

# Gene Discovery by EST Sequencing in *Toxoplasma gondii* Reveals Sequences Restricted to the Apicomplexa

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To accelerate gene discovery and facilitate genetic mapping in the protozoan parasite *Toxoplasma gondii*, we have generated >7000 new ESTs from the 5' ends of randomly selected tachyzoite cDNAs. Comparison of the ESTs with the existing gene databases identified possible functions for more than 500 new *T. gondii* genes by virtue of sequence motifs shared with conserved protein families, including factors involved in transcription, translation, protein secretion, signal transduction, cytoskeleton organization, and metabolism. Despite this success in identifying new genes, more than 50% of the ESTs correspond to genes of unknown function, reflecting the divergent evolutionary status of this parasite. A newly recognized class of genes was identified based on its similarity to sequences known only from other members of the same phylum, therefore identifying sequences that are apparently restricted to the Apicomplexa. Such genes may underlie pathways common to this group of medically important parasites, therefore identifying potential targets for intervention.

*Toxoplasma gondii* is a widespread opportunistic pathogen that causes disease in immunocompromised patients and in congenitally infected infants. *T. gondii* belongs to the phylum Apicomplexa, which consists primarily of obligate intracellular parasites that are phylogenetically ancient and related only distantly to model single-celled organisms (Gajadhar et al. 1991). The phylum Apicomplexa contains numerous parasites of medical and veterinary significance, including Plasmodium (the causative agent of malaria), Eimeria (the causative agent of coccidiosis in domestic animals), and Cryptosporidium (an opportunistic pathogen that causes diarrheal disease in animals and humans). *T. gondii* is unusual among this group of parasites in being amenable to both classical and molecular genetics (Roos et al. 1994; Boothroyd et al. 1995). Combined

with the simplicity of in vitro propagation and production of mutants, *T. gondii* provides a powerful system for experimental studies. Despite these many attributes, it suffers from a lack of gene sequence information. The January 1996 edition of GenBank contained <30 protein-coding genes from *T. gondii*.

Single-pass sequencing of cDNAs to generate ESTs has become the fastest growing segment of the public DNA databases (Boguski et al. 1993). Although incomplete and error prone, ESTs remain a powerful means of gene discovery and for generating biologically informative probes for mapping studies (Adams et al. 1995; Hillier et al. 1996). The increased power of algorithms used to compare gene sequences and corresponding predicted amino acid sequences, such as BLAST (Altschul et al. 1990), have enabled identification of a significant number of homologs by comparison of EST sequences with more complete entries in the public genome data-

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bases. Such comparisons have been used to identify ancient conserved regions—sequences that are present across diverse phyla and that represent protein domains with highly conserved structures or functions (Green et al. 1993).

One arena where EST sequencing is likely to make its greatest impact is the discovery of genes from phylogenetically distant organisms. Such systems suffer from the dual hindrance of limited scientific study and a greater degree of sequence divergence from well-studied organisms, therefore limiting gene identification by heterologous probing. Such is the situation with many parasitic organisms, which, despite their medical importance, lag behind in the application of genetics to solving important biological problems.

The feasibility of EST analysis for gene discovery in *T. gondii* was demonstrated recently by a pilot project where ~20% of ESTs identified putative homologs to known genes by conservative BLAST comparisons with the databases (Wan et al. 1995). Based on this initial success, we have undertaken a larger-scale project to sequence ~10,000 randomly isolated cDNAs from the rapidly proliferating stage (called tachyzoite) of *T. gondii*. The results demonstrate that moderate-scale EST sequencing is a powerful means for identifying genes that may mediate important aspects of intracellular parasitism by *T. gondii* and related parasites.

## RESULTS

### EST Sequencing: Rapid and Efficient Identification of New Genes

Single-pass sequencing from the 5' end of 10,000 independent PCR-amplified *T. gondii* cDNAs was used to generate more than 7000 new ESTs that were submitted to GenBank. The use of semiautomated sequencing, processing, and annotation resulted in a total cost of <\$15 per completed EST entry. Failures were primarily attributable to poor-quality sequence (2269 of 2676 or 85% of failures), whereas contaminants with vector, mitochondrial, or non-*T. gondii* sequences represented <5% of all failures. The remaining failures (~10%) were attributable to sequences that were considered too short for useful analyses.

The average read of the good-quality sequence was 285 bp, sufficient to allow robust homology searches. The ESTs were searched against the nonredundant protein and nucleic acid database of GenBank using BLAST with various search parameters to create listings of putative genes with different

match stringencies. Using a probability cut-off of  $P \leq 10^{-6}$ , ~30% (2183 out of 7165) of the ESTs were assigned putative identities at the time of submission. Following submission, homologies were listed for ~45% of the ESTs by the National Center for Biotechnology Information (NCBI) using more permissive criteria ( $P \leq 10^{-3}$ ). Both “putative assignments” designated by Washington University and “neighbors” assigned by NCBI can be viewed at the following web site: [http://www2.ncbi.nlm.nih.gov/dbST/dbest\\_query.html](http://www2.ncbi.nlm.nih.gov/dbST/dbest_query.html). Adopting a more conservative cutoff ( $P \leq 10^{-10}$ ), ~27% of ESTs had a putative identity; these were compiled into a listing of 500 nonredundant homologs of existing genes (available at <http://www.ebi.ac.uk/parasites/toxo/toxpage.html>). Although these putative homologs will require additional study to verify, it is apparent that EST sequencing is extremely cost-effective and has an excellent success rate for gene discovery.

### Distribution of EST Clusters

ESTs were clustered by sequence similarity to reveal the number of times a given sequence was encountered. The majority of sequences occurred only a single time—58% of ME49 and 50% of RH sequences were singletons. Highly redundant sequences, those occurring >10 times, made up ~34% of all successful reads. In total, 4062 unique sequences were identified by combining all singletons plus the number of unique clusters with two or more members. This number does not, however, provide a reliable estimate of the number of genes in *T. gondii* as the 5' sequences used here were not anchored; therefore nonoverlapping or minimally overlapping sequences from the same gene result in separate clusters. The relatively low rate of redundancy encountered indicates that generation of normalized libraries designed to remove abundant mRNAs may not be necessary for an EST project of this scope.

### The Most Abundant ESTs Are Similar in Both Libraries

To maximize the biological value of the data obtained, sequences were derived from two separate tachyzoite cDNA libraries from two commonly used strains that represent the clonal type I (RH) and type II (ME49) lineages (Sibley and Boothroyd 1992; Howe et al. 1996). A majority of the most abundant sequences correspond *T. gondii* genes known previously that encode a variety of surface or secretory

antigens (Table 1). Among the top 15 ESTs are a number of newly identified genes (no homology to current gene databases) that are prime candidates for further studies. Their relative abundances suggest that they encode proteins that have important roles in the biology of *T. gondii*. Whereas the frequencies of ESTs for most genes were similar between libraries, several abundant ESTs differed by three- to fivefold between the two libraries (Table 1; data not shown). When analyzed by comparative Northern blot, however, these differences proved not to be indicative of similar differences in mRNA abundances for reasons that are unknown (data not shown). The one exception was an EST family rep-

resented by the ME49 clone zy64e04 (dbEST id 571145) that detected a 6.3-kb mRNA in ME49 strain but not RH strain tachyzoites analyzed by Northern blot (data not shown). This gene occurs once in the RH strain ESTs, but is ~14 times more abundant in ME49 strain ESTs (when corrected for the total number of sequences in each library).

#### Analysis of the Quality of EST Data for Known Genes

To establish the quality of sequence data generated from the two libraries, we analyzed the ESTs obtained for several well-studied, single-copy genes of

Table 1. Summary of Most Abundant ESTs by Library as Determined by Cluster Analysis

Rank	RH tachyzoite cDNA library			ME49 tachyzoite cDNA library		
	dbEST ID no.	no.	gene/description	dbEST ID no.	no.	gene/description
1	465887	107	GRA1 p24 dense granule protein	571382	42	SAG1 p30 surface antigen
2	466589	69	GRA2 p28 dense granule protein	571076	32	GRA2 p28 dense granule protein
3	465733	68	GRA6 p32 dense granule protein	571569	29	GRA1 p24 dense granule protein
4	465767	64	SAG1 p30 surface antigen	571208	28	unknown (similar to RH 467174)
5	465877	58	GRA5 p21 dense granule protein	571108	27	SAG2 p22 surface protein
6	466942	53	SAG2 p22 surface antigen	571574	17	GRA5 p21 dense granule protein
7	467174	34	unknown (similar to ME49 571208)	571542	15	unknown (similar to RH 467213)
8	465857	29	S8 ribosomal protein	571282	15 <sup>b</sup>	unknown (similar to RH 466613)
9	466627	28	GRA3 p30 dense granule antigen	571237	11 <sup>b</sup>	unknown (similar to RH 466242)
10	466406	26 <sup>a</sup>	unknown (similar to ME49 574388)	571154	9	GRA6 p32 dense granule protein
11	465750	22	unknown (similar to ME49 571377)	571377	9	unknown (similar to RH 465750)
12	466392	21	35-kD toxoplasma polypeptide	571392	9	unknown (similar to <i>Schizosaccharomyces pombe</i> 7.67961)
13	467170	19	unknown (similar to ME49 604952)	571332	8	S2 ribosomal protein
14	465671	18	S30 ribosomal protein	571072	8	L18a ribosomal protein
15	466619	17	S7–S8 ribosomal protein	574528	8	L22 ribosomal protein

Total number of ESTs for the RH tachyzoite cDNA library was 5359; total number of ESTs for the ME49 tachyzoite cDNA library was 1806.

<sup>a</sup>EST frequency is fivefold more abundant in RH.

<sup>b</sup>EST frequency is fivefold more abundant in ME49.

*T. gondii* including *MIC2* (U62660), encoding a micronemal protein (Wan et al. 1997), *GRA1* (M26007), encoding a calcium-binding dense granule protein (Cesbron-Delauw et al. 1989), and *SAG1* (M23658), encoding the surface antigen p30 (Burg et al. 1988). The error rates for the high-quality portion of the ESTs corresponding to these genes were in the range of 1%–2%, consistent with previous reports of EST data quality (Hillier et al. 1996). To determine the distribution of sequences represented in the two libraries, the 5' end of each EST corresponding to these genes was plotted. For *SAG1* and *GRA1*, where the 5' ends of transcripts are known (Burg et al. 1988; Cesbron-Delauw et al. 1989; Soldati and Boothroyd 1995), the EST sequences were strongly biased for the true 5' ends (Fig. 1). The corresponding ESTs for the less abundant *MIC2* gene and a newly described gene, *SRS2*, were scattered across their relevant transcripts (Fig. 1). In all three cases, multiple clusters were identified for each of the genes. For *MIC2*, 13 separate ESTs were grouped into two distinct, nonoverlapping clusters that correspond to different regions of the same *MIC2* gene. Such nonoverlapping clusters derived from the same gene also occurred in the more abundant *GRA1* ESTs; however, they also defined a second al-

lele defined by at least eight amino acid changes in a total of 191 residues that was only observed in the ME49 strain (data not shown).

#### Analysis of Abundant EST Clusters Reveals Gene Families

The nuclear genome of *T. gondii* is haploid and most previously characterized genes are single-copy, including the *SAG1* gene. Multiple clusters for *SAG1* occurred in both libraries because of the presence of similar, but distinct, members of a gene family. *SAG1* encodes the major tachyzoite surface antigen p30 (Burg et al. 1988) that has distant homology to *SAG3*, which encodes a distinct surface antigen called p43 (Cesbron-Delauw et al. 1994). A second *SAG1*-related sequence (*SRS1*) has been identified recently by gene mapping experiments (Hehl et al. 1997). Analysis of the ESTs with homology to *SAG1* reveals they define a family of at least six distinct genes as summarized in Table 2. In addition to the above described *SRS1* gene, three new related genes were identified and given the names, *SRS2*, *SRS3*, and *SRS4*, respectively.

#### Classification of Conserved Protein Families

Comparison of the *T. gondii* ESTs with the P-fam database that contains 527 conserved protein domain families identified 80 nonribosomal homologs that fall into a number of different functional classes (Table 3). These homologies identify a large number of genes that have not been studied previously in *T. gondii*, therefore opening the way to direct analyses of their roles through molecular genetic studies. The absence of some conserved protein domains from the *T. gondii* ESTs is likely attributable in part to incomplete sampling of the genome (sequences obtained here come from only one of several developmental stages and are biased for highly expressed genes). Additionally, its phylogenetic position may account for an

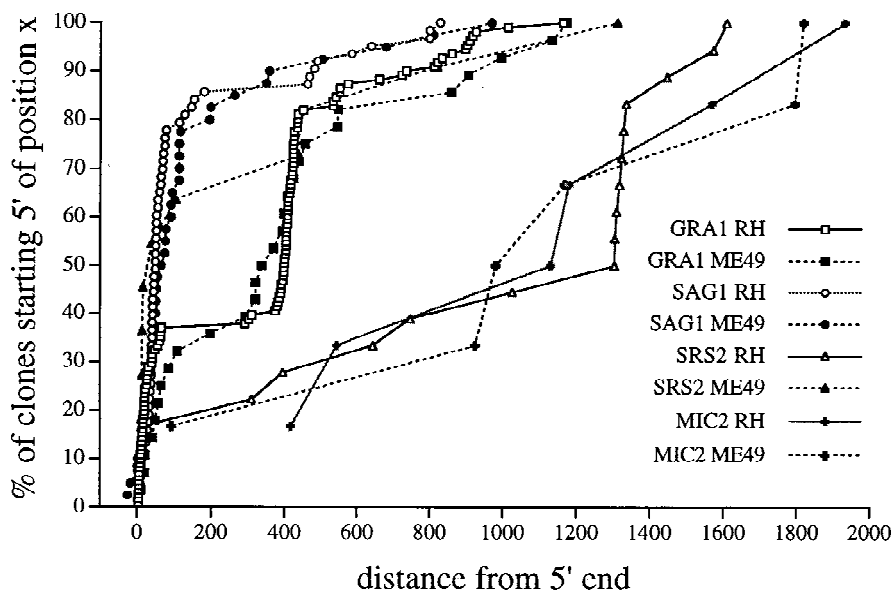


Figure 1 Plot of the 5' ends of ESTs for the genes *GRA1*, *SAG1*, *MIC2*, and *SRS2*. The majority of ESTs correspond to the extreme 5' end of the transcripts reflecting the fact that the libraries contain primary full-length cDNAs. A strong premature stop is evident in *GRA1*, 400 bp downstream, and, in the RH library only, for *SRS2* at 1300 bp downstream of the 5'-most ends. For the abundantly expressed genes, it is also possible to reconstruct an entire gene based on overlapping cDNAs.

Table 2. Identification of a Family of SAG1-Related Genes by EST Analysis

Gene Product	Alias	Size (kD) <sup>a</sup>	Number of ESTs <sup>b</sup>	Reference	GenBank accession no.
SAG1	p30	32.9	106	Burg et al. (1988)	M23658
SAG3	p43	41.8	10	Cesbron DeLauw et al. (1994)	L21720
SRS1	—	44.2	7	Hehl et al. (1997)	U77677
SRS2 <sup>c</sup>	—	39.5	29	this paper	N81803
SRS3 <sup>c</sup>	—	36.5	14	this paper	N82063
SRS4 <sup>c</sup>	—	?	1	this paper	N81407

<sup>a</sup>Size of predicted primary translation product where complete ORF is known.

<sup>b</sup>Number of essentially perfect matches in 7404 tachyzoite ESTs in dbEST.

<sup>c</sup>SRS1 stands for SAG1-related surface antigens.

absence of families restricted to plants, archaebacteria and an absence of families related to metazoan specializations.

The *T. gondii* ESTs were also compared with the yeast protein database derived from the recently completed genome sequencing project (Dujon 1996; Johnston 1996). Approximately 10% of *T. gondii* ESTs matched yeast ORFs ( $P \leq 10^{-6}$ ). Exclud-

ing ribosomal protein-coding genes, 297 putative genes were identified by this comparison (listed on the *T. gondii* web page: <http://www.ebi.ac.uk/parasites/toxo/toxpage.html>). Homologs between the yeast protein database and *T. gondii* ESTs ( $P \leq 10^{-10}$ ) were classified according to function and compared with the conserved protein families detected above (Table 3). Although similar numbers

Table 3. Functional Classification of Conserved Protein Domains vs. Yeast Proteins That Are Homologous to Toxoplasma ESTs

	Number of matching sequences <sup>a</sup>	
	P-fams	yeast proteins
Protein kinases/phosphatases	2	4
RNA/DNA binding	5	5
Translation/secretion	3	18
Signaling	4	3
Heat shock/chaperones	7	11
Amino acid metabolism	3	7
Detoxification	4	3
Glycolysis/energy metabolism	13	13
Nucleotide binding/ATP synthases	6	6
Proteases	3	3
Proteasome/ubiquitin	3	11
GTPases/ATPases	3	8
Cytoskeleton/cell adhesion/structural	7	8
Ribosomal proteins	25	67
Mitochondrial	5	10
Purine/pyrimidine metabolism	5	4
DNA/RNA replication/transcription	7	20

<sup>a</sup>Number of distinct conserved domain family members (P-fam) or yeast proteins recognized by toxoplasma ESTs.

of homologs were detected for most categories, two functional classes stand out as being more common between yeast and *T. gondii*—translation/secretion and replication/transcription. This may reflect a greater similarity between *T. gondii* and yeast among these classes or simply be attributable to the greater knowledge of these factors within yeast relative to other taxa.

### Identification of Sequences Restricted to the Apicomplexa

Identification of genes that are restricted to the phylum Apicomplexa would be of considerable interest, both in biological terms and as potential targets for drug or vaccine design. We therefore searched dbEST and the nonredundant database of GenBank to identify *T. gondii* ESTs exhibiting significant similarity to Apicomplexan sequences, while minimizing similarity to other taxa (Table 4). Several Apicomplexa-specific sequences were identified, including (1) secretory proteins associated with the specialized organelles that define this phylum (dense granules, rhoptries, micronemes) and apical antigens that are involved in adhesion (e.g., homologs of Pf-EMP1, AMA-1); and (2) genes that are expressed during specific developmental stages of the parasite life-cycle (e.g., homologs of trophozoite-, gametocyte-, and sporozoite-specific antigens in *Eimeria* and *Plasmodium*).

In addition to these Apicomplexa-specific genes, a number of ubiquitous enzymes and regulatory molecules were also identified by the above search criteria, including RNA polymerase III, HMG proteins, transhydrogenase, 14-3-3 proteins, etc. (Table 4). Based on their well-recognized functions, these would appear not to fit the criteria of phylogenetically restricted sequences. On closer examination, it was apparent these genes contain unique domains within otherwise highly conserved proteins. Therefore, although one or more ESTs matched only to other Apicomplexan sequences, other ESTs, corresponding to a different part of the same gene, were similar to a nonapicomplexan counterpart. Such comparative analysis provides a powerful means of identifying phylogenetically restricted domains within conserved proteins.

## DISCUSSION

To accelerate gene discovery in *T. gondii*, we have generated >7000 ESTs from random cDNAs by rapid, semiautomatic single-pass sequencing. In to-

tal, more than 500 genes were newly identified in *T. gondii* based on BLAST comparisons with the DNA and protein databases with the conservative probability cut-off of  $P \leq 10^{-10}$ . These genes fall into a number of families related to transcription, translation, protein secretion, and signaling. This rapid identification of new genes in *T. gondii* represents a 25-fold increase in the number of putative genes identified from this organism. In addition to providing evolutionary insight into an important group of parasites, these genes should foster studies on their roles in the biology of parasitism. Identification of phylogenetically restricted sequences of the Apicomplexa may also provide potential targets for intervention through chemotherapy or immunological means for the control of diseases caused by members of this phylum.

The use of ESTs to produce transcript maps has been heralded as a significant aid to positional cloning of genes, such as those involved in human genetic diseases (Adams et al. 1995). The ESTs generated here are being used currently to create such a genome map for *T. gondii* (J.W. Ajioka, C. Reitter, and R.M.R. Coulson, unpubl.) that will provide a framework for identification of genes involved in important biological phenotypes. Comparative EST analysis also provides a means for identifying strain polymorphisms (e.g., the *GRA1* example above) that may also be useful for genetic mapping studies. *T. gondii* has a complex life cycle that progresses from an acute stage, characterized by rapid proliferation of tachyzoites, to a slow-growing, encysted stage, the so-called bradyzoite, that is responsible for chronic infection. Further information on developmentally regulated genes should be forthcoming from a parallel effort to characterize a large number of ESTs from the bradyzoite stage of *T. gondii* (I.D. Manger, A. Hehl, S. Parmley, L.D. Sibley, M. Marra, L. Hillier, R. Waterston, and J.C. Boothroyd, in prep.).

*T. gondii* has an unusual and highly clonal population structure (Dardé et al. 1992; Sibley and Boothroyd 1992; Howe and Sibley 1995). The strain RH is the prototype for type I strains, which are all acutely virulent in mice (Sibley and Boothroyd 1992), whereas the type II strain ME49 is used commonly for models of chronic infection in animals (Hunter and Remington 1994). Type II strains are associated with the majority of human cases of toxoplasmosis both in AIDS and congenital infection (Dardé et al. 1992; Howe and Sibley 1995). One gene that has been implicated in pathogenesis is *SAG1*, which lies on chromosome VIII near a genetic locus for acute virulence in mice (Howe et al. 1996).

Table 4. Phylogenetically Restricted Sequences of the Apicomplexa

Reference (dbEST ID no.)	Contig ID <sup>a</sup> (no. of ESTs)	P value for apicomplexan genera	P value of closest non-apicomplexan	Putative identification	dbEST/ GenBank accession nos.
Apical antigens/adhesins					
467174	TQ-753 (92)	<i>Neospora</i> ( $10^{-22}$ )	$10^0$	dense granule antigen	U72991
467174		<i>Plasmodium</i> ( $10^{-3}$ )		Pf-EMP1	U27338
467347		<i>Sarcocystis</i> ( $10^{-15}$ )	$10^{-1}$	microneme protein	L08892
467347		<i>Eimeria</i> ( $10^{-14}$ )			A22655
466328	TQ-702 (2)	<i>Plasmodium</i> ( $10^{-12}$ )	$10^{-1}$	apical membrane antigen I	L27503
475702	TQ-1456 (2)	<i>Plasmodium</i> ( $10^{-14}$ )	$10^{-10}$	stippled structure-assoc. antigen	U10121
Developmental antigens					
466318	TQ-11 (8)	<i>Eimeria</i> ( $10^{-50}$ )	$10^{-1}$	19-kD sporozoite antigen	M59500
620297		<i>Plasmodium</i> ( $10^{-13}$ )	$10^{-7}$	gametocyte-specific antigen Pfs97	Z37724
489378		<i>Plasmodium</i> ( $10^{-11}$ )	$10^{-1}$	trophozoite antigen (Ser kinase?)	Z11832
487888		<i>Plasmodium</i> ( $10^{-11}$ )	$10^{-6}$		M83793
574122	TQ-254 (12)	<i>Eimeria</i> ( $10^{-31}$ )	$10^{-10}$	developmental mRNA	M98840
574122		<i>Plasmodium</i> ( $10^{-20}$ )			M29000
Metabolism, signaling, structural					
513090		<i>Plasmodium</i> ( $10^{-20}$ )	$10^{-8}$	RNA polymerase III (large subunit)	M73770
653609	TQ-1975 (4)	<i>Plasmodium</i> ( $10^{-13}$ )	$10^{-9}$	HMG protein	L31630
653609		<i>Babesia</i> ( $10^{-13}$ )			M81360
574303		<i>Plasmodium</i> ( $10^{-11}$ )	$10^{-4}$	P-type ATPase	U39298
574303		<i>Cryptosporidium</i> ( $10^{-10}$ )			U65981
487624		<i>Eimeria</i> ( $10^{-23}$ )	$10^{-14}$	transhydrogenase	L08392
467004		<i>Eimeria</i> ( $10^{-15}$ )	$10^{-6}$		
489375		<i>Eimeria</i> ( $10^{-11}$ )	$10^{-2}$		
488081	TQ-1442 (11)	<i>Neospora</i> ( $10^{-28}$ )	$10^{-4}$	14-3-3 regulatory protein	U31542
465739		<i>Neospora</i> ( $10^{-23}$ )	$10^{-16}$		
571541		<i>Neospora</i> ( $10^{-67}$ )	$10^{-33}$		
Unknown function					
467430	TQ-627 (9)	<i>Cryptosporidium</i> ( $10^{-17}$ )	$10^{-9}$	EST (identity unknown)	AA224687
465760	TQ-377 (4)	<i>Plasmodium</i> ( $10^{-39}$ )	$10^{-10}$	aspartate-rich protein	U46930

<sup>a</sup>Available from the Toxoqual database listing the consensus sequence for aligned EST contigs <http://daphne.humgen.upenn.edu/toxodb/>

Like *SAG1*, *SRS1*, *SRS2*, and *SRS3* are expressed on the surface of tachyzoites as verified by antisera raised to the recombinant proteins (Hehl et al. 1997). Consequently, this family of proteins may be involved in mediating host cell interactions particularly as *SAG1* has been implicated previously in cell adhesion (Kasper and Mineo 1994).

#### Identification of Conserved Protein Domains in *T. gondii*

Highly conserved domains were identified by comparing *T. gondii* ESTs with the yeast protein database and to the P-fam listing of conserved protein families. These similarities identify a plethora of inter-

esting genes for functional studies in *T. gondii*. Among the most abundant classes are genes with similarities to those involved in control of DNA replication/RNA transcription and protein translation and secretion. Developmental events are poorly understood in apicomplexan parasites, therefore, identification of genes involved in the machinery of replication and transcription should enable functional studies on the regulation of gene expression in *T. gondii*. Apicomplexan parasites are also highly specialized for regulated protein secretion (Carruthers and Sibley 1997). Despite the fact that yeast lacks such regulation, its secretion machinery is well understood. Identification of homologs involved in protein translation and secretion in *T. gondii* should allow studies on this important pathway in parasites. Given the enormous flexibility of genetic techniques and the functional information available about many genes in yeast, examination of these homologs may prove most fruitful using yeast as a heterologous system.

#### Identification of Sequences Restricted to the Apicomplexa

The main features that define the phylum Apicomplexa are the presence of apically specialized organelles including a unique microtubule organizing center called the conoid (Russell and Burns 1984), a novel endosymbiotic organelle called the apicoplast (Kohler et al. 1997) and three classes of secretory organelles—micronemes, rhoptries, and dense granules (Aikawa and Sterling 1974). Sequential discharge of these secretory organelles accompanies invasion of *T. gondii* into the host cell suggesting that they perform distinct functions (Carruthers and Sibley 1997). Although some cytoskeletal antigens from apicomplexans are immunologically related (Taylor et al. 1990; Morrissette et al. 1994), secretory proteins typically show no cross-reactivity at the protein or nucleic acid levels, reflecting the deep phylogenetic branches within this phylum (Gajadhar et al. 1991). The discovery of proteins/genes in common to the Apicomplexa has been hindered by the labor-intensive nature of traditional comparative means.

To identify Apicomplexa-specific genes, we have exploited the recent expansion of the gene databases, including the growing collections of ESTs for *T. gondii* (this paper; Wan et al. 1995), *Plasmodium falciparum* (Reddy et al. 1993; Chakrabarti et al. 1994), and *Cryptosporidium parvum* (R. Nelson, unpubl.). Given that the unifying features of the phylum relate to apical specialization, it is perhaps not

surprising that the phylogenetically restricted genes identified within the Apicomplexa include several proteins that may function in recognition and adhesion of host cells. For example, several *T. gondii* ESTs (including dbEST ID nos. 467174, 467408, and 466515) are similar to a conserved motif called the Duffy-binding-like (DBL) domain, which occurs in a variety of malarial proteins involved in red blood cell adhesion and in a family of malaria genes called *var*, the products of which mediate antigenic variation and sequestration of mature infected red cells (Su et al. 1995). This similarity occurs in the first of six motifs, a region defined by conserved cysteine and aromatic residues (Pettersson et al. 1995; D. Pettersson, pers. comm.) suggesting a common domain structure. Although *T. gondii* is not known to undergo either antigenic variation or sequestration, these DBL motifs may nevertheless identify proteins that participate in adhesion.

Other apicomplexan-specific genes encode developmentally regulated genes associated with the complex changes that occur during transformation of the life cycle stages. Finally, a number of unique metabolic genes were identified, including those involved in signaling and energy production. In some cases, these phylogenetically restricted domains were embedded within highly conserved proteins also found outside of the Apicomplexa. These two opposing definitions provide a powerful means for classifying protein similarities that span diverse phyla (e.g., ancient conserved regions) versus those restricted to a particular taxon. Expansion of the gene databases to include evolutionarily distant organisms will enable more refined analyses to identify phylogenetically restricted sequences, including those unique to the Apicomplexa. Application of similar searches in other organisms should likewise be a fruitful way to identify genes controlling biological traits that are of particular interest within specific taxa.

## METHODS

### Library Construction

Parasites were grown in human foreskin fibroblasts as described previously (Roos et al. 1994). Two separate laboratory strains were used for library construction—a clonal isolate of the RH strain and a clonal isolate of the ME49 strain called PDS. Freshly isolated tachyzoites were separated from host cell debris, washed in PBS, and RNAs were extracted with guanidium hydrochloride followed by phenol-chloroform treatment. cDNAs were synthesized from poly(A) mRNAs using the ZAP cDNA Synthesis Kit (Stratagene). The RH-strain library, referred to as "TgRH tachyzoite," was described previously (Wan et al. 1995) and contains cDNAs directionally cloned

into the *EcoRI* (5' end) to *XhoI* (3' end) sites of Lambda ZapII (Stratagene). A similar directional cDNA library, referred to as "TgME49 tachyzoite," was constructed from PDS tachyzoite mRNAs.

## Cycle Sequencing

Individual phage plaques grown on lawns of XL1 Blue strain *Escherichia coli* (Stratagene) were hand-picked and diluted in 20  $\mu$ l of  $\lambda$  diluent in 96-well plates. The clones were each assigned an identification number consisting of TgESTzy or TgESTzz followed by a plate number (1-99), row letter, and column number. PCR amplifications were performed on the phage eluates using T3 and T7 primers to amplify the complete inserts in MJ Research PTC 200 thermal cyclers (Hillier et al. 1996). Amplification products were diluted in water and used as templates for 5' sequencing reactions with Thermosequenase DNA polymerase and a DYEnamic ET T3-dye primer (Amersham) (Hillier et al. 1996). After thermal cycle sequencing, reactions were ethanol-precipitated, resuspended in loading buffer, and electrophoresed on ABI 373 sequencing machines.

## Processing and Annotation

Following gel image analysis and DNA sequence extraction, ABI sequence data were automatically processed to (1) assess EST quality; (2) trim flanking vector sequences; (3) mask repetitive elements; (4) remove vector, bacterial, or human mitochondrial sequences; and (5) identify ribosomal RNAs and sequences highly similar to human mRNAs. The resulting sequences were annotated with similarity information, sequence quality information (i.e., position in the sequence at which high-quality data ends), library information, and submitted to dbEST/GenBank. The details of where these procedures differ from the previous ones are as follows (Hillier et al. 1996): As in (2), above the vector sequences were trimmed using the programs VEP (Dear and Staden 1991), WEP (W. Gish, unpubl.), and BLASTN2 (W. Gish, unpubl.) where  $S$  (score) = 133,  $S_2$  (minimum reported score) = 133,  $M$  (match) = 5,  $N$  (mismatch) = -11,  $W$  (word size) = 7,  $R$  (gap extension penalty) = 11,  $Q$  (gap initiation penalty) = 11,  $E_2$  (minimal reported  $e$ -value) = 0.5. WEP also served to identify incorrect adaptor sequences. As in (3), above, repetitive elements were identified and masked using *blastx\_and\_mask* (G. Miklem, unpubl.), which uses BLASTX ( $S$  = 50) to compare with a database of *T. gondii* repeat elements (GenBank accession nos. M57916, M57917, M57918, M57919, X60240, X60241, X60242, and X75429) translated in all six frames. The programs TANDEM and INVERTED (R. Durbin, unpubl.) were used to mask local tandem and inverted repeats, and DUST (R. Tatusov and D. Lipman, unpubl.) was used to mask low entropy sequence. As in (4), above, sequences determined to be vector (BLASTN2  $S$  = 133,  $S_2$  = 133,  $M$  = 5,  $N$  = -11,  $W$  = 8 against a vector subset of GenBank), bacterial (BLASTN2  $S$  = 133,  $S_2$  = 133,  $M$  = 5,  $N$  = -11 against the bacterial division of GenBank), or human mitochondrial (BLASTN2  $S$  = 170,  $S_2$  = 150,  $M$  = 5,  $N$  = -11,  $R$  = 11,  $Q$  = 11 against GenBank:HUMMTCG, the human mitochondrion complete genome sequence) were not submitted to the public databases or included in further analysis. As in (5), above, EST similarities to ribosomal RNA were identified using BLASTN2 ( $S$  = 170,  $gapS_2$  = 150,  $M$  = 5,  $N$  = -11,  $R$  = 11,  $Q$  = 11) to compare with the RNA division of GenBank, resulting se-

quences were submitted to GenBank with the annotation of similar to ribosomal RNAs. EST similarities to human mRNAs were identified using BLASTN2 ( $S$  = 170,  $gapS_2$  = 150,  $M$  = 5,  $N$  = -11,  $R$  = 11,  $Q$  = 11) to search against a nonredundant human mRNA database (Boguski and Schuler 1995). These sequences were annotated as "similar to human mRNAs." Fourteen ESTs were >95% identical to human mRNAs at the nucleotide level and these were annotated as "probable human contaminants."

## Identification of Gene Homologies

At Washington University, similarities to proteins were identified using BLASTX ( $M$  = PAM120,  $S$  = 100) searches against SWIR, which is a nonredundant protein database containing sequences culled from PIR, SWISS-PROT, and a database of predicted *Caenorhabditis elegans* proteins called WORMPEP (E. Sonnhamer, unpubl.). These identities were annotated at the time of entry and appear in the field "putative IDs" on the NCBI entries.

Following submission, *T. gondii* ESTs were compared with the NCBI nonredundant nucleotide database using BLASTN ( $W$  = 12,  $E_2$  =  $10^{-6}$ , the theoretical query size = 1000, theoretical database size = 100,000,000) and to the NCBI nonredundant protein database (August, 1996) using BLASTX ( $W$  = 3, matrix: BLOSUM62,  $T$  (threshold) = 11, two-hit window = 40 (two hits of threshold 11 must be found no farther than 40 apart),  $E_2$  = 0.01, theoretical query size = 300, theoretical database size = 20,000,000). The resulting top matches are listed in the "neighbors" field of the NCBI entries and are updated periodically.

## Cluster Analysis for Grouping Similar ESTs

The ESTs were clustered using the program "est cluster" built around the icaass suite of tools (Parsons 1995). The algorithm compares each new EST longer than 100 bp with a growing set of representative ESTs clusters. Sequences were included with a cluster if the score was at least 50 (where matches are scored with +1 and mismatches are scored with -1). If the new sequence did not match an existing entry, then a new cluster was created. If the sequence was equivalent to more than one existing cluster, it was added to both.

## EST Alignment and Construction of a Nonredundant Consensus Sequence Database

*T. gondii* EST sequences were also assembled into overlapping sequences using the "cap2" contig assembly program (Huang 1996) to align sequences with a high degree of sequence identity. Starting with 7869 quality EST sequences of >100 bp in length (includes all *Toxoplasma* ESTs in dbEST as of June 1997), 868 aligned clusters were identified, incorporating 4653 EST sequences. Each EST is represented only once in the aligned cluster database and a consensus sequence was determined for each contig defined (available at <http://daphne.humgen.upenn.edu/toxodb/toxodb.html>). The entire "toxqual" data set (including consensus sequences and all unaligned ESTs) was compared with itself using BLASTN to identify similarities with  $P \leq 10^{-10}$ .

## Analysis of Gene Families

Identification of sequences similar to *SAG1* was performed by TBLASTN analysis of the dbEST database (NCBI server de-

faults; expect = 10, matrix = BLOSUM62) using coding sequences of the known SAGs [SAG1 (M23658), SAG3 (L21720), and SRS1 (U77677); see Table 2] to identify matching ESTs. After exclusion of ESTs yielding low BLAST scores because of overall short or poor-quality sequence, ESTs were assigned to one of the known SAGs based on similarity. ESTs that did not match existing SAGs, were analyzed further by BLASTN and FASTA comparisons to dbEST and the resulting matches were assembled into families of overlapping sequences using GCG (I.D. Manger, A.B. Hehl, and J.C. Boothroyd, unpubl.).

### Comparison of ESTs to Yeast Database

Each of the *T. gondii* ESTs was masked for low entropy sequence using DUST (R. Tatusov and D. Lipman, unpubl.) and compared with the 6218 yeast-translated ORFs/genes (Dujon 1996; Johnson 1996) using BLASTX with the following parameters: M = PAM120, T (word-score threshold) = 17, W = 4, V (no. of alignments reported) = 10000 filter seg. The yeast protein database was obtained from www-genome.stanford.edu (yeast\_orfs.fasta).

### Comparison to Conserved Protein Families

The 527 available seed alignments for protein families were obtained from release 2.0 of Pfam (Sonnhammer et al. 1997). Hidden Markov Models (HMM) were built using the hmmer-1.9j package with the following parameters: hmmb -d -R -PBLOSUM62. The -d option allows for maximum discrimination; the -R option allows that some family members may be fragmentary as occurs in EST data. The program HMMFS was then used to search the resulting HMMs against the entire set of ESTs, translated in all six frames using the program "orfer" (S. Eddy, unpubl.). A cutoff score of 20.0 bits was used to determine whether a given clone belonged to a specific family. This is a log-odds score; a score of 20 indicates that a sequence is 2<sup>20</sup>-fold more likely to represent an authentic match than to occur by chance.

### Identification of Phylogenetically Restricted Sequences

BLASTN and BLASTX searches of the nonredundant GenBank and dbEST databases were carried out using the GCG (v. 9.0 UNIX, December 1996) for each member of the consensus "toxqual" dataset and phylogenetic information was retrieved by matching the genus field for each match with  $P \leq 10^{-10}$  with a look-up table (generated from the GSDB Taxonomy table) to assign a kingdom and phylum for each match. All entries in the toxqual dataset that showed no high probability match outside of the phylum Apicomplexa ( $P > 10^{-10}$  and at least 1000-fold greater than matches to apicomplexan sequences) were examined manually to facilitate annotation.

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