Chromosome-size variation in *Giardia lamblia*: the role of rDNA repeats

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ABSTRACT

*Giardia lamblia* trophozoites contain at least five sets of chromosomes that have been categorized by chromosome-specific probes. Pulsed field separations of *G. lamblia* chromosomes also demonstrated minor bands in some isolates which stained less intensely with ethidium than the major chromosomal bands. Two of the minor bands of the E11 clone of the ISR isolate, MBa and MBb, were similar to each other and to chromosomal band 1 by hybridization to total chromosomal DNA and by hybridization of specific probes. In order to determine the extent of this similarity, I have developed a panel of probes for many of the Pael restriction fragments and have shown that most of the Pael and NotI fragments found in MBa are also present in MBb. The differences are found in both telomeric regions. At one end, MBb contains a 300 kb region not found in MBa. At the other end of MBb is a 160 kb region containing the rDNA repeats which is bounded on one end by the telomeric repeat and on the other by sites for multiple enzymes that do not digest the rDNA repeats. The corresponding region of MBa is 23 kb in size. The size difference is consistent with the eightfold greater number of rDNA repeats in MBb than MBa and suggests that 30% of the size difference is accounted for by different numbers of copies of the rDNA repeat. MBa of another ISR clone (ISR G5) is 150 kb larger than MBa of ISR E11. The data suggest that MBa and MBb are homologous chromosomes of different sizes and that a portion of the size difference is accounted for by different copy numbers of the rDNA repeat.

INTRODUCTION

*Giardia lamblia* is a flagellated protozoan parasite that colonizes the small intestine of susceptible hosts and causes diarrhea. It is a common cause of diarrhea in humans in developed and developing countries and is the most commonly diagnosed parasitic infection.

*G. lamblia* trophozoites each contain two nuclei that are equivalent in all ways that have been examined (1, 2). At least five distinct chromosomes have been identified by pulsed field gel electrophoresis (PFGE), and characterized by their hybridization to 16 chromosome-specific probes (3). The JH isolate contained five distinct bands and two to five specific probes were identified for each of the five bands. Other isolates contained chromosome-sized DNA that stained less intensely with ethidium than other chromosomal bands of comparable size. A cloned line of the ISR isolate (ISR E11) contained four intensely stained bands and two less intensely stained bands, MBa and MBb (Fig 1; Ref 3). The smallest intense band of ISR E11 (1400 kb) hybridized to probes for JH bands I and II and is presumed to represent at least two distinct comigrating chromosomes (I/II). Total chromosomal DNA from JH Band I as well as random probes specific to JH Band I hybridized to MBa (smaller than I/II), MBb (larger than I/II), and I/II, suggesting significant similarity among chromosomes, MBa, MBb and I (3). Bands I/II and MBb were constant in size among different cloned lines of ISR, while MBa demonstrated size variability. For example, MBa of ISR G5 was approximately 150 kb larger than MBa of ISR E11 (Fig 1; Ref 3). One interesting possibility is that these bands represent size variants of homologous chromosomes.

In related work, we showed that rDNA repeats are present on multiple chromosomes and are variable among different *G. lamblia* isolates (4). At least some of the rDNA repeats were telomeric with an abrupt transition from rDNA to telomeric sequence. An rDNA probe hybridized much more intensely to ISR E11 MBb than to MBa on a chromosomal blot, suggesting that MBa and MBb differ in the number of rDNA repeats. In order to accurately define the differences and similarities between MBa and MBb, I have physically mapped MBa and MBb and shown that they contain long regions of identity with each other, rather than just sharing smaller regions of similarity. The difference in the size of the region containing the rDNA repeat accounts for approximately 30% of the size difference between MBb and MBa.

METHODS

Source and growth of organisms

The cloned lines (E11 and G5) of the ISR isolate have been described (3). Trophozoites were grown in TYI-S-33 with bile (5) and were harvested in late log phase.

PFGE

Total chromosomal DNA was prepared and separated by PFGE using an orthogonal field alternating gel electrophoresis (OFA GE) apparatus (6) as previously described (3). Restriction digests
of individual chromosomal bands were performed as described (3). PacI digests were preincubated with enzyme overnight at 4°C before incubation. The digested fragments were separated in a contour-clamped homogeneous electric field (CHEF) pulsed field electrophoresis unit (7). DNA was transferred to a nylon membrane by alkaline transfer in 0.4 M NaOH after depurination for ten minutes in 0.25 M HCl. End labeling of NotI-digested chromosomes was performed by replacing the restriction enzyme buffer with Klenow buffer containing Klenow, dGTP, and ³²P-labeled dCTP for one hour followed by washing in TE and separation by CHEF electrophoresis. After electrophoresis, the gel was fixed in 10% methanol and 10% acetic acid, dried, and exposed to film. Densitometric measurements of autoradiographs were made using a video densitometer (BioRad, Richmond, CA, USA).

Chromosome-specific probes
The rDNA probe was a gift from Tom Edlind (8). The telomere probe (4) and probes pF11 and pSD2 (3) have been previously described. A chromosome-specific library was constructed using a modification of the method described by Wellems, et al (9). MBb chromosomal DNA was excised from PFGE separations and the ethidium bromide extracted with sec-butanol saturated with 1 M NaCl. The DNA was digested with AccI and electroeluted from the agarose with the gene-elutor (Invitrogen). The ends of the AccI-digested DNA were filled in using Klenow and all four deoxynucleotides, followed by ligation of BstXI adapters to the blunt ends. The DNA was size-selected on an agarose gel, followed by electroelution and ligation to the vector, pcmDNAQ, and transformation of competent cells. Probes were selected by hybridizing transformants to PFGE separations of ISR ElI clones and NotI or PacI digests of MBb. In general, probes were selected only if they did not hybridize to bands III, IV, or V, thereby limiting the number of probes with highly repetitive DNA. Plasmid DNA was isolated by alkaline lysis (10). DNA probes were labeled by random priming (11) and unincorporated nucleotides removed by G50 Sephadex column chromatography. Hybridization was performed in 4×SSC at 50°C and washing in 0.2×SSC at 50°C. When necessary, probes were stripped from the blots in 0.2 M NaOH, 0.1% SDS.

RESULTS
If MBa and MBb contain long regions in common, then the restriction patterns should be similar or identical through those regions. Conversely, dissimilar restriction patterns would imply greater differences between the two chromosomal bands. Therefore, the restriction enzyme patterns of MBa and MBb were compared using NotI and PacI, two enzymes that digest G. lamblia DNA infrequently.

NotI Fragments
NotI digests of MBa and MBb chromosomal DNA were end-labeled with Klenow and radiolabeled dCTP and separated on a CHEF gel (Fig 2). This method allowed detection of the smaller fragments which cannot be seen clearly on ethidium-stained PFGE separations. In addition, the intensity of labeling is independent of size and should be similar for all sizes of nontelomeric fragments. Thus, comigrating fragments can more easily be identified. A comparison of MBa and MBb indicates that nearly every fragment of MBa is also contained in MBb. Band I/II also contains fragments the same size as those in MBa in addition to numerous additional fragments (data not shown), consistent with the suggestion that Band I/II contains two chromosomes, one of which is similar to MBa and MBb (3). In contrast, the NotI fragments of the other chromosomes are very different (data not shown).

Differences between MBa and MBb with respect to NotI digests include the presence of 80 kb (one or two fragments), 75 kb, 45 kb (two fragments) and a 10 kb fragment not present in MBa, accounting for 255 to 300 kb of the size difference between MBa and MBb. In addition, there is a slight size difference between the bands found at 145 kb on Fig 2. On a gel that gave better separation in the 97 to 194 kb size range, the size was estimated.
at 160 kb (MBa) and 175 kb (MBb) (data not shown). Several random probes hybridized to the 160 and 175 kb fragments, indicating their similarity; however, the telomere probe hybridized to the 160 MBa fragment, but not to the 175 kb MBb fragment (data not shown). In addition to the qualitative differences, there is a much greater number of 5 kb NotI fragments in MBb than MBa.

rDNA Repeats
The *G. lamblia* rDNA genes contain two NotI sites, yielding 138 and 5428 bp fragments (12). Previous work demonstrated the telomeric location of at least some of the rDNA genes in *G. lamblia* and much greater intensity of hybridization of an rDNA probe to MBb than MBa (4). This suggested that the greater number of 5 kb NotI fragments in MBb than in MBa may be due at least in part to a difference in the number of rDNA repeats. In order to further analyze the regions containing the rDNA repeats, DNA from MBb and MBa was digested with several enzymes containing no sites in the rDNA genes (BclI, EcoRV, EcoRI, HindIII, ClaI), and the blots were probed with the telomere repeat sequence, stripped and reprobed with the rDNA probe. In each case, the telomeric repeat and the rDNA probe hybridized to a 23 kb fragment on MBa and a 160 kb fragment on MBb (The BclI digest is shown in Figure 3). These results indicate that the rDNA genes are telomeric in MBa and MBb and that approximately 140 kb of the size difference between MBa and MBb is accounted for by the difference in the telomeric region containing the rDNA repeats. Since an accurate quantitation of the small amount of MBa DNA cut from a gel cannot be obtained, an actual copy number of the rDNA repeats on MBa and MBb was not obtained. The maximum number of copies of the rDNA repeat was estimated from the sizes of these fragments to be approximately four for MBa and 25 to 30 copies for MBb. However, the copy number could be smaller if there is a sizable region between the rDNA repeats that contains no sites for BclI, EcoRV, EcoRI, HindIII, or ClaI. An independent method of comparing the number of rDNA repeats on MBa and MBb is to compare the amount of hybridization of the rDNA probe to MBa and MBb on the blot of a pulsed field gel. Scanning densitometry of the autoradiograph showed that the rDNA probe hybridized to MBb with an intensity eight times greater than that of MBa, a ratio which would be expected if most of the telomeric region consists of rDNA repeats.

PacI fragments and physical map of MBb and MBa
Twelve PacI fragments of MBb were identified on an ethidium-stained CHEF gel and by hybridization of total MBb DNA to the Southern blot (data not shown). Eight of these fragments were

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**Figure 3.** BclI digest of MBa and MBb separated by CHEF electrophoresis. The blot was hybridized with the telomere probe, stripped and hybridized with the rDNA probe. Size markers (kb) are on the left. The telomere and rDNA probes both recognize a 23 kb fragment of MBa and a 160 kb fragment of MBb. The fainter bands recognized by the rDNA probe at 80 kb (MBa and MBb) and 160 kb (MBa) represent DNA from Band I/II. The gel was electrophoresed at 160 V in 1% agarose for 24 hours with a switching interval of 20 seconds.

**Figure 4.** PacI digests of ISR E11 MBa, MBb, and Band I/II separated by CHEF electrophoresis. Size markers (kb) are on the left. Three identical sets of PacI digests were separated on the same gel, blotted, probed, stripped and reprobed as needed to evaluate the individual probes. The fainter bands at 240 kb and 175 kb in the MBa lane probed with rDNA represent contaminating DNA from Band I/II. The gel was electrophoresed at 160 V in 1% agarose for 38 hours with a switching interval of 15 seconds.

**Table 1.** PacI fragments of MBa and MBb.

<table>
<thead>
<tr>
<th>Size</th>
<th>Probes</th>
</tr>
</thead>
<tbody>
<tr>
<td>260 kb</td>
<td>pAD2, pAA12, pAF6</td>
</tr>
<tr>
<td>200</td>
<td>pCB2</td>
</tr>
<tr>
<td>185</td>
<td>pCB2</td>
</tr>
<tr>
<td>110</td>
<td>pDF7</td>
</tr>
<tr>
<td>80</td>
<td>pAG5, pEF9</td>
</tr>
<tr>
<td>60</td>
<td>pCB21</td>
</tr>
<tr>
<td>40</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td></td>
</tr>
<tr>
<td>MBa only</td>
<td>telomere, rDNA</td>
</tr>
<tr>
<td>240</td>
<td>pAE12, pCE8</td>
</tr>
<tr>
<td>185</td>
<td>pAE12, pCE8</td>
</tr>
<tr>
<td>140</td>
<td>telomere, pAE12, pCE8</td>
</tr>
<tr>
<td>100</td>
<td>pEF11, pDA7, pBC10</td>
</tr>
<tr>
<td>MBb only</td>
<td>telomere, rDNA</td>
</tr>
<tr>
<td>130</td>
<td>pDA7, pBC10</td>
</tr>
<tr>
<td>120</td>
<td>telomere, rDNA</td>
</tr>
<tr>
<td>23</td>
<td>telomere, pBC10</td>
</tr>
</tbody>
</table>

1. pCB2 hybridized predominantly to the 40 kb fragment and less intensely to the 185 kb and 200 kb fragments.
also present in MBa (260, 200, 185, 110, 80, 60, 40, and 20 kb) and probes were identified for all except the 80 and 20 kb fragments. Specific probes were identified for some of the fragments (260, 110, and 60 kb fragments) while probe pCB2 hybridized most intensely to the 40 kb fragment and less intensely to the 185 and 200 kb fragments (Fig 4; Table I).

Four PacI fragments were present only in MBb (240, 185, 140, and 100 kb) and MBa contained three fragments not present in MBb (130, 120, and 23 kb). Note that two 185 kb fragments were identified, one present on both chromosomal bands and one unique to MBb. The two 185 kb fragments were suggested by the greater intensity of ethidium-staining of the 185 kb than the 200 kb fragment of MBb. The hybridization of pCB2 and total MBb DNA to 185 kb fragments of MBa and MBb identified the common fragment while the hybridization of other probes to a 185 fragment only on MBb (see below) identified the fragment unique to MBb. The rDNA and telomere probes hybridized to the 240 kb and 120 kb fragments of MBb and MBa, respectively, consistent with the 135 kb difference between MBa and MBb in the length of the telomeric rDNA repeat region that was suggested by other restriction enzymes.

The random probes that recognized the 160 kb telomeric NotI fragment of MBa and the 175 kb NotI fragment of MBb all hybridized to 23 kb telomeric and/or 130 kb subtelomeric PacI MBa fragments and to a 100 kb PacI fragment of MBb. The pAE12 probe (Fig 4) and pCE8 probe (data not shown) hybridized to a 140 kb telomeric fragment and the 185 kb fragment unique to MBb, but did not hybridize to MBa, placing the 185 kb fragment adjacent to the 140 kb telomeric fragment in a region not found on MBa, resulting in the physical map shown in Figure 5.

The sizes of MBa and MBb can be estimated by adding the sizes of the PacI fragments, resulting in a size of 1225 kb for MBa and 1620 kb for MBb. The size estimate for MBa is in close agreement with the previous size estimate for MBa of 1100 kb (3) and suggests that all or most of the PacI fragments were identified. Of the 395 kb size difference between MBa and MBb, 120 to 135 kb (30%) is accounted for by the region containing the rDNA repeats.

Comparison of MBa from ISR E11 and G5

In order to determine the basis for the size differences of MBa of ISR G5 and ISR E11, the chromosome ends were compared. The rDNA and telomeric probes hybridized to a 23 kb HindIII fragment of E11 MBa and a 180 kb HindIII fragment of G5 MBa (Fig 6), suggesting a greater number of copies of the rDNA repeats on G5 MBa than E11 MBa. On the other chromosome end, p4F11 and the telomeric probe hybridizes to a 160 kb NotI fragment of E11 MBa and G5 MBa (data not shown). Thus, most of the difference in the sizes of MBa from ISR clones E11 and G5 is found in the telomeric region containing the rDNA repeat.

DISCUSSION

Ploidy of G.lamblia

Previous work using PFGE separations of G.lamblia chromosomes identified at least five sets of chromosomes (3), which were categorized by the specific hybridization to five groups of probes. The total number of chromosomal DNA molecules per trophozoite was estimated at 30 to 50 by densitometric comparison of a known number of G.lamblia trophozoites with an equal number of haploid Plasmodium falciparum organisms. These data suggest that G.lamblia trophozoites are polyploid (six to ten copies of each chromosome/trophozoite). The alternative explanation is that each of the PFGE bands contains more than one set of chromosomes. In fact another report suggested greater numbers of chromosomes separated by PFGE (13); however, the additional chromosomal bands were not categorized by chromosome-specific probes and may represent size variants of similar chromosomes.

The two possibilities could be distinguished if accurate estimates of genome size and complexity were available. However, estimates of genome complexity have varied widely. The values obtained by CoT analysis have been 3 x 10^7 (14) and 8 x 10^7 (15), while an estimate obtained by adding the sizes of the NotI fragments was 1.1 x 10^7 (16). The latter estimate is consistent with the value of 1.2 x 10^7 obtained by adding the sizes of the five chromosomes separated by PFGE (3) and is most consistent with the existence of five polyploid chromosomes. However, the larger estimates obtained by CoT analysis are more consistent with a much greater number of chromosomes.

In previous studies, the two faintly staining chromosomal DNA bands, MBa and MBb, were estimated to be present in approximately two copies per trophozoite. They demonstrated...
considerable similarity to each other and to Band I of the JH isolate and Band I/II of the ISR isolate. The estimated sizes are 1225 kb for MBa, 1400 for Band I, and 1620 kb for MBb. The present report demonstrates that MBa, MBb, and Band I contain a region at least 955 kb in length which has identical-sized PacI and NotI restriction fragments.

The substantial similarity of MBa and MBb (and Band I) suggests that they are homologous chromosomes of different sizes. (In organisms that reproduce by a sexual cycle, homologous chromosomes are those that pair during meiotic division. The meaning of homologous chromosomes is less clear in organisms without a known sexual cycle, such as *G. lamblia*, but as a working definition, I have taken the approach that if two chromosomes are identical throughout a majority of their length, they are homologous chromosomes.) Since MBa and MBb have each been estimated to be present in two copies per trophozoite, the data suggest that *G. lamblia* trophozoites are polyploid, at least for this chromosome. If Band I/II also contains two copies of Chromosome I, *G. lamblia* trophozoites would contain at least six copies of Chromosome I with MBa, Band I, and MBb, representing size variants of Chromosome I. These size variants can then be represented as different subscripts of Chromosome I (eg. Ia for MBa, Ib for Band I, and Ic for MBb. 

Etiology of chromosome size variation in *G. lamblia*

The rDNA genes of *G. lamblia* are present on several chromosomes and variation in the number of copies of the rDNA repeat accounts for some of the size variation. The rDNA repeats are telomeric in at least some of the chromosomes (4, 17, 18), and the variability of location among and within isolates suggests increased mobility of the rDNA genes. The rDNA repeat hybridizes to a 160 kb telomeric region of MBb bounded on the chromosome-internal end by sites for BclI, EcoRV, EcoRI, HindIII, or ClaI enzymes that have no recognition site in the rDNA repeat. The corresponding region of MBa is 23 kb. Since these are not rare cutting enzymes for *G. lamblia*, it is likely that most of the 160 kb and 23 kb regions consist of rDNA repeats. The densitometric scanning result which indicated that MBb contains approximately eight times as many rDNA repeats as MBa is consistent with the suggestion that most of this telomeric region is made up of rDNA repeats and that the size difference between MBa and MBb is accounted for by a difference in the number of rDNA repeats. The telomeric region containing the rDNA repeats may also account for all or most of the size difference between MBa of the two cloned lines of ISR, E11 and G5.

The CCCCGG sequence has been found at the rDNA/telomere junction of all three junctions that have been reported (4, 18) and could possibly be the site of telomere healing after chromosome breakage. The remaining 70% of the size difference between MBa and MBb is found in the other telomeric region. This difference may possibly have resulted from a subtelomeric deletion or by a telomeric deletion followed by telomere healing.

Chromosome size variation in other protozoans

*G. lamblia* is similar to other protozoans in the plasticity of its genome and chromosome structure. Size variation of chromosomes has been documented in other protozoans, including *P. falciparum* (19), *Trypanosoma brucei* (20), and *Leishmania sp.* (21) and may result from deletion, amplification, or from unequal crossing-over. In a genetic cross of two *P. falciparum* isolates, one of the resultant organisms contained a chromosome intermediate in length between the two parent organisms and occurred as a result of unequal crossing-over in the subtelomeric region (19). Chromosome breakage followed by telomere healing has also been documented as a cause of size variation of *P. falciparum* chromosomes (22, 23). Further research into the mechanism of chromosome and genetic variability of *G. lamblia* and other protozoans will shed further light on this diverse set of organisms.

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