.

Xanthine phosphoribosyltransferase (XPRT) and adenine phosphoribosyltransferase (APRT) reactions were performed similarly, using PRT reaction mixes containing TMD buffer with 1 mM phosphoribosyl pyrophosphate, 0.3 μCi of [14C] xanthine (57 Ci mol−1) or 1.3 μCi of [14C]-adenine (56 Ci mol−1), and appropriate volumes of crude lysate. Reactions were terminated by spotting 10 μl of the reaction mix onto DEAE anion-exchange filters. Filters were washed 3× in water and once in ethanol, dried completely at 60 °C; radioactivity was assessed by liquid scintillation counting. Measurements were taken over a 120-min time course.

RESULTS

Defined Genetic Knockouts of Purine Salvage Enzymes—Successful genetic knock-outs of either the AK or HXGPRT loci have been described previously and shown to be deficient in the corresponding purine salvage activities (9, 10). The ease of generating such mutants prompted efforts to delete both loci in a single parasite. A summary of the results from numerous

2 M. Nishi and D. S. Ross, manuscript in preparation.

The following are available on the World Wide Web: Plasmodium falciparum are from //PlasmoDB.org; T. gondii is from //ToxoDB.org; Eimeria tenella and Theileria annulate are from ftp://ftp.sanger.ac.uk/pub/pathogens; Cryptosporidium parvum is from www.parvum.mic.vcu.edu; Cryptosporidium hominis is from www.cbc.umn.edu/ResearchProjects/AG/Ag/index.htm; Tetrahymena thermophila is from www.tigr.org/tdb/e2k1/T.thermophila/; P. chabaudi, P. vivax, P. knowlesi, T. gondii, E. tenella, and Theileria parva are from ftp://ftp.sanger.ac.uk/pub/pathogens; and Theileria parva genome was obtained from www.tigr.org/db/ek2/T.para1.
such experiments is provided in Table I. Deletion of either individual locus was readily achieved, using AK-KO or HXGPRT-KO constructs (see “Materials and Methods”). In contrast, in three independent experiments, we were unable to delete the AK locus in the ΔHXGPRT background (based on adenine arabinoside selection of parasites transfected with the AK-KO construct), or to delete the HXGPRT locus in the ΔAK background (based on 6TX selection of parasites transfected with the HXGPRT-KO construct). Parasites resistant to both adenine arabinoside and 6TX were occasionally isolated in the latter experiment (using 6TX selection), but DNA hybridization analysis revealed no obvious lesion at the HXGPRT locus, and Western blotting showed normal levels of HXGPRT protein. These parasites are presumed to harbor substrate specificity mutations at the HXGPRT locus or mutations at other loci that affect 6TX toxicity.

The inability to delete both HXGPRT and AK suggests that the double-knockout phenotype may be lethal for *T. gondii*. To further address this hypothesis, we attempted to genetically delete both loci, while providing AK activity in *trans* (producing a double knock-out genotype that is nevertheless AK<sup>+</sup>). The HXGPRT-KO construct was modified to contain the coding region of *T. gondii* AK (driven by the *T. gondii* dihydrofolate reductase promoter), generating construct HXGPRT::AK. 6TX-resistant parasites were readily obtained after transfection of ΔAK parasites, and the resulting clones were tested by Southern analysis to assess their genotype and enzyme assays to assess purine salvage activities, as shown in Fig. 1.

As expected, probing with AK cDNA demonstrates genomic EcoRI fragments of 2.1 and 3.8 kb in wild-type and ΔHXGPRT parasites and deletion of the larger fragment in the ΔAK mutant (10). This same pattern was observed in the HXGPRT::AK mutant, demonstrating maintenance of the ΔAK genotype. In addition, a larger band was also observed of the size expected from integration of the HXGPRT::AK plasmid at the HXGPRT locus (Fig. 1, *arrowhead*). The same size band was observed with an HXGPRT probe, which also shows that the endogenous HXGPRT locus (observed in wild-type parasites and ΔAK mutants) was successfully disrupted in the HXGPRT::AK mutant. The smaller size of the HXGPRT locus in this mutant relative to the ΔHXGPRT clone is attributable to molecular manipulations associated with construction of the HXGPRT::AK vector (see “Materials and Methods”).

Enzyme assays demonstrate no AK activity in the ΔAK parent but show activity comparable with that of wild-type parasites (and ΔHXGPRT mutants) when AK was targeted to the HXGPRT locus in the ΔAK background. XPRT assays show normal activity in the ΔAK parent but none in HXGPRT:AK parasites, similar to the ΔHXGPRT mutants characterized previously (9). Taken together, these results demonstrate that it is possible to disrupt both the AK and HXGPRT genetic loci simultaneously, but only if one of these activities is provided in *trans* (in this case, by expression of AK at the disrupted HXGPRT locus).

**APRT Activity and Parasite Sensitivity to Subversive Substrates of APRT**—The inability to generate ΔHXGPRT, ΔAK double-knock-out parasites is somewhat surprising, given the previous identification of APRT activity in *T. gondii* (3), which could provide for an alternative route of purine assimilation for the parasite. Consistent with prior reports, RH-strain *T. gondii* cultivated in normal human foreskin fibroblasts were susceptible to both 2-FA (IC<sub>50</sub> = 75 ng/ml) and 8-azaA (IC<sub>50</sub> = 1.5 μg/ml), as shown in Fig. 2 (*white symbols*). These adenine analogs are known to function as subversive substrates of APRT, and both have been shown to produce APRT-dependent toxicity in other systems (23–25). When parasites were grown in APRT-deficient cells (16), however, IC<sub>50</sub> increased to >100 μg/ml, a decrease in sensitivity of ~2.7 × 10<sup>3</sup> and 67-fold for 2-FA and 8-azaA, respectively (Fig. 2, *black symbols*). These results suggest that toxicity of 2-FA and 8-azaA is dependent upon host, rather than parasite, APRT activity.

Furthermore, although variable levels of APRT activity were observed in crude cytosolic extracts of parasites cultivated in wild-type human foreskin fibroblasts (data not shown), we were completely unable to detect phosphoribosylation of radio-labeled adenine when parasites were grown in APRT-deficient fibroblasts (Table II), supporting the suggestion that parasites lack an endogenous APRT activity. In addition, *T. gondii* tachyzoites grown in APRT-deficient host cells incorporate only very low levels of [<sup>14</sup>Cl]adenine (see Fig. 3B).

**Expression of L. donovani APRT in T. gondii**—To further probe purine salvage pathways in *T. gondii*, we attempted to

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**Table I. Summary of attempts to knock out AK and HXGPRT loci**

| Parental genotype          | Transfection construct | Selection | Resistant parasites? | Deletion of targeted gene? |
|----------------------------|------------------------|-----------|----------------------|---------------------------|
| RH, wild-type              | AK-KO                  | AraA      | Yes                  | Yes                       |
| RH, wild-type              | HXGPRT-KO              | 6TX       | Yes                  | Yes                       |
| ΔHXGPRT                    | AK-KO                  | AraA      | No                   | No                        |
| ΔAK                        | HXGPRT-KO              | 6TX       | Yes                  | No                        |
| ΔHXGPRT, LdAPRT<sup>+</sup> | AK-KO                  | AraA      | No                   | No                        |
| ΔAK, LdAPRT<sup>+</sup>    | HXGPRT-KO              | 6TX       | Yes                  | No                        |
express *L. donovani* APRT in wild-type, ΔAK, and ΔHXGPRT *T. gondii*. An *LdAPRT-HA* fusion construct (see “Materials and Methods”) was expressed in the parasite cytosol (Fig. 3A), and the resultant protein possesses APRT activity both in vivo (Fig. 3B) and in vitro (Fig. 3C and Table II). Wild-type parasites incorporate very little [3H]adenine into acid-precipitable material when grown in APRT-deficient host cells, in contrast to transgenic parasites expressing *L. donovani* APRT (Fig. 3B). APRT activity was virtually undetectable in cell-free extracts of wild-type RH-strain parasites (grown in APRT-deficient host cells), but significant levels of activity were seen in RH-*LdAPRT, ΔAK-LdAPRT*, and ΔHXGPRT-*LdAPRT* transgenics (Fig. 3C and Table II).

Analysis of other purine salvage activities in parasites expressing high levels of *LdAPRT* indicates that HXGPRT and AK activities are unaffected by the presence of the transgenic APRT activity (Table II). Interestingly, the relative values of these salvage activities suggest that adenosine utilization in *T. gondii* is significantly more efficient than for other parasites, whereas the activity of transgenic APRT is detectable but considerably lower than either AK or HXGPRT.

To explore whether APRT-expressing parasites might be amenable to genetic deletion of both the AK and HXGPRT loci, ΔAK-*LdAPRT* and ΔHXGPRT-*LdAPRT* parasites were transfected with HXGPRT-KO or AK-KO vectors (respectively) and subjected to selection for deletion of either locus. As indicated in Table I, no double knock-outs have been identified to date.

**Fitness Assays for ΔAK and ΔHXGPRT Parasites—**Previous studies on ΔAK and ΔHXGPRT mutants showed no dramatic differences in growth rates as compared with the wild-type parasites from which they were derived (9, 10). More recently, however, we have found that sensitive competition assays enable the detection of subtle fitness defects in other mutants (22). As shown in Fig. 4, both the ΔAK and ΔHXGPRT knockout lines showed significant fitness impairment, with ΔAK parasites displaying a fitness defect of 7.6% per generation (Fig. 4A) and ΔHXGPRT exhibiting a defect of 3.7% per generation (Fig. 4B). These findings indicate that both AK and HXGPRT play an important role in parasite metabolism and fitness, although the presence of neither gene is essential for viability, as noted above. The larger defect observed in ΔAK supports the suggestion that this enzyme plays the more important role in purine salvage.

**Comparative Genomic Analysis of Purine Salvage Pathways in Related Species—**Taking advantage of the large-scale genomic datasets that have recently become available for various apicomplexan species (26–28), we have constructed a detailed model for purine salvage pathway in these parasites, as shown in Fig. 5. The ciliate *T. thermophila* was also included in this analysis, as ciliates, dinoflagellates, and apicomplexans are thought to be sister taxa, grouped in the superphylum *Alveolata* (29). No genes encoding *de novo* purine synthetic enzymes are evident in any of these protozoa, suggesting that all are incapable of purine biosynthesis. This is true even for the free-living ciliate *T. thermophila*, which supports previous studies on the incorporation of [14C]glycine and formate (30). Many of these genes are evolutionarily well conserved, and redundant synthetic pathways have not been described, making it unlikely that purine synthetic pathways were missed in this analysis.

Purine salvage requires the ability to transport nucleosides and/or nucleobases, and equilibrative-type transporters...
ways to the purine economy of T. gondii and related organisms. T. gondii possesses two functionally redundant salvage enzymes, HXGPRT and AK, and previous results have shown that this parasite can survive elimination of either activity alone, i.e. neither HXGPRT nor AK is essential for T. gondii survival (9, 10). Attempts to knock out both HXGPRT and AK in a single parasite were unsuccessful, providing circumstantial evidence that functional expression of at least one of these two enzymes is essential (Table I). Direct support for this hypothesis is provided by the ΔAK and HXGPRT-ΔAK parasites (Fig. 1), where expression of AK activity in trans permitted simultaneous disruption of the endogenous genomic loci for both AK and HXGPRT. These experiments show that AK and HXGPRT provide the only two physiologically relevant routes for purine acquisition in T. gondii. ΔAK parasites exhibited a greater fitness defect than ΔHXGPRT mutants (Fig. 4), arguing that flux through AK is probably greater than HXGPRT.

Previous biochemical studies have noted APRT activity in T. gondii (3), with parasite extracts exhibiting higher specific activity of adenine phosphoribosylation than that of guanine (but less than hypoxanthine or xanthine). In reviewing these data, however, we note that radiolabeled adenine was incorporated into nucleotide pools with a distribution virtually identical to that observed for hypoxanthine (3), suggesting that adenine may have been deaminated to hypoxanthine and incorporated via HXGPRT, rather than APRT (Fig. 5). Alternatively, the high levels of APRT present in mammalian host cells (35) may have resulted in inadvertent contamination of parasite extracts. In our hands, parasites grown in wild-type host cells were susceptible to toxic adenine analogs, but those grown in APRT-deficient host cells (16) were not (Fig. 2). Moreover, we found no APRT activity in wild-type RH-strain parasites or parasite extracts when APRT-deficient host cells were used for parasite cultivation (Fig. 3). Expression of a heterologous APRT allowed transgenic parasites to incorporate low levels of radiolabeled adenine (Fig. 3), although this activity was insufficient to permit inactivation of both HXGPRT and AK (Table I).

APRT activity has also been reported in Plasmodium (36, 37), Eimeria (38), and Cryptosporidium (39). However, immucillin H (which inhibits parasite nucleoside phosphorylase, and hence the acquisition of purines via HXGPRT) is lethal to P. falciparum (40), arguing against a biologically significant role for APRT. No APRT genes were evident in the genome of any apicomplexan parasite (Fig. 5), and it seems unlikely that any such genes were missed, as all known APRT genes are highly conserved in primary sequence (and no alternative enzymes are known to phosphoribosylate adenine). We conclude from the available genetic, biochemical, and genomic data that APRT is lacking from all of these apicomplexan parasites.

Interestingly, the T. thermophila genome encodes a putative APRT, suggesting that the last common ancestor of the Alveolata may have possessed three purine salvage pathways, utilizing APRT, AK, or HXGPRT. Phylogenetic analysis (not shown) provides no evidence for horizontal transfer of any of these genes, in contrast to Cryptosporidium IMPDH (41). Biochemical studies indicate that this ciliate also exhibits other differences from most of the apicomplexa, including a nucleoside hydrolase and the lack of XPRT and GMP synthetase activity (42); these observations are supported by the genome sequence. Genomic analysis also reveals a gene likely to encode GMP reductase, explaining the observation that Tetrahymena can survive on guanine or guanosine as a sole purine source (30). A probable GMP reductase was also observed in Theileria species, but this enzyme seems to have been lost in other apicomplexans.
In sum, HXGPRT and/or AK seem to provide the sole routes for purine assimilation in apicomplexans, but different species may possess one, the other, or both of these activities. *Toxoplasma* and *Eimeria* parasites contain genes predicted to encode both enzymes, as well as many interconverting enzymes indicated by arrows in Fig. 5. Adenosine deaminase was not readily identifiable in these parasite genomes, but this gene is not highly conserved, and the observed conversion of adenosine into inosine (3, 10, 43) suggests that it is probably present. Biochemical studies also indicate an adenine deaminase in *Eimeria* (43), although this was not evident in the genome. Biochemical and pharmacological studies (44) were unable to detect AMP deaminase in the *T. parva* or *T. annulata* genomes (see supplementary material), but presume that this activity must be present as the sole means for guanylate nucleotide production. *Plasmodium* expresses a highly active and well characterized HXGPRT activity (45) but lacks AK.

Several subversive purine analogs and inhibitors have shown efficacy against protozoan pathogens (46–48), but functional redundancy in purine salvage pathways poses a potential impediment to rational drug design, as effective therapies may have to inhibit multiple activities to maximize potency and reduce the likelihood of resistance. The lack of an APRT activity in *T. gondii* indicates that only HXGPRT and AK would have to be blocked, and both of these enzymes have previously been characterized biochemically and structurally (9, 11, 13, 49). The significant fitness effect of genetically deleting either locus (Fig. 4) suggests that this may be a viable drug target, even in *T. gondii*. The more highly reduced purine salvage pathways observed in other parasites makes these targets even more attractive. For example, inhibiting any of the five activities leading from host cell adenosine to GMP in the *Cryptosporidium* panel in Fig. 5 (adenosine transport, adenosine kinase, AMP deaminase, IMP dehydrogenase, GMP synthetase) would have to be blocked.
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the parasite. The necessary framework in terms of structural and biochemical data for both the parasite and mammalian enzymes is available (9, 11–13, 45, 50–54) and should expedite the development of antiparasitic drugs with high therapeutic potential.

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Purine Salvage Pathways in the Apicomplexan Parasite *Toxoplasma gondii*
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