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Frequent rearrangements of rRNA-encoding chromosomes in *Giardia lamblia*

Sylvie M.Le Blancq, Stanley H.Korman and Lex H.T.Van der Ploeg*
Department of Genetics and Development, College of Physicians and Surgeons of Columbia University, 701 West 168th Street, New York, NY 10032, USA

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ABSTRACT

The ribosomal RNA (rRNA) genes in *Giardia lamblia* are present as short tandem arrays of a 5.6 Kb repeat unit on at least six telomeres. Four of these telomeres have the same overall organisation comprising a domain ranging in size from 25 to 300 Kb, delineated chromosome internally by a conserved island of restriction enzyme sites. Cloned lines of *G. lamblia* derived from the WB strain contain polymorphic subsets of chromosomes encoding rRNA genes. However, changes in the size of the rRNA telomere domains of these polymorphic chromosomes alone cannot account for the total size changes in the chromosomes. The rearrangement events are very frequent: 60% of subcloned lines had discrete rearranged karyotypes that differed from each other, suggesting either an estimated rearrangement rate that may be as high as 3% per division or a cloning-induced rearrangement event. The extreme plasticity of the genome has obvious implications for the maintenance of a functional genome and the control of gene expression in *Giardia*.

INTRODUCTION

Giardia lamblia is an aerotolerant anaerobic protozoan that is a common enteric parasite of man. The life cycle of *G.lamblia* consists of vegetative trophozoites which parasitize the upper intestinal tract of various mammals and infective cysts that are shed in the faeces. Giardiasis can be associated with malabsorption and acute diarrhoea (1); however, the pathogenesis is poorly understood. Most infected individuals are asymptomatic (2) and the factor(s) that modulate the symptoms are not known. *Giardia* exhibits antigenic variation (3, 4, 5) which has been proposed to facilitate evasion of the gut's immune surveillance system. This has broad implications in terms of understanding the pathology and assessing the potential efficacy of future vaccines.

Giardia represents the earliest diverging extant eukaryotic lineage according to small subunit ribosomal RNA sequence homology (6). Several standard eukaryotic organelles, i.e. Golgi apparatus and mitochondria are absent in *Giardia* (7) while it has the unusual feature of containing two equally sized, transcriptionally active, nuclei (8).

Four chromosome size classes, ranging from 1–3 Mb, have been identified in *G.lamblia* using pulsed field gradient gel electrophoresis (PFG) (9, 10). Karyotype analysis suggests that there are from 30 to 50 chromosomes in *G.lamblia* (9). The haploid genome contains 8×10^7 bp (11) and predicts the minimum number of chromosomes to be approximately 40. The ploidy of each trophozoite is not known, neither is the nature of the distribution of the chromosome complement between the two identically sized nuclei. It has been suggested that each nucleus contains the haploid genome complement and each nucleus has been shown to encode ribosomal RNA (rRNA) genes (8).

The genomes of protozoa are plastic and gene rearrangments are known to be involved in the control of differential gene expression (12, 13). Adam *et al* (14) presented evidence for gene rearrangements involving a surface antigen gene family and described polymorphic bands in the karyotypes of a series of clones (9).

To investigate the nature and extent of genome plasticity in *G.lamblia* we analyzed the genomes of a series of cloned lines. Rearrangement events involving rRNA encoding chromosomes occurred at a high frequency—60% of progeny subclones had discrete karyotyes, suggesting an estimated mutation rate of 3% per division. Alternatively, rearrangement events may be induced by cloning of the organisms by limiting dilution. The rRNA genes are present in short tandem arrays on at least six telomeres in *G.lamblia*. These rRNA encoding telomere domains also undergo rearrangements and the polymorphic telomeres are components of the polymorphic chromosomes.

MATERIALS AND METHODS

Cell strains and culture

Giardia lamblia strain WB (ATCC 30957) was obtained from the American Type Culture Collection. Trophozoites were grown at 36–37°C in screw-capped borosilicate tubes (20 or 16 ml) filled with modified TYI-S-33 medium (15). The yield of trophozoites from late-log cultures was about 2×10^6/ml. Trophozoites were harvested from confluent monolayers by

* To whom correspondence should be addressed

chilling the tubes for 10 minutes in ice-water. Cultures were passaged every 3–4 days. Cells were not kept in continuous culture, instead they were cryopreserved and introduced into culture as necessary. Cryopreservation involved decanting the medium and non-adherent trophozoites from the culture tube, leaving the monolayer of trophozoites; the tube was refilled with fresh medium containing 10% dimethyl sulphoxide. The trophozoites were released from the glass by chilling the tube for 10 min in ice-water. Aliquots in Nunc cryopreservation vials were frozen at −20°C for two hours, then overnight at −90°C and then stored at −135°C. Frozen stocks were recovered by warming the tube to 37°C rapidly and inoculating into fresh medium (1:200 dilution). The viability of the stabilate was checked by monitoring the mobility of trophozoites on thawing. High recovery efficiency was routinely observed.

Cloning by limiting dilution

WB trophozoites were cloned using the limiting dilution method (16). Trophozoites were harvested and serially diluted in fresh medium to give 0.5 to 0.1 cells per ml in different experiments. Cloning was performed in parallel using 10 times the concentration of cells (1–5 cells/ml) to assess the viability of the trophozoites. 0.2–0.3 ml aliquots were placed into each well of sterile, flatbottomed, 96 well microtitre plates. Microscopic analysis of *G. lamblia* trophozoite suspensions used for cloning revealed no evidence of cell-aggregation that could have affected our calculation of the cloning efficiencies. This method ensures a >90% probability that each population is indeed clonal. For example, the WB-F9 clone was generated by decanting off the culture medium and diluting the remaining adherent trophozoites to a concentration of 0.1 cells/ml and placing 0.2 ml aliquots into microtitre wells; using this procedure, the probability that F9 is derived from a single organism is 96.8% (16). The plate was covered and sealed into an anaerobic environmental chamber (Marion Biobag, type A.) and incubated at 36–37°C for 4–6 days. In each case approximately the expected number of positive wells was seen.

Trophozoites from positive wells were established in culture by chilling the plate for 15 min on ice-water and transferring the contents of the well into a culture tube with fresh medium. In one subcloning experiment, 0.1 ml aliquots of diluted trophozoite suspension were inoculated into 1.9 ml of fresh medium in each of 100 sterile, 2 ml-capacity, rubber-stoppered plain glass vacuum tubes (Vacutainer^{R}, Becton Dickinson, New Jersey) to give a final concentration of 0.04 cells/ml (manuscript in preparation). Subsequent processing of culture-positive tubes was as for the microtiter well cloning experiments.

Clone names include their derivation and clone number: for instance WB is the parental population, WB-D9 is the clone D9 and WB-D9-B10 is the subclone B10 obtained from WB-D9. Approximately 32 generations of growth separate each limiting dilution cloning step.

Pulsed field gradient gel-electrophoresis

Chromosome-sized *Giardia* DNA was prepared by harvesting trophozoites and washing them three times with ice-cold TSE (10 mM Tris/Cl, pH 8.0, 100 mM NaCl, 50 mM EDTA). The pellet was resuspended in TSE to give 5×10^{8} cells/ml, warmed to room temperature and mixed with an equal volume of 1% low-melting point agarose in TSE at 42°C. 75 μl blocks were formed by dropping the trophozoite/agarose mixture into a chilled perspex block-maker. The agarose was allowed to gel briefly and the blocks were put into 10 ml of lysis mix (0.5 M EDTA pH 9, 1% sarkosyl, 2 mg/ml proteinase K) and incubated for 2 days at 51°C with gentle shaking. The blocks were stored in the lysis mix at 4°C. 1/6 of a block per lane was loaded into pulse field gradient (PFG) gels and run as described previously (17, 18). Size markers of *Trypanosoma brucei* variant 118 clone 1 and phage Lambda ladders were kindly provided by Dr. A.Rattray and Dr. K.Gottesdeiner. Each *Giardia* chromosome size class runs as a single band on PFG gels. Changing the PFG running conditions allows resolution of particular bands into their components while creating compression zones containing the remaining size classes. Pulse-frequency, voltage and run duration were varied according to the size of the molecule to be resolved; specific conditions used are given in the figure legends. After electrophoresis, gels were stained with ethidium bromide (2.5 μg/ml).

Southern blotting and DNA probes

DNA probes were prepared from gel-purified fragments of plamids using random priming (Random Prime Kit, Boehringer Mannheim) with either α^{32}PdATP or α^{32}PdCTP or α^{32}PdGTP. DNA separated on PFG agarose gels was transferred to nylon filters using standard procedures (19). Southern blots were hybridized in 6×SSC or SSPE, 5×Denhardt's solution, 0.5% SDS and 100 μg/ml sheared salmon sperm DNA, at 65°C overnight. The blots were washed once in 2×SSC for 15 min at 65°C; twice in 2×SSC/0.1% SDS for 15 min at 65°C and to a final stringency of 0.1×SSC/0.1% SDS for 20 min at 65°C.

Bacterial strains and plasmids

The ribosomal RNA gene repeat unit of *G.lamblia*, (pGRPI), cloned into the Sma I site of pUC 8, was provided by Dr. C.C.Wang (10). pGC1 is a *G.lamblia* telomere cloned into the Sma I/Xba I site of Bluescript (20). pGC1ΔN, a subclone of pGC1 with the 6 Kb Not I fragment deleted was used as a source of the telomere-specific repeat probe. *E. coli* strains DH5α and HB101 were used. Plasmid manipulations were carried out using standard protocols.

Restriction endonuclease digestions

Restriction endonucleases were used according to the manufacturer's instructions. Digestion of chromosome-sized DNA was achieved by digesting the DNA in PFG blocks. The blocks were washed twice in 10 ml TE/1 mM PMSF (phenylmethyl sulphonyl fluoride) with gentle shaking for 1 hr, followed by 3×1 hr washes in TE alone. Blocks were sliced into 3 or 4 pieces and each one added to a final volume of 100 μl restriction enzyme digestion mix and 30 U of enzyme were added. The reactions were incubated overnight at the appropriate temperature, then loaded onto a PFG. The uncut controls underwent the whole procedure but the enzyme was ommitted from the incubation mixture.

Determining the chromosomal location of restriction fragments

Blocks of chromosome-sized DNA from subclones WB-D9-B10 and WB-E10-C4 were loaded onto 1% low melting point agarose PFG gels in duplicate lanes and run to separate the polymorphic rRNA-containing chromosomes (600 sec pulse-frequency, 150 V, 6 day run). A lane of each clone was cut out, washed with TE and stored at 4°C. The remainder of the gel was stained with ethidium bromide, blotted onto nylon membranes and hybridized with the rRNA probe (2 kb EcoRI/Pst fragment from pGRPI).

Those regions of the stored lanes that corresponded to the rDNA-hybridizing chromosomes were cut into 1 mm slices and each slice was digested with Spe I as described above. DNA in blocks was also digested in parallel to serve as markers. The slices and blocks were loaded onto PFG gels and run at a pulse-frequency of 40 sec at 330 V for 40 hr, then the DNA was tranferred to nylon membranes and hybridized with a rRNA probe.

RESULTS

Cloning of *G. lamblia*: generation of a clonal series

We cloned *G.lamblia* by limiting dilution and compared the karyotypes of randomly selected clones, subclones and sub-subclones. We have analysed 38 different clones, including 5 clones, 27 subclones and 6 sub-subclones. The lineages of some of the clones are indicated in Fig. 1. Please note that in the figures in this paper we present the comparison of a limited size range of chromosomes only. The rationale for this is that in any particular PFG gel optimal separation of only a limited number of chromosomes can be achieved. Given the large number of cloned lines and chromosomes to be analysed we concentrated on chromosomes in the size range between several hundred kilobase pairs and about 1.5 Mb. Hybridisation of these PFG blots with a rRNA probe revealed discrete polymorphic rRNA-containing chromosomes among the different cloned lines (Fig. 1 and data not shown). Firstly, all five clones had an rRNA distribution pattern that differed from the WB parent strain (Fig. 1A shows the example of clone WB-F9 compared to WB parent line and Fig. 1B shows a comparison of WB parent line and clones WB-D9, WB-B4 and WB-E10). Secondly, of 27 different subclones, 16 had a different karyotype (Fig. 1A shows six different WB-F9-derived subclones). Thirdly, 2 of 6 sub-sub clones also had different karyotypes (data not shown). Overall chromosome size changes of several hundreds of kilobasepairs were seen for the rRNA hybridizing chromosomes with the appearance and disappearance of one or more bands (for example Fig. 1A lanes 5 and 7). β-tubulin (21) and cysteine-rich cell-surface protein coding genes (14) were not present on rRNA-containing chromosomes and probes for these gene families did not reveal any additional chromosomal heterogeneity (data not shown). Furthermore, the ethidium bromide (EtBr) staining patterns of these gels only revealed new bands that hybridized with the rRNA probe (data not shown). These new bands stained relatively faintly with EtBr when compared to the major EtBr staining bands, indicating that they are present at a low copy number per cell.

We conclude that rRNA genes are located on a subset of hypervariable chromosomes.

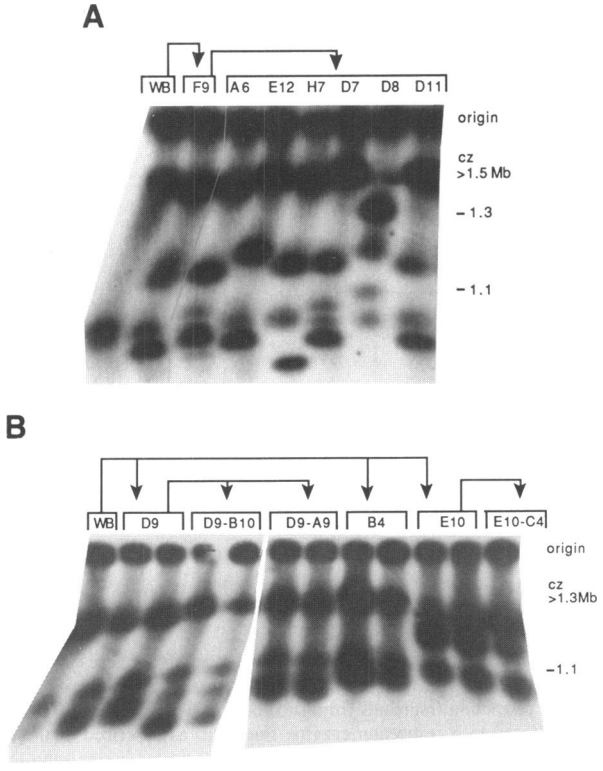

Fig. 1. Rearranged chromosomes containing rRNA genes in a series of *Giardia* clones. Pulsed field gradient electrophoresis (PFG) gels comparing the chromosome-sized DNA of parent, clones, subclones and sub-subclones from two series of cloned lines of the *G.lamblia* WB strain were blotted onto nylon filters. The lineage of the clones are indicated above each blot. Panel A (pulse frequency 650 sec, 150 V, 5 days) was hybridised with the rRNA repeat unit (Eco RI/HindIII 6kb insert from pGRPI). Panel B (pulse frequency 600 sec, 150 V, 5 days) was hybridised with a rRNA probe (1.2 kb Bam HI/Eco RI fragment from pGRPI). cz is the compression zone. *Trypanosoma brucei* chromosomes were used as size markers (34).

Characterisation of rearranged rRNA-encoding chromosomes

In order to understand the nature of the DNA rearrangement events that generated the rearranged molecules and to be able to address their biological significance, we analysed the structure of several clones from the series shown in Fig. 1B. We first wanted to see whether these newly generated rRNA-hybridizing bands were linear or circular. The presence of the telomere-specific [TAGGG] repeat array on these polymorphic chromosomes would indicate that they were linear molecules with functional chromosome ends (20). A PFG gel separating some of the rearranged rRNA hybridizing molecules is shown in Fig. 2 with hybridization with a rRNA probe shown on the left and

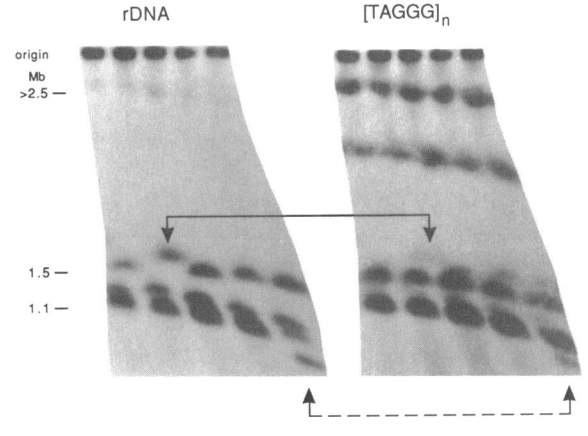

Fig. 2. Identification of rearranged chromosomes as linear molecules. PFG gel (pulse frequency 950 sec, 150 V, 5.5 days) separating the chromosomes of a series of subclones (lane 1, WB-F9-A6; lane 2, WB-F9-E12; lane 3, WB-F9-G3, lane 4, WB-F9-H4; lane 5, WB-F9-H7) was blotted onto nylon and hybridised with a rRNA probe (1.2 kb Bam HI/Eco RI fragment from pGRPI) (Panel A). The signal was removed and the filter was hybridised with the telomere repeat probe $[TAGGG]_n$ (530 bp Sma I/Pst fragment from pGCIΔN) (Panel B). The arrows indicate individual polymorphic bands that hybridise with both rDNA and telomere repeat probes.

hybridization with the telomere repeat probe on the right. Each of the rRNA hybridizing bands hybridizes with the telomere probe, although the intensity of the signal from the rearranged molecules is faint. This is consistent with a short telomere repeat array (0.5 to 1 Kb) at the end of *Giardia* chromosomes (20). We therefore concluded that the rearranged molecules are linear and that the low hybridization intensity indicates that they are present at a low copy number per cell.

Telomeric DNA sequences in many different eukaryotes can undergo large size changes (13, 22, 23, 24). The newly generated rRNA-hybridizing molecules in *G. lamblia* have telomeres and they differ in size from the parental chromosomes by hundreds of kilobase pairs. The telomere repeat arrays in the *G. lamblia* genome are short ranging in size from 0.5 to 1 Kb, on average (20), therefore the changes in chromosome size are unlikely to be accounted for by changes in the length of the telomere repeat arrays alone. Similarly, while comparison of the signal intensity of the rRNA hybridization (Figs. 1 and 2) suggests slight changes in rRNA gene copy number, it is unlikely that rRNA amplification and/or deletion account for the total size changes (Figs. 1 and 2 and data not shown). This conclusion is based on the lack of change in the overall intensity of rRNA hybridization on PFG blots and quantitative comparison of the intensity of rRNA-hybridizing bands in normal Southern blots in which most rRNA genes are confined to a single restriction fragment (data not shown). We therefore generated physical maps of a sample of the rearranging rRNA -encoding chromosomes, to begin to understand the mechanism and biological significance of the rearrangement events.

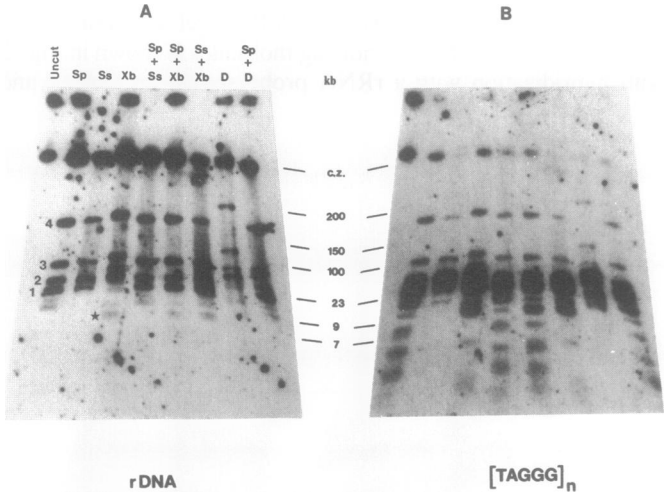

Fig. 3. Four telomeres in *G.lamblia* have the same overall organisation and contain rRNA genes. Chromosome-sized DNA from *G.lamblia* subclone WB-D9-B10 in PFG blocks was digested with different enzymes: Sp, Spe I; Ss, Ssp I; Xb, Xba I; D, Dra I or combinations of enzymes (see Materials and Methods) and was loaded and run in a PFG gel (pulse-frequency 15 sec, 330 V, 24 hr). Uncut DNA is shown in the lane marked 'uncut'. Phage lambda ladders and phage lambda digested with the restriction enzyme HindIII were used as size markers. Sizes in kb are shown between the two panels. The gel was blotted onto a nylon membrane and hybridized with an rRNA probe (2 kb EcoRI/Pst fragment from pGRPI) (Panel A). The asterisk indicates the component that was cloned as pGC1 in (20). The strongly hybridizing fragments are labeled 1–4. C.z. is the compression zone. The signal was removed and the filter was hybridized with the telomere repeat probe $[TAGGG]_n$ (Panel B).

Physical mapping of rRNA gene loci in *G.lamblia*

Chromosome-sized DNA from two subclones, WB-D9-B10 and WB-E10-C4 was digested with a series of restriction endonucleases that do not cut within the rRNA gene repeat (i.e. Spe I, Ssp I, Xba I, Dra I). The DNA was separated on PFG gels and hybridized with an rRNA probe. Fig. 3 shows the data for the subclone WB-D9-B10 (data for WB-E10-C4 not shown). Ethidium bromide staining of this gel indicated that the majority of the digested DNA ranged in size from 7 to 50 Kb (smaller fragments were spread out over the lowest portion of the gel) and gave a different pattern with each enzyme. Surprisingly, digestions of the DNA with the different restriction enzymes each generated four strongly hybridizing rRNA- encoding fragments of about the same size (labelled 1–4; Fig. 3A) independent of the restriction enzyme that was used. Additional faintly hybridizing bands were also detected in the digestions using Spe I, Xba I and Dra I. The band representing the cloned telomere (20) is indicated by the asterisk in the Xba I lane. The sizes of the restriction enzyme fragments differed slightly according to the restriction enzymes used: Dra I generated the largest and Spe I and Ssp I the smallest fragments. Double digestions always resulted in generation of the smaller of the two potential restriction enzyme fragments. These mapping data indicate that the physical map of these fragments is compatible with a telomeric derivation of the rRNA-hybridizing fragments. Indeed, the restriction

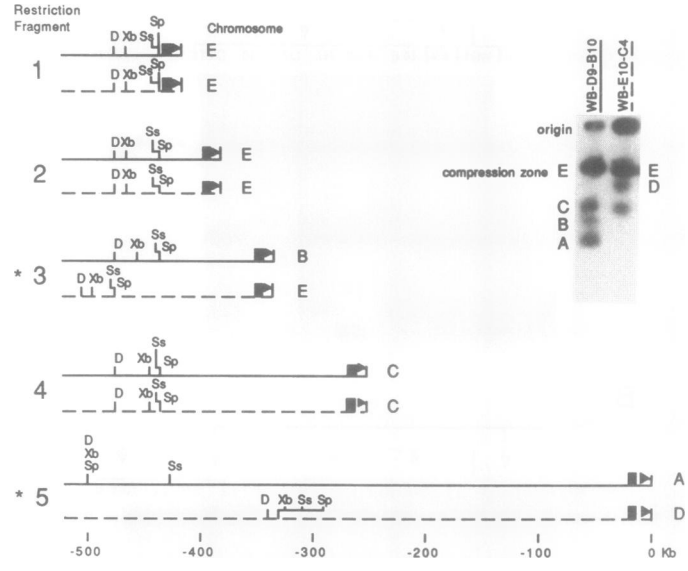

Fig. 4. Physical maps of the five telomeres that contain rRNA genes in two *Giardia* clones. Summary of restriction digestion and hybridization data for telomeric rRNA containing restriction fragments from subclones WB-D9-B10 and WB-E10-C4. The number of each restriction enzyme fragment, as described in the text, is indicated on the left. WB-D9-B10 sequences are indicated by the solid line and WB-E10-C4 by the broken line. The solid blocks are rRNA gene arrays; their precise position in the telomeric fragment is not known. The solid arrowhead represents the telomere repeat array. The restriction sites are D, Dra I; Xb, Xba I; Ss, Ssp I; Sp, Spe I. Non-rDNA sequences to the right of the restriction site island may be simple-sequence DNA. The asterisks indicate those fragments which differ from the parental WB configuration. Inset: a PFG gel (pulse-frequency 600 sec, 150 V, 5 days) separating the chromosomes of two WB subclones: WB-D9-B10 and WB-E10-C4, blotted and hybridized with an rRNA probe (0.3 kb Pst fragment from pGRPI) (see Fig. 1B). The rRNA-containing chromosomes are labelled A, B, C and D, and E is the compression zone. The rRNA-containing chromosome from which the fragments are derived are shown on the right of each physical map.

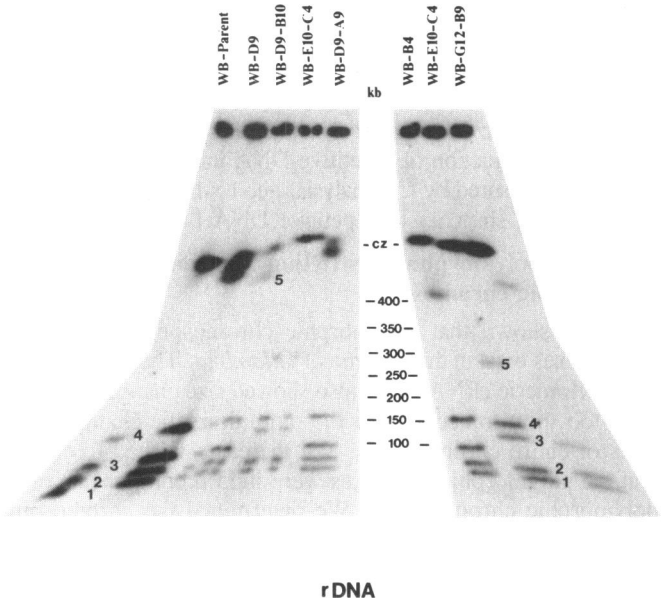

Fig. 5. Comparison of the rRNA containing fragments in WB parent and several cloned lines. Chromosome-sized DNA was digested by Spe I and run out on a PFG gel (pulse-frequency 40 sec, 330 V, 40hr), blotted onto nylon and hybridized with an rRNA probe (2 kb EcoRI/Pst fragment from pGRPI). The rRNA-containing fragments are labeled 1–5. Phage lambda ladders were used as size markers.

enzyme fragments 1-4 hybridized with the telomere repeat probe $[TAGGG]_n$ (Fig. 3B; the hybridization of restriction fragments 1 and 2, obscured by the intense hybridization of other coincident telomeric fragments in this experiment, was seen after fractionation of chromosomes in two-dimensional PFGs (data not shown)). The fact that each of these rRNA-containing restriction fragments is located at a telomere facilitated the generation of physical maps of these chromosomes (Fig. 4 and see next sections for a more detailed discussion of the physical mapping strategy).

We digested the DNA of several of the rearranged *G.lamblia* cloned lines with the same series of restriction endonucleases and compared the physical maps of their telomeric rRNA-containing fragments. Fig. 5 shows the result of digestions of DNA from the parent WB and different cloned lines with the restriction enzyme Spe I, followed by hybridization of the DNA with a rRNA probe. The numbering of the restriction enzyme fragments is the same as in Fig. 3, but here an additional very large (400–500 Kb) fragment, labeled 5, has been separated from the compression zone. The WB parent contains four rRNA-hybridizing restriction fragments as well as the compression zone (Fig. 5, WB-parent lane). The clone (WB-D9) and subclones (WB-D9-A9 and WB-D9-B10) retained the parental configuration of bands 1–4 while band 5 is clearly of a different size in these clones. Lighter exposures show that WB-D9 contains three bands in the 'cz' region. We can, therefore, make a direct comparison of the physical maps of telomeric rRNA-encoding restriction enzyme fragments in parental and cloned *G.lamblia* lines. Additional Ssp I, Dra I and Xba I restriction enzyme digestions generated the same five bands which hybridized with the rRNA probe (Fig. 6A and data not shown) and the telomere repeat probe

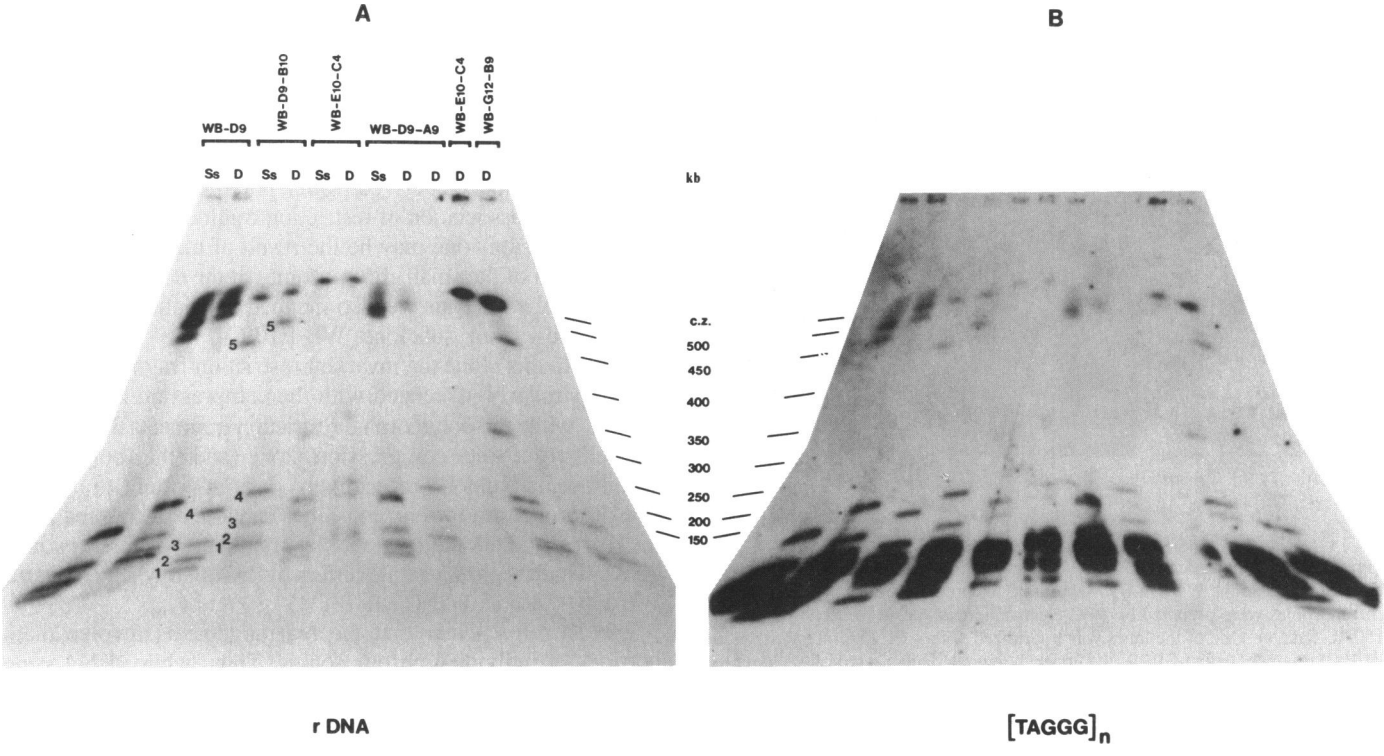

Fig. 6. Telomeres containing rRNA genes are rearranged in different *G.lamblia* cloned lines. Chromosome-sized DNA from a series of *G. lamblia* WB cloned lines (listed at the top of panel A) was digested with either Ssp I (Ss) or Dra I (D) and run out on a PFG gel (pulse-frequency 40 sec, 330 V, 40hr), blotted onto nylon and probed with a rRNA probe (2kb Eco RI/Pst fragment from pGRPI) (Panel A). The signal was removed and the filter was hybridized with the telomere repeat probe $[TAGGG]_n$ (Panel B). C.z. is the compression zone. Phage lambda ladders were used as size markers.

(Fig. 6B) revealed the telomeric location of these rRNA genes. Some of these fragments remained the same size among the different clones (i.e. fragments 1,2 and 4), while other restriction enzyme fragments (i.e. 3 and 5) differed in size (compare the subclones WB-D9-B10 and WB-E10-C4 (Figs. 5 and 6)). We have not analysed the additional rRNA-containing fragments at the compression zone.

Fig. 4 summarizes the physical mapping data of the DNA from the subclones WB-D9-B10 (continuous line) and WB-E10-C4 (broken line). The physical maps of the restriction fragments 1, 2 and 4 have the same relative organisation in both clones, comprising telomere repeats, rRNA genes and Spe I + Ssp I, Xba I and Dra I sites, respectively. The physical maps differ from one another in the distance between the island of restriction sites and the telomere repeats. The exact position of the rRNA genes relative to the telomere repeats or the restriction site island is not known. The size of fragment 3 differs between the two clones and while the order of restriction enzyme sites has remained unaltered, the distance between them has changed. In contrast, the physical map of fragment 5 in subclone WB-D9-B10 differed entirely from that of fragments 1−4 and the island of restriction enzyme sites could not be found. Extensive modifications in the primary DNA sequence must therefore have occurred in the region around the island of restriction enzyme sites in fragment 5. These rearranged regions comprise a large domain of the chromosome, measuring hundreds of kilobase pairs in size. The intensity of hybridization of each restriction enzyme fragment is similar when compared between the different cloned lines, suggesting that each of them contains an equivalent number of rRNA repeat units (estimated to be 4−5). The non-rRNA component of these telomeres may consist of simple sequence DNA, since sites for six restriction enzymes are absent (data for Cla I and Xmn I not shown). [The fraction of repetitive DNA in the *Giardia* genome is 14% as measured by C_0t analysis, i.e. 1×10^7 bp, (11) allowing for such long stretches of repetitive DNA.]

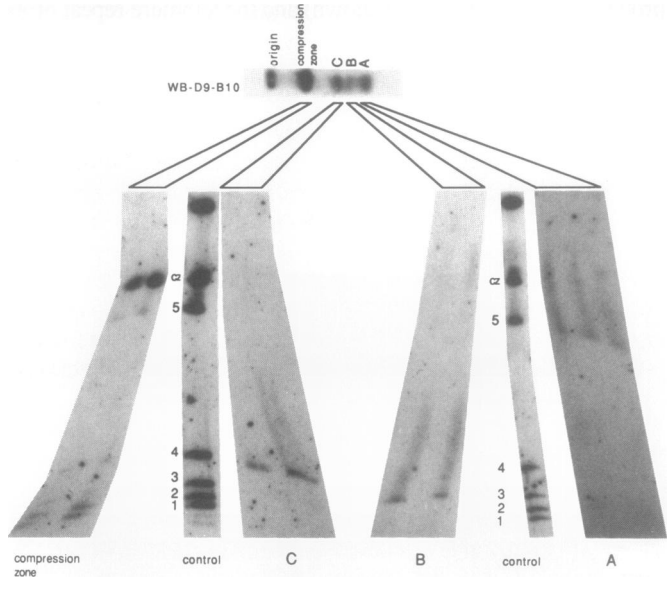

Fig. 7. Chromosomal derivation of telomeres containing rRNA genes. Duplicate lanes of the subclone WB-D9-B10 separating the polymorphic rDNA-containing chromosomes were prepared by PFG electrophoresis (pulse-frequency 600 sec, 150 V, 5 days). One of the lanes was hybridized with a rRNA probe (2 kb Eco RI/Pst I fragment from pGRPI) (the upper, horizontal lane is a typical separation) and regions of the duplicate lane corresponding to the rRNA-containing chromosomes were cut into 1mm slices and each one digested separately by the restriction endonuclease Spe I (see Materials and Methods). DNA in blocks was digested in parallel as controls. The slices and blocks were loaded onto PFG gels (pulse-frequency 40 sec, 330 V, 40hr), blotted and hybridized with a rRNA probe (2 kb Eco RI/Pst I fragment from pGRPI). Representative lanes from the second dimension PFG gels are shown aligned in the lower portion of the figure. The tails of hybridization signal represent sheared DNA. cz is the compression zone. Phage lambda ladders were used as markers (see Fig. 5).

Locating polymorphic restriction enzyme fragments to polymorphic chromosomes

We have shown that polymorphic chromosomes that encode rRNA genes exist in the genome of *G. lamblia*. The physical maps of the telomeric rRNA genes also showed size changes, but these were too short to account for the more dramatic changes in the sizes of entire chromosomes. We therefore wished to confirm that the polymorphic telomeres were indeed components of the polymorphic chromosomes. We determined the chromosomal location of each of the rRNA-hybridizing restriction enzyme fragments 1−5 in subclones WB-D9-B10 and WB-E10-C4. PFG gels were run under conditions that separated some of the rearranged chromosomes while the majority of the chromosomes were confined to the compression zone (see the inset in Fig. 4, and Fig. 7; chromosome-sized DNA has been numbered A−E). The size-fractionated chromosomes were next cut from the gel and digested with the restriction enzyme Spe I after which the fragments were size-separated in a second PFG gel and hybridized with a rRNA probe (Fig. 7). The faint smears of hybridization signal extending into the high molecular weight range behind each band represent sheared and partially digested DNA. First, we could show that all five restriction fragments characterized were associated with the compression zone that contained the majority of the chromosome complement. However, restriction fragments 1 and 2 were only derived from the compression zone. Restriction fragment 4 was associated with PFG band C; restriction fragment 3 with PFG band B and restriction fragment 5 was associated with band A in WB-D9-B10. Restriction fragment 5, the telomeric restriction enzyme fragment that had changed in size, was therefore indeed derived from a polymorphic chromosome, band A. The association of restriction fragments 3, 4 and 5 with the compression zone may be the result of mechanical trapping of a fraction of the small chromosomes at the compression zone.

In a similar analysis we also mapped restriction fragments to PFG bands from subclone WB-E10-C4 (as summarized in Fig. 4). In this clone the invariant restriction fragments 1, 2 and 4 were similarly associated with the compression zone (E) and band C, while the polymorphic restriction fragments 3 and 5 were derived from the compression zone and the polymorphic chromosome band D, respectively. It is important to note that size differences in the chromosomes cannot be accounted for by the rearranged telomeres alone, ie. fragment 3 differs in size by 40 Kb while its chromosome differs by >200kb when comparing WB-E10-C4 and WB-D9-B10.

It is therefore clear that the rearrangements involve major portions of individual chromosomes. Thus, while rRNA genes were used as the markers of this process, other regions of the chromosomes were involved.

DISCUSSION

We have shown that the genome of the protozoan *G. lamblia* can undergo genomic reorganisation involving a subset of chromosomes encoding ribosomal RNA genes. These events

occur at a high frequency and involve segments of DNA, hundreds of kilobasepairs in size, comprising a large portion of individual chromosomes. 60% (16/27) of progeny subclones had discrete karyotypes which not only differed from the karyotype of the source population, but also from one another. Sub-subclones also had new karyotypes.

The WB strain is an uncloned isolate and therefore may be considered to be a mixed population. Hence the heterogeneity seen among the clones after the first cloning step may reflect extensive size heterogeneity of the rRNA-encoding chromosomes in individual trophozoites selected from the mixed parent WB population. Each clone would represent a small percentage of the cells in the WB population with each different karyotype contributing to a background smear, indeed a smear was a consistent component of the WB parent population karyotype (Fig 1A). However, subclones prepared from these clones also showed discrete chromosome size polymorphisms: for example, in one experiment 61% (14/23) of the subclones of clone WB-F9 had rearranged chromosomes. The new karyotypes seen in the subclones and sub-subclones are the result of chromosome rearrangements that must have occurred either during the selection of the individual trophozoite at the cloning step, its expansion or the subsequent cloning step that generated the subclone. Two hypotheses can be invoked to explain this hypervariability in the rRNA-containing chromosomes. Firstly, these events could have accumulated during the expansion of the cloned line prior to the limiting dilution cloning step that generated the subclones (approximately 32 generations of growth). An estimated total rearrangement rate of 3% per division could account for this. This is a minimum figure since we have only analysed a limited size range of chromosomes so polymorphisms confined to the larger chromosomes would have gone unnoticed. These rearranged karyotypes would not be visible as a component of the source population since each different karyotype will comprise a small fraction of the population. A similar high rate (1%) has been reported in a rearranging retrotransposon in the mini-exon locus of *Crithidia fasciculata* (25).

An alternative explanation is that the rearrangements occurred during the cloning process perhaps due to stress. *Giardia* trophozoites are killed by prolonged exposure to atmospheric oxygen tensions, even in the presence of reducing agents (26), however, oxygen stress is unlikely to be involved in the generation of the rearrangements since limiting dilution cloning performed in the presence of the antioxidant butylated hydroxyanisole (5 μM) did not affect the proportion of clones with rearranged karyotypes. Further analysis will allow us to test these hypotheses. Furthermore, it remains to be determined whether the chromosomal rearrangements only occur *in vitro* or whether they also occur during the infectious cycle of *Giardia*. Given the extensive differences in the karyotypes of related *Giardia* isolates it is likely that these rearrangement events have their corollary *in vivo* (S.H.K., S.M.L.B. and L.H.T.V.D.P., manuscript in preparation).

An interesting feature of the rearranging chromosomes is that they have telomeric rRNA genes. The complement of rRNA genes in *G. lamblia* is estimated to contain about 60 copies arranged in tandem arrays (11, 27). rRNA genes in most organisms analysed to date are located in the vicinity of chromosome ends (28). A rRNA array is located within 150 Kb of a telomere in *Plasmodium falciparum* (23). We have shown that most of the rRNA genes in *G. lamblia* exist as short arrays on at least six telomeres. Four of these telomeric fragments have the same overall organisation (Fig. 4) delineated proximally by an island of restriction enzyme sites and distally by telomere repeats. The DNA separating the island of restriction sites and the telomere repeats ranges in size from 25–300 kb and contains the short array of rRNA genes and hitherto uncharacterized sequences. The paucity of restriction enzyme sites suggests that this may comprise simple sequence DNA, or perhaps subtelomeric repeats. The precise position of the rRNA gene arrays in relation to the telomere repeat arrays is not known although close proximity is suggested by a cloned telomere where a truncated rRNA gene repeat is juxtaposed to the telomere array (20).

The mechanisms underlying the rearrangement events are still unclear. We do not know whether the polymorphic chromosomes represent homologues or whether they merely share telomeres containing rRNA genes. Possible recombinational mechanisms thus include, unequal sister chromatid exchange, non-sister chromatid exchange and/or gene conversion mediated perhaps by rRNA repeats or subtelomeric repeats. These events may be mitotic since meiosis has not been demonstrated in *G.lamblia*, gametes have not been seen and there is no genetic or morphological evidence of conjugation. It is of interest to note that homologous recombination in the yeast *Saccharomyces cerevisiae* occurs by unequal sister chromatid exchange (29), where the mitotic unequal crossover rate in the rRNA locus was estimated to be 1%.

The telomeric location of the rRNA genes and the telomeric restriction fragment length polymorphisms indicate that these sequences may play a role in the rearrangement events. Chromosome size polymorphisms in *Plasmodia* are generated by homologous recombination within subtelomeric repeat sequences (23, 24, 30, 31). Evidence for subtelomeric translocation between nonhomologous human chromosomes has been described (32). Independent chromosome markers will facilitate analysis of the nature of the recombination events in *G. lamblia*. Alternative mechanisms to explain the chromosome size polymorphisms involving extrachromosomal elements containing rRNA (33) are less likely since we do not have evidence for the presence of episomal rRNA genes or extensive rRNA gene amplification, associated with the rearrangment events (Le Blancq and Van der Ploeg, unpublished observations).

Chromosome rearrangements have been described in numerous protozoan species whose genomes are plastic. In both *Plasmodia* and trypanosomes these rearrangements are associated with changes in the surface antigen repertoire and its expression. Antigenic variation has also been demonstrated in *G.lamblia in vitro* and *in vivo* (3, 4, 5) although it does not appear to persist during chronic infections. A family of cysteine-rich surface protein genes is present in the *G. lamblia* genome and its genomic organisation differs among clones expressing different surface antigens (14). We detected polymorphisms in the physical map of this gene family among the cloned lines studied in this paper (data not shown). However, members of this surface antigen gene family were not present in the rearranging *Giardia* telomeres and were not located on polymorphic chromosomes.

rRNA genes are present in both nuclei in *G. lamblia* (8). It is therefore probable that the genome may be organised differently in the two nuclei since it is unlikely that identical rearrangement events would occur simultaneously in both nuclei. This would suggest that the genomic content of each nucleus may diverge over time and that homogenisation could only be achieved by inter-nuclear sequence information exchange (the nuclear membranes remain intact throughout the cell cycle in *Giardia*).

Our data suggest that DNA rearrangements of rRNA-encoding chromosomes occur at high frequency in *G. lamblia* and we would like to understand the basis for this potentiation of the recombination machinery and the possible impact of genome plasticity on the control of differential gene expression in *Giardia*.

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