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Improved separation of chromosome-sized DNA from *Trypanosoma brucei*, stock 427-60

Lex H.T.Van der Ploeg, Cassandra L.Smith[1], Ramona I.Polvere and Keith M.Gottesdiener

Department of Genetics and Development and [1]Departments of Microbiology and Psychiatry, College of Physicians and Surgeons, Columbia University, 701 West 168 St., New York, NY 10032, USA

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ABSTRACT.

Separation of chromosome-sized DNA from the parasitic protozoan Trypanosoma brucei had previously resulted in the fractionation of DNA molecules that ranged in size from 50 kb up to roughly 1.5 Mb. The number of larger chromosomes and their size, accounting for 80% of the DNA of T.brucei remained unclear. We have now size separated these larger DNA molecules by pulsed field gel electrophoresis (PFG) and resolve a total of 20 bands, accounting for roughly 120 chromosomes, ranging in size from 50 kb up to the size of the largest, 5.7 Mb chromosome of Schizosaccharomyces pombe. Three different VSG gene expression sites were located to chromosomes of 430 kb, 1.5 Mb and 3 Mb, respectively. We have not been able to identify additional, previously cryptic DNA rearrangements, that could explain the activation or inactivation of the expression sites.

INTRODUCTION.

The plasma-dwelling protozoan parasite Trypanosoma brucei escapes immune-destruction by antigenic variation. Antigenic variation results from the sequential expression of different VSG genes that can each encode an antigenically distinct cell-surface coat (4, 5). The repertoire of separate VSG genes consists of about one thousand silent, basic copy (BC) VSG genes. These BC VSG genes are located in tandem arrays at chromosome internal positions as well as at chromosome ends or telomeres (6, 7). The different VSG genes are sequentially expressed in a more or less ordered manner and the process of VSG gene switching occurs independent of the immune response, at a frequency of about 10^{-6} to 10^{-7} per division (8). The activation of a BC VSG gene can occur through its transposition into a telomerically located VSG gene expression site, generating a transcribed expression linked copy (ELC) of the VSG gene. Antigenic switches can also result from the inactivation

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of the old expression site and the activation of a new expression site located on a different chromosome. The mechanism which controls the differential transcription of multiple expression sites is unclear. In a few cases the activation and inactivation of the expression site correlated with chromosomal rearrangement events that moved the site to a different chromosome (9-12). The study of antigenic variation requires knowledge of the number and size of the chromosomes as well as an overview of the chromosomal location of VGS genes. Initially, only a fraction of the genome of T.brucei could be size separated by PFG (1, 13). Recently the size range of DNA molecules amenable to fractionation by PFG was expanded to about 10 Mb (3, 14, 15). Using these techniques we have now separated most of the chromosomes from T.brucei and present a detailed overview of the number and size of chromosomes in T.brucei.

MATERIALS AND METHODS.

All trypanosomes used were of T.brucei stock 427-60; the different antigenic variants have been described by: Cross, 1975 (5), variants 117a, 118a and 221a; Michels et al., 1982, 1983, 1984 (16-18), variant 1.8a, 1.8c, 118a', 118b and 1.208; and Lee and Van der Ploeg, 1987 (19), variant 118 clone 1 and variant 118 clone 4.

Schizosaccharomyces pombe strain 972, obtained from Dr. U. Yanagida, was used as a size standard (2) as well as the yeast Saccharomyces cerevisiae strain YN295 obtained from Dr. R. Davis and wild type phage lambda (48.5 kb; 21, 22, 33). Chromosome-sized DNA was prepared essentially as described (1, 20, 23, 24). Two ug of trypanosome DNA was loaded per lane, except for gels in which smaller chromosomes (up to 600 kb) were size-separated where 10 ug of DNA was loaded.

All size separations were performed in 20 cm square 1% agarose gels at 15°C. Specific conditions were as noted in the figure legend. The apparatus used was the Pharmacia LKB Pulsaphor, a 33 cm square, inhomogeneous submarine electrophoresis unit with cathode electrode arrays on the North and West and a single anode electrode on the South and East corners. After electrophoresis the gels were stained with ethidium bromide and transferred to nitrocellulose filters as described (1). The different probes for hybridization and the hybridizational and post-hybridizational conditions are as described in the legend to the figure and as previously published (1).

Size separations in figure 1 were performed under the following conditions: In panel A, DNA was size separated at a pulse-frequency of 45 seconds at 330 Volts for 40 hours; In panel B, a pulse-frequency of 900 seconds at 150 Volts for 110 hours was used; in panel C, a pulse frequency of 2400 seconds at 100 volts for 120 hours; in panel D a pulse-frequency of 4500 seconds at 99 volts for 200 hours; in panel E a pulse-frequency

of 4500 seconds, 99 volts, for 269 hours; in panel 1F a pulse frequency of 4500 seconds and 99 volts for 169 hours; and in panel G the most left hand two lanes samples were run at 2700 seconds, 60 volts, for 135 hours.

Hybridizations were performed at 65°C for 12 hours in hybridization mixtures with dextran sulfate (28). Post-hybridization washes were at 65°C and 0.1 x SSC except for the most right hand panel of figure 1 F, which was washed at 65°C and 3 x SSC.

RESULTS.

Size separation of high molecular weight T.brucei DNA.

In figure 1 (panels A through E) representative samples of different ethidium stained PFG gels are presented showing the separation of chromosome-sized DNA from T.brucei into 20 bands. The most left hand panel (Figure 1A) shows the six different bands (bands numbered, 1-6) whose separation had been described previously (1, 9, 25). By applying longer pulse frequencies to size separate the larger chromosomes, up to five additional molecules had been separated that ranged up to roughly 1.5 Mb (11, 26). However, these size-separations suffered from a decreased efficiency of recovery of the large DNA molecules.

S.pombe chromosomes are 3.5 Mb, 4.7 Mb and 5.7 Mb (2). These S.pombe DNA molecules may be fractionated using very long pulse times and running times with low voltages of electrophoresis (3). When the trypanosome samples are run under similar conditions and are standardized against phage lambda multimers, yeast and S.pombe chromosome-sized DNA, a total of 20 bands can be identified. About one hundred mini-chromosomes in band 1 (50-150 kb); five bands range in size from 200-430 kb (bands 2-6); and nine bands range in size from over 680 kb to roughly 3 Mb (bands 7-15). These bands migrate between the phage lambda marker of 680 kb, and the smallest S.pombe chromosome measuring roughly 3.5 Mb. Finally, 4 additional bands are detected, the largest (band 19) co-migrates with the 5.7 Mb S.pombe chromosome-sized DNA molecule; Band 17 co-migrates with the 4.7 Mb S.pombe band; and band 16 migrates slightly slower then the 3.5 Mb S.pombe molecule. Additional DNA (band 20), stays at or stays close to the well of the PFG gels. We have no evidence for the presence of just a single large chromosome at this location and we infer from the data that band 20 contains several

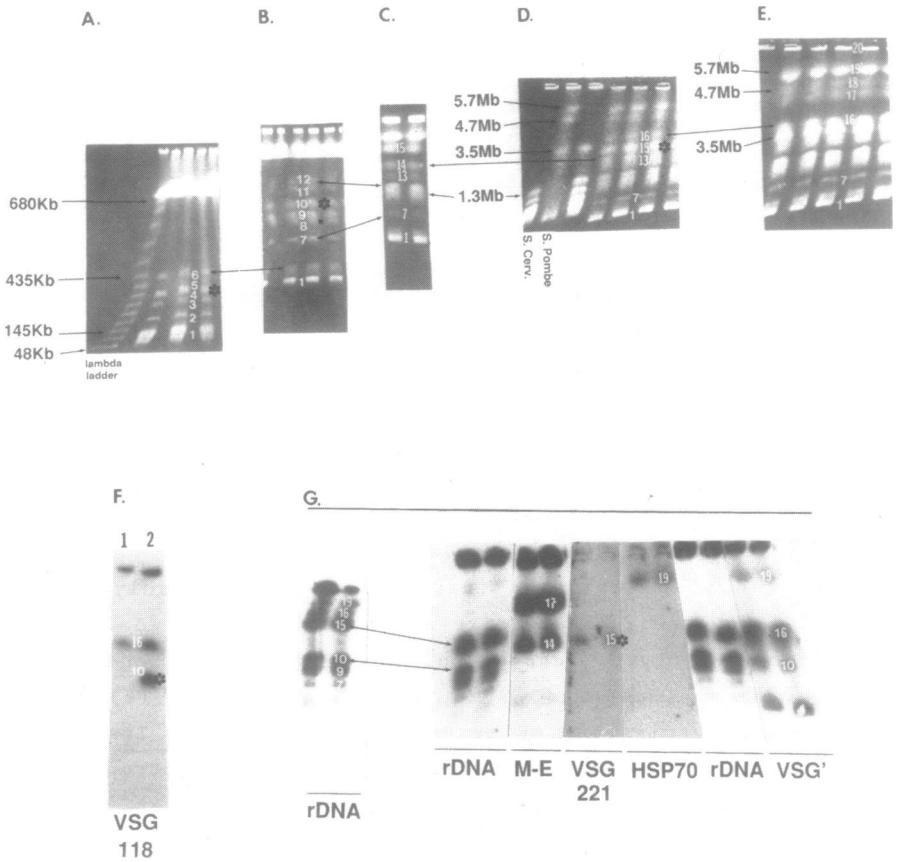

Figure 1. Size separation of T.brucei chromosome-sized DNA.
All trypanosome samples in panels A through E are ethidium stained DNA of variant 118 clone 1; panel 1F contains variant 221a (lane 1) and variant 118 clone 1 (lane 2); panel 1G, the left hand two lanes contain variant 1.208 and variant 118a DNA all other lanes are hybridizations to DNA from panel E. Panel D contains, S.cerevisiae, S.pombe and Not I digested S.pombe DNA size standards. The different ethidium stained bands are numbered 1-20. Bands denoted "no'" stain non-stoichiometrically with ethidium bromide. Horizontal arrows connect identical ethidium stained bands in adjacent panels.
The probes used are: VSG 118, a 5' cDNA probe of the VSG 118 gene (36), rDNA, a clone containing an entire rDNA repeat of T.brucei (37); M-E, the 1.4 kb mini-exon repeat unit (38); VSG 221, a 5' cDNA sub-probe of VSG 221 (39); HSP 70, an hsp 70 coding sequence probe (40); and VSG', the 3' end of VSG 118 cDNA which detects many cross hybridizing, related VSG genes (27, 41). Separation conditions are described in the Materials and Methods.

different molecules among which are the kinetoplast DNA network and maxi-circle DNA (1) as well as chromosome-sized DNA molecules which are non-specifically trapped (see next sections). In the remainder of the text we will not discuss the hybridization of the different 32P labeled probes with the DNA at the well (band 20) of the PFG gels since it is unclear whether band 20 presents additional chromosomes.

Location of expression sites and VSG genes.

A representative sample of hybridizations with different DNA probes is shown in Figure 1F and 1G. We determined the location of BC VSG genes on these chromosomes. Since all VSG genes share conserved sequences at their C-termini, one can visualize the abundance of VSG genes using DNA probes derived from the C-terminus in hybridizations that are performed under non-stringent conditions (27, 28). In the most right hand panel of figure 1G the hybridization with a probe encoding the C-terminal sequences of VSG gene 118 (panel labeled VSG') is shown. A hybridization signal is detected in band 1 which contains many telomeric VSG genes (1, 25). Three larger bands hybridize as well and must contain the tandem arrays of internally-located BC VSG genes (bands 10, 16 and 19). The fact that the conserved C-terminal probe, which detects hundreds of related genes, is spread over only three bands and the mini-chromosomes confirms our earlier observations of clustering of the VSG gene families (27, 28).

At least three bands (marked with rosettes in Figure 1) contained VSG gene expression sites: double band 4, 5 (340 kb, variant 1.208, as previously published ref. 10), band 10 (roughly 1.5 Mb), which migrates slightly slower then the largest 1.3 Mb S.cerevisiae chromosome-sized DNA molecule (variant 118, Figure 1F) and band 15, (roughly 3 Mb, variant 221, Figure 1G, and data not shown). The location of these expression sites and the location of BC VSG genes on different chromosomes show that many of the duplicative transpositions of VSG genes occur inter-chromosomally. Finally, the data confirms that the mutually exclusive transcriptional control of expression sites occurs between distinct chromosomes of

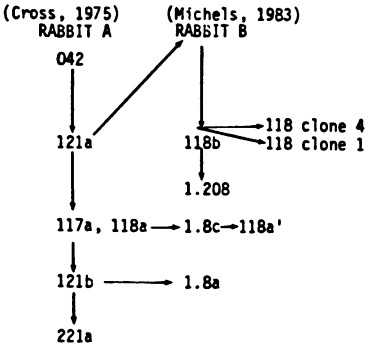

Figure 2. Family tree of antigenic variants.
The lineage of different trypanosome variants is schematically represented. Cross, 1975 is described in reference 5 and Michels et. al., 1983 reference 17. Variants 042, 121a and 121b have not been analyzed.

drastically different sizes which are therefore unlikely to represent homologues (also see Figure 2).

Approximately one hundred ribosomal RNA encoding genes (rDNA) genes are spread over at least six different bands (Figure 1G, bands, 7, 9, 10, 15, 16, 19). Only one band (number 19) hybridizes with a representative house keeping, hsp 70 gene (Figure 1G); and 2 bands (13/14 and 17) hybridize with the two hundred fold repetitive, mini-exon donor genes.

The fact that the well of the PFG gels hybridizes with few probes only (compare the signals with the rDNA and mini-exon probe to those obtained with the VSG 221 or hsp 70 probes) indicated that only specific chromosomes are confined to the well. This was confirmed by our previous observation with wild type and dys-kinetoplastid strains, showing that the molecules at the well of the PFG gels specifically contained the network of mitochondrial mini- and maxi-circles, but none of the mini-chromosomes (1).

However, non-specific trapping of molecules at the well of the PFG gels occurs as well. We had previously shown that the use of dys-kinetoplastid trypanosome strains did not affect the chromosome trapping (1). That trapping still occurs under the improved separation conditions is made clear by the fact that

the 118 VSG gene probe is expected to hybridize to a single band in variant 221, since one basic copy VSG 118 gene exists per cell (29). This probe, however, detects two bands in variant 221 (figure 1F, lane 1, band 16 and hybridization at band 20 in the well of the PFG gels). In figure 1F lane 2 (variant 118 clone 1) the same probe should hybridize to the VSG 118 basic copy and the VSG 118 ELC only. Again hybridization at the well of the PFG gels occurred in addition to the hybridization at band 10 (118 ELC) and band 16 (118 BC). Trapping of DNA must therefore contribute to the banding patterns, but only some chromosomes seem to be affected.

Chromosome rearrangements and expression site transcriptional control.

Figure 2 presents the family tree of the different variants, analyzed under the improved PFG conditions that are described above. The chromosome separation pattern of each of these variants was identical (Figure 1, and data not shown). In addition, we reexamined the location of VSG gene expression sites which were previously shown to be activated or inactivated in situ. We studied the location of the expression site with the single copy 221 VSG gene and determined that independent of the transcriptional activity of the 221 expression site it was always located on band 15 (indicated with an asterisk in figure 1D and 1G). We also analyzed the expression site which contains the transcribed VSG gene in variant 118a, 118b, 118a', 118 clone 1, 118 clone 4 and variant 117 clone 6. In each of these variants the expression site always located to band 10 (Figure 1F and data not shown). Since the physical map of the expression sites with VSG 118a, 118a' and 118b, VSG 118 clone 1 and 118 clone 4, had previously been shown to be identical, we assume that these VSG genes are activated by duplicative transposition into the same expression site (17, 28). This expression site also remained in band 10 whether it was in an active form (variant 118a, and 118b) or in an inactive form, as a lingering expression site in variants 1.8c and variant 1.208 (10, 11, 18, and data not shown). Still, since band 15 and band 10 are likely to be a double band, as judged from their non-stoichiometric staining intensity we cannot exclude that chromosome

rearrangements with activation or inactivation of the expression sites, occurred among the molecules in band 10 or 15.

One of the telomeric 1.8 VSG genes is always located at band 8 (band indicated with a black dot in figure 1B (as published previously, 11). We also re-examined three independent activations of the 1.8 VSG genes in variants, 1.208, 1.8a, and variant 1.8c. In the activation of the 1.8 VSG gene in variant 1.208 the gene on band 8 served as the basic copy for the generation of the VSG 1.8 ELC now located in band 5 (as previously published, 9-11). We now compared the chromosomal location of the expression linked copy of the 1.8 gene in the variants 1.8a and 1.8c. Both variants 1.8a and 1.8c have one additional telomeric 1.8 VSG gene when compared to variant 1.208 (1.8 genes in variants 1.8a and 1.8c are located at bands 8, 12 and 16). All VSG 1.8 genes retained their chromosomal positions and DNA rearrangement events could not be detected at activation or inactivation of variant 1.8 (data not shown).

DISCUSSION.

The amount of DNA in the diploid T.brucei nucleus was measured by cytophotometry at 0.097 pg (29). Measurements of the complexity, which were are less accurate, indicated that 68% of this DNA is single copy DNA with a complexity of 2.5×10^7 bp (30). The haploid amount of DNA of the T.brucei nucleus was thus calculated to be 0.041 pg, which is in reasonable agreement with the total amount of 0.097 pg for the diploid T.brucei nucleus (31). The diploid nucleus of T.brucei should thus contain roughly 70,000 kb of DNA. Assuming the presence of one hundred mini-chromosomes with an average molecular weight of one hundred kb (1); assuming that the non-stoichiometric staining in bands 9, 10, 15 and 19 represents the presence of more than one chromosome per band; and assuming that all these molecules are linear, the accumulated sizes of the 19 bands (ranging from 50 kb up to 5.7 Mb) add up to over 58,000 kb. These bands thus account for over 80% of genome content of T.brucei. If our assumptions are correct we have, therefore, separated most or all T.brucei chromosome-sized DNA molecules, retaining only a small fraction of the DNA at the well of the PFG gels. The

improved separation will greatly facilitate the genetic analysis of this protozoan parasite.

The accuracy of the sizes of these chromosomes can indirectly be deduced from a comparison of the size of the E.coli chromosome, measured by physical mapping at 4.65 Mb (32) and the largest similarly measured S.pombe chromosome of 5.7 Mb (2). The relative migrational behavior in PFG and the sizes of these two molecules are consistent with each other and a variety of other DNA molecules in this size range that have been accurately sized using different methods (Smith, C.L. unpublished data and 33, 34, 35). Thus, it is reasonable to assume that the larger molecules are sized accurately.

For several chromosomes (bands 2-6) we were able to show that the bands actually consisted of a single chromosome and do not contain homologues (1, 10). The 10,000 kb of DNA in mini-chromosomes and about 1600 kb of DNA in bands 2-6 could thus represent aneuploid chromosomes. Since the accumulated sizes of the remaining bands almost account for the entire genome of T.brucei it is possible that homologous chromosomes in T.brucei are of different sizes.

Finally, we have not been able to detect new DNA rearrangement events, affecting the chromosomal location of expression sites at their activation or inactivation. The data, therefore, indicates that the transcription at several expression sites can be coordinately controlled without the involvement of inter-chromosomal rearrangement events. The chromosomal rearrangement events identified previously (9-11) could thus reflect a different mode of expression site transcriptional control.

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