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Insertional tagging of at least two loci associated with resistance to adenine arabinoside in *Toxoplasma gondii*, and cloning of the adenosine kinase locus

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Abstract

A genetic approach has been exploited to investivate adenylate salvage pathways in the protozoan parasite *Toxoplasma gondii*, a purine auxotroph. Using a new insertional mutagenesis vector designed to facilitate the rescue of tagged loci even when multiple plasmids integrate as a tandem array, 15 independent clonal lines resistant to the toxic nucleoside analog adenine arabinoside (AraA) were generated. Approximately two-thirds of these clones lack adenosine kinase (AK) activity. Parallel studies identified an expressed sequence tag (EST) exhibiting a small region of weak similarity to human AK, and this locus was tagged in several AK-deficient insertional mutants. Library screening yielded full-length cDNA and genomic clones. The *T. gondii* AK gene contains five exons spanning a ~3 kb locus, and the predicted coding sequence was employed to identify additional AK genes and cDNAs in the GenBank and dbEST databases. A genomic construct lacking essential coding sequence was used to create defined genetic knock-outs at the *T. gondii* AK locus, and AK activity was restored using a cDNA-derived minigene. Hybridization analysis of DNA from 13 AraA-resistant insertional mutants reveals three distinct classes: (i) AK^- mutants tagged at the AK locus; (ii) AK^- mutants *not* tagged at the AK locus, suggesting the possibility that another locus may be involved in regulating AK expression; and (iii) mutants with normal AK activity (potential transport mutants).

Keywords: Molecular parasitology; Purine metabolism; Adenosine kinase; Adenosine transport; Insertional mutagenesis; Gene targeting; Knock-out mutants

Abbreviations: AraA, adenine arabinoside; AK, adenosine kinase; EHNA, erythro-9-(2-hydroxy-3-nonyl)-adenine; EST, expressed sequence tag; HFF, human foreskin fibroblasts; HXGPRT, hypoxanthine–xanthine–guanine phosphoribosyltransferase; PBS, phosphate buffered saline; UPRT, uracil phosphoribosyltransferase.

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1. Introduction

The obligate intracellular parasite *Toxoplasma gondii* is a common source of congenital birth defects, and has become a significant threat to immunocompromised individuals [1]. The standard treatment for acute toxoplasmosis (and many other infectious diseases) targets pyrimidine metabolism, employing synergistic doses of pyrimethamine and sulfonamides to inhibit the folate pathway necessary for TMP synthesis [2]. Complications such as pyrimethamine-resistance and the adverse effects of prolonged sulfonamide treatment have emerged, however, providing the motivation for developing new drugs and exploring new metabolic targets [3].

Like all intracellular parasites, *Toxoplasma* is unable to carry out *de novo* purine biosynthesis [4–6]. The absolute reliance of these parasites on purine salvage pathways therefore offers an excellent opportunity for chemotherapeutic intervention. Among the enzymes of purine salvage in *T. gondii*, adenosine kinase (AK; EC 2.7.1.20) provides the most attractive target, as AK activity is 10-fold higher than the next most active purine salvage enzyme [6–8]. In contrast to mammalian cells, deamination of adenosine to inosine and subsequent cleavage to hypoxanthine (by adenosine deaminase and purine nucleoside phosphorylase, respectively) is not a highly active pathway in *T. gondii* [6]. Structure-activity analysis and metabolic studies indicate that the substrate specificity of *T. gondii* AK differs significantly from that of the human host: 6-substituted-9-β-D-ribofuranosylpurines were among the best ligands, exhibiting selective toxicity against the parasite enzyme [9,10].

Unfortunately, investigation of AK as a potential target for parasiticidal chemotherapy has been hampered by difficulties in cloning the gene. The paucity of AK sequences in GenBank [11–13] complicates sequence-based strategies, and the lack of suitable mutants in other systems precludes complementation cloning. Recent studies have demonstrated the feasibility of employing molecular genetic techniques to investigate metabolic pathways in *Toxoplasma*, however, and molecular tools for transient and stable expression, allelic replacement, and insertional mutagenesis are now available for the haploid tachyzoite form of this pathogen [14,15]. Insertional mutagenesis procedures, conceptually analogous to transposon tagging schemes commonly exploited in other systems [16], have allowed the cloning and characterization of several *T. gondii* loci of chemotherapeutic interest, including the genes encoding uracil phosphoribosyltransferase (UPRT) and hypoxanthine–xanthine–guanine phosphoribosyltransferase (HXGPRT) [17–19].

T. gondii mutants lacking AK activity are resistant to the toxic adeonsine analog adenine arabinoside (AraA) [7]. AK-deficient mutants are still viable, however [5], as purines can also be salvaged via HXGPRT, and IMP can be converted to AMP via adenylosuccinate. Adenine phosphoribosyltransferase activity has also been reported in *T. gondii* [6], although the presence of this enzyme is controversial. We have therefore employed insertional mutagenesis, combined with selection for resistance to AraA, to identify at least two independent loci associated with adenosine metabolism. Approximately 70% of insertional mutants resistant to AraA were deficient in AK activity, and the majority of these were shown to be tagged at the AK locus. The AK locus was also identified via an informatics approach, exploiting the recently-released *T. gondii* expressed sequence tag (EST) database [20,21]. Definitive proof of function comes from molecular genetic manipulation: a defined knock-out mutant was produced by targeted homologous recombination, and AK activity was restored in these mutants by expression of an AK cDNA under the control of a heterologous promoter.

2. Materials and methods

2.1. Parasite cultivation

The virulent *T. gondii* strain RH was used for all experiments described in this report. Parasites and many other reagents for the molecular manipulation of *T. gondii* are available from Ogden Bioservices Corporation, Rockville MD (e-mail: obcaids@ix.netcom.com; tel./fax: + 1-301-340-

0245H; fax-9245). Cultivation in primary human foreskin fibroblasts (HFF cells), mutagenesis, clonal isolation, and other relevant procedures have been described previously [14].

2.2. Insertional mutagenesis

Tachyzoites ($\sim 2 \times 10^7$) were electroporated with 50 μg of insertional mutagenesis plasmid (see below), linearized with *Hin*d III as previously described [14,17], and inoculated into 25-cm^2 T-flasks containing a confluent monolayer of HFF cells and culture media supplemented with 1 μM pyrimethamine. Upon lysis of the host cells, the emerging parasite tachyzoites were passed into multiple 175-cm^2 T-flasks containing both pyrimethamine and 110 μM AraA (Sigma, St. Louis, MO). Flasks that showed evidence of viable parasites were passed for an additional cycle of AraA selection, and clones were isolated by limiting dilution in 96-well plates. Each of the 15 mutants described below was obtained from an independent 25-cm^2 T-flask, and therefore represents an independant clone.

Five AraA-resistant mutants were isolated from parasites transfected with plasmid *p*TgDHFR-TSc3/M2M3 (Ogden catalog #2854), which contains a cDNA-derived DHFR-TS minigene mutated to confer pyrimethamine resistance [14]. Ten additional AraA-resistant mutants were produced using a modified insertional mutagenesis vector, designated *p*c3ABP. This vector was created by ligation of *Pml* I- and *Nhe* I-digested *p*TgDHFR-TSc3/M2M3 to a *Sma* I (blunt)–*Xba* I (*Nhe* I-compatible) fragment derived from plasmid *p*NEB193 (New England Biolabs):

Asc I *Pac* I
5′-GGGGGCGCGCCGGATCCTTAATTAAGT-3′
3′-CCCCCGCGCGGCCTAGGAATTAATTCAGATC-5′
*Bsc*H II *Bam*H I

Prior to the reaction, *p*NEB193 was also digested with *Eco*R I, to prevent ligation of the plasmid backbone into the transfection vector. Sequencing across the junction region confirmed that the desired fragment, containing rare-cutter restriction sites *Asc* I, *Bss*H II and *Pac* I in addition to a *Bam*H I site, was introduced upstream of the DHFR-TS promoter in *p*c3ABP (restriction sites are underlined above). See Section 3 for further discussion of this vector.

2.3. Enzyme assays

Freshly harvested parasite tachyzoites were isolated by passage through a 3 μm polycarbonate filter (Nuclepore) and pelleted by centrifugation at 2500 rpm for 20 min at 4°C. After washing once in cytomix [22], the pellet was resuspended in 0.5 ml ice-cold 50 mM Tris pH 7.4 and sonicated 2×10 s on ice. An aliquot of the crude extract was tested by Bradford assay [23] and samples normalized for equal protein concentration. AK activity was assayed by measuring the conversion of [8-^{14}C]adenosine (NEN) to AMP in crude parasite extracts. Reactions were carried out at 37°C in 50 μl volumes containing 20 μM [8-^{14}C]-adenosine (10 Ci/mol), 2.5 mM ATP, 5.0 mM $MgCl_2$, 10 mM NaF, and various concentrations of extract. In some reactions, 10 μM erythro-9-(2-hydroxy-3-nonyl)-adenine (EHNA; Sigma) was added to inhibit adenosine deaminase activity [24]. Ten μl aliquots of the reaction mix were removed at various time points, boiled 1 min, and spotted on Silica Gel G plates (Fischer) for thin layer chromatography in 7:4:4:3::*n*-butyl alcohol:ethyl acetate:ammonium hydroxide:methanol [9]. Adenosine migrates with an R_f of ~ 0.58 in this solvent system, while phosphorylated products remain at the origin.

2.4. Molecular methods

Amplification of putative AK sequences from genomic DNA was carried out by polymerase chain reaction on ~ 1 μg of RH strain genomic DNA, using primers 5′-TGCGGTCCGCGTCAGTGACAGAC-3′ (sense) and 5′-TCCCCTCTTGAGGAAGAACTCGTC-3′ (antisense), as predicted from EST #466677 (GenBank ID #N60952). Amplification entailed 30 cycles of the following program: 30 s at 94°C; 90 s at 68°C; 90 s at 72°C. The single prominent 150 bp product was purified from an agarose gel using the MerMaid system (Bio101), radiolabeled by random priming, and used to probe a λZAP-II cDNA library (Ogden #1896) according to standard procedures. *p*SK phagemids were recovered using the ExAssist/SOLR system (Stratagene) and submitted to the University of Pennsylvania Cancer Center Sequencing Facility for automated sequencing on an ABI-PRISM unit using T3 and T7 primers.

AK cDNA was used to screen a genomic λDASH-II library (Ogden #2862), and an 11.5 kb genomic *Xba* I fragment spanning the AK locus was subcloned into *p*KS+. A targeting construct for producing genomic knock-out mutants was created by deleting an internal 1.7 kb *Stu* I–*Bst*X I fragment which includes essential AK coding sequence. Wild-type parasites were transfected with 30 μg of plasmid DNA (linearized with *Spe* I), and subjected to AraA selection. Resistant clones were examined by Southern analysis to confirm organization of the AK locus.

A *T. gondii* AK expression vector was constructed based on the *p*minCAT plasmid (Ogden #2850) [14], placing a cDNA-derived AK open reading frame under control of the *T. gondii* DHFR-TS promoter. The AK open reading frame was amplified by polymerase chain reaction (PCR) using PFU polymerase (New England Biolabs) and primers 5′-ATCG<u>AGATCT</u>AAAATG-GCAGTCGATTCCTCC-3′ (sense) and 5′-ATTTAA<u>ATGCAT</u>TCAACACGGAAGT-GAAG-3′ (antisense), introducing engineered *Bgl* II and *Nsi* I restriction sites (underlined). The 1.1 kb product was digested with *Bgl* II and *Nsi* I (compatible with *Pst* I) and ligated into *Bgl* II- and *Pst* I-digested *p*minCAT to produce plasmid *p*minAK.phd. Sequencing across the amplified region confirmed fidelity of the PCR reaction. This plasmid (50–100 μg) was transfected into AK-deficient parasites by electroporation [14], followed by inoculation into 25-cm^2 T-flasks containing confluent HFF monolayers.

3. Results

3.1. Insertional mutants resistant to AraA

In order to circumvent the difficulties of other strategies for cloning the *T. gondii* AK gene, a genetic approach was developed, employing insertional mutagenesis techniques [19] to produce AraA-resistant mutants [25] in which the resistance locus is tagged by transgene integration. A first round of insertional mutagenesis experiments yielded five independent AraA-resistant mutants (designated RD1-5). Four of these mutants displayed dramatic decreases in AK activity as compared to wild-type (Table 1). Unfortunately, DNA hybridization analysis of transgene organization revealed that none of these mutants was suitable for plasmid rescue [19]. Several of the mutants were produced by transformation with circular plasmids, which integrated into the parasite genome in such a way that genes essential for bacterial replication were disrupted. Other mutants were generated by transfection with linearized DNA, which integrated without rearrangement, but contained multiple transgenes—often integrated in a tandem array, as shown in Fig. 1. Attempts to rescue these transgenes invariably yielded only the original insertional mutagenesis vector itself rather than flanking DNA (Fig. 1A).

Table 1
Adenosine kinase analysis and RFLP analysis of adenine arabinoside (AraA)-resistant *Toxoplasma gondii* mutants[a]

Strain	AK activity	AK RFLP	RD3 RFLP
RH (wild-type)	+	−	−
RD1	−	nd[b]	nd
RD2	−	+	−
RD3	+	−	+
RD4	−	+	−
RD5	−	nd	nd
BS2	−	+	−
BS3	−	−	−
BS4	−	−	−
BS6	+	−	+
BS7	−	−	−
BS9	+	−	+
BS12	−	−	−
BS15	−	+	−
BS16	+	−	+
BS20	−	+	−

[a] Activity and restriction analysis as described in Section 2; see Figs. 3 and 5.
[b] nd, not determined (clones lost).

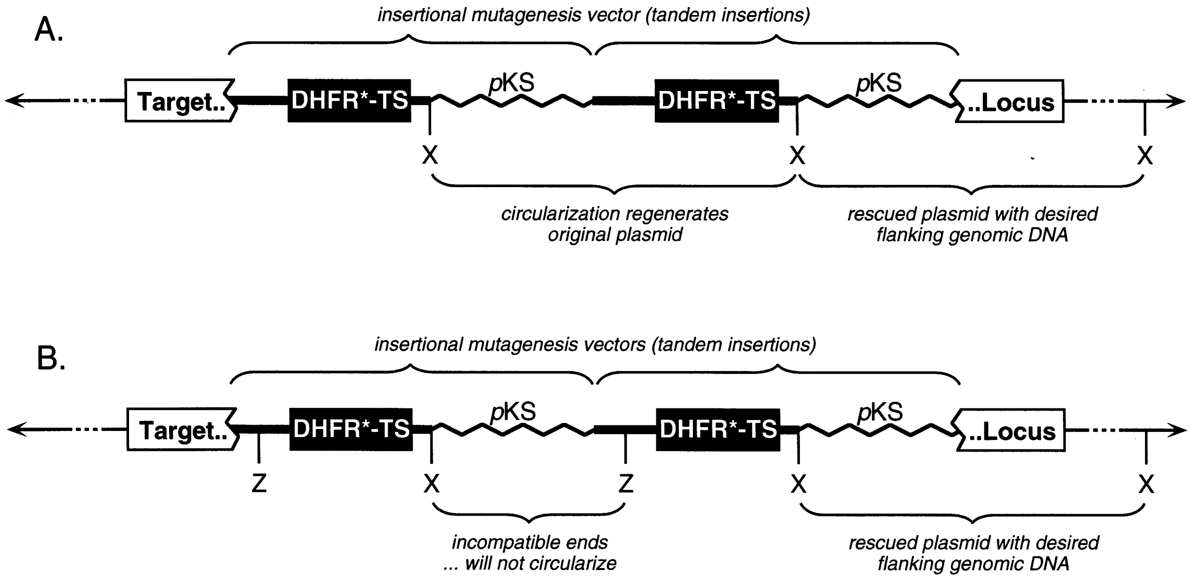

Fig. 1. Diagram of insertional mutagenesis and plasmid rescue using second-generation vectors (see text). Rescue of bacterial sequences integrated into the parasite genome following insertional mutagenesis involves: (i) digestion with a restriction enzyme (X) that separates the plasmid (*p*KS) from the parasite selectable marker (DHFR*-TS); (ii) recircularization by ligation in dilute solution; and (iii) transformation of *Escherichia coli* [14]. Panel A: insertional mutagenesis vectors commonly integrate as tandem head-to-tail arrays. Integration of two plasmids is shown here, but larger arrays are also observed. Unfortunately, rescue of the desired flanking sequences is usually less common than rescue of the original transformation plasmid. Panel B: double digestion of transgenic parasite DNA with restriction endonucleases 'X' and 'Z' prevents rescue of the original transformation plasmid. In the modified insertional mutagenesis vector *p*c3ABP (see text), *Asc* I, *Pac* I, or *Bss*H II restriction sites are present at site 'Z', and digestion with these enzymes yields ends that are incompatible with ends produced by *Eco*R I (site 'X'). It is highly unlikely that all of the rare restriction sites introduced at site 'Z' will occur before the next site 'X' downstream of the transgene, so digestion at 'Z' should not interfere with rescue of the desired genomic DNA.

To facilitate the rescue of flanking sequences from tandemly-integrated transgenes, a second-generation insertional mutagenesis vector was employed, in which a polylinker containing multiple rare restriction sites was introduced upstream of the *T. gondii* promoter (plasmid *p*c3ABP; see Section 2). This plasmid was designed to facilitate the rescue of genomic sequences flanking tandem insertions, as shown in Fig. 1B: digestion with restriction enzymes *Asc* I, *Pac* I, or *Bss*H II (in combination with digestion with *Eco*R I or other enzymes suitable for plasmid rescue) prevents recircularization of the original vector, due to the incompatibility of restriction fragment ends. These infrequent-cutters are unlikely to cut nearby in the flanking genomic sequence, permitting DNA to be rescued from the tagged locus. Ten independent AraA-resistant insertional mutants were generated using mutagenesis vector *p*c3ABP, and seven of these lack AK activity (Table 1).

3.2. cDNA clones encoding T. gondii adenosine kinase

In parallel with the production of insertional mutants, an EST (dbEST ID #466677; GenBank ID #N60952) was identified that exhibited very low-level similarity to the protein sequence of human AK [11]. The BLASTx *P*-value [26] of this putative AK EST was only $\sim 10^{-4}$, but closer inspection revealed that sequence similarity was restricted to the extreme N-terminus of AK, immediately downstream of the start codon, suggesting that the poor *P*-value might be attributable to insufficient overlapping sequence. PCR primers

```
                                                                           -644 gcacgagctttttt -630
ctggcgtccaaactttcgcgcttcacgtgtgccatagaccagctgtcttcgttgcttcgatggagtgcgtgtcccacgagggtcgtccac -540
cccacaacaacagggaggacgggagtttctgcgtccgaaccgacttggcgtcggatgcaaagcttagccgagacagatccgcggcgatg -450
cggacatagcggagcccgactttgtgccagaagtctccgcgcgtttcgtcttcttccgctcgttccgcttggtttcatggccgactacca -360
gattcgtcatctttatcgcgagtcggcgaacttcttccaaggacgtcccttgcctctaagacccatccaacggggccagcaagcgcatgc -270
agcgtcgcggcggtggcaacatcccttgtgcatcacgaacggcttttcccgaccgtcgaagatcaagaacaggtctccagattttggagg -180
gtagaggacggtacatcggaatttccgttgtttttcgccgacactctcaactcgtcagtcagaccatggctcctgcggaaccacatcta - 90
cagaggtcccgcatcaaggctctccaggggttcatcttggtcttgtctctcgctttcctttctccggcctccgcttcagtgacagacaga -  1

 M  A  V  D  S  S  N  S  A  T  G  P  M  R  V  F  A  I  G  N  P  I  L  D  L  V  A  E  V  P
ATGGCAGTCGATTCCTCCAACTCGGCGACGGCCCCATGAGAGTGTTCGCGATAGGGAATCCGATTCTGGACCTCGTCGCCGAGGTGCCC   90

 S  S  F  L  D  E  F  F  L  K  R  G  D  A  T  L  A  T  P  E  Q  M  R  I  Y  S  T  L  D  Q
TCTTCCTTCCTCGACGAGTTCTTCCTCAAGAGGGGAGACGCGACTCTCGCGACTCCGGAACAAATGCGCATCTACTCCACTCTTGACCAG  180

 F  N  P  T  S  L  P  G  G  S  A  L  N  S  V  R  V  V  Q  K  L  L  R  K  P  G  S  A  G  Y
TTCAATCCGACTTCCCTGCCTGGTGGCAGTGCACTCAACTCTGTTCGTGTCGTCCAGAAACTCTTGAGAAAGCCTGGCAGCGCTGGGTAC  270

 M  G  A  I  G  D  D  P  R  G  Q  V  L  K  E  L  C  D  K  E  G  L  A  T  R  F  M  V  A  P
ATGGGAGCCATTGGCGATGACCCCCGTGGCCAGGTGCTAAAAGAACTCTGCGACAAGGAAGGCCTCGCCACTCGCTTCATGGTCGCTCCA  360

 G  Q  S  T  G  V  C  A  V  L  I  N  E  K  E  R  T  L  C  T  H  L  G  A  C  G  S  F  R  L
GGTCAGAGCACCGGAGTGTGTGCCGTGTTGATCAACGAGAAAGAACGGACGCTCTGCACGCATCTCGGCGCATGCGGTTCTTTCCGACTG  450

 P  E  D  W  T  T  F  A  S  G  A  L  I  F  Y  A  T  A  Y  T  L  T  A  T  P  K  N  A  L  E
CCTGAGGACTGGACTACCTTCGCTTCCGGAGCGCTCATCTTTTATGCCACCGCCTACACACTTACTGCGACCCCAAAGAACGCGCTTGAG  540

 V  A  G  Y  A  H  G  I  P  N  A  I  F  T  L  N  L  S  A  P  F  C  V  E  L  Y  K  D  A  M
GTAGCGGGGTATGCTCATGGCATACCGAACGCCATCTTTACGCTGAACTTGTCGGCTCCGTTCTGCGTTGAGCTGTACAAGGACGCGATG  630

 Q  S  L  L  L  H  T  N  I  L  F  G  N  E  E  E  F  A  H  L  A  K  V  H  N  L  V  A  A  E
CAGTCTTTGCTTCTCCATACGAACATTCTCTTTGGAAACGAAGAGGAGTTCGCGCATTTGGCGAAAGTTCACAATCTCGTGGCTGCTGAG  720

 K  T  A  L  S  T  A  N  K  E  H  A  V  E  V  C  T  G  A  L  R  L  L  T  A  G  Q  N  T  G
AAGACGGCGCTGTCCACTGCGAACAAAGAACACGCAGTGGAAGTGTGCACGGGCGCCTTGCGTCTGCTCACTGCCGGTCAGAACACGGGC  810

 A  T  K  L  V  V  M  T  R  G  H  N  P  V  I  A  A  E  Q  T  A  D  G  T  V  V  V  H  E  V
GCGACGAAGCTCGTTGTCATGACACGGGGTCACAACCCCGTCATTGCTGCTGAGCAAACAGCCGACGGAACTGTGGTCGTCCACGAAGTT  900

 G  V  P  V  V  A  A  E  K  I  V  D  T  N  G  A  G  D  A  F  V  G  G  F  L  Y  A  L  S  Q
GGTGTCCCGGTGGTTGCTGCGGAAAAAATTGTGGACACCAACGGCGCGGGCGACGCGTTTGTCGGCGGTTTTCTCTATGCACTCTCGCAG  990

 G  K  T  V  K  Q  C  I  M  C  G  N  A  C  A  Q  D  V  I  Q  H  V  G  F  S  L  S  F  T  S
GGAAAAACGGTGAAACAGTGCATCATGTGCGGCAACGCTTGTGCTCAGGATGTGATTCAGCATGTAGGATTTAGTCTCAGCTTCACTTCA 1080

 L  P  C  *
CTTCCGTGTTGAactggttgatctgcagcatgtacacccttgaaagggggggattggggaatcccatacacggtccaaagagttcgctt 1170
ctttgaacggccgtcttttgccaggcgtcaacgtgtgccctgtgctcaactgtacagaaatcctcagtttagtttcgccgtttggctctt 1260
ccgaacccatgtacattagccttgcagggtcgtgtttcccagtgctttccgccgtgatgcgaccgtgtggcagtctccgtgtgtttgagg 1350
tggaagttcgttcggtccaaaatggtgtggcagtgaaatggtgcattcacgggaacgtagtcccacagtttcgctccatacgccgcgtgt 1440
attggccaaaatagtcgttgcagaactcgtttgacaagtcaaatgacggattcagttccAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
```

(A)

Fig. 2. *Toxoplasma gondii* adenosine kinase. Panel A (above): sequence of a full-length adenosine kinase (AK) cDNA clone, with predicted translation for the single large open reading frame. Sequence of the expressed sequence tag (EST) used to identify this locus is underlined. The location of four introns is noted by arrowheads (cDNA and genomic sequences spanning the AK locus are available from GenBank, with accession numbers AF128275 and AF128274, respectively). Panel B (right): alignment of predicted coding sequence for various adenosine kinase genes: *Tg*, *Toxoplasma gondii*; *Dd*, *Dictyostelium discoideum* (slime mold); *Hs*, *Homo sapiens* (human); *Cg*, *Cricetulus griseus* (hamster); *Ce*, *Caenorhabditis elegans* (nematode); *Sc*, *Saccharomyces cerevisiae* (budding yeast); *Sp*, *Schizosaccharomyces pombe* (fission yeast); *Pp*, *Physcomitrella patens* (moss); acquisition numbers are shown at the end of each listing. Discrepancies between various published human sequences are indicated in lower case; the *Dictyostelium* sequence is a composite assembled from several genomic and cDNA clones. Additional sequences available from GenBank or dbEST include complete sequences for *Mus musculus*, *Rattus rattus* and *R. norvegicus;* and partial sequences for *Orysza sativa*, *Arabidopsis thaliana*, *Zea mays*, *Medicago trunculata*, *Saccharum* sp., *Drosophila melanogaster*, and *Brugia malayi* (not shown). Function has been demonstrated for human, rodent and *T. gondii* clones only ([11–13] and this manuscript); other assignments are based solely on sequence similarity. Alignments were generated by ClustalW [38,39], with manual readjustment. Underlined residues in the *T. gondii* sequence are conserved in at least one other species; underlined residues in the consensus line are not conserved in *T. gondii*. Bullets (•) indicate where *T. gondii* residues differ from the universal consensus of other species, and asterisks indicate residues Leu-215 and His-216 (a frame-shift at this position would yield the highly conserved residues Pro-Tyr; see text). In the consensus line, upper case indicates universally conserved residues, and lower case indicates conservation in the majority of known sequences; brackets indicate motifs conserved in microbial ribo- and fructo-kinases [11].

```
       Toxoplasma gondii  mavdssnsatgpMRVFAIGNPILDLVAEVPSSFLDEFFLKRGDATLAT----PEQMRIYSTLDQ-FNP
  Dictyostelium discoideum           MSNIKILCAGNPLLDLSTHVEMAILDKYELKLGNAILAE----DKHLPLYGEIKS-GKV
            Homo sapiens           mtsvrenilfgMGNPLLDISAVVDKDFLDKYSLKPNDQILAE----DKHKELFDELVKKFKV
      Cricetulus griseus                      MGNPLLDISAVVDKDFLDKYSLKPNDQILAE----EKHKELFDELVRKFKV
  Caenorhabditis elegans           msplpentligMCNPLLDIQTTVEKAFLDKWGLKENDAILCD----DKHNDMFTELTRDFKV
Saccharomyces cerevisiae            MTAPLVVLGNPLLDFQADVTAEYLAKYSLKENDAILVDAKSGDAKMAIFDELLQMPET
Schizosaccharomyces pombe           MSSYILFGLENPLLDYYVGGETATLEKYGLKSNDAVLAS----ESQMGIYKEP----CV
     Physcomitrella patens          MASEGVLLGMGNPLLDISCVVDDAFLEKYGLTLNNAILAE----DKHLPMYKELAANPDV
              CONSENSUS:                      mgNPlLDisavvd  fL k   K ndailae    dkh   fdel   fkv

Tg  TSLPGGSALNSVRVVQKLLRKPG-SAGYMGAIGDDPRGQVLKELCDKEGLATRFMVAPGQSTGVCAVLINEK--ERTLCTHLGACGSFR-
Dd  EYIPGGAAQNTSRVCKWMLKDKQ-TVCYTGCVGTDENATILKTATESNGVVTIYQVDSSAPTGACAVLINHK--ERSMVTNLGAANNFKI.
Hs  EYHAGGSTQNSIKVAQWMIQQPHKAATFFGCIGIDKFGEILKRKAAEAHVDAHYYEQnEQPTGTCAACITGD--NRSLIANLAAANCYKK
Cg  EYHAGGSTQNSIKVAQWMIQKPHKAATFFGCIGIDKFGEILKSKAAEAHVDAHYYEQNEQPTGTCAACITGD--NRSLVANLAAANCYKK
Ce  EYIPGGAAQNSLRVAQWILNAPN-RTVFFGAVGKDQYGDLLASKAKEAGVNVHYQINETVKTGTCAALINGT--HRSLCAHLAAANTFT-
Sc  KLVAGGAAQNTARGAAYVL-GAG-QVVYFGSVGKDKFSERLLNENEKAGVKSMYQVQNDIGTGKCAALITGH--NRSLVTDLGAANFFT-
Sp  SYSAGGAAQNSCRAAQYVL-PPN-STVFAGCVGQDKFADMLLESNEKAGLRSEFSVDPTTPTGVCAVVLSNNNKNRSLCTNLGAANNYK-
Pp  EYIAGGATQNTIRIAQWML-GESNATSYFGCVGKDEYGDRMFKLASEGGVNIRYDVDEDLPTGTCGVLVVKG--ERSLVANLSAANKYK-
    ey aGGatqNs rvaqwmlq p  at ffGciG Dk ge lk  a  agv  hyy     ptGtCaa i      RslvanL Aan

Tg  -----LPEDWTTFASGALIFYATAYTLTATPKNALEVAGYAHGIPNAIFTLNLSAPFCVELYKDAMQSLLLHTNILFGNEEEFAHLAKVH
Dd  AHFQT...          ...LVGYFLTVSPDSAVHLGKHAAEN-DKPFLYGLAAPFLIDFFFDKVSELLPYVDIVFANESEAATLGRKM
Hs  EKHLDLEkNWMLVEK-ARVCYIAGFFLhVSPESVLKVAHHASEN-NRIFTLNLSAPFISQFYKESLMKVMPYVDILFGNETEAATFAREQ
Cg  EKHLDLENNWVLVEK-ARVYYIAGFFLTVSPESVLKVARYAAEN-NRIFTLNLSAPFISQFFKESLMEVMPYVDILFGNETEAATFAREQ
Ce  QDHLQKEENQKIIEQ-AKYFYVTGFFITVCPPAILQLASHSAEF-NKTFTLNLSAPFISQFFFDKLSEIIPLVDVLFGNEDEAAAFAKAN
Sc  PDHLDK--HWDLVEA-AKLFYIGGFHLTVSPDAIVKLGQHAKEN-SKPFVLNFSAPFIPHVFKDALARVLPYATVIIANESEAEAFCDAF
Sp  LKDLQQPNVWKFVEE-AKVIYVGGFHLTVSPESMLCLAQHANEN-NKPYIMNLSAPFLSQFFKEQMDSVIPYCDYVIGNEAEILSYGENH
Pp  IDHLKKPENWAFVEK-AKYIYSAGFFLTVSPESMMTVAKHAAET-GKYYMINLAAPFICQFFKDPLMELFPYVDFIFGNESEARAFAQVQ
      hl    nw lvek A v YiagffltvsPes l va haaen n  ftlnLsAPFisqffkd l  v pyvdilfgNE Eaa far q

Tg  NLVAAEKTALSTANKEHAVEVCTGALRLLTAGQNTGATKLVVMTRGHNPVIAAEQTADGTVVVHEVGVPVVAAEKIVDTNGAGDAFVGGF
Dd  NW-GEDLTVIA------------------EKLAAWEKVNTKRTRTVVFTQGPDATFVFQNGVLTKYNPIKVATEDILDLNAAGDSFCGGF
Hs  GFETKDIKEIA------------------KKTQALPKMNSKRQRIVIFTQGRDDTIMATESEVTAFAVLDQDQkEIIDTNGAGDAFVGGF
Cg  GFETKDIKEIA------------------KKAQALAKVNSKRPRTVVFTQGRDDTVVATENEVMAFAVLDQNQKEIIDTNGAGDAFVGGF
Ce  GWETTCVKEIA------------------LKAAALPKK-STKPRLVVFTQGPEPVIVVEGDKVTEFPVTRLPKEEIVDTNGAGDAFVGGF
Sc  QLDCANTDLEA-------------------IAQRIVKD-SPVEKTVIFTHGVEPTVVVSSKGTSTYPVKPLDSSKIVDTNGAGDAFAGGF
Sp  GIKSTDVQEIA------------------LALSSVEKVNKKRTRVVVITQGADATIVAKDGKVTTYKPNRVPSEEIVDTNGAGDAFAGGF
Pp  GWETEDTKVIA------------------VKLAALPKAGGTHKRVAVITQGTDPTIVAEDGKVTEFPVTPIPKEKLVDTNAAGDSFVGGF
    q et d keia                   k qalpkvnskr rtv ftqgrd tiva    vt fpv     eeivdtNgAGDaFvGGF

Tg  LYALSQGKTVKQCIMCGNACAQDVIQHVGFSLSFTSLPC*   (AF128274,AF128275)
Dd  LAAYSNGQEIAKCVEAGHYASWEIIRQNGATVPASEPKIQF* (C24399,C24400,C91105)
Hs  LSQLVSDKPLTECIRAGHYAASIIIRRTGCTFP-EKPDFH*  (U50196,U33936,U90339)
Cg  LSQLVYNKPLTECIRAGHYAASVIIRRTGCTFP-EKPDFH*  (U26588)
Ce  LSQFIQGKGVEASVTCGSYAAQEIIKKHGCTVP-SVCKYH*  (Z81107)
Sc  MAGLTKGEDLETSIDMGQWLAALSIQEVGPSYPSEKISYSK* (P47143)
Sp  IAALSQGQGIDYAVTLGHWLGQECIKVSGTTLPLPKKQFPLP* (D89173)
Pp  LSQLVLGKDIAQCVRAGNYAASVIIQRSGCTFP-SKPSFESQ* (Y15430)
    lsqlv gk    ciraGhyaa viI r Gct p ekp
```

(B)

Fig. 2. (*Continued*)

were synthesized based on the EST sequence and used to amplify a 150 bp fragment from parasite genomic DNA, which was used to probe a Southern blot containing DNA from the AraA-resistant parasite lines generated by insertional mutagenesis. Several of these mutants were found to be tagged at the same locus, strongly suggesting that the AK gene had been tagged (data not shown, but see below for a more extentive analysis by Southern blotting).

The PCR-amplified fragment was next used to screen a *T. gondii* cDNA library. Six cDNA clones were obtained, and sequence analysis of a single full-length clone revealed a 1089 nt open reading frame (Fig. 2A; GenBank #AF128275). Sequences upstream of the first ATG are consistent with the *T. gondii* consensus (cAaa/ATG) [27], and the codon usage is unremarkable [28]. The predicted 363 amino acid protein exhibits unequivocal similarity to mammalian AK enzymes [11–13]. Surprisingly few AK sequences have been described to date, but comparison of the *T. gondii* and human sequences with the GenBank and dbEST databases reveals several putative AK genes from other species (Fig. 2B).

The putative *T. gondii* AK sequence is highly diverged from AK homologs in other species, sharing only ~23% identity with its nearest neighbor, *Dictyostelium discoideum* (the only other protozoan sequence available). Less than half of all residues (173/363) are shared with any other species, and only 44% (106/240) of the consensus amino acids derived from a multiple sequence alignment are conserved in *T. gondii* (Fig. 2B). The *Toxoplasma* coding sequence differs from the consensus at 15 loci that are conserved in all other species for which AK sequence information is available. The high level of divergence between *T. gondii* and other species suggests that the parasite enzyme will differ substantially from human (and other) AK enzymes, which bodes well for structure-based drug design.

To test whether the *T. gondii* cDNA encodes a functional AK enzyme, transient transfection studies were performed using a *T. gondii* expression vector. The AK open reading frame was amplified by PCR to introduce suitable flanking restriction sites, and ligated between 5′ and 3′ untranslated regions derived from the *T. gondii* dihydrofolate reductase-thymidylate synthase (DHFR-TS) gene [14]. Electroporation of an AK^- insertional mutant (clone #2) with 50 μg of plasmid *p*minAK.phd restored AK activity to these parasites, as shown in Fig. 3. Radiolabel was also incorporated into inosine, but this labelling could be blocked by the addition of an adenosine deaminase inhibitor [24] (lanes 6–8). Using the endogenous AK promoter did not increase transient AK expression over the levels presented here; the low level of activity observed is attributable to the transient assay, as stable transgenics exhibit levels of AK activity comparable to wild-type (data not shown).

3.3. The genomic AK locus and creation of an AK knock-out mutant

Probing of an RH-strain genomic λ library with AK cDNA identified three overlapping clones, and restriction mapping and sequence analysis reveals a gene containing five exons (Fig. 4A; genomic sequence spanning the AK locus is available from GenBank, with accession #AF128274). All putative intron/exon boundaries conform to the consensus defined from previously-sequenced *T. gondii* genes [29]. Intron location in the *Toxoplasma* AK gene is distinct from the position of introns in the putative *Caenorhabditis elegans* gene; no introns are present in the putative AK gene of *Saccharomyces cerevisiae*, and genomic sequence has not been reported for any other eukaryotic AK gene to date.

In order to better characterize AK function in *T. gondii*, genetically-defined knock-out mutants were generated by targeted homologous recombination [14,30]. A knock-out construct was produced by deleting a 1.7 kb *Stu* I–*Bst*X I fragment from the AK genomic locus, completely eliminating exons III and IV and truncating exon V (Fig. 4A). This AK targeting construct retains ~3 kb of genomic sequence 5′ of the deletion and 6.5 kb of 3′ sequence (sufficient for allelic replacement via homologous recombination [31]). AraA-resistant mutants were selected following electroporation with 30 μg of either circular or linearized plasmid and cloned by limiting dilution.

Examination of *Eco*R I-digested DNA obtained from four AraA-resistant clones revealed that three of these mutants (lanes 2–4 in Fig. 4B) lack the *Stu* I–*Bst*X I fragment which had been deleted from the targeting construct (compare with wild-type controls). Reprobing this blot with a 3.8 kb *Eco*R I fragment spanning the deletion

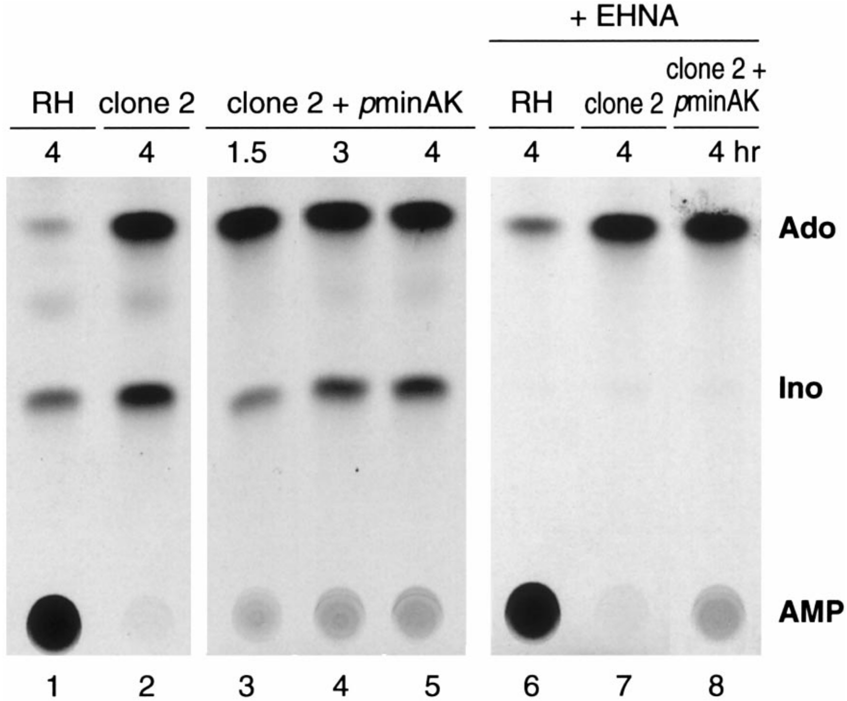

Fig. 3. Transient adenosine kinase (AK) expression in AK-deficient insertional mutants. Thin layer chromatography was used to assay the conversion of radiolabeled adenosine substrate (Ado) to AMP product by AK in sonicated extracts of RH strain *T. gondii*, as described under Section 2. Lane 1, wild-type; lane 2, AK-deficient insertional mutant clone #2; lanes 3–5, clone 2 mutants transiently transfected with plasmid *p*minAK.phd (1.5, 3, and 4 h reactions). Lanes 6–8 show 4 h reactions carried out with wild type, mutant, and transfectant extracts in the presence of the adenosine deaminase inhibitor erythro-9-(2-hydroxy-3-nonyl)-adenine (EHNA) [24], confirming that the band of intermediate R_f is inosine (Ino).

(Fig. 4C) confirmed that the 3.8 kb fragment characteristic of wild-type parasites was reduced to 2.1 kb in the mutants, due to deletion of the 1.7 kb *Stu* I–*Bst*X I fragment. Mutant 1B2 appears to contain both wild-type and mutant alleles of AK, probably organized as a pseudodiploid [30,31]. As this clone is resistant to AraA, the apparently normal allele probably harbors a subtle mutation not resolvable on this gel (similar mutations introduced as a result of transgene integration have previously been observed at the UPRT locus [17]). Transient AK activity was restored to knock-out mutant 1B3 by electroporating 50 μg of the *p*minAK expression vector, as shown in Fig. 4D, fulfilling the molecular equivalent of Koch's postulates [32] to formally prove that the locus under study is necessary for AK activity in *T. gondii*.

3.4. Tagging of additional loci in AraA-resistant insertional mutants

A full-length AK cDNA clone was used to probe the organization of AraA-resistant insertional mutants, identifying three distinct classes of clones (Fig. 5 and Table 1). The first group consists of AK-deficient mutants harboring transgenes at the AK locus (clones #2, 15, 20, RD2 and RD4), as characterized above (additional digests confirm that subtle shift observed in clone 20 is indeed the result of tagging the AK locus; not shown). Insertional mutagenesis tagged a 2.0 kb *Eco*R I fragment in mutants #2, 15 and RD2, and a 3.8 kb *Eco*R I fragment in mutants #20 and RD4 (Fig. 4A).

A second group of insertional mutants consists of AK^- parasites that were not tagged at the AK locus (clones #3, 4, 7 and 12; question marks in Fig. 5). More extensive probing with genomic clones spanning >12 kb of genomic sequence confirmed that no transgene was integrated at the AK locus in these mutants (data not shown). Sequence analysis reveals no mutations in the AK coding sequence of these mutants, suggesting that they may define an essential regulator of AK expression or activity. Cloning of DNA sequences flanking the integration site(s) in these mutants is presently underway.

A third group of insertional mutants (clones #6, 9, 16 and RD3) consists of AraA-resistant clones that exhibit normal AK activity, and were not tagged at the AK locus. Hybridization of a Southern blot with DNA flanking the transgene integration in clone RD3 reveals that the same locus was tagged in all four clones (Fig. 5B and

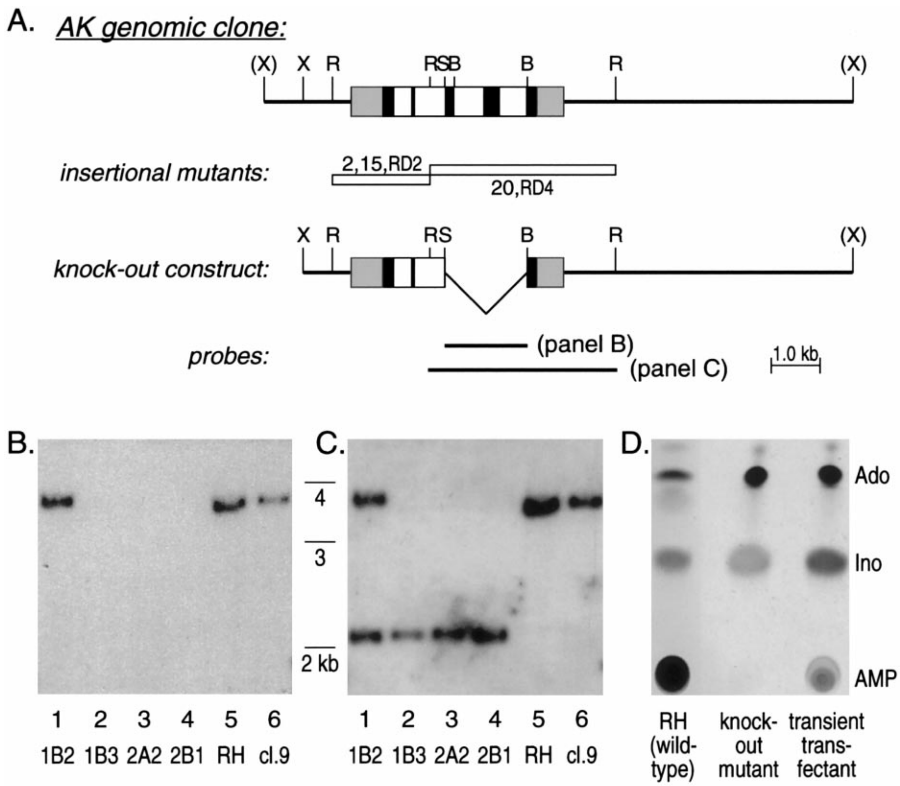

Fig. 4. Construction of a defined genetic knock-out at the *Toxoplasma gondii* adenosine kinase (AK) genomic locus. Panel A: map of the genomic AK locus, based on a λDASH-II genomic clone (two additional λ clones extending 9 kb upstream were also obtained; not shown). Black boxes indicate predicted protein coding sequence, introns are shown in white, and 5′ and 3′ untranslated regions are shaded. Transfected plasmids integrated into the 2.0 kb (upstream) *Eco*R I fragment in insertional mutants #2, 15 and RD2, and into the 3.8 kb (downstream) fragment in mutants #20 and RD4 (see Fig. 5A). No other insertional mutants were tagged at the AK locus. A 1.7 kb *Stu* I–*Bst*X I fragment was deleted from an 11.5 kb subclone spanning the AK locus to produce a plasmid suitable for targeted gene knock-outs. B, *Bst*X I; R, *Eco*R I; S, *Stu* I; X, *Xba* I. Panel B: Southern analysis of wild-type parasites and putative AK genomic knock-outs. Probing with the 1.7 kb *Stu* I–*Bst*X I fragment that was deleted to produce the AK targeting construct shows loss of these sequences from the genome in clones 2B1, 2A2 and 1B3 (but not 1B2). Panel C: Southern analysis of the same blot, stripped and probed with a 3.8 kb genomic *Eco*R I fragment spanning the AK knock-out. The three knock-out mutants (2B1, 2A2 and 1B3) exhibit the expected 2.1 kb band; clone 1B2 is probably a subtly mutated pseudodiploid [14]. Panel D: restoration of AK activity in a defined AK knock-out mutant. Thin layer chromatography was used to assay AK activity in crude extracts from wild-type parasites, genomic AK knock-out mutant 1B3, and mutant 1B3 after transient transformation with 50 μg expression vector *p*minAK.phd.

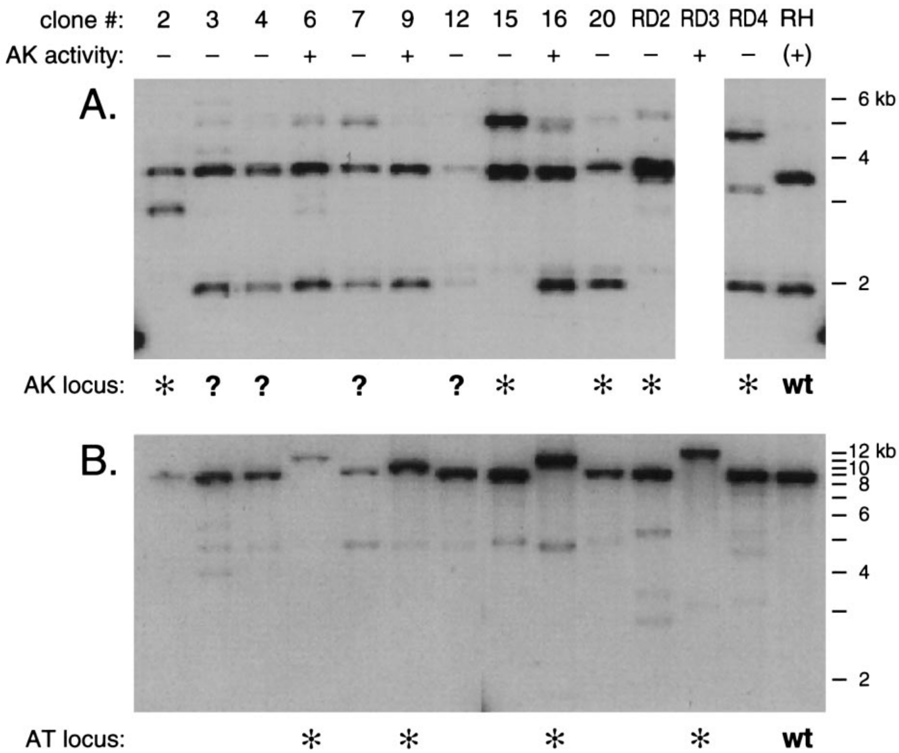

Fig. 5. Southern analysis of adenine arabinoside (AraA)-resistant insertional mutants. Genomic DNA was prepared from wild-type parasites (RH) and various AraA-resistant insertional mutants, digested with *Eco*R I, separated by gel electrophoresis, and transfered to nylon membranes. Results from adenosine kinase (AK) assays for each mutant are also shown. Panel A: hybridization with a radiolabeled AK cDNA sequences reveals disruption of the AK locus in five of nine AK-deficient clones (*). More extensive probing with sequences spanning the entire AK locus reveals no additional information (not shown). Thus the nature of the remaining AK-deficient clones (?) is a mystery. Panel B: hybridization with a probe rescued from the tagged locus in clone RD3 (Chiang et al., in preparation) indicates that the same locus was tagged in all four AK^+ clones that were resistant to AraA (*).

Table 1). Recent studies indicate that this phenotype results from insertional inactivation of the parasite's adenosine transporter (Chiang et al., in preparation).

4. Discussion

A combination of genome informatics approaches and insertional mutagenesis have been employed to identify at least two loci associated with adenosine metabolism in the protozoan parasite *T. gondii*. One major advantage of genetic approaches (such as insertional mutagenesis) is the potential to identify multiple loci that confer a given phenotype (in this case resistance to AraA). For example, in addition to the AK locus originally sought in this study, an adenosine transporter (Chiang et al., in preparation) and a third class of as-yet-uncharacterized mutants have also been identified (Table 1 and Fig. 5). In future, the new vectors described in this study (Fig. 1) should facilitate the rescue of tagged loci in insertional mutants.

The parasite's adenosine kinase gene was also identified based on weak sequence similarity to a random cDNA clone from the dbEST database. The unconvincing *P*-value obtained when searching dbEST with the human AK sequence ($\sim 10^{-4}$ by tBLASTn search) was found to be attributable to insufficient overlap: most of the *T. gondii* EST entry consists of 5′ non-coding sequence (Fig. 2A). In constructing cDNA libraries, every effort is generally made to clone full-length cDNA. As a result, EST databases (which are often compiled from single pass sequencing from the 5′ end of directionally-cloned cDNAs) are biased in favor of 5′ untranslated sequences. Noncoding sequences are poorly conserved across species boundaries, however, and it is therefore likely that a search algorithm that specifically seeks matches between the 3′ ends of EST sequences and the N-termini of known proteins could enhance EST identification.

Despite the considerable attention focused on enzymes involved in nucleotide metabolism in a wide range of organisms, the sampling of AK genes is very poor (the first AK sequences have only recently reported [11–13]). The *T. gondii* sequence provides the first confirmed microbial sequence, facilitating the identification of other putative AK genes (Fig. 2B) and the identification of evolutionarily-conserved motifs likely to be important in enzyme function.

Possible pathways for purine salvage in *T. gondii* are shown in Fig. 6. As purine auxotrophs, intracellular pathogens must salvage nucleobases and/or nucleosides (or, conceivably, nucleotides) from the host cell [4]. It has recently been shown that *T. gondii* expresses a single HXGPRT gene that is responsible for all salvage of hypoxanthine, xanthine and guanine within the parasite [18]. Deletion of the HXGPRT locus is not lethal, however, as redundancy is provided by the ability to deaminate AMP to IMP. Similarly, a deletion at the AK locus is also viable (Fig. 4), because IMP can be converted to AMP via adenylosuccinate [5,6]. There has been debate about whether or not *T. gondii* also possesses an adenine phosphoribosyltransferase. Activity has been detected by some investigators [6], but parasites are insensitive to toxic adenine analogs (E.R. Pfefferkorn, J.A. Darling and D.S. Roos, unpublished results). The availability of molecular reagents, including constructs for targeted gene knock-outs at both the AK and HXGPRT loci ([18] and this paper), should permit resolution of this question.

Purine salvage pathways have been suggested as targets for parasite-specific chemotherapy [5,9,10,33,34], and cloning of the *T. gondii* AK gene should permit purification of recombinant enzyme for biochemical and structural analysis, complementing recent studies on *T. gondii* HXGPRT [34,37]. AK provides a particularly promis-

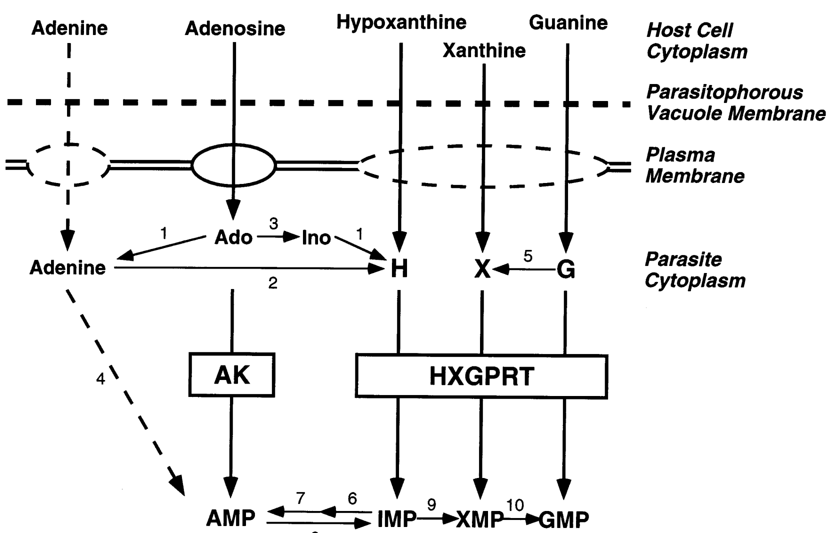

Fig. 6. Model of purine salvage in *Toxoplasma gondii*. See text for discussion of purine transport and salvage pathways in *Toxoplasma*. 1, nucleoside hydrolase, purine nucleoside phosphorylase, or adenosine phosphorylase; 2, adenine deaminase; 3, adenosine deaminase; 4, adenine phosphoribosyltransferase; 5, guanine deaminase; 6, adenylosuccinate synthetase; 7, adenylosuccinate lyase; 8, AMP deaminase; 9, IMP dehydrogenase; 10, GMP synthetase.

ing target enzyme, as flux through this pathway is thought to dominate purine metabolism in *Toxoplasma* [5,6,8], and the substrate specificity of *T. gondii* adenosine kinase is distinct from that of the human enzyme [9,10]. Although neither AK nor HXGPRT is essential for *T. gondii* survival, subversive substrates provide possible lead compounds for drug design, analogous to the successful use of allopurinol for treating cutaneous leishmaniasis and Chagas' disease [35,36]. Substrate specificity differences between parasite and host permit the design of inhibitors that are not toxic to the host (such as nitrobenzylthioinosine and thioxanthine). Alternatively (or in addition), it may be possible to simultaneously target both the adenylate and guanylate sides of the salvage pathway with a combination of inhibitors.

5. Note

Expression of recombinant *T. gondii* AK has permitted enzyme purification for kinetic analysis, as described in the accompanying report [40].

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References

[1] Luft BJ, Remington JS. Toxoplasmic encephalitis in AIDS. Clin Infect Dis 1992;15:211–22.
[2] Brooks RG, Remington JS, Luft BJ. Drugs used in the treatment of toxoplasmosis. Antimicrob Agents Annu 1987;2:297–306.
[3] Laughon BE, Allaudeen HS, Becker JM, Current WL, Feinberg J, Frenkel JK, Hafner R, Hughes WT, Laughlin CA, Meyers JD. Summary of the workshop on future directions in discovery and development of therapeutic agents for opportunistic infections associated with AIDS. J Infect Dis 1991;164:244–51.
[4] Perotto J, Keister DB, Gelderman AH. Incorporation of precursors into *Toxoplasma* DNA. J Protozool 1971;18:470–3.
[5] Schwartzman JD, Pfefferkorn ER. *Toxoplasma gondii:* purine synthesis and salvage in mutant host cells and parasites. Exp Parasitol 1982;53:77–86.
[6] Krug EC, Marr JJ, Berens RL. Purine metabolism in *Toxoplasma gondii*. J Biol Chem 1989;264:10601–7.
[7] Pfefferkorn ER, Pfefferkorn LC. The biochemical basis for resistance to adenine arabinoside in a mutant of *Toxoplasma gondii*. J Parasitol 1978;64:486–92.
[8] Schwab JC, Afifi MA, Pizzorno G, Handschumacher RE, Joiner KA. *Toxoplasma gondii* tachyzoites possess an unusual plasma membrane adenosine transporter. Mol Biochem Parasitol 1995;70:59–69.
[9] Iltzsch MH, Uber SS, Ankersley KO, el Kouni MH. Structure-activity relationship for the binding of nucleoside ligands to adenosine kinase from *Toxoplasma gondii*. Biochem Pharm 1995;49:1501–12.
[10] el Kouni MH, Guarcello V, Al Safarjalani ON, Naguib FNM. Metabolism and selective toxicity of 6-nitrobenzylthioinosine in *Toxoplasma gondii*. FASEB J 1998;12:A126.
[11] Spychala J, Datta NS, Wakabayashi K, Fox IH, Gribbon T, Mitchell BS. Cloning of human adenosine kinase cDNA: sequence similarity to microbial ribokinases and fructokinases. Proc Natl Acad Sci USA 1996;93:1232–7.
[12] Singh B, Hao W, Wu Z, Eigl B, Gupta RS. Cloning and characterization of cDNA for adenosine kinase from mammalian (Chinese hamster, mouse, human and rat) species: high frequency mutants of Chinese hamster ovary cells involve structural alterations in the gene. Eur J Biochem 1996;241:564–71.
[13] McNally T, Helfrich RJ, Cowart M, Dorwin SA, Meuth JL, Idler KB, Klute KA, Simmer RL, Kowaluk EA, Halbert DN. Cloning and expression of the adenosine kinase gene from rat and human tissues. Biochem Biophys Res Commun 1997;231:645–50.
[14] Roos DS, Donald RGK, Morrissette NS, Moulton ALC. Molecular tools for genetic dissection of the protozoan parasite *Toxoplasma gondii*. Methods Cell Biol 1994;45:23–65.
[15] Boothroyd JC, Kim K, Pfefferkorn ER, Sibley LD, Soldati D. *Toxoplasma* as a paradigm for the use of genetics in the study of protozoan parasites. Methods Mol Genet 1995;6:1–29.
[16] Way JC, Davis MA, Morisato D, Roberts DE, Kleckner N. New Tn10 derivatives for transposon mutagenesis and for construction of lacZ operon fusions by transposition. Gene 1984;32:369–79.
[17] Donald RGK, Roos DS. Insertional mutagenesis in a protozoan parasite: direct cloning of the uracil phosphoribosyltransferase gene from *Toxoplasma gondii*. Proc Natl Acad Sci USA 1995;92:5749–53.

[18] Donald RGK, Carter D, Ullman B, Roos DS. Insertional tagging, cloning and expression of the *Toxoplasma gondii* hypoxanthine–xanthine–guanine phosphoribosyltransferase gene: characterization of a new genetic marker for stable transformation. J Biol Chem 1996;271:14010–9.

[19] Roos DS, Sullivan WJ Jr., Striepen B, Bohne W, Donald RGK. Tagging genes and trapping promoters in *Toxoplasma gondii* by insertional mutagenesis. Methods 1997;13:112–22.

[20] Brunk, B.P., Bohne, W., Overton, G.C., Roos, D.S., 1997. Mining data from dbEST—querying the *Toxoplasma gondii* expressed sequence tag (EST) database < http://daphne.humgen.upenn.edu:1024/toxodb/ver_2/ toxodb.html > .

[21] Ajioka J, Boothroyd JC, Brunk BP, Hehl A, Hillier L, Manger ID, Overton GC, Marra M, Roos DS, Wan KL, Waterson R, Sibley DL. Gene discovery by EST sequencing in *Toxoplasma gondii* reveals sequences restricted to the Apicomplexa. Genomic Res 1998;8:18–28.

[22] van den Hoff MJB, Moorman AFM, Lamers WH. Electroporation in 'intracellular' buffer increases cell survival. Nucleic Acids Res 1992;20:2902.

[23] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.

[24] Schaeffer HJ, Schwender CF. Enzyme inhibitors 26. Bridging hydrophobic and hydrophilic regions on adenosine deaminase with some 9-(2-hydroxy-3-alkyl)-adenines. J Med Chem 1974;17:6–8.

[25] Pfefferkorn ER, Pfefferkorn LC. Arabinosyl nucleosides inhibit *Toxoplasma gondii* and allow the selection of resistant mutants. J Parasitol 1976;62:993–9.

[26] Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool (BLAST). J Mol Biol 1990;215:403–10.

[27] Seeber F. Consensus sequence of translational initiation sites from *Toxoplasma gondii* genes. Parasitol Res 1997;83:309–11.

[28] Ellis J, Griffin H, Morrison D, Johnson AM. Analysis of dinucleotide frequency and codon usage in the phylum Apicomplexa. Gene 1993;126:163–70.

[29] Roos DS. Primary structure of the dihydrofolate reductase/thymidylate synthase gene from *Toxoplasma gondii*. J Biol Chem 1993;268:6269–80.

[30] Donald RGK, Roos DS. Gene knock-outs and allelic replacements in *Toxoplasma gondii*: HXGPRT as a selectable marker for 'hit-and-run' mutagenesis. Mol Biochem Parasitol 1998;91:295–305.

[31] Donald RGK, Roos DS. Homologous recombination and gene replacement at the dihydrofolate reductase/thymidylate synthase locus in *Toxoplasma gondii*. Mol Biochem Parasitol 1994;64:243–53.

[32] Falkow S. Molecular Koch's postulates applied to microbial pathogenicity. Rev Infect Dis 1988;10(Suppl. 2):S274–6.

[33] Ullman B, Carter D. Hypoxanthine–guanine phosphoribosyltransferase as a therapeutic target in protozoal infections. Infect Agents Dis 1995;4:29–40.

[34] Naguib FNM, Iltzsch MH, el Kouni MM, Panzica RP, el Kouni MH. Structure-activity relationship for the binding of ligands to guanine or xanthine phosphoribosyltransferases from *Toxoplasma gondii*. Biochem Pharmacol 1995;50:1685–93.

[35] Gallerano RH, Sosa RR, Marr JJ. Therapeutic efficacy of allopurinol in patients with chronic Chagas' disease. Am J Trop Med Hyg 1990;43:159–66.

[36] Martinez S, Marr JJ. Allopurinol in the treatment of American cutaneous leishmaniasis. N Engl J Med 1992;326:741–4.

[37] Schumacher MA, Carter D, Roos DS, Ullman B, Brennan RG. Crystal structures of *Toxoplasma gondii* HXGPRTase reveal the catalytic role of a long flexible loop. Nat Struct Biol 1996;3:881–7.

[38] Schuler GD, Altschul SF, Lipman DJ. A workbench for multiple alignment construction and analysis. Proteins 1991;9:180–90.

[39] Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 1994;22:4673–80.

[40] Darling JA, Sullivan WJJr, Carter D, Ullman B, Roos DS. Recombinant expression, purification and characterization of *Toxoplasma gondii* adenosine kinase. Mol Biochem Parasitol 1999;103:15-23.