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Acute Virulence in Mice Is Associated with Markers on Chromosome VIII in *Toxoplasma gondii*

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***Toxoplasma gondii* has an unusual population structure consisting of three widely distributed clonal lineages. Acute virulence in mice is strictly observed in type I strains, indicating that a genetic determinant(s) unique to this lineage controls acute pathogenesis. We have analyzed several naturally occurring recombinant strains of *T. gondii* that carry allele 1 at the *SAG1* locus; this allele is characteristic of the type I strains and was previously found to be 100% correlated with the acute virulence phenotype. Recombinant strains G622-M and ROD both had a predominantly type III genotype, with the significant exception of allele 1 at the *SAG1* locus. Although these two strains had virtually identical multilocus genotypes, they differed in their virulence in mice. Strain ROD was virulent whereas strain G622-M was nonvirulent, thus demonstrating that the presence of allele 1 at *SAG1* is not alone sufficient to confer acute virulence. Several sequence polymorphisms upstream of *SAG1* were found to be highly correlated with the acutely virulent lineages. Collectively, these results suggest that acute virulence is regulated by a region linked to the *SAG1* locus on chromosome VIII in *T. gondii*.**

Toxoplasma gondii is an intracellular protozoan parasite that infects a wide variety of vertebrate hosts, including humans. The life cycle of *T. gondii* is complex, involving mitotic division in a wide variety of intermediate hosts and meiotic division which occurs exclusively within the intestinal epithelial cells of cats (7). *T. gondii* infections in humans are extremely common yet typically asymptomatic. Between 10 and 40% of the adult population in the United States are chronically infected with *T. gondii*, and the prevalence is significantly higher in Latin America, Africa, and parts of Europe (22). Although infections are usually benign, substantial morbidity and mortality can occur with congenital infections (25) and in immunocompromised individuals, particularly in cancer (14), transplant (21), or AIDS (22) patients. Even among these susceptible individuals, there is wide variation in the severity of disease. Host genetics are likely important in determining susceptibility and severity of infection, as shown in studies using inbred mice (1, 26, 38, 40). However, disease severity is also influenced by the genotype of the infecting parasite (13, 34, 37), although the genes influencing pathogenesis have yet to be identified.

The population structure of *T. gondii* is clonal, and virtually all strains that have been analyzed by multilocus isoenzyme or restriction fragment length polymorphism (RFLP) analyses can be placed in one of three distinct clonal lineages (6, 5, 13, 34). The type I lineage was shown to exclusively contain those strains of *T. gondii* that are highly virulent in mice (34). Strain RH, and those strains that are genetically similar to it, exhibit a 100% lethal dose (LD_{100}) (i.e., concentration at which infection always results in death) of a single viable parasite. In contrast, nonvirulent strains exhibit an LD_{50} of greater than or equal to 10^2 parasites, and chronic infections are easily established in mice. The basis for the difference in virulence is unknown, but it is likely that a genetic factor(s) unique to the type I lineage is responsible. In a previous study, it was found that a specific allele at the *SAG1* locus (*SAG1-1*, found in strain RH) was 100% correlated with the acute virulence phenotype (34). *SAG1* is a single-copy gene that encodes the major surface antigen of *T. gondii* tachyzoites, SAG1 (also referred to as p30) (4), and several studies have shown that SAG1 participates in attachment and invasion of host cells by tachyzoites (9, 27, 28).

Despite the remarkably clonal population structure, the three lineages of *T. gondii* are not separate species (13). Although rare, strains with mixed genotypes are observed, and it is likely these were derived from naturally occurring meioses. The fortuitous isolation of three recombinant strains that carry allele 1 at the *SAG1* locus allowed us to directly analyze the role of this gene in contributing to acute virulence in mice.

MATERIALS AND METHODS

***T. gondii* strains.** Strains RH (32), PLK (17), and CEP (30) represent the type strains for lineages I, II, and III, respectively (13). Strain ROD was isolated from a congenital case of toxoplasmosis in the United States and was obtained from J. Remington (Palo Alto Medical Foundation, Palo Alto, Calif.); strain G622-M was isolated from a dove in Panama and was obtained from J. Frenkel (University of Kansas, Kansas City, Mo.); strain P89 was isolated from a pig in the United States (8) and was obtained from J. P. Dubey (U.S. Department of Agriculture, Beltsville, Md.). *T. gondii* strains were grown as tachyzoites in human foreskin fibroblast host cells maintained in Dulbecco's modified minimal essential medium supplemented with 1 mM glutamine, 10% fetal bovine serum, and 20 μg of gentamicin per ml.

Determination of acute virulence in mice. Female CF-1 outbred mice (>25 g) (Charles River Laboratories, Inc.) were used for experimental infections. *T. gondii* tachyzoites were harvested by syringe passage through 25-gauge needles and filtration through 3.0-μm-pore-size filters (36). Parasites were counted in a hemacytometer and diluted to the appropriate concentrations in Hanks' balanced salt solution. For each inoculum, five mice were infected by intraperitoneal (i.p.) injection, and cumulative percent mortality and time to death were recorded for 30 days postinfection.

At the end of the 30-day observation period, surviving mice were bled by cardiac puncture, and the sera were tested by Western blotting (immunoblotting) for antibodies against *T. gondii*. Tachyzoites (strain RH) were pelleted and lysed by boiling for 2 min in sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer containing the protease inhibitors E64, *p*-aminophenylmethyl sulfonyl fluoride, leupeptin, and *N*α-*p*-tosyl-L-lysine chloromethyl ketone, and the lysates were separated on 10% polyacrylamide gels (20). Gels were transferred to nitrocellulose membranes by semidry electrophoretic transfer in Tris-glycine buffer (pH 8.3). Membranes were blocked in phosphate-buffered saline (PBS) containing 1% nonfat dry milk (NFDM) and 0.05% Tween 20 and incubated in mouse sera that were diluted 1:50 in PBS containing 0.1% NFDM. After rinsing, membranes were incubated with ^{125}I-goat anti-mouse immunoglobulin G (Am-

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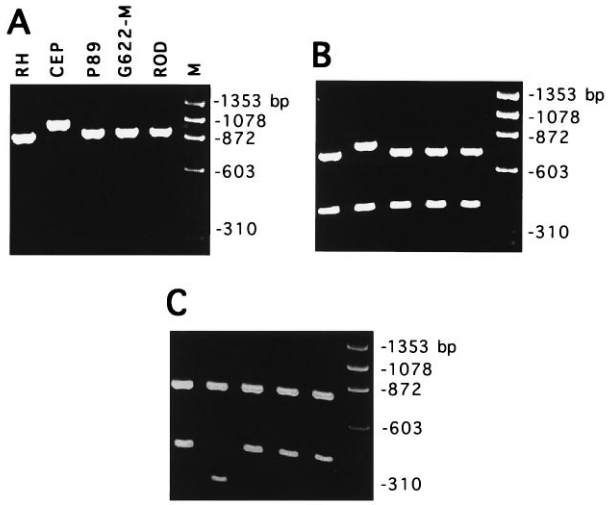

FIG. 1. Restriction patterns for the *SAG1* locus from *T. gondii* strains. The *SAG1* gene was amplified from each of the five strains and digested with *Dde*I (A), *Sau*96I (B), or *Hae*II (C). Two alleles are distinguished by these analyses; allele 1 is found in RH, P89, G622-M, and ROD, and allele 2 is found in CEP. Fragments were resolved in 1.5% agarose. Molecular weight marker is ϕX174 DNA digested with *Hae*III.

ersham, Inc.), rinsed in PBS–0.05% Tween 20–0.1% NFDM, and exposed to X-ray film.

Genetic analyses. The genotypes of *T. gondii* strains were determined by using gene probes selected from a collection of previously described genetic markers (33, 36). With the exception of the four smallest linkage groups (chromosomes IA, IB, II, and III), at least two representative genetic markers from each of the 11 chromosomes were used for analysis. Probes were labeled with [α-^{32}P]dCTP by using a random-primed labeling kit (Boehringer Mannheim) and hybridized to Southern blots of endonuclease-digested genomic DNA as described previously (36). Genetic markers *SAG1*, *SAG2*, *ROP1*, *850*, *L328*, and *62* were amplified by PCR and analyzed by endonuclease digestion and agarose gel electrophoresis as described previously (12, 13, 34).

Analysis of regions flanking *SAG1*. Sequences immediately flanking the *SAG1* gene were analyzed by digesting genomic DNA with restriction endonucleases that do not cut within the *SAG1* coding region and hybridizing with a 9-kb *Cla*I-*Eco*RI genomic fragment that contains *SAG1* (4).

To further analyze the region upstream of *SAG1*, oligonucleotides were selected from the nucleotide sequence of a *Kpn*I-*Hin*dIII restriction fragment designated KHB (10). The KHB fragment was amplified by PCR using the forward oligonucleotide KHB.F (5′-ACGGACGTGATCAAGATGC-3′) and the reverse oligonucleotide KHB.R (5′-ACGATCTGCTTCATGGTGAC-3′). The resulting products were ligated into *Eco*RV-digested pBluescript vector (Stratagene) and transformed in *Escherichia coli* XL1-Blue (23). Nucleotide sequence was obtained by Sanger dideoxynucleotide sequencing (23) from both ends of the KHB fragments in strains RH, CEP, P89, G622-M, and ROD.

RESULTS

PCR-RFLP analysis at the *SAG1* locus. To identify the allele carried at the *SAG1* locus in each of the strains, three separate restriction endonuclease digestions were performed on PCR-amplified *SAG1* DNA. Strains P89, G622-M, and ROD exhibited identical restriction patterns to allele 1, previously identified in strain RH (34), and differed from allele 2, found in strain CEP, for all three restriction digests (Fig. 1).

Determination of acute virulence. To establish the virulence of strains P89, G622-M, and ROD, female outbred mice were injected i.p. with different doses of tachyzoites of each strain, and the mortality and time to death were observed over a 30-day period. For comparison, the dose-dependent mortality of the type I strain RH (virulent) and the type III strain CEP (nonvirulent) were evaluated in parallel. Virulence assays for each strain were repeated two or more times, and the data presented are representative of one experiment. Infections with strain RH resulted in 100% mortality at an inoculum of 10^1 tachyzoites and higher, and partial mortality (20%) was seen at an inoculum of 10^0 (Fig. 2A). Mice that survived the 10^0 inoculum had negative serology at a serum dilution of 1:50, indicating that they had not been infected (data not shown). Mortality of 100% was observed with strain CEP at doses of 10^3 and 10^4 tachyzoites, and the LD_{50} for this strain was approximately 10^2 tachyzoites (Fig. 2B). Mice surviving injection with strain CEP had positive serology at a serum dilution of 1:50, indicating that they had been infected (data not shown).

Strains P89 and G622-M exhibited virulence similar to that observed for strain CEP (Fig. 2C and D). For strain P89, 100% mortality occurred at an inoculum of 10^4 tachyzoites, and the

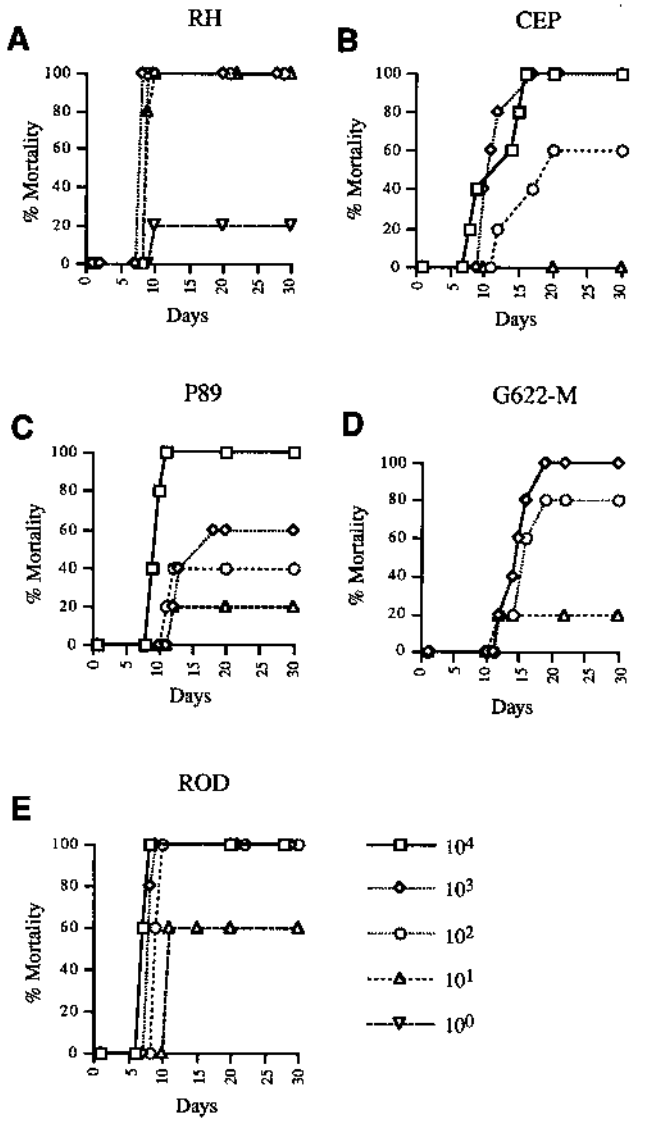

FIG. 2. Mortality of female outbred (CF-1) mice after i.p. injection with different strains of *T. gondii*. Infections with strain RH or ROD resulted in very high mortality and rapid time to death. Mice surviving inoculation with strain ROD showed negative serology to *T. gondii*, indicating that infection had not been established. Infections with strain CEP, P89, or G622-M resulted in moderate mortality and an extended time to death relative to RH and ROD. Mice surviving inoculation with strain CEP, P89, or G622-M showed positive serology to *T. gondii*, indicating that these mice had been infected. The key applies to all five panels, although a 10^0 inoculum was used only with strain RH and a 10^4 inoculum was not used with strains RH and G622-M.

TABLE 1. Genotypes at loci distributed across the 11 different chromosomes of *T. gondii*

Strain	Allele(s)[a]										
	IA[b] *G1A*[c] S96[d]	IB *cS8* HI	II *cB7* RI	III *cB22* RI	IV *c16A*, *cA5* PII, RI	V *L353*, *850*, *cS1* S3A, S96, RV	VI *c20B*, *L339* RI, NI	VII *L328*, *cS10A* MI, PII	VIII *SAG1* DSH	IX *L376*, *62A*, *RDN1* RV, HfI, RsI	X *c6A*, *c12*, *L53* PII, RV, S96
RH88	1	1	1	1	1, 1	1, 1, 1	1, 1	1, 1	1	1, 1, 1	1, 1, 1
PLK	1	1	2	2	1, ND	2, 2, 1	1, 1	1, ND	2	2, 1, 2	1, 2, 2
CEP	2	3	3	3	2, 2	2, 3, 2	2, 2	2, 2	2	3, 2, 2	2, 3, 3
P89	2	3	3	**1**	2, 2	**1**, 3, 2	**1**, 2	2, 2	**1**	3, 2, **3**	**3**, 3, **2**
G622-M	2	3	3	3	2, 2	**1**, 3, 2	2, 2	2, 2	**1**	3, 2, **1**	2, 3, **2**
ROD	2	3	3	3	2, 2	**1**, 3, 2	2, 2	2, 2	**1**	3, 2, 2	2, 3, **2**

[a] Numbers in boldface indicate loci where the recombinant strains do not carry a type III-associated allele. ND, not done. S96, *Sau*96I; HI, *Hha*I; RI, *Eco*RI; PII, *Pvu*II; S3A, *Sau*3AI; RV, *Eco*RV; NI, *Nco*I; MI, *Msp*I; DSH, *Dde*I, *Sau*96I, and *Hae*II; HfI, *Hin*fI; RsI, *Rsa*I.
[b] Chromosome.
[c] Locus.
[d] Restriction enzyme.

LD_{50} for this strain was approximately 10^3 tachyzoites. Injection of 10^4 and 10^3 tachyzoites of strain G622-M resulted in 100% mortality with delayed time to death as for CEP, and the LD_{50} for this strain was approximately 10^2. Mice surviving injections with strains P89 and G622-M had positive serology to *T. gondii*, indicating that they were chronically infected (data not shown).

In contrast to strains P89 and G622-M, recombinant strain ROD was highly virulent in mice (Fig. 2E). Mortality of 100% was observed at doses of 10^2 tachyzoites and higher, and 60% mortality was seen at a dose of 10^1 tachyzoites (Fig. 2). As for strain RH, time to death was very rapid. The serum from one of the two surviving mice did not react to *T. gondii* by Western blotting, indicating the mouse was not infected (the other surviving mouse died after the 30-day observation period and before blood could be obtained).

Analysis of loci on separate chromosomes. To determine the extent of genetic recombination among the tested strains, representative markers for each of the chromosomes were used to analyze genomic DNA digests (Table 1). The 20 genetic markers analyzed here encompass 75% of the mapped genome (110 of 147 map units) (36). The markers covering the remaining 25% of the genome do not show RFLPs between type I and type III strains. The absence of RFLPs in these regions suggests that they are similar in the type I and type III strains, and it therefore seems less likely that a virulence determinant is harbored in these regions. Strains G622-M and ROD had alleles that are consistent with a type III genotype at 17 of the 20 analyzed loci, with the exceptions of allele 1 at *SAG1* and *L353*, allele 2 (characteristic of type II strains) at *L53*, and in G622-M, allele 1 at the *RDN1* locus. In strain P89, alleles that are characteristic of type III strains were observed at 13 of the 20 loci analyzed, and alleles that are characteristic of type I strains (those found for strain RH) were observed at four loci (including *SAG1* and *L353*). Allele 2 (characteristic of type II strains) was observed at *L53*, and unique alleles were observed at *RDN1* and *c6A*.

Analysis of loci on chromosome VIII. Because acute virulence has previously been associated with *SAG1*, we examined the genotypes of the recombinant strains at additional loci also located on chromosome VIII (Fig. 3) (36). In strains G622-M and ROD, chromosome VIII is comprised of alleles that are characteristic of type III strains at all of the loci tested with the exception of *SAG1-1*. Strain P89 had alleles that are characteristic of type III strains at four loci and carried allele 1 at both the *SAG1* and *ROP1* loci. These results indicate that chromosomes VIII in strains G622-M and ROD are likely the products of single genetic crossovers on the distal *SAG1*-containing end of the chromosome, while at least two recombinations were involved in forming chromosome VIII in P89.

Analysis of *SAG1* flanking regions. Although the recombinant strains G622-M and ROD both have the virulence-associated *SAG1-1* allele, they have contrasting phenotypes, suggesting that a genetic difference between these strains (e.g., due to differences in the point of crossover) may lie in the regions flanking the *SAG1* locus. To analyze sequences outside the *SAG1* coding region, genomic DNA digests were probed with a 9-kb *Cla*I-*Eco*RI genomic fragment containing the *SAG1* gene (4). Southern blots of *Ava*I- or *Xba*I-digested DNAs revealed identical restriction patterns for strains RH, P89, G622-M, and ROD, and these differed from the pattern obtained from strain CEP (Fig. 4). These results are consistent with the above interpretation that the three recombinant strains (P89, G622-M, and ROD) have acquired *SAG1-1* via a genetic recombination involving the gene and the regions that flank it and are not due to point mutations within the gene (i.e., convergence). Southern blot analysis of *Pvu*II genomic digests revealed an important RFLP linking the two virulent strains

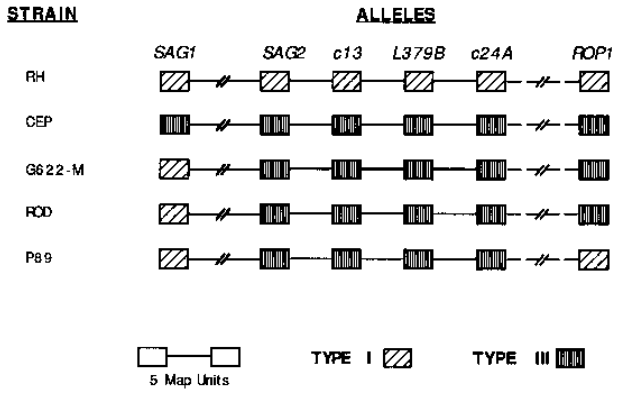

FIG. 3. Schematic representation of loci located on chromosome VIII in strains of *T. gondii*. The genotypes found for RH and CEP represent the alleles that are characteristic of the type I and the type III lineages, respectively. Strains G622-M and ROD contain a chimeric chromosome composed of primarily type III alleles with the exception of the type I allele at *SAG1* on the distal portion of the chromosome. Likewise, P89 contains a chimeric chromosome composed of type III alleles with the exceptions of type I alleles at *SAG1* and *ROP1* on either end of the chromosome. The *ROP1* locus was mapped to the end of chromosome VIII by analysis of an *Ava*I RFLP in 19 recombinant F_1 progeny (34).

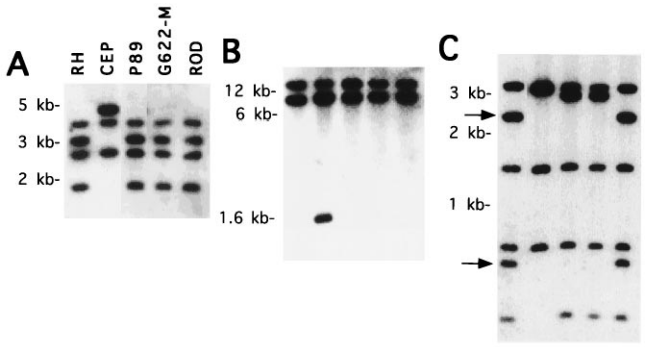

FIG. 4. Genomic restriction enzyme analysis of the regions flanking the *SAG1* gene in strains of *T. gondii*. The restriction patterns observed after digestion with *Ava*I (A) or *Xba*I (B) are identical for strains RH, P89, G622-M, and ROD, while the *Pvu*II (C) digests reveal a polymorphism that is shared by the two virulent strains RH and ROD. Genomic DNAs were digested with the indicated restriction enzymes and hybridized with a 9-kb ^{32}P-labeled *Cla*I-*Eco*RI fragment that contains the *SAG1* gene.

RH and ROD by a shared restriction site not found in the nonvirulent strains P89 and G622-M (Fig. 4).

To identify the basis for this polymorphism, the *Pvu*II site was mapped to within approximately 1 kb upstream of the *SAG1* ATG initiator codon (Fig. 5). Sequence analysis of this region in the parasite strains identified five nucleotide changes (designated F1 and R1 to R4), three of which specifically distinguished the virulent strains RH and ROD from the nonvirulent strains CEP, PLK, P89, and G622-M (Fig. 5 and Table 2). These sequence polymorphisms lie immediately downstream of a second open reading frame designated as *SUSI* that encodes an apparent SAG1 homolog (Fig. 5) (10). The nucleotide sequence in the region containing the polymorphisms did not have a significant open reading frame and did not show any significant matches in the protein or nucleotide sequence databases, suggesting that they lie in a region of noncoding DNA.

To determine the degree to which the *Pvu*II restriction site polymorphism was associated with the virulent type I lineage, the KHB fragment was amplified by PCR from a collection of strains (13) and digested with *Pvu*II. This restriction site was found to be highly associated with virulence, as 18 of 19 type I virulent strains (including RH) had the *Pvu*II restriction site, and 16 of 16 type II and type III nonvirulent strains (including CEP and PLK) did not have the restriction site (data not shown). The single exception was strain MAS, which has previously been shown to have a multilocus genotype that differs from the type I lineage by having unique alleles at multiple loci (6, 13). Although strain MAS has an LD_{50} of between 10^1 and 10^2, the sera from surviving mice showed no reactivity to *T. gondii*; hence, this strain is considered virulent (11).

DISCUSSION

Virulent strains of *T. gondii* were previously shown to share a single common multilocus genotype (34). While these strains are certainly not identical, they share a majority of alleles and represent a distinct clonal lineage. These type I virulent strains differ genetically by 1% or less from the type II and type III nonvirulent strains (13), and the expression of the virulence phenotype is likely due to genetic information contained within this very limited difference. Prior to this study, few recombinant strains had been identified (6, 13), and none had been critically examined for their virulence phenotypes. We have analyzed three naturally occurring recombinant strains of *T. gondii* which carry the virulence-associated *SAG1-1* allele in a genetic background of nonvirulence-associated alleles. Our findings suggest that *SAG1-1* is not alone sufficient to account for the high virulence observed in type I strains but instead may be closely linked to a virulence determinant(s) on chromosome VIII.

Previous studies indicate that the SAG1 protein is important for infection by *T. gondii* (9, 27, 28), and it seemed possible that the allele of *SAG1* carried by a specific strain might influence its virulence in mice. The SAG1 protein is a highly abundant surface antigen of 30 kDa that is attached to the surface of the parasite by a glycosylphosphotidylinositol anchor (16, 29, 39). Because of its strong immunogenicity, SAG1 has been favored as a candidate antigen for the development of diagnostic tests and subunit vaccines (3, 18, 19). Previous studies on the SAG1 surface protein showed that attachment and invasion of host cells by *T. gondii* could be blocked by antibodies against SAG1, thereby implicating this protein in the process of invasion (9, 27, 28). In addition, chemically mutagenized parasites expressing altered SAG1 (PtgA and PtgC) showed a reduced ability to bind host cells compared to wild-type strains (27).

The three recombinant strains P89, G622-M, and ROD differ from one another in their virulence in mice, despite all having the *SAG1-1* allele. Strain ROD exhibits a phenotype that is most similar to that of strain RH (Fig. 2) and is thus

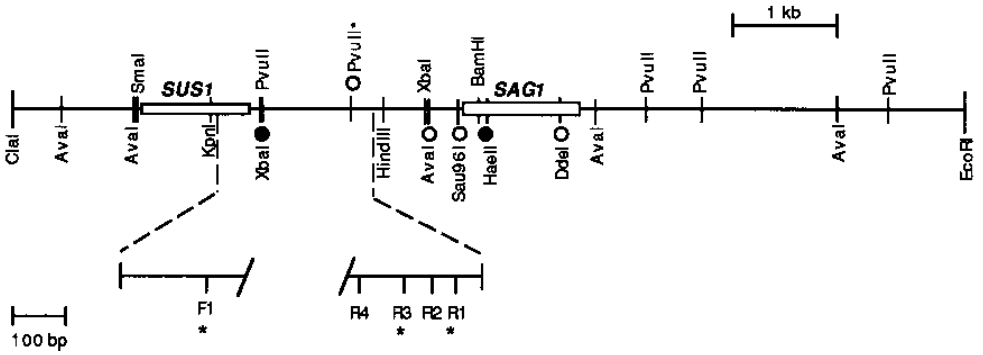

FIG. 5. Restriction map of the 9-kb *Cla*I-*Eco*RI fragment containing the *SAG1* and *SUS1* genes. Boxes indicate the open reading frames for the *SAG1* and *SUS1* genes. The lower map represents the regions that were sequenced from the 1.5-kb KHB fragment; nucleotide polymorphisms are designated F1 and R1 to R4. The *SUS1* termination codon lies approximately 35 nucleotides upstream of the F1 polymorphism (10). The restriction sites associated with *SAG1-1* are designated with an open circle. Restriction sites associated with *SAG1-2* are designated with a filled circle. Nucleotide changes that specifically identify the virulent strains RH and ROD are indicated with an asterisk.

TABLE 2. Sequence comparison of the KHB fragment from strains of *T. gondii*[a]

Strain	Sequence				
	F1[b]	R4	R3[b]	R2	R1[b]
RH	...GGTACGT...	...GACGCGG...	...CAGCTGC...	...CTGCGAT...	...CTCTGAC...
ROD	...GGTACGT...	...GACGCGG...	...CAGCTGC...	...CTGCGAT...	...CTCTGAC...
CEP	...GGTGCGT...	...GACGCGG...	...CAGATGC...	...CTGGGAT...	...CTCCGAC...
PLK	...GGTGCGT...	...GACGCGG...	...CAGATGC...	...CTGGGAT...	...CTCCGAC...
P89	...GGTGCGT...	...GACACGG...	...CAGATGC...	...CTGCGAT...	...CTCCGAC...
G622	...GGTGCGT...	...GACACGG...	...CAGATGC...	...CTGCGAT...	...CTCCGAC...

[a] Nucleotide changes are presented in sequential order (5′-3′) as they occur in the KHB fragment (see Fig. 5).
[b] Nucleotide changes that are shared by the two virulent strains RH and ROD. The R3 change creates the *Pvu*II polymorphism.

considered a virulent strain. In contrast, strains P89 and G622-M exhibit phenotypes that approximate that seen for the nonvirulent strain CEP. Not only is the LD_{50} similar to that of CEP, but P89 and G622-M also behave like the nonvirulent strains in two other important criteria: delayed time to death and positive serology in the surviving mice.

The relative pathogenicity of *T. gondii* can be influenced by route of inoculation, genotype of the host, and life cycle stage of the parasite (15, 24, 38). For example, oral or subcutaneous inoculation of bradyzoites or oocysts of strain P89 was previously found to cause high mortality in mice (8). In contrast, we have shown that i.p. inoculation of tachyzoites in Swiss-Webster mice causes low mortality, a result that is also seen in BALB/c mice (data not shown). The apparent difference in pathogenicity observed for strain P89 is likely due to differences in the procedures used for characterizing this strain. However, i.p. challenge with a carefully quantified inoculum of tachyzoites (the stage that mediates the acute phase of disease) provides the greatest experimental control and the most reliable method for determining the acute virulence of *T. gondii* strains and has been used previously to establish the association of the parasite genotype with the virulence phenotype (6, 13, 34).

Additionally, the phenotype observed for virulent strains is not simply a reflection of the number of passages the parasites have undergone in culture. Strain CEP has been continually maintained by serial passage for more than 10 years, yet it retains a nonvirulent phenotype. Additionally, we have tested low-passage-number (i.e., soon after isolation) type I strains and found that they are acutely virulent like strain RH (11). Nonvirulent strains often cause little or no mortality in mice when they are initially isolated but may increase in their relative virulence after acclimation to in vitro culture (11). Strain G622-M was initially avirulent in mice (no deaths at the 10^4 inoculum) (data not shown), but after continuous passage in human foreskin fibroblasts, its virulence increased to an LD_{50} of about 10^2, which has now remained stable for over 6 months of in vitro growth. During this transition, there was no change in genotype at the single-copy loci *SAG1* and *SAG2* (data not shown). Despite the observed increase in pathogenicity of nonvirulent strains, the LD_{50} of type II and type III strains plateaus at approximately 10^2 parasites, and these strains do not acquire a virulent phenotype, as defined here (LD_{100} = a single viable parasite).

Collectively, the data indicate that the presence of *SAG1-1* is not alone sufficient to confer the acute virulence phenotype. All three recombinant strains carry allele 1 at the *SAG1* locus, but two of these strains (P89 and G622-M) are nonvirulent, indicating that the presence of this allele does not impart a virulent phenotype. In support of this observation, we have transfected CEP parasites with a cosmid containing *SAG1* and *SUS1* from the virulent RH strain and found no increase in the virulence of this transfected clone (11).

The difference in virulence between G622-M and ROD is surprising since these two strains have nearly identical genotypes (Fig. 3 and Table 1). Their disparate phenotypes suggest that these strains differ at some genetic locus which controls virulence. Both strains have genotypes characteristic of nonvirulent strains (predominantly type III alleles) with the exception of the distal end of chromosomes V (*L353-1*), VIII (*SAG1-1*), and IX (*RDN1-1*). Of these three, *SAG1-1* remains the most likely candidate for linkage to the virulence phenotype based on the prior association with acute virulence in over 100 strains (13, 34). Additionally, three sequence polymorphisms upstream of *SAG1* were associated with the virulent strains RH and ROD. One of these sequence differences, which creates a *Pvu*II polymorphism, was correlated with acute virulence in 38 of 39 strains tested. Although these nucleotide changes lie in a region of noncoding DNA (10) and are therefore unlikely to be directly responsible for the phenotype, they indicate the proximate presence of a virulence determinant on the distal end of chromosome VIII.

Virulent strains of *T. gondii* comprise less than 10% of the strains found in nature, yet they are associated with a substantial proportion of AIDS and congenital toxoplasmosis cases (19 and 27%, respectively) in humans (13). Identification of genetic factors involved in acute virulence in mice may provide insight into the pathology associated with acute toxoplasmosis. The combination of classical (33, 36) and molecular genetic (reviewed in references 2, 31, and 35) approaches that are now available for *T. gondii* will be useful for identifying genes involved in pathogenicity, including acute virulence.

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