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The *Giardia lamblia* trophozoite contains sets of closely related chromosomes

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ABSTRACT

Size variations in homologous chromosomes from six Giardia lamblia isolates have been demonstrated. Four or five intensely stained (major) bands as well as a variable number of lightly stained (minor) bands are present in pulsed field gradient separations. Southern blot analysis with total chromosomal DNA as well as chromosome specific probes indicates that each minor band cross-hybridizes with a major band. Minor bands of doubly cloned organisms appear identical to those of parent clones, indicating that the minor bands do not reflect the presence of variant members within the total population of trophozoites. Densitometric comparisons of chromosome bands from known numbers of Plasmodium falciparum ring stage forms and known numbers of Giardia trophozoites suggest that minor bands MBa and MBb are present in each Giardia trophozoite. Comparison of Not I restriction fragments from the major and minor bands reveals common restriction fragments. Taken together, the data imply that sets of closely related chromosomes occur in the Giardia trophozoite.

INTRODUCTION

Giardia lamblia is a flagellated protozoan parasite that is a common cause of diarrhea in countries throughout the world (1, 2). Its life cycle consists of two stages, the trophozoite and the cyst. The trophozoite replicates in the small intestine and can cause disease in the susceptible host. A portion of the trophozoites encyst in the small intestine and are excreted in the feces. Other hosts are subsequently infected by ingestion of the cysts.

Trophozoites have two nuclei that are morphologically identical and divide almost simultaneously (3). During encystment, each nucleus of the trophozoite apparently divides to form two cyst nuclei, producing a cyst with four nuclei that are morphologically identical. During excystment, a trophozoite with four nuclei is formed from the cyst and undergoes fission to produce two trophozoites with two nuclei each (4). Whether the two nuclei of the trophozoites possess homologous or totally different chromosomes is unknown as is the ploidy of each nucleus.

Genetic analysis of Giardia is hindered by the inability to study sexual recombination in this organism. In addition, the small size of the chromosomes makes it impossible to study Giardia chromosomes using classical cytogenetic techniques. A previous report presented light microscopy drawings that demonstrated four chromosomes in each nucleus of Giardia trophozoites (probably G. muris) (4), but that work has not been confirmed by electron microscopy (5). In this report, we describe studies of Giardia chromosomes by pulsed field gradient gel electrophoresis (PFGE), which has proven to be a powerful method of karyotype analysis in yeast (6 - 8) and protozoa (9 - 12). We demonstrate size polymorphism of homologous chromosomes among different isolates and present evidence that sets of closely related chromosomes occur in the Giardia trophozoite.

MATERIALS AND METHODS

Organisms

The G. lamblia isolates used in this study have been described (13), including five human (JH, ISR, WB, GS/M, P-1) and one cat (CAT-1) isolate. The GS/M, P-1, and CAT-1 isolates are labeled GS, P, and CAT, respectively. Trophozoites were grown in modified TYI-S-33 medium (14) and harvested in late log or early stationary phase except where noted otherwise. Trophozoites were harvested by centrifugation at 4000×g for 10 minutes, washed with TSE (NaCl 100 mM, EDTA 50 mM, Tris HCl, pH 8, 10 mM) and centrifuged at 4000×g for 10 minutes. PFGE blocks were prepared and digested as described (15) in EPS (EDTA 500 mM, Proteinase K 2 mg/ml, Sarkosyl 1%). Plasmodium falciparum XP5 (15) and Saccharomyces cervisiae [strain BWGI-7a provided by Dr. Leonard Guarente (16)] were prepared as described (15, 17). Yeast blocks were digested overnight in lyticase (Sigma, St. Louis, MO; 2 mg/ml) before digestion in EPS.

Giardia trophozoites were cloned by limiting dilution (18). The medium from cultures was decanted and replaced with fresh medium to select only organisms adherent to the culture tubes. Tubes were chilled for at least 10 minutes in ice water and inverted vigorously to shake loose adherent organisms. Trophozoites were counted, diluted to 0.5 and 1 organism per ml, plated on 96 well plates at 200 µl/well, and incubated at 37°C in an anaerobic environment (Bio-Bag Environmental Chamber Type A, Marion Scientific, Kansas City, MO).

When used to estimate chromosome number per organism, Giardia trophozoites (ISR clones E11 and G5) were quantitated with a Coulter counter and

diluted to 1.25×10^8 organisms/ml (2.5×10^8 nuclei/ml). Cultures of P. falciparum clones Dd2 (Wellems et al., unpublished) and 3A-B4 (Panton, et al., unpublished) were synchronized with 5% sorbital (19) and harvested when 10 - 15% ring forms were present 6 hours after completion of reinvasion. Parasite counts were determined from examination of 50 - 100 fields throughout a blood smear prepared at the time of harvest. Schizonts and trophozoites in no instance accounted for more than 1% of the parasites. Parasite numbers were obtained from the product of the infection rate and the total number of red cells collected. The cells were pelleted, washed with TSE, lysed with saponin as described (15), and recovered by centrifugation at 6000×g for 30 minutes at 4°C. All erythrocyte ghosts were included in the suspension to ensure quantitative recovery of parasites. The suspension was cast into PFG blocks at a final concentration of 2.5×10^8 organisms/ml.

Pulsed field gradient gel electrophoresis (PFGE) of chromosomes

PFGE was performed for 8 days in the apparatus described by Carle and Olson (7) with switching times of 11 minutes at 80V for 2 days, 25 minutes at 65V for 2 days, and 1 hour at 50V for 4 days.

Probes

DNA from individual bands was obtained from preparative PFGE gels using low melt agarose (SeaPlaque by FMC). The bands were cut out, melted at 65°C, and digested for 2 hours with Eco RI at 37°C. DNA was purified using Elutip-d columns (Schleicher and Schuell, Kenne, NH) by recommended procedures. Band-specific DNA probes were isolated from a WB Giardia plasmid genomic library (13) by colony hybridization using DNA from individual bands as probes. The specificity was confirmed by probing Southern blots of PFGE gels of the JH isolate.

DNA was labeled with ^{32}P dCTP by the random primer method (20) and the unincorporated isotope was removed using G50 Sephadex column chromotography. Southern blotting and hybridization were performed as described (21) and blots were washed at high stringency (65°C, 0.1X SSC, 0.1% SDS).

Restriction enzyme digests

Bands were cut from preparative PFGE gels and the ethidium bromide extracted with sec-butanol (15). After equilibration in TE (Tris pH 7.5 10 mM, EDTA 1 mM), blocks were equilibrated overnight in 1X Not I enzyme buffer at 4°C. Digestion with Not I (New England Biolabs, Beverly, MA) was performed at 37°C for 4 hours in 200 µl buffer with 20 U enzyme added initially and 20 U added after 2 hours. PFGE was performed at 160V for 40 hours using a 1.5%

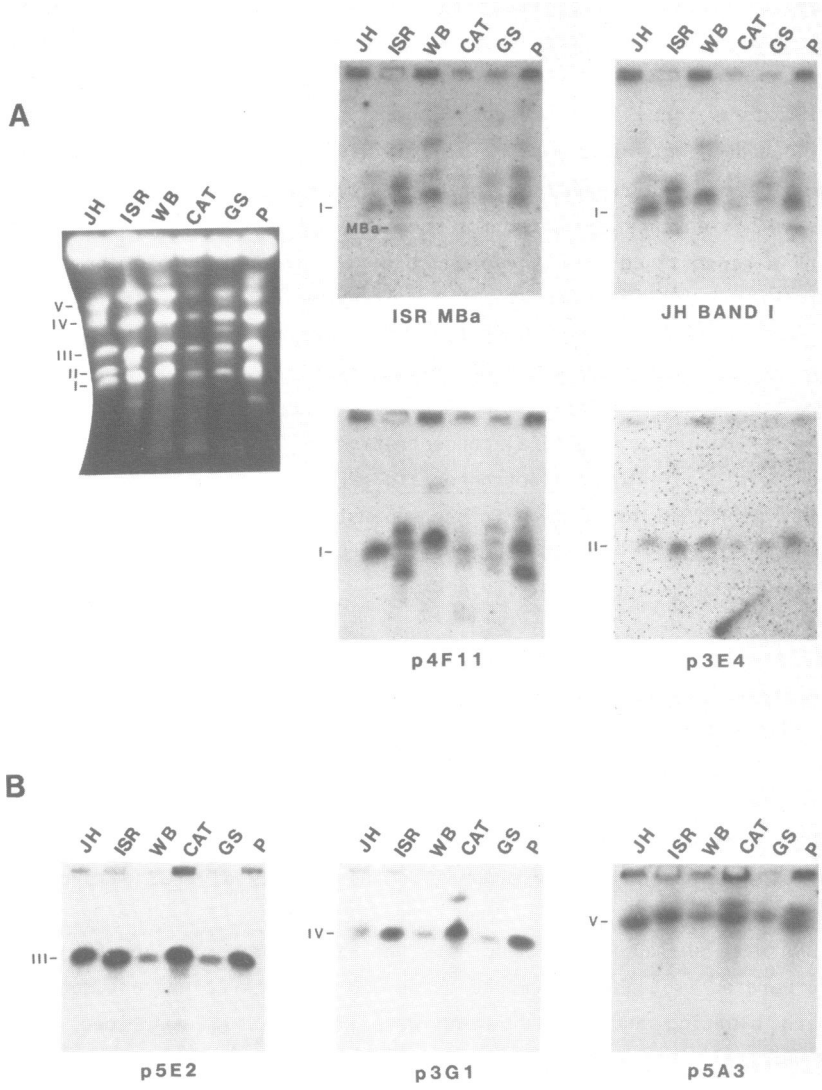

Figure 1

A. PFGE of six Giardia isolates. Major bands are labeled I through V. The DNA was transferred to nitrocellulose and probed successively with DNA from the lower minor band of ISR (MBa), band I of the JH isolate, p4F11 (specific for band I of JH) and p5E5 (specific for band II of JH). The blot was stripped with NaOH (15) between hybridizations.

B. Blots of the same six isolates hybridized with probes specific for bands III (p5E2), IV (p3G1), and V (p5A3). (See Table I).

agarose gel and switching times of 60 or 15 seconds. λ ladders were used for size standards (15).

RESULTS

Six Giardia isolates were analyzed by pulsed field gradient gel electrophoresis (PFGE) (Figure 1A). All had four (ISR, WB, CAT-1, GS/M, P-1) or five (JH) intensely stained bands (major bands), and many had one or more lightly stained bands (minor bands). Labeled total chromosomal DNA from individual bands and band-specific probes (Table I) were used to define homologous chromosomes for the various isolates. All probes specific to either JH band I or II hybridized to the lowest major band of each of the other

Table I. Band-Specific Probes Hybridization

Probe	Band of JH	Not I Frag Size (kb)
p4F11	1	170
p5D2	1	320
p3E4	2	300
p4A8	2	300
p4E11	2	
p5E5	2	150
p3C1	2	
p3F1	3	
p5E2	3	
p3G1	4	
p4E10	4	
p3D1	4	
p4B7	5	
p5A3	5	
p5D5	5	
p6E11	5	

Recombinant WB Giardia DNA probes specific for JH bands I through V. Some of the probes were hybridized to Not I digests of individual bands of the JH isolates (right column).

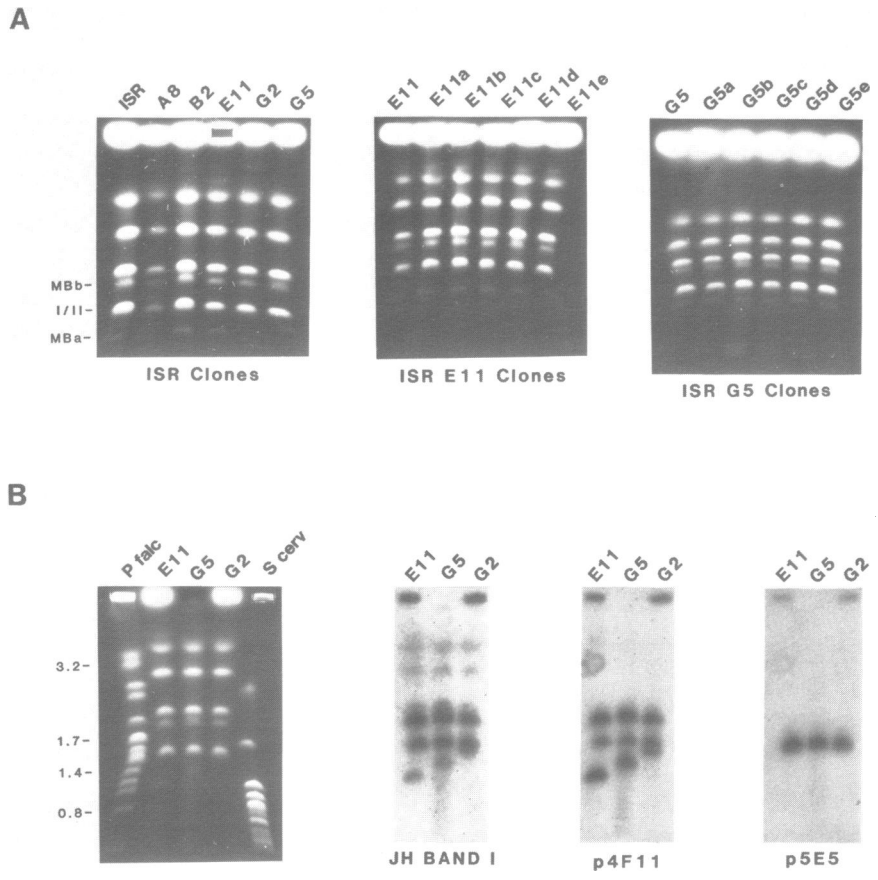

Figure 2

A. Left-PFGE of ISR (uncloned) and five clones of ISR, middle-PFGE of the ISR E11 clone and five subclones (doubly cloned organisms). Right-PFGE of the ISR G5 clone and five subclones.

B. PFGE of three ISR clones (E11, G5, G2) with P. falciparum and S. cervisiae for comparison. The DNA was transferred to nitrocellulose and probed successively with JH band I, p4F11 (specific for JH band I), and p5E5 (specific for JH band II). The blot was stripped with NaOH (15) between hybridizations. Note the ethidium stained "halo" surrounding the embedded agarose plugs which is not present in the P. falciparum or S. cervisiae lanes. The halo itself probably does not arise from DNA for the following reasons: 1) it is diffused in all directions from the well rather than migrating with the electrical current; 2) the material in the halo labeled poorly when excised and random primed; in contrast, the residual material from the well itself was labeled to high specific activity and hybridized proportionately to the well and each of the chromosomal bands (data not shown).

isolates. In addition, the lowest major band was stained with ethidium more intensely than the band above it in the isolates with four major bands, providing further evidence that the lowest major band of these isolates contained DNA complementary to both JH bands I and II. Therefore, the bands were labeled I through V according to the JH isolate, and the lowest major band of the other isolates was labeled band I/II.

A probe specific for band I of isolate JH (p4F11) hybridized to band I of all six isolates and, in addition, yielded signals of comparable intensity from minor bands of several isolates (Figure 1A). Identical results were obtained when a different band I-specific probe (p5D2) was used (data not shown). Similar patterns were found when DNA from the lower minor band of the ISR isolate (ISR MBa) or band I of the JH isolate were used as probes (Figure 1A). Although there was some cross-hybridization with chromosomal bands II through V when Isr MBa and JH band I were used as probes, the hybridization to band I and the minor bands was much more intense. The high degree of similarity between band I and MBa thus indicated that band I contains DNA which is closely related to the DNA in MBa as well as some other minor bands, but differing in size or structure.

In contrast, p3E4 (specific for band II) hybridized only to JH band II and band I/II of the other isolates (Figure 1A). Probes for bands III, IV, and V (Figure 1B) showed less heterogeneity than band I; however, the CAT-1 isolate had a lightly stained minor band near the top of the PFG separation that hybridized with a band IV-specific probe, and several isolates had other minor bands that hybridized with a band V-specific probe (Figure 1B). Blots from these six isolates were probed with at least two distinct probes specific for each band (data not shown) and in each case, results were the same.

In order to investigate whether the minor bands were size polymorphisms of a chromosome in subpopulations of Giardia, trophozoites from the ISR isolate were cloned by limiting dilution and five of the clones were randomly chosen for further investigation. Minor bands were observed in all clones, but different mobilities were seen (Figure 2) for the lower minor band (MBa). Clones A8, B2, and E11 yielded identical patterns, while MBa in clone G5 migrated more slowly, and MBa in clone G2 could not clearly be distinguished from band I/II by ethidium staining. The pattern of the upper minor band (MBb) was the same for each clone (Figure 2).

Two clones, E11 and G5, were recloned and five clones from each were chosen randomly for further investigation. Each showed the same pattern as

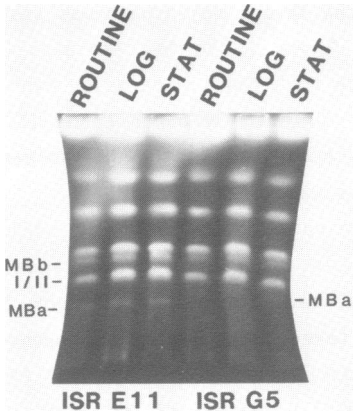

Figure 3
PFGE of routinely prepared organisms, log phase, and stationary phase organisms from ISR clones; clone E11, left 3 lanes, and clone G5, right 3 lanes. No differences are seen between the minor bands of log and stationary phase organisms.

its immediate parent (Figure 2A). This demonstrated that minor bands indeed occurred in individual organisms and did not merely represent diversity of mixed populations. The minor bands were stable for the 20 generations required from cloning to recloning; however, four different clones of the WB isolate that had been in continuous culture for 50 to 100 generations did show diversity when probed with p4F11 (data not shown). Therefore, size or structural polymorphisms of the minor bands can develop within 50 to 100 generations in axenic culture.

To investigate whether the minor bands are present only during a particular phase of the growth cycle, we prepared PFGE blocks from the logarithmic and stationary phases of growth for ISR clones E11 and G5. Log phase organisms were harvested at concentrations of 1.6 and 1.3 x 10^5 organisms/ml, and stationary phase organisms at 1.9 and 1.8 x 10^6 organisms/ml, respectively. When these preparations were subjected to PFGE, there was no discernable difference between organisms from log or stationary phase (Figure 3).

Information regarding the stoichiometry of the minor bands was obtained by comparing equal numbers of Giardia and P. falciparum nuclei. Since the intensity of DNA staining is proportional to the quantity of DNA represented by an ethidium stained band (15), laser densitometry can be used to estimate relative amounts of DNA contained in different bands. Early erythrocyte ring

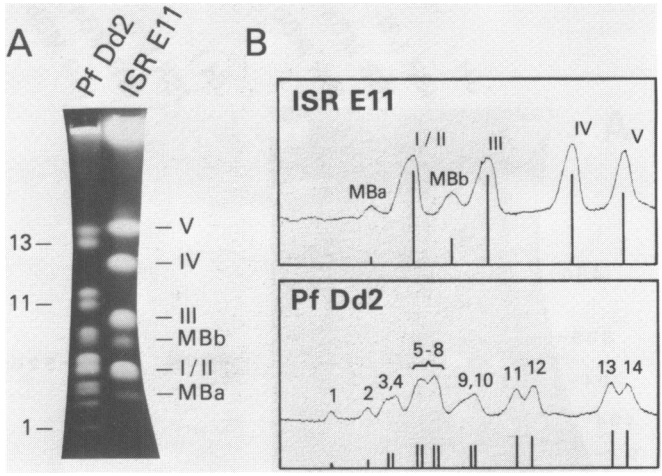

Figure 4

A. PFGE separation of equal numbers of nuclei from P. falciparum Dd2 clone (left lane) and G. lamblia ISR clone E11 (right lane). The intensities of ISR MBa and MBb are similar to that of comparably sized chromosomes of P. falciparum.

B. Densitometric tracings of relative areas under each peak are noted by bars under the peaks. The peaks for minor bands, MBa and MBb, have areas similar to those for comparably sized P. falciparum chromosomes, while the peaks for the major bands are much larger. Values for P. falciparum 3AB4 and G. lamblia ISR clone G5 were within 25% of the values shown for P. falciparum Dd2 and G. lamblia ISR clone E11, respectively (data not shown). Because residual ethidium staining detected in the wells may indicate entrapped chromosomal DNA that could affect the densitometric analysis, the gels were blotted to nitrocellulose and probed with chromosome specific DNA. The chromosome 4-specific probe pH31 (Sinnis and Wellems, unpublished) and the MBa probe, p4F11, in each case, showed 15 - 20% hybridization to the well when compared with the PFG band (data not shown), indicating that residual DNA in the well did not significantly distort the stoichiometric comparison.

stages of P. falciparum which, as for P. berghei (22), we take to contain the haploid complement of DNA, were embedded in PFGE blocks at 2.5×10^8 organisms/ml. Likewise, Giardia trophozoites were embedded at 1.25×10^8/ml (2.5×10^8 nuclei/ml). Upon electrophoresis and densitometric comparison of ethidium stained bands, the minor bands were found to have the same intensity as comparably sized individual chromosomes from P. falciparum (Figure 4). Thus, the densitometric data are most consistent with a copy number of two per trophozoite for the chromosomes in MBa and MBb and a greater number of copies of chromosomal DNA molecules in the major bands. These data are

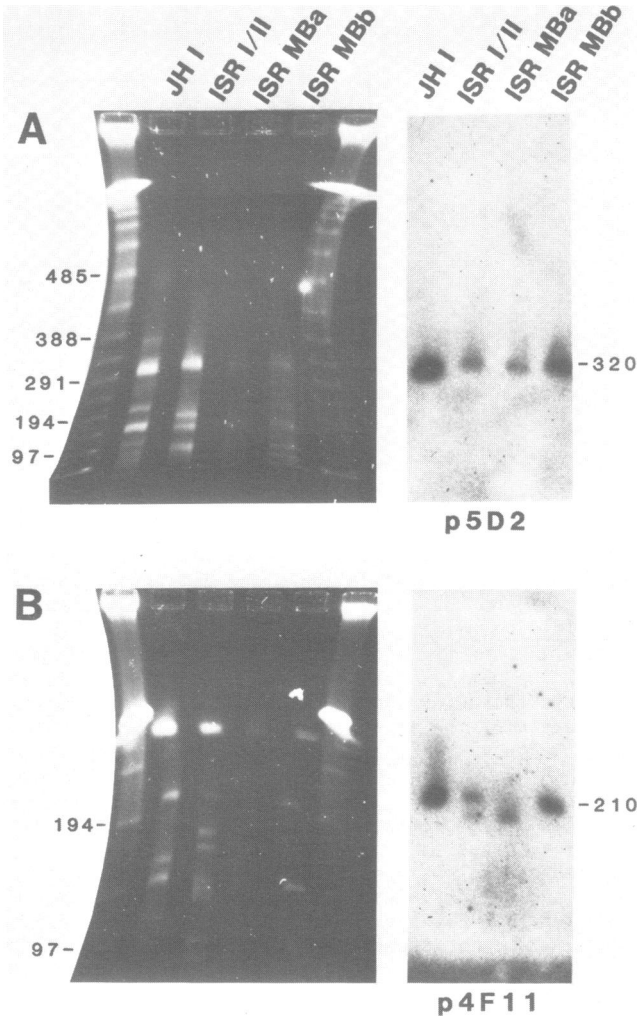

Figure 5

PFGE of Not I digests of JH band I, ISR (G5 clones) band I/II, MBa and MBb.

A. Ethidium bromide stained gel with λ ladders (15) as size standards (size in kb is noted on left). Switching interval was 60 seconds. The DNA was transferred to nitrocellulose and probed with p5D2, which recognized a 320 kb fragment on each band.

B. PFGE of the same bands with a 15 second switching interval to provide better separation in the 100 to 250 kb size range. The DNA was transferred to nitrocellulose and probed with p4F11, which hybridized to a 210 kb fragment from JH band I and ISR MBb, and a 200 kb fragment from ISR MBa. p4F11 hybridized primarily to a 210 kb fragment and less intensely to a 200 kb fragment from ISR band I/II.

not precise enough to give an exact stoichiometry, but do confirm the data from the cloning experiments suggesting that the minor bands are present in each organism and not merely a subpopulation.

Three of the ISR clones (E11, G5, G2) were compared with P. falciparum and S. cervisiae (Figure 2B) to estimate the sizes of the ISR minor bands. Size estimates for the Giardia chromosomes were approximately 1.1 and 1.4 million base pairs (Mbp) for MBa in clones E11 and G5, respectively, 1.6 Mbp for band I/II, and 1.9 Mbp for MBb. MBa from the G2 clone could not readily be distinguished from band I/II by ethidium staining, but was identified as a separate band when probed with JH band I and p4F11 (Figure 2B). Bands III, IV, and V were estimated to have sizes of 2.3, 3.0, and 3.8 Mbp, respectively.

Relative mobilities of the minor bands as compared to band I/II did not change with variations in switching time during PFGE (data not shown), as has been reported for circular DNA molecules (23, 24). In addition, variation of temperature (6°C vs. 26°C) had no effect on the relative mobilities of the minor bands. To further evaluate the relationships between band I and minor bands MBa and MBb, Not I restriction enzyme digests of JH band I, and ISR G5 bands I/II, MBa, and MBb were compared (Figure 5). Fragments ranging in size from 300 to <20 kb were obtained. Although the exact number of restriction fragments could not be determined accurately, size ranges of 0.9 - 1.3 Mbp and 1.3 - 1.9 Mbp. were obtained for MBa and MBb, respectively, by estimating the number and sizes of the individual Not I fragments. The range between the upper and lower values is reflective of the difficulty in determining the number of restriction fragments in the more brightly stained bands. These ranges are consistent with the sizes of 1.4 Mb and 1.9 Mb predicted by comparison with the mobility of the P. falciparum chromosomes. Southern blot analysis of Not I restriction fragments was performed with band I-specific probes. ISR band I/II, MBa, and MBb all contained a 320 kb Not I fragment that hybridized to probe p5D2 (Figure 5A). Probe p4F11 identified 200 and 210 kb fragments from band I/II, a 200 kb fragment from MBa, and a 210 kb fragment from MBb (Figure 5B). These results indicate that band I/II contains large restriction fragments in common with both MBa and MBb.

DISCUSSION

In this work, we have separated and analyzed the chromosomal DNA molecules of Giardia lamblia trophozoites from one cat and five human isolates. In

all cases, the DNA molecules are clearly separated free of the compression zone by the prolonged switching times and low voltages that have been used to separate the molecules of P. falciparum (15) and Schizosaccharomyces pombe (25). The PFGE separations reveal four or five intensely stained (major) bands and a variable number of faintly stained (minor) bands, ranging in size from approximately 1 - 4 million base pairs. The size polymorphism observed is reminiscent of that reported for other protozoans (9 - 12, 15) and yeast (6 - 8).

Since size estimates of the minor bands determined by restriction with Not I are consistent with the sizes of P. falciparum chromosomes of comparable mobility, and since changes in switching time or temperature do not significantly alter relative mobilities of the different bands, we conclude that the minor bands contain linear chromosomal DNA molecules. Stoichiometric comparisons of known quantities of cloned Giardia and P. falciparum trophozoites indicate that the chromosomes of the minor bands are present in each trophozoite (our very approximate measurements suggest two copies of each per trophozoite) while the major bands represent a greater number of chromosomal DNA molecules. Whether each major band contains multiple copies of one or more than one chromosome is not known. Although the densitometric analysis is not sufficiently accurate to enumerate precisely the total number of chromosomal DNA molecules per trophozoite, we estimate the number to be approximately 30 to 50. The estimates of the size and number of chromosomes in each trophozoite thus yields an approximate DNA content per organism of $8 - 12 \times 10^7$ base pairs. In comparison, previous estimates of the genome complexity performed by COt analysis have yielded values of $3.0 - 3.2 \times 10^7$ and 8×10^7 base pairs (13, 26). A more accurate assessment of the genome size remains to be determined.

The cross-hybridization data and Not I restriction analysis of the ISR MBa, MBb, and I/II bands reveal close similarity between large regions of DNA. Such similarity may be explained by extensive crossing over between chromosomes or by the development of multiple chromosomes from a single ancestor. Alternatively, the minor and major bands may represent homologous chromosomes of different sizes. Homologous chromosomes pair during meiosis; however, a sexual cycle has not been identified in Giardia. If appropriate markers such as drug resistant organisms or nutritional auxotrophs were available, genetic crosses between different Giardia isolates could be attempted, in order to determine whether Giardia does have a sexual cycle. In the absence of such studies, electron microscopic visualization of kine-

tochores in the nuclei may help to enumerate the chromosomes, and in situ hybridization could address whether the nuclei of each trophozoite contains the same or different complements of chromosomes.

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