Generation of a Restriction Fragment Length Polymorphism Linkage Map for *Toxoplasma gondii*

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ABSTRACT

We have constructed a genetic linkage map for the parasitic protozoan, *Toxoplasma gondii*, using randomly selected low copy number DNA markers that define restriction fragment length polymorphisms (RFLPs). The inheritance patterns of 64 RFLP markers and two phenotypic markers were analyzed among 19 recombinant haploid progeny selected from two parallel genetic crosses between PLK and CEP strains. In these first successful interstrain crosses, these RFLP markers segregated into 11 distinct genetic linkage groups that showed close correlation with physical linkage groups previously defined by molecular karyotype. Separate linkage maps, constructed for each of the 11 chromosomes, indicated recombination frequencies range from approximately 100 to 300 kb per centimorgan. Preliminary linkage assignments were made for the loci regulating sinefungin resistance (*snf-1*) on chromosome *IX* and adenine arabinoside (*ara-1*) on chromosome *V* by linkage to RFLP markers. Despite random segregation of separate chromosomes, the majority of chromosomes failed to demonstrate internal recombination events and in 3/19 recombinant progeny no intramolecular recombination events were detected. The relatively low rate of intrachromosomal recombination predicts that tight linkage for unknown genes can be established with a relatively small set of markers. This genetic linkage map should prove useful in mapping genes that regulate drug resistance and other biological phenotypes in this important opportunistic pathogen.

TOXOPLASMA gondii is an obligate intracellular parasite that is widespread in animals and humans, infecting up to 50% of the world's population. Toxoplasma exists as a single species that is capable of infecting most vertebrates wherein it parasitizes a wide range of host cell types. While chronic infection is not normally symptomatic, Toxoplasma has recently emerged as a major opportunistic pathogen in AIDS patients (LEVY, BREDESEN and ROSENBLUM 1985; MILLS 1986; LUFT and REMINGTON 1988). Combined with its better known role in producing congenital defects in humans (DESMONTS and COUVREUR 1974), this development has necessitated a greater understanding of the biology of this organism as well as a need for the identification of new targets for chemotherapy.

In vivo, tachyzoites replicate rapidly during a short-lived acute infection that stimulates a vigorous and protective immune response (KRAHENBUHL and REMINGTON 1982). A proportion of tachyzoites differentiate into slow-growing bradyzoites contained in tissue cysts within skeletal muscle, the central nervous system, and other tissues (REMINGTON and CAVANAUGH 1965; DUBEY 1977). These long-lived cysts present the source of reactivation of acute infection during immune deficiency states and form the infective stage for transmission through the food chain. The sexual cycle occurs only within enterocytes in the gut of members of the cat family (Felidae) where macrogametes and microgametes fuse to form a diploid zygote. This zygote is shed into the environment within a maturing oocyst where it undergoes division into 8 haploid progeny called sporozoites. When ingested, these sporozoites invade host cells where they differentiate into asexually dividing haploid tachyzoites (for details of the life cycle see DUBEY and FRENKEL 1972).

Unlike most medically important protozoan parasites, Toxoplasma offers the potential for development of genetics as a powerful investigational tool. Toxoplasma replicates rapidly as a haploid stage called a tachyzoite that is easily cloned and propagated *in vitro* in a variety of vertebrate cell lines. This flexibility has been used to construct a number of drug-resistant mutants by chemical mutagenesis and selection (PFEFFERKORN 1981). Although a number of genes have been isolated from Toxoplasma, the lack of a DNA transformation system for this organism has limited the ability to identify and clone genes that regulate phenotypes of interest by molecular genetic techniques.

Classical genetic crosses, between separate drug-

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resistant mutants produced in an isogenic background, have indicated that sporozoites are the product of a single round of classical meiotic division (PFEFFERKORN and PFEFFERKORN 1980). These intrastrain crosses have revealed that individual clones undergo both self-mating and cross-mating to yield recombinants in about one half of the progeny (PFEFFERKORN and KASPER 1983). Infections initiated from a single haploid tachyzoite are capable of completing the entire sexual cycle in the cat culminating in production of infective oocysts (PFEFFERKORN, PFEFFERKORN and COLBY 1977; CORNELISSEN and OVERDULVE 1985), indicating there is no predefined mating type. Classical genetic analysis has not been extensively developed in Toxoplasma due to the expense and relative difficulty in producing and characterizing the numerous phenotypic markers that would be necessary to perform linkage studies.

The general utility of RFLPs in providing markers for physical and genetic mapping has been demonstrated in a number of systems where genetic approaches are well developed (COULSON *et al.* 1986; CHANG *et al.* 1988). Additionally, RFLPs have provided a means of rapidly identifying markers that has accelerated the development of emerging genetic systems for a number of agricultural crops and animals. RFLP-based mapping has also provided unique approaches for mapping of virulence traits of pathogens (TZENG *et al.* 1992), for quantitative trait mapping (LANDER and BOTSTEIN 1989), and for mapping the basis of human genetic diseases (GREEN and OLSEN 1989). The development of genetics in medically important parasites would have a significant impact in addressing questions of virulence, pathogenicity and drug resistance in this group of organisms; however, most of these systems are hampered by an inability to perform sexual crosses. In the malaria parasite, *Plasmodium falciparum,* the recent development of an RFLP based genetic map has enabled genetic mapping of the locus involved in chloroquine resistance (WELLEMS, WALKER-JONAH and PANTON 1991), a clinically important problem of global concern. This is a significant development for the malaria field; however, there are limitations to the routine use of this system imposed by the biology of the organism which requires both a breeding mosquito colony and chimpanzees to perform each cross.

Due to the relative ease in constructing and propagating mutants, performing genetic crosses, and analyzing the resulting progeny, we are interested in developing Toxoplasma as a model parasitic system for genetic analysis of important biological phenotypes. As a first step, we have recently developed a molecular karyotype for Toxoplasma by separating chromosomes using pulsed-field gel electrophoresis and mapping 57 low copy number DNA probes to individual chromosomes by hybridization (SIBLEY and BOOTHROYD 1992). This karyotype consists of 9 distinct bands that range in size from approximately 2 Mb to greater than 6 Mb and includes 1 or more chromosomes too large to enter the gel. We have used these same probes, combined with additional markers, to define strain-specific RFLPs as a rapid means of acquiring a large number of markers for physical and genetic mapping. In the present report, the inheritance of these randomly selected markers among 19 recombinant progeny from two parallel interstrain crosses was used to construct a working genetic map of the Toxoplasma genome.

MATERIALS AND METHODS

Parasite culture: Two cloned lines of *T. gondii* were used for genetic analysis. Strain ME49, initially isolated from a sheep in California, was cloned by limiting dilution in LLOYD KASPER's laboratory to obtain P strain (KASPER and WARE 1985) (Dartmouth Medical School, Hanover, New Hampshire); it is referred to here as PLK. Strain C, isolated from a naturally infected cat in New Hampshire, was cloned in the laboratory of ELMER PFEFFERKORN (Dartmouth Medical School) by limiting dilution and microscopic identification of a microwell containing a single plaque (PFEFFERKORN, PFEFFERKORN and COLBY 1977); it is referred to here as CEP. Toxoplasma tachyzoites were propagated in human fibroblast (HF) monolayers and harvested for DNA analyses as described previously (BURG *et al.* 1988; SIBLEY and BOOTHROYD 1992). These strains were chosen based on their likelihood of completing the meiotic phase of the life cycle in the cat, a property that is frequently lost in laboratory passaged strains (FRENKEL, DUBEY and HOFF 1976). In some cases a third strain, RH (SABIN 1941) was included in the RFLP screens for comparative purposes only.

RFLP markers: Toxoplasma DNA probes were randomly selected from one of three sources: (1) lambda gt11 cDNA libraries (BURG *et al.* 1988; PRINCE *et al.* 1989), (2) a genomic *Hin*dIII/*Eco*RI insert library constructed in the plasmid Bluescript (Stratagene, Inc.) or (3) a partial *Sau*3A genomic cosmid library (BURG *et al.* 1988). Many of these same markers have been previously used for molecular karyotype analysis and the naming of markers here follows previous conventions (SIBLEY, PFEFFERKORN and BOOTHROYD 1991; SIBLEY and BOOTHROYD 1992). Briefly, genes of known coding function are referred to by genetic names following nomenclature rules similar to those used in yeast (SIBLEY, PFEFFERKORN and BOOTHROYD 1991). Randomly isolated cDNAs are named simply by clone name. Randomly isolated plasmids are named with the prefix L followed by a clone number. Randomly isolated cosmid clones are named with a prefix c followed by a clone number. In all cases these libraries and respective clones are derived from RH strain Toxoplasma DNA. The probes used here are listed in Table 1 along with their source and coding function, where known.

To identify informative RFLPs between PLK and CEP strains, genomic DNAs were digested with a variety of restriction endonucleases and resolved by agarose gel electrophoresis followed by standard Southern blotting (MANIATUS, FRITSCH and SAMBROOK 1982). For cDNAs and plasmid clones the following enzymes were used in screening: *Aha*II, *Alu*I, *Ase*I, *Ava*I, *Ava*II, *Bam*HI, *Bcl*I, *Bgl*II, *Bst*EII, *Bst*NI, *Cla*I, *Dde*I, *Eco*RI, *Eco*RV, *Fnu*4HI, *Hae*II, *Hae*III, *Hin*dIII, *Hin*cII, *Hin*fI, *Hha*I, *Hpa*II, *Mae*I, *Mae*III,

*Mbo*I, *Mse*I, *Msp*I, *Nco*I, *Nde*I, *Pst*I, *Pvu*I, *Pvu*II, *Rsa*I, *Sac*I, *Sac*II, *Sal*I, *Sau*3AI, *Sau*96I, *Sma*I, *Ssp*I, *Taq*I, *Xho*I and *Xba*I. For cosmid clones the following restriction enzymes were used for screening: *Ase*I, *Bam*HI, *Cla*I, *Dde*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Hha*I, *Msp*I, *Pst*I, *Pvu*II, *Rsa*I, *Sal*I, *Sac*I, *Sac*II, *Sau*3AI, *Sau*96I and *Sma*I. Enzymes were obtained from New England Biolabs, Inc., and used according to the manufacturers instructions. To minimize partial digestion products, digests were conducted with 10–20 units/μg genomic DNA for 2 hr at 37° followed by replenishment with fresh enzyme for a second 2-hr incubation at 37°.

Probes were labeled with [α-^{32}P]dCTP using a random-primed labeling kit (Boehringher Mannheim) and used for hybridization as described previously (SIBLEY and BOOTHROYD 1992). Briefly, hybridizations were conducted overnight at 42° in 6 × SSPE containing 50% formamide, 10 × Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS) and 200 μg/ml calf thymus DNA. Blots were washed to final stringency of 0.1× SSPE, 0.5% SDS at 60° (±5°) for 1 hr. Autoradiographs were scored for informative RFLPs and probes that detected polymorphisms were retained for further analysis. For repeated probings with different probes, blots were stripped with 0.2 M NaOH 0.1% SDS (5 min, 25°) and neutralized with 0.5 M Tris-HCl, pH 7.5 (10 min, 25°).

Genetic cross and isolation of recombinant progeny: To easily identify the products of interstrain mating, a mutant clone of parental strain CEP that was resistant to sinefungin and adenine arabinoside was chosen to cross with the wild-type PLK strain. This clone of CEP, designated as CEP Ara^R Snf^R, was the product of a previously reported genetic cross between singly resistant parental clones of the CEP strain that were obtained by chemical mutagenesis and selection of clones (PFEFFERKORN and KASPER 1983). The CEP Ara^R Snf^R clone is fully resistant *in vitro* to sinefungin (Calbiochem) at a level of 2.7×10^{-7} M and to ara-A (Sigma) at a level of 1.3×10^{-4} M; levels that fully inhibit growth of wide-type Toxoplasma strains.

To perform genetic crosses, BALB/c mice were given an intraperitoneal injection with approximately 10^4 tachyzoites grown *in vitro*, and infections were allowed to progress to a chronic state (approximately 2 months). Chronically infected mice were sacrificed, the brains removed, and cyst abundance was estimated by homogenizing a small section of brain in phosphate-buffered saline and examining microscopically. A mixture of brain homogenate containing approximately equal numbers of cysts from the two parental strains was fed to two separate kittens as described previously (PFEFFERKORN and PFEFFERKORN 1980). Kittens were locally bred and raised under conditions that precluded previous infection with Toxoplasma (Dartmouth Medical School, Animal Research Facility). Immature oocysts were harvested 4–8 days later, isolated by flotation and allowed to sporulate at room temperature. Mature oocysts were chemically induced to hatch and the resulting sporocysts and sporozoites were used to infect new monolayers of HF cultures (PFEFFERKORN and PFEFFERKORN 1980). Singly resistant progeny were selected by expansion first in the presence of a single drug then cloned by limiting dilution and microscopic identification of single plaques in microwell cultures. Individual clones were expanded and tested for sensitivity to the second drug by plaque assay (PFEFFERKORN and PFEFFERKORN 1976). Representative clones were cryopreserved in liquid nitrogen storage (PFEFFERKORN and PFEFFERKORN 1976) and expanded by *in vitro* culture in HF monolayer cultures for isolation of genomic DNA.

Polymerase chain reaction (PCR) analysis: Progeny clones isolated from the crosses were also analyzed using two unlinked markers to determine their recombinant status by PCR-based genotyping. Individual clones were grown in HF monolayers in 96 well plates until confluent lysis occurred. A sample of cells and media was removed equal to approximately 10% of the total volume and containing approximately 10^4 Toxoplasma cells. Cells were lysed and DNA denatured by heating to 95° for 5 min and PCR amplification was performed separately for the two loci *850* and *SAG1*. Following amplification, samples were extracted with phenol/chloroform, ethanol precipitated, and subjected to restriction enzyme digestion typing by gel electrophoresis and blotting.

Probe 850 represents a randomly selected cDNA that maps to chromosome *V* and is highly polymorphic with respect to distinguishing PLK *vs.* CEP strains (SIBLEY and BOOTHROYD 1992). The PCR primers used for amplification of the 850 locus from genomic DNA were: 5′AAGGACCTGGTAACAGTCC3′ and 5′TCAAGGCTTGGATGTTTCG3′ which are based on the sequence of this gene in the RH strain as determined by dideoxy sequencing (Sequenase, U.S. Biochemical Corp.). Amplification was performed for 40 cycles of denaturation at 94° for 30 sec, annealing at 50° for 2 min, and extension at 72° for 2 min. Restriction endonucleases *Dde*I, *Rsa*I and *Sau*96I were used to distinguish PLK from CEP strain alleles based on RFLP differences that were first identified by genomic Southern blots.

Probe p30.5c is a single copy gene, encoding the major surface antigen p30 (SAG1) (BURG *et al.* 1988), located on chromosome *VIII* (SIBLEY and BOOTHROYD 1992). Upstream of this gene are a series of 27 nucleotide repeats (BURG *et al.* 1988) of unknown function. Genomic Southern blots were initially used to identify a RFLP identified by *Rsa*I sites that flank these repeats in the genome. This upstream region was subcloned as a *Hin*dIII/*Bam*HI 900-bp fragment from a cosmid containing this genomic region (BURG *et al.* 1988) and the ends of the clone were sequenced. Oligonucleotides used to amplify the region surrounding the repeats and the *Rsa*I RFLP were: 5′AGCTTCAAAGACTGGTCGTTGCGA3′ and 5′CATACAACCGTGTGTTTACACGAC3′ (5′ C of this second oligo corresponds to position 314 within the sequence provided in BURG *et al.* 1988). Amplification was performed for 40 cycles of denaturation at 94° for 30 sec, annealing at 60° for 1 min, and extension at 72° for 1 min.

RFLP analysis and genetic mapping: Individual recombinant clones were genotyped by performing genomic Southern blot analyses using the set of 64 RFLP markers each matched with a respective informative restriction digest (Table 1). Linkage analysis was performed by testing for cosegregation of markers that was significantly different from the expected frequency of 0.5 using the X^2 test with a significance level of $P \leq 0.01$ (1 d.f., corrected for $N < 25$) (SOKAL and ROHLF 1969). The power of this test is such that the likelihood of concluding that two markers are linked when in fact the data may have arisen by chance is 1 in 100. Pairwise comparisons were conducted for all possible cosegregating loci and linkage groups were constructed. Simple linear maps of the loci mapped to each chromosome were constructed by estimating the minimum number of crossovers that would explain the inheritance data from all 19 progeny clones.

RESULTS

Identification of RFLP markers: Initially, a number of cDNAs, available in this lab or provided by

TABLE 1

DNA probes and the specific restriction endonucleases used to detect RFLPs identifying PLK and CEP strain alleles in an F_1 progeny of a genetic cross

Chromosome	Probe[a]	Enzyme(s)[b]	Source/reference
I	G1A	*Sau*96I [2.0 kb][c]	K
	c22	*Pst*I	K
	c29	*Bam*HI	K
	cS8	*Hha*I	
	cS9B	*Sau*96I [2.0 kb]	
II	L31	*Dde*I	K
	L351	*Rsa*I	K
	L379A	*Sau*3A	K
	G1B	*Sau*96I [multiple bands]	K
	c18B	*Eco*RV [5.0 kb]	K
	cB7	*Eco*RI	
	cB19	*Pst*I	K
	cS6B	*Bam*HI	K
	cS9A	*Sau*96I [1.4 kb]	
III	c28	*Pst*I, *Msp*I	K
	cA16	*Pst*I	
	cB21	*Bam*HI	K
	cB22	*Eco*RI, *Eco*RV, *Pst*I	
IV	226	*Hha*I	K
	Bc18	*Mbo*I	K[d]
	cA5	*Eco* RI	K
	cA15	*Pvu*II	K
	cB8	*Hin*dIII	K
	cB9	*Eco*RI	
	c16A	*Pvu*II	K
	c18A	*Eco*RV [1.8 kb]	K
	c20A	*Hin*dIII	K
V	Tb10 (*HSP1*)	*Sau*3A	K[e]
	850	*Dde*I	K
	GR	*Sau*3A	K
	L52	*Rsa*I	
	L353	*Sma*I	
	L354	*Bcl*I	
	L358	*Mbo*I	K
	F	*Dde*I	
	G	*Msp*I	
	c21	*Pst*I	K
	c27	*Eco*RI	K
	cS1	*Eco*RV	K
VI	L339	*Pvu*II, *Hin*dIII	K
	L363A	*Sau*3A	K
	c20B	*Eco*RI	K
VII	L328	*Rsa*I	K
	c16B	*Bam*HI, *Eco*RV	K
	c25A	*Eco*RI, *Eco*RV, *Pst*I	K
	cS4	*Eco*RV	
	cS7	*Pst*I	K
	cS10A	*Pvu*II	K
VIII	c88 (*SAG2*)	*Hha*I	K[f]
	p30.5c(*SAG1*)	*Rsa*I	K[g]
	L357	*Hha*I	
	L379B	*Hha*I	K
	c13	*Eco*RI	K
	c24A	*Eco*RI, *Eco*RV, *Pst*I	K

TABLE 1

Continued

Chromosome	Probe[a]	Enzyme(s)[b]	Source/reference
IX	TBT1 (*TUB2*)	*Hin*dIII	K[h]
	Tg4B(*RDN1*)	*Eco*RI	K[i]
	B1	*Hha*I	K[j]
	62A	*Pvu*II, *Msp*I	K
	L375	*Eco*RI	K
	L376	*Eco*RV	
	BS	*Msp*I	
	c1	*Sma*I	K
	c10	*Bam*HI	K
	cS2	*Bam*HI	K
X	L53	*Rsa*I	K
	L366	*Sau*3A	
	c5	*Pvu*II	K
	c6A	*Dde*I, *Pvu*II	
	c12	*Eco*RV, *Sma*I	K
	c19	*Bam*HI	K
	c3013	*Pst*I	K

K Probes mapped to individual chromosomes by DNA hybridization (SIBLEY and BOOTHROYD 1992).
[a] Clone names are provided in each case. Genetic names for probes with known coding function are given in parentheses where known.
[b] Refers to enzymes used for analysis of F_1 clones in the present study.
[c] In cases where the same restriction enzyme detects two separate RFLPs that map to distinct loci, the approximate fragment sizes are given.
[d] Obtained from the laboratory of JACK REMINGTON, Stanford University.
[e] MUHICH and BOOTHROYD (1988).
[f] PRINCE *et al.* (1989).
[g] BURG *et al.* (1988).
[h] NAGEL and BOOTHROYD (1988).
[i] JOHNSON, ILLANA and DUBEY (1987).
[j] BURG *et al.* (1989).

others, were analyzed by Southern blot to identify RFLPs between PLK and CEP strains. In addition to genes of known coding function, a number of anonymous cDNAs were included in this group. The majority of genes recognized by these probes were apparently low copy or single copy with the notable exceptions of probes detecting the rDNA (JOHNSON, ILLANA, and DUBEY 1987), a heterologous probe for *Trypanosoma brucei* HSP-70 (MUHICH and BOOTHROYD 1988), and two cDNAs of unknown function, B1 (BURG *et al.* 1989) and BS. To obtain additional random genomic inserts, further probes were selected from a genomic library containing inserts ranging from 500 to 4000 bp constructed in the plasmid Bluescript. The frequency of RFLPs varied, with low/single copy number cDNAs being the least polymorphic (Table 2). In general, only 1 in 12 restriction enzymes revealed a polymorphism with this set of probes. Low/single copy number random genomic inserts were slightly more polymorphic, however, with an average of 6.5 digests required to identify one RFLP between PLK and CEP. In contrast, multicopy cDNAs were far more polymorphic, identifying useful RFLPs in one-half of enzymes tested (Table 2). In all, 31 RFLP markers were identified among these two groups of probes, the majority being single or low copy number probes. While repetitive probes offer an advantage of easily identifiable RFLPs, these probes were not abundant in either the cDNA library or the genomic library and hence additional markers of this sort were not readily obtainable.

TABLE 2

Frequency of RFLPs between PLK and CEP strains detected by cDNAs, plasmid clones and cosmid clones

Source	No. probes tested	No. probes w/RFLP[a]	No. enzymes tested[b]	Percent enzymes w/RFLP[c]
cDNAs				
Single copy	16	9	24.4 (6–40)	8
Multicopy	4	4	6.75 (5–11)	57
Plasmid clones	25	18	17.5 (6–35)	15
Cosmid clones	60	47	6.4 (2–14)	45

[a] Number of probes where at least one RFLP was detected.
[b] Mean number of restriction enzymes used (range).
[c] Percentage of enzymes which detect at least one RFLP. Includes all probes tested.

To obtain probes with more easily identifiable RFLPs, we investigated the use of probes randomly picked from a genomic cosmid library (BURG *et al.* 1988). These probes average 35–40 kb in size and are thus more than 10 times the average size of the previous group of probes. A representative Southern blot for cosmid c3013 is shown in Figure 1. While the

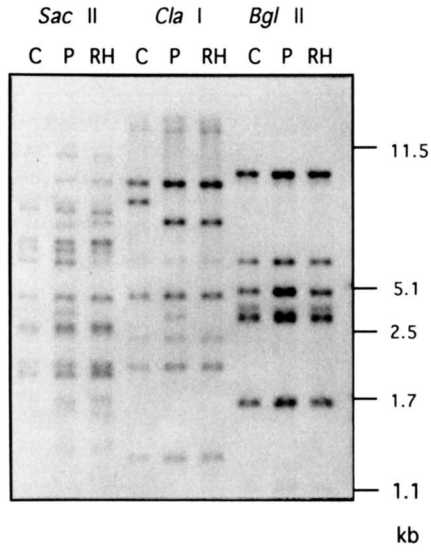

FIGURE 1.—Genomic southern blot analysis of three strains of Toxoplasma. While a majority of bands are shared between strains, informative RFLPs are readily identified using a cosmid, c3013, for a probe. Genomic DNAs were digested with the indicated restriction endonucleases, resolved by agarose gel electrophoresis and transferred to membranes for blotting. C refers to CEP strain, P refers to PLK strain, and RH to the RH strain of Toxoplasma.

majority of DNA fragments are of similar size in the three strains examined, RFLPs between PLK and CEP are easily identified. Using this method, RFLPs were rapidly identified for 47 out of 60 separate cosmid probes and approximately one-half of all restriction enzyme digests identified RFLPs (Table 2).

In all, only four cases of repetitive elements were identified among the 105 probes examined, as judged by the complexity of bands revealed by genomic Southern blotting. Three of these cases were due to different cosmid probes that each contained short tandemly repeated segments and in one case a dispersed repetitive element was identified by a cosmid probe. Restriction enzymes, *Bam*HI, *Eco*RI, *Eco*RV, *Hin*dIII, *Pst*I and *Pvu*II provided the appropriate complexity of bands for identifying RFLPs with cosmid probes. All of the DNA probes examined here detected similar restriction digestion patterns in Southern blots of both PLK and CEP strains, emphasizing the overall similarity of these two strains.

Isolation of recombinant progeny: A genetic cross between CEP and PLK strains was performed in duplicate using separate kittens as experimental hosts to obtain F_1 progeny for analysis. Because the parental strain CEP contained two drug resistance markers, recombinant progeny were easily selected by identifying clones that were singly resistant. Twelve clones were obtained from oocysts collected from the first infected kitten and these bear the prefix S followed by a clone number (Table 3). An additional nine clones were similarly isolated from a second kitten and these bear the prefix CL followed by a clone number. In three cases, CL12, CL14 and CL15,

TABLE 3

Recombinant F_1 clones were selected from progeny of two parallel crosses between PLK (Ara^S Snf^S) and CEP (Ara^R Snf^R) by analysis of drug resistance phenotypes or by PCR analysis at two unlinked loci

Clone	Phenotype drug selection		Genotype PCR loci	
	Snf[a]	AraA[b]	*850*	*SAG1*
S1T	S	R	P	C
S2T	S	R	C	C
CL17	S	R	P	P
CL18	S	R	C	P
CL11	R	S	P	P
CL13	R	S	P	P
CL16	R	S	C	C
CL19	R	S	C	C
CL21	R	S	C	C
S21	R	S	C	C
S22	R	S	P	P
S23	R	S	P	C
S25	R	S	P	P
S26	R	S	P	P
S27	R	S	P	C
S28	R	S	P	C
S30	R	S	P	C
CL12	R	R	P	C
CL14	R	R	C	C
CL15	S	S	P	C

R, resistant; S, sensitive. P refers to allele from PLK parent, C refers to allele from CEP parent.

[a] Sinefungin was used at a concentration of 2.7×10^{-7} M.

[b] Adenine arabinoside was used at a concentration of 1.3×10^{-4} M.

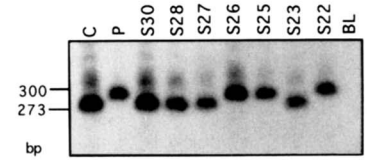

FIGURE 2.—PCR-based analysis of progeny clones based on the *SAG1* locus. Whole cell lystates were used to amplify a 900-bp fragment of the *SAG1* locus that was digested with *Rsa*I to reveal a strain specific RFLP. C refers to CEP parent, P refers to PLK parent, individual progeny are indicated by clone numbers, BL refers to a no DNA blank.

clones with parental drug sensitivities were retained for further analysis.

All of the clones isolated by drug screening (listed in Table 3) were subsequently checked by PCR to establish their genotype at two unlinked loci. An example of PCR based typing is shown in Figure 2 for amplification of the *SAG1* locus digested with *Rsa*1. In each of the progeny, a single allele is detected corresponding to the genetic pattern from either the CEP or PLK parent (Figure 2). The second locus analyzed was that identified by an unknown cDNA probe, 850 (see methods). The segregation of these two loci was random with respect to each other, confirming that the chromosomes bearing these two loci segregated independently (Table 3). In addition,

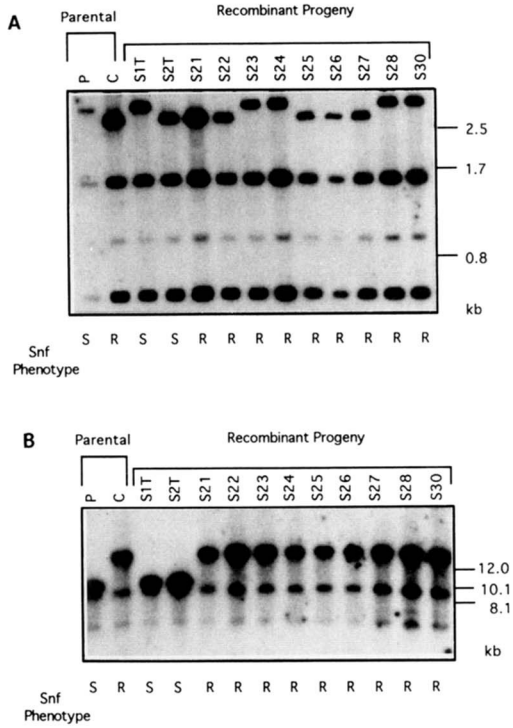

FIGURE 3.—Inheritance of parental alleles in recombinant progeny was scored by genomic southern blot analyses. Phenotypes are indicated as R, resistant or S, sensitive for the drug sinefungin (Snf). (A) The randomly isolated plasmid clone L328 was used to probe a Southern blot of genomic DNAs digested with *Rsa*I. This probe demonstrates random segregation with respect to Snf phenotype. (B) The cDNA TBT1 encoding the β-tubulin locus (*TUB2* gene) was used to probe a southern blot of genomic DNAs digested with *Hin*dIII. This probe demonstrates linkage with resistance to Snf.

SAG1 segregated randomly with respect to either drug sensitivity. While *850* segregated independently of Snf, it was associated with Ara resistance at a frequency greater than expected by chance alone. In two cases, where progeny had parental phenotypes (*i.e.*, doubly drug-sensitive or doubly drug-resistant), PCR analysis confirmed that these clones were in fact recombinants (CL12 and CL15) (Table 3). Clone CL14 proved to be nonrecombinant by PCR analysis of RFLPs at both the *850* and *SAG1* loci (Table 3) and by genomic blot analysis with an additional six RFLP markers (data not shown). This clone apparently represents a product of self-mating of the CEP Ara^R Snf^R strain and consequently was not analyzed further.

Inheritance data from F_1 progeny: The inheritance of RFLPs was examined in all 19 recombinant progeny by performing genomic Southern blots and determining the inheritance of parental alleles. In most cases the RFLP marker segregated independently with respect to drug resistance. An example of this inheritance is shown in Figure 3A for probe L328 which is a randomly selected plasmid clone containing a 2.8-kb insert apparently present as a single copy in the Toxoplasma genome. In contrast, other RFLP markers were clearly linked to drug resistance as demonstrated for probe TBT1, a single copy gene encoding β-tubulin (*TUB2* locus) (NAGEL and BOOTHROYD 1988), which is closely linked to the gene regulating Snf resistance (*snf-1*) (Figure 3B). In all, 10 cDNAs, 17 genomic plasmid clones, and 37 genomic cosmid clones were examined independently to establish the inheritance pattern of RFLPs among the 19 progeny. All progeny were found to have either the PLK or CEP allele at each respective locus and no examples of duplication or unequal crossover were observed. In seven cases, two independently segregating RFLPs were detected by the same probe and these have been named as separate loci by appending the letter A or B to the clone name as described previously in mapping probes by physical hybridization (SIBLEY and BOOTHROYD 1992). Presumably these probes contain sequences that are present in two or more homologous segments of the genome. Consequently, the 64 probes used here identify 71 independent genetic loci used in the construction of genetic maps below.

Linkage analysis for mapping drug resistance: To verify linkage, pairwise comparisons were made to statistically test the hypothesis that unlinked markers segregate independently. Based on the expected frequency of 0.5 for unlinked markers, two markers that cosegregate in 16 or more of the 19 progeny are considered linked ($p \leq 0.01$). The construction of linkage groups and orientation of RFLP markers within a group is most easily illustrated for the mapping of drug resistance to Snf which shows tight linkage to markers on chromosome *IX*. In Figure 4A, the inheritance patterns of RFLP markers on chromosome *IX* are shown for each of the 19 progeny as being inherited from PLK parent (solid circles) or CEP parent (open circles) along with the corresponding drug-resistance phenotype shown for each progeny clone. A linear chromosome map shown in Figure 4B depicts the distance between markers based on genetic units where 1 map unit is representative of approximately 1% recombination. These distances and the relative order of probes were calculated by estimating the minimal number of crossovers necessary to explain the inheritance data for all 19 progeny. For markers that share the same genotype for all 19 progeny the order of arrangement is arbitrary. Consequently while L375 is tightly linked to *snf-1*, probe TBT1 (*TUB2* locus) differs by a single recombination event in progeny clone CL15. Presuming the 1/19 ratio would hold for a larger sample of progeny clones, this places TBT1, approximately 5 map units from both *snf-1* and probe L375.

Construction of genetic maps: When all possible pairwise comparisons were examined, a set of 11 linkage groups emerged as consistent with the data. The inheritance data for the 19 recombinant progeny are shown in Figure 5 where probes have been

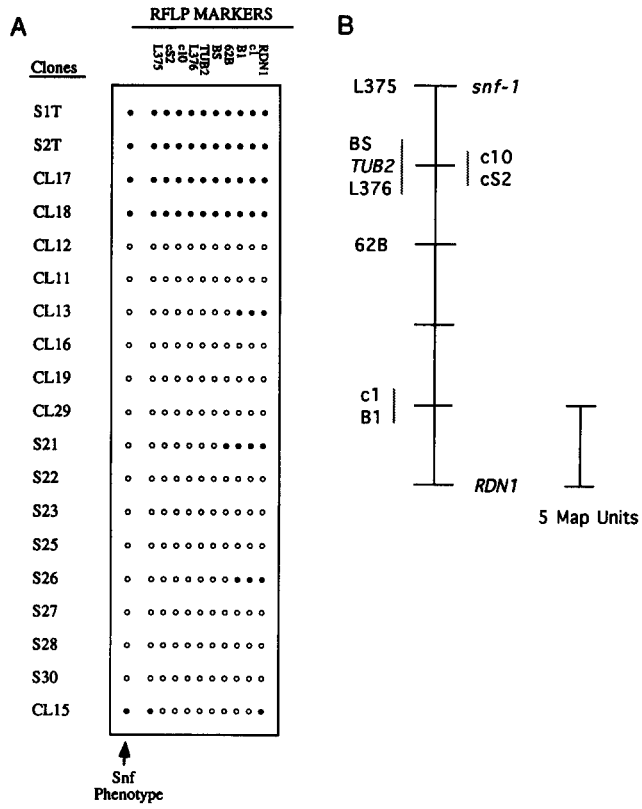

FIGURE 4.—Linkage analysis of RFLP markers on chromosome *IX* with resistance to Snf. (A) The inheritance of RFLP markers for individual progeny clones are indicated diagrammatically using solid circles to represent the PLK parental allele and open circles to represent the CEP parental allele. Individual markers are placed in linear order based on recombination events. The phenotype of resistance to Snf is also plotted using similar symbols where solid circles represent sensitivity (PLK parent) and open circles represent resistance (CEP parent). RFLP marker L375 shows complete correlation with resistance/sensitivity to Snf. (B) The same data in A has been used to construct a linear chromosome map that places RFLP markers along the chromosome based on recombination events. In this representation, a single recombination event in 1 of 19 progeny represents approximately 5 map units or one node along the linear map. The probes have been ordered to minimize the number of recombinations necessary to explain the inheritance data in all 19 progeny. Where different RFLP markers share the same genetic data for all 19 progeny (*i.e.*, c1 and B1) they are placed at the same point on the genetic map and their relative order in A is arbitrary. ○ = C strain Snf^R Ara^R; ● = P Strain Snf^S Ara^S.

grouped into their respective linkage groups. These linkage groups are numbered based on the prior mapping of probes by DNA hybridization to chromosomes separated by pulsed-field gel electrophoresis (SIBLEY and BOOTHROYD 1992). The markers are relatively equally distributed among the chromosomes with the exception of *VI* which has fewer markers than the others based on relative size estimates (SIBLEY and BOOTHROYD 1992). Based on the criteria presented above, a linear map for each chromosome was constructed showing the order and relative genetic distances separating markers (Figure 6). In cases where two or more probes co-segregate, they are listed as lying together at the same junction on the linear genetic maps and their order in Figures 5 and 6 is arbitrary. As an aid to the reader and to facilitate identification of further chromosome markers, a number of DNA probes that have been mapped solely by physical hybridization (SIBLEY and BOOTHROYD 1992) are indicated in boxes beneath their respective chromosomes in Figure 6.

The maximum recombination distance allowable between any two adjacent markers on the genetic map is approximately 15 map units as defined by the statistical limitations of the X^2 test. There were only two cases where the genetic distances exceeded this cutoff value for establishing linkage (shown in parentheses in Figure 6). In the case of drug sensitivity to Ara, the best linkage observed was approximately 20 map units from marker L353 on chromosome *V*. The gene encoding the major surface antigen, *SAG1* on chromosome *VIII*, also involves a gap of approximately 20 map units from the RFLP marker most tightly associated to it, L357. The mapping of these two markers therefore must be considered preliminary pending the analysis of additional progeny and/or the identification of more closely linked markers.

When data from the individual chromosomes are considered separately, the majority exhibited the expected 50/50 segregation of parental alleles. As expected, chromosomes *V* and *IX*, which are linked to drug resistance markers, differed from this 50/50 ratio as a result of the majority of clones having the phenotype of Snf^R Ara^S. The only exception was seen in the two smallest linkage groups, IA and IB, where 15 out of 19 progeny have inherited the CEP allele. Despite this unequal inheritance, neither group is linked to drug resistance or to each other and consequently the reason for this bias is unclear.

Surprisingly, evidence for intrachromosomal recombination was evident in no more than 3 of the 11 chromosomes in the majority of progeny. In examining the markers on chromosome *IX* (Figure 4), 15 of 19 progeny appear to have inherited this chromosome from a single parent without apparent crossover. This pattern was not limited to chromosomes linked to the drug sensitivity phenotypes as crossovers appeared with roughly equal frequency among the majority of chromosomes (Figure 5). On several chromosomes, crossovers were exceedingly rare or absent. The most extreme cases being IA, IB, where no crossovers are detected; although, this may be due to the low number of genetic markers mapped to these groups. In three cases, CL17, S23 and S25, progeny are seen to inherit all of their chromosomes without apparent intrachromosomal crossovers (Figure 5).

DISCUSSION

We have constructed a working genetic map for *T. gondii* by analyzing the segregation of 64 RFLP mark-

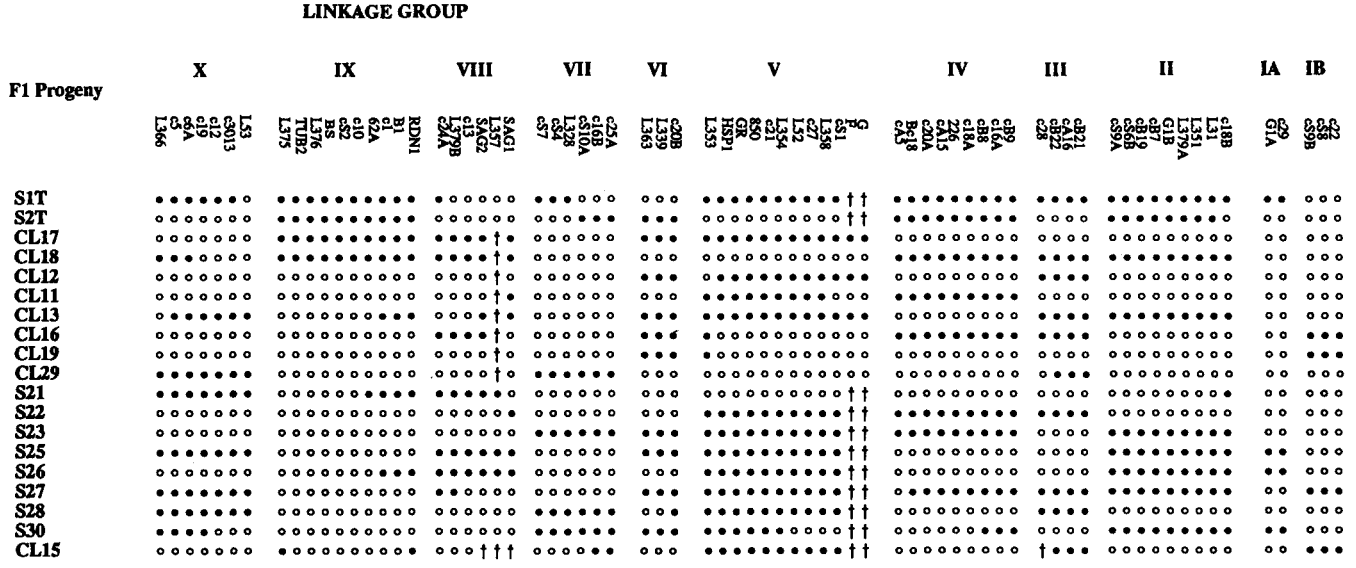

FIGURE 5.—The inheritance of PLK *vs.* CEP alleles at each of 71 loci is shown for the 19 recombinant progeny as defined by linkage analysis. Markers are ordered based on the minimum number of recombinational events consistent with the genotypes of all 19 progeny. Where more than one marker cosegregates, their relative order is arbitrary. Solid circles refer to the PLK allele and open circles refer to the CEP allele. Dagger indicates not done.

ers among 19 recombinant progeny from two parallel genetic crosses. The genetic map indicates the Toxoplasma genome consists of 11 distinct chromosomes totaling at least 147 centimorgans. A total of 48 of the probes used here were previously mapped to individual chromosomes separated by pulsed-field gel electrophoresis (SIBLEY and BOOTHROYD 1992). In all cases, there is agreement between the placement of probes into linkage groups by physical mapping and by genetic analysis. Furthermore, this comparison indicates that each of the gel-separated bands constitutes a single genetic linkage group with the exception of the fastest migrating band I. Based on earlier estimates of ethidium bromide staining, this band is expected to be at least a doublet. Genetic analysis indicates there are at least two distinct genetic linkage groups that comigrate in band I, denoted here as IA and IB. In the physical karyotype analysis, one group of probes hybridized exclusively to the plug material, indicating the presence of one or more molecules too large to enter the gel (SIBLEY and BOOTHROYD 1992). When 7 RFLP markers from this group were mapped by segregation analysis, only one tightly linked group was evident, denoted here as chromosome *X*.

For all of the RFLP markers, each progeny inherited either the PLK or CEP allele with no cases of unequal crossover, nondisjunction, or aneuploidy being detected. The majority of chromosomes are well represented having from 4 to 10 individual RFLPs that span 10–25 map units. The exception to this is chromosome *VI*, where only three RFLPs were identified despite the relatively large size of this molecule (estimated at 4.0 Mb) (SIBLEY and BOOTHROYD 1992). This may be due to a higher than average similarity between strains PLK and CEP on this chromosome, that would reduce the likelihood of obtaining RFLP markers from this region. For four of the markers mapped to chromosome *VI* by physical hybridization (see Figure 5), extensive screening with restriction enzymes failed to identify any RFLPs between PLK and CEP strains. Apart from this specific case, the use of cosmids as RFLP probes generally provided a rapid means of identifying new markers with a small number of restriction digests. While RFLP markers were often difficult to identify for individual single copy cDNAs it should be possible to readily identify RFLPs that are closely linked by first selecting for cosmid clones that contain the cDNA of interest.

RFLP markers from different chromosomes were observed to segregate independently and in the majority of cases no bias in parental inheritance was observed. An exception to this was observed for linkage groups IA and IB, where a higher than expected number of progeny inherited the CEP allele. The reasons for this bias are presently not clear; however, as the two parental strains differed slightly in growth characteristics, with CEP being the more vigorous, this bias could reflect a slight growth advantage during expansion of the progeny.

In seven cases, a single probe detected two different RFLPs that segregated independently and these have been treated as separate loci. A similar finding was previously observed when mapping probes by hybridization to gel-separated chromosomes (SIBLEY and BOOTHROYD 1992). There is agreement between these two methods, with all examples of probes that detect two distinct genetic loci also being confirmed

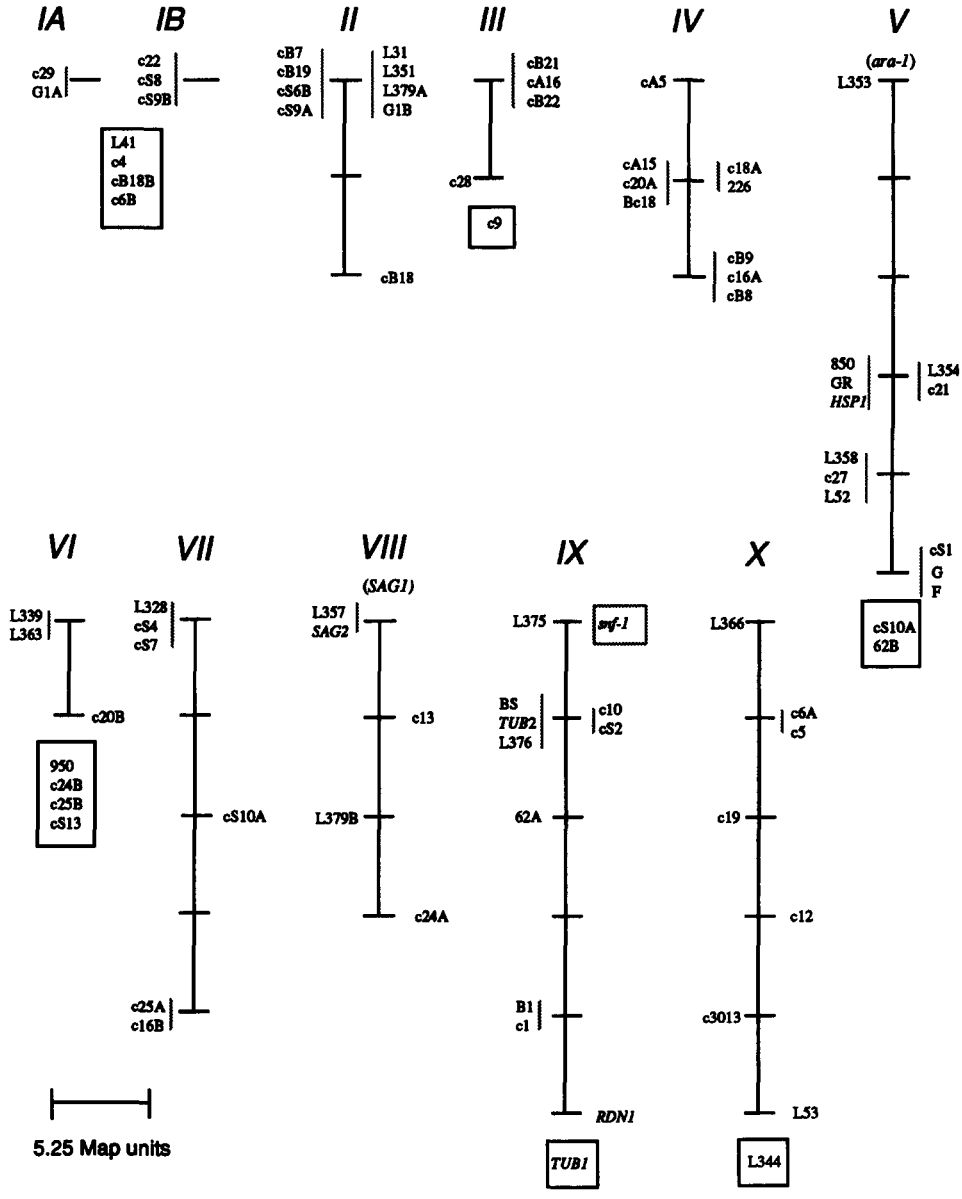

FIGURE 6.—Linear genetic maps are shown for each of the 11 chromosomes in the Toxoplasma nuclear genome. Probes have been ordered based on the minimum number of recombination events that explain the inheritance in all 19 progeny. Probes that have been mapped solely by hybridization to gel-separated chromosomes are indicated in a solid line box below the genetic map of their respective chromosomes. The locus that controls Snf resistance (*snf-1*) maps to chromosome *IX*. The two markers that were mapped with a statistical accuracy of only $P \leq 0.05$ are indicated in brackets (*ara-1* and *SAG1* on chromosomes *V* and *VIII*, respectively). All other linkages are supported by a statistical significance of $P \leq 0.01$.

by physical hybridization to these same two chromosomes. While we have not examined these probes in detail, a simple explanation is that these constitute DNA segments that are repeated with the second copy lying on a distinct chromosome. While these low copy number repeats are observed in approximately 10% of randomly selected probes, very little evidence was found for more abundant classes of repetitive DNA. In a total of 60 cosmids and 45 plasmid or cDNA probes screened, only a single dispersed repeat element was identified. This repeat has been described elsewhere and constitutes a class of mitochondrial-like pseudo-genes dispersed in the nuclear genome of Toxoplasma (OSSORIO, SIBLEY and BOOTHROYD 1991). The low prevalence of repetitive elements encountered in using randomly isolated cosmids for RFLP analysis is of practical advantage that should facilitate both genetic and physical mapping strategies in this system.

The majority of chromosomes exhibited low to moderate intrachromosomal recombination that allowed mapping of the loci regulating drug resistance by linkage analysis. Sinefungin is thought to act by inhibiting methylation reactions involving S-adenosylmethionine (FULLER and NAGARAJAN 1978; BORCHARDT *et al.* 1979). The biochemical basis of the mutation conferring resistance to Snf in the parental CEP strain used here is not known and this clone grows normally both *in vitro* and *in vivo*. By analyzing the segregation of drug sensitivity in the 19 progeny, the locus governing this phenotype, *snf-1*, was mapped to RFLP marker L375 on chromosome *IX*. The identification of this correlated marker provides a physical landmark that may be useful in the identification and

isolation of a gene(s) likely to be involved in methylation reactions in Toxoplasma.

In the case of Ara, less precise mapping was obtained and the best correlation with drug sensitivity was observed with RFLP L354 on chromosome V. The biochemical basis for Ara resistance in the CEP parental strain has previously been shown to result from lack of adenosine kinase (PFEFFERKORN and PFEFFERKORN 1978) which normally phosphorylates adenine arabinoside, allowing this toxic metabolite into the nucleic acid biosynthesis pathway. Toxoplasma is a purine auxotroph and must salvage purines from the host. However, this Ara^R mutant grows normally indicating that adenosine kinase is nonessential, probably due to the presence of redundant purine salvage pathways in Toxoplasma (PFEFFERKORN and PFEFFERKORN 1978; KRUG, MARR and BERENS 1989). The development of genetic systems in Toxoplasma may offer the best potential for identifying the adenosine kinase gene and for examining the interactions of *ara-1* mutations with other purine salvage pathways in this system.

In developing genetics as an experimental approach in Toxoplasma, we are interested in estimating the basic genetic parameters of this system as guidelines for future application. In addition to mapping genes to RFLP markers on individual chromosomes, the "map unit" provides the most generally useful estimate of recombination distances. For using RFLP markers as correlates of unknown genes, this estimate is particularly important for identifying markers that are successively closer to an unknown gene of interest. In order to estimate the recombination frequency in Toxoplasma, we made the following assumptions: (1) the outermost RFLP markers on any given chromosome lie at or near the true ends of the chromosome (supported by the random selection of the markers used here) and (2) the size of individual chromosomes is accurately estimated by gel migration as previously reported (SIBLEY and BOOTHROYD 1992). Based on these assumptions, the average recombination frequency is estimated to range from 120 kb (*e.g.*, chromosome *V*) to 300 kb (*e.g.*, chromosomes *IV* and *VII*) per centimorgan (cM); as with other genetic systems, this value varies considerably from one chromosome to another. Alternatively, if the map unit is estimated based on the total genome size of 80 Mb (CORNELISSEN, OVERDULVE and VAN DER PLOEG 1984), there is on average, 0.5 Mb per cM. The recombination distance (map unit) is large in Toxoplasma relative to yeast (1–4 kb/ cM) and is about 10-fold higher than in the malaria parasite *P. falciparum* (WALKER-JONAH *et al.* 1992), but is comparable to that observed in organisms with similar sized genomes such as *Arabidopsis thaliana* (CHANG *et al.* 1988) and *Caenorhabditis elegans* (COULSON *et al.* 1986).

The above estimates of the map unit in Toxoplasma are obviously preliminary and will require further physical and genetic mapping to refine. In order to establish the approximate physical distances between RFLP markers, we have begun constructing a large scale physical map of chromosome *V* using restriction endonucleases that cleave the Toxoplasma genome into 100–500-kb fragments. These preliminary studies indicate that the RFLP markers on chromosome *V* span at least 2.5 Mb in total size (L. D. SIBLEY, unpublished) corresponding with the size estimate of 3.6 Mb based on gel separation (SIBLEY and BOOTHROYD 1992). This result suggests that failure to identify markers closely linked to *ara-1* on chromosome *V* may have resulted from having too few markers in this region of the chromosome.

In the case of both chromosomes *V* and *VIII*, the true genetic size is likely larger than that estimated in the genetic map provided in Figure 6. In both cases, one additional marker has been tentatively mapped to these chromosomes with a statistical validity of $P \leq 0.05$. While this is beyond the cut-off value for linkage as defined here ($P \leq 0.01$) these markers do not show even partial linkage to any other group and in both cases have been mapped to their respective chromosomes by physical hybridization (SIBLEY and BOOTHROYD 1992). Additionally, the relatively small number of markers on chromosome *VI* likely underestimates its true genetic size. The further mapping of additional DNA markers and mutant phenotypes will be useful for completing these portions of the genetic map.

From the data presented here it is clear that preliminary linkages will be easily established for newly selected DNA markers or for mapping phenotypes of interest. However; identifying markers that are progressively closer to unknown genes of interest may require the examination of large numbers of progeny in order to identify crossovers that are informative. There are several features of Toxoplasma that make this approach feasible. First, a single cross yields $>10^7$ oocysts that can be stored for several years prior to analysis. Second, the use of PCR for identifying recombinant clones saves considerable time, as individual progeny can be typed from small numbers of cells grown in microwell cultures, eliminating the need for expansion of stocks that are not of interest. PCR based screening, as demonstrated here using two RFLP markers adapted for PCR analysis, could easily be applied to any set of RFLP markers flanking a genetic region of interest.

In a majority of progeny, crossovers in a majority of chromosomes were not observed (*i.e.*, 83% of all chromosomes failed to show evidence of crossovers) and consequently all of the RFLP markers on a given chromosome were inherited from a single parent.

This was most dramatic in clones CL17, S23 and S25 where no crossovers were observed in any of the 11 chromosomes. This low degree of crossing over was not simply a result of selecting clones by virtue of their recombinant drug phenotypes, as clones CL12 and CL15, which had parental phenotypes, exhibited similar low levels of recombination. Similarly, in recent crosses of the malaria parasite, *P. falciparum*, some chromosomes were inherited without apparent crossover (WALKER-JONAH *et al.* 1992), although much less frequently than observed here in Toxoplasma. The low rate of crossover observed in Toxoplasma may explain previous microscopic observations that failed to find evidence for synaptonemal complexes in *Eimeria tenella*, a closely related protozoan parasite that causes disease in poultry (CANNING and ANWAR 1968). Despite the lack of apparent crossovers in Toxoplasma, individual chromosomes segregated independently and no occurrences of unequal crossover or recombination between non-homologous chromosomes were observed.

The low rate of intrachromosomal crossover in Toxoplasma is somewhat surprising in light of models which suggest that synaptonemal complexes and recombination may be essential for proper sorting of chromosomes during meiosis. The evidence for this model comes largely from yeast mutants with defects in chromosomal pairing that fail to undergo proper sorting in meiosis I and consequently produce inviable spores (BHARGAVA, ENGEBRECHT and ROEDER 1992). However, studies using yeast artificial chromosomes have indicated that even nonhomologous artificial chromosomes can pair at meiosis I, and their extent of recombination does not affect the sorting fate of these molecules (DAWSON, MURRAY and SZOSTAK 1986; MANN and DAVIS 1986). In these studies, a recombination-independent system is implicated in sorting molecules which either have no homolog or have not formed a chiasma and undergone recombination. Recombination-independent sorting typically involves much lower levels of crossover, yet is remarkably accurate in distributing chromosomes to daughter cells with greater than 90% fidelity (DAWSON, MURRAY and SZOSTAK 1986; MANN and DAVIS 1986).

Whereas in yeast, recombination-independent sorting appears to be a less favored pathway, such a system may predominate in Toxoplasma. While this would explain the observed absence of recombination in most chromosomes, the physical location of the RFLP probes here has not been established and conceivably these markers may be clustered in a way that underestimates the frequency of recombination.

There are several feature of the Toxoplasma genome that should facilitate application of classical genetic analyses for the identification and isolation of unknown genes. The genome is relatively stable with continued mitotic passage and has chromosomes that are easily resolved by pulsed-field gel electrophoresis. Several laboratory strains that have been examined are relatively similar allowing mapping data to be readily compared between strains (L. D. SIBLEY, unpublished). Randomly selected cosmid DNA probes provide easily identifiable RFLPs with minimal complication from repetitive DNA sequences. There is a low to moderate recombination rate that facilitates genetic mapping. Because all the progeny of meiosis are believed to be contained within a single oocyst, it should be feasible to perform tetrad analysis in Toxoplasma. Hence, Toxoplasma provides a readily addressable experimental system for the application of genetics toward understanding aspects of intracellular parasitism. In addition to mapping the basis of drug resistance phenotypes involved in key biochemical pathways, the genetic map establishes a set of landmarks for genetic and physical mapping of genes that regulate important biological phenotypes including virulence and drug resistance.

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