Spontaneous chromosome rearrangements in the protozoan *Giardia lamblia*: estimation of mutation rates

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Received May 27, 1992; Revised and Accepted August 11, 1992

ABSTRACT

Subcloned lines of the WB strain of *Giardia lamblia* contain polymorphic ribosomal RNA (rRNA) encoding chromosomes (Le Blancq *et al.*, Nucl. Acids Res. 1991, 19, 4405–4412). We show that in a continuously propagated culture of *G. lamblia* trophozoites the proportion of trophozoites with rearranged rRNA encoding chromosomes gradually increases, consistent with the high mutation rate of about 1% per cell per division cycle. This conclusion is based on the finding in one experiment that after about 8 division cycles 20% of the population consisted of independent mutants, while after ~100 division cycles 87.5% of the population were independent mutants. In a second experiment, approximately 38% and 71.5% of the trophozoites were independent mutants after ~9 and ~100 division cycles, respectively. The data show that the genome of the WB strain of *G. lamblia* has a highly recombinogenic phenotype. Extensive karyotype heterogeneity has also been observed among recently isolated *G. lamblia* strains obtained from a defined geographic area (Korman *et al.*, J. Clin. Invest. 1992, 89, 1725–1733) suggesting that a high mutation rate might also occur *in vivo*.

INTRODUCTION

Giardia lamblia is a protozoan parasite of man and other mammals. Its life cycle consists of binucleated trophozoites, that parasitize the upper intestinal tract and replicate by binary fission, and quadrinucleated infective cysts which are passed out in the faeces. The majority of *G. lamblia* infections in man are asymptomatic but some cases can be associated with diarrhea and malabsorption; the factors responsible for virulence are unknown. Extensive heterogeneity has been seen in the number and size of the chromosomes (karyotype) among different isolates of *G. lamblia* from within a single geographic area (1), indicating that karyotype variability may be a feature of *G. lamblia*.

We have previously shown that the genome of the WB strain

of *G. lamblia* is plastic: subcloned lines contained a polymorphic subset of hypervariable chromosomes encoding the ribosomal RNA (rRNA) genes (2). The rearranged chromosomes were linear molecules with polymorphic telomeres encoding rRNA genes. These telomeric polymorphisms were not readily attributable to either expansion and contraction of telomere repeats or to changes in the copy number of the rRNA genes (2, 3). The rearranged chromosomes differed in size by hundreds of kilobase pairs of DNA and this size heterogeneity was only partly attributable to the polymorphic telomere domains. Such chromosome rearrangements involve heritable changes in the primary sequence of the DNA and are therefore mutations by definition. It is likely that recombination is the primary mutation process since point mutations alone could not achieve such size changes.

In our initial experiments 60% of subcloned lines had new karyotypes, suggesting either a very high mutation rate of approximately 3% per division, or cloning-induced rearrangement events (2). Theoretically a constant mutation rate will result in a progressively increasing proportion of mutated individuals in a proliferating culture, since new mutants are constantly being added to the exponentially growing population of old ones (assuming that the growth rates of mutant and wild type cells are equivalent [4, 5]). Hence the proportion of mutated cells in the population will increase with time (Fig. 1, solid line). In contrast, if the mutations are induced by an exogenous factor they will only occur at induction; following the induction, the proportion of mutated individuals in the culture will remain constant with time (Fig. 1, dashed line). Moreover, if the sampling method itself, i.e. limiting dilution cloning, is the inducer of mutation, then the proportion of mutated cells will be the same in each sample irrespective of the age of the sampled population. Hence we set out to distinguish between spontaneous and cloning-induced mutations as the source of the karyotype variability by comparing the proportion of mutated versus wild type cells in proliferating populations of *G. lamblia* trophozoites.

In this paper we show that the proportion of *G. lamblia* trophozoites with rearranged (mutated) karyotypes increases with

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Figure 1. Discriminating spontaneous mutations from cloning-induced mutations: an idealized version. A spontaneous mutation rate results in the accumulation of mutations in a growing culture since new mutants are continuously being added to the exponentially growing population of old ones (4). Hence, the proportion of mutants increases with time such that the proportion of mutants = number of division cycles $\times$ mutation rate (assuming that the growth rate of mutant and wild type is the same) (solid line). In contrast, in a culture where mutations are induced by an exogenous factor, mutants only accumulate while the induction is applied. In a case where the sampling method itself is the inducer e.g. limiting dilution cloning, the proportion of mutants will be the same in each sample irrespective of the age of the sampled population (dashed line).

time. This observation supports the contention that *G.lamblia* rRNA-encoding chromosomes are subject to spontaneous mutations. We calculate the probability of mutation per cell per division cycle to be $\sim 1\%$.

MATERIALS AND METHODS

Cell strains and culture

G.lamblia strain WB (ATCC 30957) was obtained from the American Type Culture Collection. The mutational analyses were initiated with individual trophozoites from subcloned lines of the WB strain to avoid any possible carry over of preexisting heterogeneous karyotypes from the parental population. Briefly, the parent WB strain was cloned by limiting dilution using microtiter plates (6). Two clones WB-F9 and WB-D9 were recloned to generate the subclones WB-F9-H7 and WB-D9-B10, respectively (Fig. 2). The probability that each cloned line was indeed derived from a single trophozoite is 96.8% for the WB-F9 series, and $>90\%$ for the WB-D9 series. Trophozoites were grown in modified TYI-S-33 medium (7) with antibiotics. Cryopreservation and recovery of frozen stocks were as described (2).

Limiting-dilution cloning of *G.lamblia*

Microtiter plate cloning. Trophozoite suspensions were diluted in chilled fresh medium to a final concentration of 0.25 cells/ml. Aliquots of 0.25 ml were transferred to 96-well microtitre plates which were incubated anaerobically at 37°C as previously described (2, 6). Positive wells were scored after 4–5 days.

Vacutainer cloning. This method of limiting dilution cloning was used because we needed to recover the majority of the trophozoites and in our experience trophozoites detach from glass much more easily than from tissue culture microtiter plates. The

medium was aspirated from a midlog phase culture, removing any aggregates of trophozoites, and replaced with fresh chilled medium. The tubes were chilled in ice water to detach adherent trophozoites. The suspension of trophozoites was serially diluted to a final concentration of 0.6 cells/ml. Aliquots of 0.1 ml of this 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 0.06 cells/tube. The probability that each positive tube is derived from a single organism is 96.8% (6). Aliquots of 0.1 ml of the penultimate suspension in the serial dilution (6 cells/ml) were inoculated into 10 vacuum tubes containing 1.9 ml fresh medium to verify the dilution series. The vacuum tubes were incubated at 37°C for 72 hours (approximately 8 division cycles) to give a predicted population of ~ 256 trophozoites (the generation time has previously been shown to be 9–10 hr [8] a value that closely matched our observations [see next sections]). Each tube was examined under an inverted microscope for the presence of trophozoites. Positive tubes were chosen and processed as described below. The remaining tubes were incubated for a week to verify the early scoring of positive and negative tubes (confluent monolayers of trophozoites are easily visualised).

Generating and sampling *G.lamblia* populations

A vacuum tube containing the progeny of a single trophozoite after ~ 8 division cycles (~ 256 trophozoites; see above) was chilled in icewater for 10 minutes and inverted several times to release the adherent trophozoites. Trophozoites readily detach from glass when cooled, and hence the majority of the cells will be present in suspension after chilling in ice water (9). The cell suspension was removed from the vacuum tube and the tube was rinsed with 2 ml of fresh chilled medium to ensure maximal recovery of trophozoites. The cell suspension was divided in two: one half constituted the sample of the population at ~ 8 division cycles. These ~ 128 cells were inoculated into 480 ml fresh medium preparatory to limiting dilution cloning, (see above for methods involved in the microtiter plate cloning). The other half of the original cell suspension constituted the population: the ~ 128 cells were inoculated into 14 ml of fresh medium in a 16 ml screw-capped borosilicate tube which was incubated at 37°C and then propagated by serial passage every 2 days. Serial passage consisted of chilling a late log phase culture tube in icewater for 10 minutes, inverting it to mix and inoculating a 0.5 ml aliquot of the resulting trophozoite suspension ($\sim 7 \times 10^5$ cells) into 19.5 ml of fresh medium in a 20 ml borosilicate tube. The population was propagated for approximately 100 division cycles at which point the population was sampled again by subjecting an aliquot to limiting dilution cloning (microtiter plate cloning) as described above.

Preparation of chromosome-sized *G.lamblia* DNA

Late logarithmic phase cultures were chilled and harvested by centrifugation at 4°C as described previously (2), except that the 3 washes were replaced by a single wash in 1 ml ice cold TSE (10 mM Tris-HCl pH 8, 100 mM NaCl, 50 mM EDTA). Blocks were prepared as previously described (1, 2).

Pulsed field gradient gel-electrophoresis (PFGE)

PFGE was performed as previously described (10). Please note that in PFGE each set of running conditions will give optimal separation of a specific chromosome size class; molecules outside

of this size range will appear compressed together. The specific running conditions (pulse time [sec], voltage and run duration [days]) chosen allow separation of chromosomes ranging in size from about 50 kb to 2 megabase pairs (Mb). *Trypanosoma brucei* variant 118 clone 1 chromosomes (11) and phage lambda were used as size markers. After electrophoresis, gels were stained with ethidium bromide (2.5 µg/ml). Southern transfer of DNA to nylon filters was as previously described (1).

DNA probes and hybridisation

The following DNA probes were used: *Ribosomal RNA (rRNA)*: a 0.5 kb Pst I fragment including the 3' end of the spacer and 5' end of the large subunit rRNA gene, from pGRPI (kindly provided by Dr. C.C. Wang [12]). *Surface Antigen: D2dS*, a 1.4 kb Sma I/Kpn I from the coding region of TSA 417 (kindly provided by Dr. F.D. Gillin [13]). *Surface Antigen: M2-1*, a 1 kb fragment including two complete copies of the 195 bp tandem repeat from the coding region of CRP170 (kindly provided by Dr. R. Adam [14]).

Plasmid manipulations were carried out using standard protocols (15). DNA probes were prepared from gel-purified restriction fragments using the random priming method (Random Prime Kit, Boehringer Mannheim) with [α^{32} P]dCTP. Southern blots were hybridised in 10% dextran sulphate, 3×SSC, 10×Denhardt's reagent and 0.1% SDS with 100 µg/ml sheared and denatured salmon sperm DNA at 65°C over night. Post-hybridisation washes were: once in 2×SSC for 15 min at 65°C, twice in 2×SSC, 0.1% SDS for 15 min at 65°C and finally in 0.1×SSC, 0.1% SDS at 65°C. (The final post hybridization washes for the experiment shown in Figure 4 was 0.2×SSC, 0.1% SDS at 65°C.) Prior to rehybridisation, filters were stripped in 0.4 M NaOH for 30 min at 45°C, washed in 0.2 M Tris-HCl pH 7.5, 0.1×SSC, 0.1% SDS at 45°C and exposed for autoradiography to evaluate any residual signal.

Mutation rates

The mutation rate (m , defined as the probability of mutation per cell per division) can be calculated from the formula:

$$p = dm$$

where p is the proportion of mutants and d is the number of division cycles (5).

When the number of mutants at two time intervals is known then the mutation rate can be assessed from the formula:

$$m = 2 \log_2(M_2/N_2 - M_1/N_1)/d$$

where m = mutation rate per division cycle, M_1 and M_2 , and N_1 and N_2 are the number of mutants (M) and total cells (N) at times 1 and 2, respectively and d is the number of division cycles (5). For these experiments numerical values for M and N per se were not available and the proportion of mutants in the population at times 1 and 2, (p_1 and p_2 , respectively), was used directly.

RESULTS

We measured the proportion of trophozoites with mutant karyotypes in samples from two proliferating populations of the WB strain of *G. lamblia*. These samples were taken after each population had undergone ~ 8 and ~ 100 division cycles. The

Figure 2. Experimental design for distinguishing spontaneous mutations and cloning induced mutations in *Giardia lamblia*. The two subclones WB-F9-H7 and WB-D9-B10 were derived by limiting dilution cloning using microtiter plates. In each experiment a population derived from a single trophozoite via vacutainer cloning is sampled after ~ 8 and ~ 100 division cycles. * indicates a cryopreservation and recovery step interrupting the continuous propagation in Experiment I. There was no cryopreservation step in Experiment II. Sampling consists of i. expanding an aliquot of trophozoites representative of the total population, harvesting the cells for karyotype analysis using PFGE; and ii. cloning individual trophozoites via limiting dilution cloning, expanding the clones for harvesting and karyotype analysis (solid flat arrowheads indicate clones).

karyotypes were analysed by PFGE and mutations were scored based on the occurrence of polymorphic chromosomes that hybridized with a rRNA probe.

Measurement of the proportion of mutant trophozoites in samples of a population (C15) taken at two time points (Experiment I)

A schematic outline of this experiment is given in Figure 2. The population C15 was derived from a single trophozoite which was sampled after 72 hours of growth by subjecting half of the trophozoite suspension to limiting dilution cloning. Eighty-three clones were established indicating that, as expected, between 7 to 8 division cycles had occurred ($2^8 = 256$). It should be noted that the population was dividing asynchronously. Forty of these clones were chosen at random, expanded and harvested for PFGE

Figure 3. Karyotypes of a *G. lamblia* population C15 sampled after ~8 and ~100 division cycles. Panel A: Southern blot of a PFGE gel comparing the chromosome-sized DNA of ten clones derived from population C15 after ~8 division cycles (lanes 1–5 and 9–13) and the population itself (C15 (8d); lane 7). Lane 6 is the subclone WB-F9-H7 from which C15 (lane 7) and B2 (lane 8) were derived via vacutainer cloning. The blot was hybridised with an rRNA probe. The white arrow indicates the polymorphic rRNA encoding chromosome in lane 4. Panel B: Southern blot of a PFGE gel comparing the chromosome-sized DNA of ten clones derived from population C15 after ~100 division cycles (lanes 1–5 and 8–12) and the population itself at ~100 division cycles (C15 (100d); lane 6). C15 (8d) (lane 7) is the population sampled earlier on. The blot was hybridised with an rRNA probe. Electrophoresis conditions in Panel A and B were: 650 sec pulse time, 7.5 V/cm, 6 days. *T. brucei* chromosomes (not shown) were used as size markers. c.z. is the compression zone.

analysis. An aliquot of cells from C15 itself was also expanded and harvested for PFGE analysis, designated C15 (8d) (Fig. 2). Population C15 was cryopreserved after it had been propagated for ~20 division cycles. An aliquot of this cryopreserved material was recovered and propagated for a further ~80 division cycles, to give a total of ~100 division cycles. The possibility that this cryopreservation step might have affected the outcome of the experiment is unlikely, since no significant difference in outcome was observed when this step was omitted in a second experiment (see next section). Population C15 was sampled after a total of ~100 division cycles by subjecting an aliquot of trophozoites to limiting dilution cloning. Forty clones were chosen at random, expanded and harvested for PFGE analysis. Cells

were also harvested directly from population C15 for PFGE analysis of the total population, designated C15 (100d).

PFGE was performed on all of the clones to examine and compare the karyotypes of their rRNA-encoding chromosomes with that of the C15 (8d) and C15 (100d) populations (see Fig. 3). The same PFGE conditions were used throughout and those selected allowed good separation of the majority of the rRNA-encoding chromosomes. We could detect size changes ranging from 40–600 kb in the well-fractionated chromosomes. The karyotype of the founding trophozoite (C15) is wild type and was represented by the karyotype of the total population i.e. C15(8d) and C15(100d) (see below). Karyotypes of the clones were scored as being mutant when a detectable size difference was seen in one or more chromosome bands or if a chromosome band(s) was missing when compared to the wild type karyotype. Intensity differences among chromosomes were not scored.

The karyotypes of 10 of the 40 clones examined from the population sampled after 8 division cycles are shown in Figure 3A. One out of these 10 clones had a mutant karyotype (lane 4, note the extra band marked with a white arrow). In total, 8 of the 40 clones had mutant karyotypes and each of the mutant karyotypes was different from the others. (Please note that these mutations involved chromosome size changes measuring hundreds of kilobase pairs.)

In contrast, 35 of the 40 clones from the population sampled after 100 division cycles had mutant karyotypes. Ten of the clones with mutant karyotypes are shown in Figure 3B. The polymorphic and non-rearranged chromosomes show an equivalent range of rRNA hybridisation signal intensities (compare lanes 3, 6 and 12 in Fig. 3B, for example). While it appears that some chromosomes are more likely to be mutated than others, mutations have been seen in all of the rRNA-encoding chromosomes, including the 1.1 Mb chromosome which is not mutated in any of the clones shown in Figure 3B. We cannot distinguish between mutation from wild type and secondary mutations in cells that already had a previous mutation. The presence of multiple differences in some karyotypes (e.g. lane 5, Fig. 3B) suggests that they are the result of more than a single mutation event. The karyotype of the total population after 100 division cycles, C15 (100d) (lane 6, Fig. 3B) shows an increased background smear of hybridization when compared to the karyotype of the younger population (C15 (8d); lane 7, Fig. 3B). This is consistent with an increasingly heterogeneous population containing a multiplicity of polymorphic chromosomes contributed by each individual mutant in the population. Nonetheless, the wildtype karyotype still dominates even after one hundred division cycles because not all of the chromosomes in each mutant are mutated and each new mutant represents only a small fraction of the total number of cells in the population. The data are summarised in Table I.

Measurement of the proportion of mutant trophozoites in samples of population A6 at two time points (Experiment II)

Experiment II followed the same methodology as Experiment I (Fig. 2) except that a cryopreservation step which had been introduced in Experiment I, did not interrupt the population's growth. Population A6 was derived from a single trophozoite and the sample taken after 72 hr of propagation yielded 168 clones. Hence this population had undergone at least one more doubling than population C15, i.e. approximately 9 division cycles. This could either reflect a shorter doubling time, a

Table I. Summary of the number of clones with mutant karyotypes derived from *G.lamblia* populations sampled after ~8 and ~100 division cycles.

Experiment	Population	Division cycles	Number of clones analysed	Number of mutant karyotypes*	Estimated mutation rates [†] $m = p/d$	$m = 2 \log_e 2(p_2 - p_1)/d$
I	C15	~ 8	40	8/40 (20%)	0.025 (2.5%)	0.01 (1%)
		~ 100	40	35/40 (87.5%)	0.00875 (0.875%)	
II	A6	~ 9	40	13/40 (38%)	0.042 (4.2%)	0.005 (0.5%)
		~ 100	35	25/35 (71.5%)	0.00715 (0.715%)	

* Based on polymorphic ribosomal RNA-encoding chromosomes.

[†] The mutation rate, m , was calculated as follows: $m = p/d$ where p is the proportion of mutants and d is the number of division cycles; and $m = 2 \log_e 2(p_2 - p_1)/d$ where p_1 and p_2 are the proportion of mutants at times 1 and 2, respectively (5).

decreased lag time for the first cell division or the unlikely possibility that the population was founded by two trophozoites.

Thirteen of the 40 clones from the population sampled after 9 generation cycles had mutant karyotypes (Table I and data not shown). However, two mutant karyotypes were represented by 5 clones. This suggests that these mutations occurred during one of the early division cycles and therefore represented a greater fraction of the total population than other mutants that had occurred at a later stage. This is likely to account for the increased number of mutant clones when compared to population C15. Twenty-five out of 35 clones from the population sampled after 100 division cycles had mutant karyotypes, all of which were different from one another. The data and estimated mutation rates are summarized in Table I.

Polymorphisms in genomic organization of surface antigen gene sequences

The polymorphic chromosomes were initially identified with rRNA genes as a marker sequence. We wanted to determine whether other genes were also affected by the high mutation rate. We therefore examined the karyotypes of all the clones from population C15 for evidence of heterogeneity in two cysteine-rich surface antigen gene families: TSA 417 and CRP170. Three out of 40 and 22 out of 40 of the clones from the ~8 and ~100 division cycle samples, respectively, showed differences in the distribution of sequences hybridizing with the TSA 417 gene probe (Figure 4 (faintly hybridizing bands; examples are marked with asterisks in lane 8); and data not shown). All of the clones which had heterogeneous distribution of TSA 417 sequences also had polymorphic rRNA-encoding chromosomes. The karyotypes of 10 clones hybridized with the TSA 417 probe are shown in Figure 4. Eight of the clones show heterogeneity and 2 are wild type (lanes 1 and 12). The majority of chromosomes of the WB strain of *G.lamblia* tend to comigrate and distribute into a few size classes on PFGE gels (16); hence the non-stoichiometric ethidium bromide staining intensities in Figure 4A and the relatively faint ethidium bromide staining of the polymorphic chromosomes. The TSA 417 hybridisation signal is primarily distributed among chromosomes that fractionate into three size classes, one of which is in the compression zone in Figure 4, with only a small component present in polymorphic chromosomes (e.g. bands marked with an asterisk in lane 8 of Fig. 4B). The TSA 417 gene family comprises at least 4 or 5 different copies presumably accounting for the strongly hybridizing bands (F.D. Gillin and S.B. Aley personal communication). Therefore, the sequences that hybridize only

Figure 4. Distribution of TSA 417 sequences in *G.lamblia* clones. PFGE gel comparing the chromosome-sized DNA of ten clones derived from population C15 after ~8 and ~100 division cycles and the population itself (C15 (8d) and C15 (100d) lanes 7 and 6, respectively). Electrophoresis conditions used were: 650 sec pulse time, 7.5 V/cm, 6 days. *T.brucei* chromosomes (not shown) were used as size markers. c.z. is the compression zone. Panel A: ethidium bromide stained PFGE gel. Panel B: southern blot of the gel in panel A hybridised with the surface antigen TSA 417 (D2dS) probe. The clones in lanes 1 and 2 were from the ~8 division cycle sample; the other clones (lanes 3–5 and 8–12) were from the ~100 division cycle sample (none of these clones are included in Figure 3). The asterisks (*) indicate examples of polymorphic chromosomes hybridising with the TSA 417 probe in lane 8.

weakly in the polymorphic chromosomes may represent pseudogenes, partial copies or crosshybridizing sequences. The polymorphic chromosomes containing TSA 417 sequences comigrated with polymorphic rRNA-encoding chromosomes (data not shown), strongly suggesting that they were encoded in the same molecules. It should be noted, however, that TSA 417 sequences were not detected in every rRNA-encoding chromosome.

The CRP170 gene family of surface antigen genes was primarily restricted to chromosomes that remained in the

compression zone in these PFGE gels (data not shown); hence, although no chromosome-size polymorphisms were detected in the distribution of the CRP170 gene family, this may merely reflect the detection limits of these gels rather than a genuine absence of heterogeneity. Indeed, restriction fragment length polymorphisms (RFLPs) were detected in a Pvu II fragment hybridising with the CRP170 gene probe in 1 of 4 clones analysed (data not shown); moreover a ladder of Pvu II RFLPs ranging in size from 3.5 to 5 kb was seen in the C15 population itself. This parallels the observation of Adam et al (17) who attribute the unit size differences to changes in copy number of a 95-bp repeat region of CRP170.

DISCUSSION

We have demonstrated that the proportion of trophozoites with mutant karyotypes increases with time in proliferating populations of the WB strain of *G. lamblia*. This observation is consistent with the hypothesis that *G. lamblia* rRNA-encoding chromosomes are subject to spontaneous mutations. We estimate that the mutation rate for the rRNA-encoding chromosomes is ~ 1% per cell per division cycle. We can not exclude the possibility that a small number of the rearrangements was induced by limiting dilution cloning nor can we exclude the possibility that the periodic manipulation of the cells during expansion of the trophozoite populations (i.e. chilling to 4°C) served as an inducer of mutation.

The chromosome size changes ranged from tens to hundreds of kilobase pairs. It is important to note that these size changes are not merely due to telomere repeat associated rearrangement events. This conclusion is based on the findings that the average size of the telomere repeat array in *G. lamblia* is 0.5–1 kb (3) and that the telomere repeat arrays at both ends of the 1.1 Mb rearranged chromosome in the WB-F9-H7 subclone (2) did not exceed 1 kb in size (Le Blancq and Van der Ploeg, unpublished). Therefore the mutations lie elsewhere in the chromosomes. We do not know whether the polymorphic chromosomes are homologues or merely share homologous regions (ie. rRNA genes), indeed the ploidy of *G. lamblia* trophozoites has not been ascertained. Thus while changes in ploidy may occur, recombination must be involved in generating the size polymorphisms we observed. The polymorphic chromosomes contain rRNA-encoding sequences in large telomere domains whose paucity of sites for digestion by restriction endonucleases suggested that they comprised simple sequence DNA (2). The presence of sequences that hybridized to one of the cysteine-rich surface protein gene families (TSA 417) suggests that these chromosomes may include other sequence classes.

The estimated values for the mutation rates all show a remarkably high value but also reveal considerable variation, depending on the formula applied, the population examined and our estimation of the number of division cycles (Table I). We assume that the second method for calculating the mutation rate is more accurate since it compares the fraction of mutated cells at two different time points. Also given that the potential error is largest when comparing mutation rates after very few division cycles, the more accurate measurements range from 0.5% to 0.715%, 0.875% and 1%. However, all of these estimations of the mutation rate are subject to error. Firstly, we know that we have underestimated the total number of mutants since we only analysed chromosomes up to 2 Mb in size; we were not able

to detect mutants with chromosome size changes that were less than ~40 kb; and we assumed that each change in the karyotype was the result of only a single mutation. We also assumed that the growth rate of mutant and wild type cells were the same and constant, while it is likely that some mutants will either be inviable or slow growers and hence be underrepresented in the population, thus leading to an underestimation of the mutation rate (our data indicate that it is unlikely that any particular mutants grew very rapidly and were overrepresented in the population since no particular mutant was seen to predominate, or to accumulate with time). Secondly, in such analyses it is usually assumed that the mutation rate is very low so that the number of wild type organisms available to generate new mutants is almost the same as the total number of cells. In our case the mutation rate is unusually high and hence mutants will accumulate in the population rapidly such that the proportion of wild type cells will diminish and the rate of mutation from wild type to mutant will appear to decrease with time. Thirdly, errors can arise from statistical fluctuations in the number of mutants developing during early generations; this may explain the disparity in the proportion of mutants seen in population A6 when compared to population C15. Fourthly, it is possible that individual trophozoites vary in their intrinsic mutation rate, hence the differences seen between populations C15 and A6 could be due to differences between the two founding trophozoites. Notwithstanding the variability in the calculated values, it is clear that the mutation rate is at least 1% per cell per division cycle, an unusually high value.

Proper interpretation of the mutation rate in *G. lamblia* requires its comparison with mutation rates in other organisms. This, however, is not straightforward since mutation rates in most organisms are extrapolated from data involving point mutations at single loci and calculated as either rates per base pair or per genome (18): an average mutation rate of 0.0033 (0.33%) per genome per DNA replication has been described for DNA based microbes (both prokaryotes and eukaryotes) (18). We do not have any information on the rate of spontaneous point mutations in *G. lamblia* and therefore can not directly compare these data. However, by their very nature the chromosome polymorphisms described here must include other mutation processes. Chromosome rearrangements leading to similar size polymorphisms occur in other protozoa such as *Plasmodia* (19, 20) and *T. brucei* (21) but mutation rates have not been measured.

It is of interest to note that similar mutation rates have been described in the rRNA locus in *Saccharomyces cerevisiae* (1%, [22]) and tandem repetitive minisatellite regions in humans (0.4–5%, [23]). Those mutations involved rearrangements of tandemly arrayed repetitive DNA. We have speculated that the rearrangements in *G. lamblia* may also arise by recombinational processes involving tandemly arrayed repetitive elements (2); the mechanisms however remain unknown. If direct repeat recombination is involved it is obvious that the considerable length changes observed (tens to hundreds of kilobasepairs of DNA) would require substantially staggered unequal exchanges. It is instructive that RFLPs in the CRP170 gene family involve changes in the copy number of a repeat domain which is present as an array of 15–30 tandem repeats (17 and this paper). A 1% mutation rate was also reported for a rapidly rearranging retrotransposon within the minixon locus of *Crithidia fasciculata* (24) where active transposition was thought to play a role. We do not have evidence for the presence of transposable elements in the *G. lamblia* genome and the very large size changes observed are uncharacteristic of transposition-mediated rearrangements.

Although the subset of chromosomes encoding rRNA genes has been the focus of our analysis the hypervariability we observed may not be restricted to these chromosomes. Genomic rearrangements involving the CRP170 gene family, which is encoded elsewhere in the genome, are frequent (14, 17 and this paper). Hence the whole genome of the WB strain may be subject to a highly recombinogenic phenotype. This could complicate attempts to correlate gene rearrangements with the control of gene expression. Interestingly, genomic rearrangements are not restricted to the WB strain of *G.lamblia*: cloned lines from the Portland 1 strain (ATCC 30888) had polymorphic rRNA encoding telomeres (unpublished data) and one out of 13 subclones (taken from a cloned line which had undergone ~35 division cycles) of a recently isolated strain from Israel (J₂) had a mutant karyotype (1). Furthermore, extensive karyotype heterogeneity has been identified among *G.lamblia* isolates obtained from within a single geographic area. This observation suggests that high levels of mutation may exist *in vivo* and that the entire genome is affected (1).

ACKNOWLEDGEMENTS

We thank Hui-Min Chung and Alison Rattray for their invaluable assistance and Richard Deckelbaum for his support and encouragement. We thank Frances Gillin and Stephen Aley for communicating unpublished results. We thank Rob Krauss, Richard Deckelbaum, Keith Gottesdiener, Hui-Min Chung, Sabine Kohler and Turan Urmenyi for critical reading of the manuscript, C.C. Wang, Rodney Adam and Frances Gillin for kindly providing us with cloned *Giardia* genes and David Figurski, James Cox and Chantelle Nerette for helpful discussions. This work was supported by N.I.H. grant A126497 in the International Collaborative Infectious Disease Research Program to L.H.T.V.D.P. and by a grant from the John D. and Catherine T. MacArthur Foundation to L.H.T.V.D.P. who is a Burroughs Wellcome Scholar in Molecular Parasitology.

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