The genetic map and comparative analysis with the physical map of *Trypanosoma brucei*

Annette MacLeod¹,*, Alison Tweedie¹, Sarah McLellan², Sonya Taylor², Neil Hall³, Matthew Berriman³, Najib M. El-Sayed⁴, Michelle Hope¹, C. Michael R. Turner¹,² and Andy Tait¹

¹Wellcome Centre for Molecular Parasitology, Anderson College Complex, University of Glasgow, 56 Dumbarton Road, Glasgow G11 6NU, UK, ²Division of Infection and Immunity, Institute of Biomedical and Life Sciences, Joseph Black Building, University of Glasgow G12 8QQ, UK, ³Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Hinxton CB10 1SA and ⁴The Institute for Genomic Research, 9712 Medical Center Drive, Rockville, MD 20850, USA

Received September 5, 2005; Revised October 20, 2005; Accepted November 8, 2005

ABSTRACT

*Trypanosoma brucei* is the causative agent of African sleeping sickness in humans and contributes to the debilitating disease ‘Nagana’ in cattle. To date we know little about the genes that determine drug resistance, host specificity, pathogenesis and virulence in these parasites. The availability of the complete genome sequence and the ability of the parasite to undergo genetic exchange have allowed genetic investigations into this parasite and here we report the first genetic map of *T. brucei* for the genome reference stock TREU 927, comprising of 182 markers and 11 major linkage groups, that correspond to the 11 previously identified chromosomes. The genetic map provides 90% probability of a marker being 11 cM from any given locus. Its comparison to the available physical map has revealed the average physical size of a recombination unit to be 15.6 Kb/cM. The genetic map coupled with the genome sequence and the ability to undertake crosses presents a new approach to identifying genes relevant to the disease and its prevention in this important pathogen through forward genetic analysis and positional cloning.

INTRODUCTION

*Trypanosoma brucei* is a diploid zoonotic protozoan parasite transmitted by tsetse flies. This species has been further subdivided into three morphologically identical subspecies, *T. b. gambiense* and *T. b. rhodesiense* cause sleeping sickness in humans whereas the third subspecies, *T. b. brucei*, is not infective to humans. *T. brucei* also infects cattle and is one of three trypanosome species that causes the economically important disease ‘Nagana’ in sub-Saharan Africa. There is significant variation both between and within the subspecies in a range of important phenotypes, such as drug resistance and virulence. Identifying genes involved in these phenotypes would be a considerable advance in the study of this important pathogen.

The development of a genetic map for *T. brucei* is crucial to our understanding of the genetic system in this pathogen and opens up the possibility of using forward genetic analysis as a tool to identify genes that determine traits of importance in the transmission, treatment and pathogenesis of the disease (1). Genetic analysis in other parasitic protozoa has been central to identifying the genes and loci that determine drug resistance (2–6) and virulence (7,8).

As no chromosome condensation has been observed in any life cycle stage and no gamete stages identified, the main approach in determining whether *T. brucei* has a sexual cycle and undergoes meiosis has been to undertake classical genetic analysis. Previous work (9–11) has shown that when the tsetse fly vector is co-infected with two genetically different lines of the parasite, the resultant parasites comprise a mixture of the original two parental lines together with hybrids that, by marker analysis, are the equivalent of F1 progeny (12). Recently, further marker analysis on a large number of progeny clones from two genetic crosses, between two *T. b. brucei* strains and between a *T. b. brucei* and a *T. b. gambiense* strain (STIB 247 × TREU 927 and STIB 247 × STIB 386,
from genomic DNA, under the following conditions: 95°C differences. To do this, microsatellite markers were amplified in the parental stocks for loci that were heterozygous for allele size variation. The primers were used to screen the sequence data (19) to identify microsatellites and PCR primers were designed to the sequence flanking each locus, using the primer design program, PRIDE (http://pride.molgen.mpg.de/pride.html, PRIDE 1.2). The primers were used to screen the available sequence data (20). The products were separated on 3% Nusieve agarose gels and visualized under UV. Minisatellite markers, including MS42, 292 and CRAM, were analysed as described previously (20).

RESULTS AND DISCUSSION

The 25 Mb genome of T.brucei has been sequenced on a chromosome by chromosome basis by two sequencing centres, The Wellcome Trust Sanger Institute and The Institute for Genome Research (TIGR) and covers all the 11 megabase chromosomes (17). These results mean that T.brucei is amenable to genetic mapping, linkage analysis and positional cloning. Here we describe the generation of the first genetic map for T.brucei.

MATERIALS AND METHODS

Generation of hybrids

A number of independent F1 progeny clones had been generated previously (12,13) from a genetic cross between an isolate from a tsetse fly (TREU 927) and one from a hartebeest (STIB 247). In total 39 F1 progeny of independent mating events were generated from the STIB 247 × TREU 927 cross, which were screened for new informative microsatellite markers spanning the genome. Complete lists of hybrid progeny from this cross is given in ref (13).

Identification of markers and the screening of hybrids

Tandem repeat finder (18) (http://tandem.bu.edu/trf/trf.html, Tandem repeat finder 3.21) was used to screen the available sequence data (19) to identify microsatellites and PCR primers were designed to the sequence flanking each locus, using the primer design program, PRIDE (http://pride.molgen.mpg.de/pride.html, PRIDE 1.2). The primers were used to screen the parent stock for loci that were heterozygous for allele size differences. To do this, microsatellite markers were amplified from genomic DNA, under the following conditions: 95°C for 50 s, 50°C for 50 s and 65°C for 50 s for 30 cycles, using primer concentrations and the PCR buffer described elsewhere (20). The products were separated on 3% Nusieve (flowgen) agarose gels and visualized under UV. Minisatellite markers, including MS42, 292 and CRAM, were analysed as described previously (20).

Construction of the genetic map

The map has been based on segregation of alleles in the F1 progeny for loci heterozygous in the TREU 927 parent only. This is because the parent stock STIB 247 was found to be homozygous for 94% of markers. The segregation data for all heterozygous loci from TREU 927 are given in Supplementary Tables 1–11 and http://www.gla.ac.uk/centres/wcmp/research/macLeod/gmpnov.html, (Genetic map of T.brucei) and used to construct the genetic map of TREU 927 using the program MapManager (21) with a Haldane map function, at the highest level of significance for linkage criteria, with a probability of type I error \( P = 1 \times 10^{-6} \). Each marker was linked to the adjacent marker with a LOD score of 5.5 or greater.
Figure 1. Linkage maps corresponding to the 11 megabase chromosomes (I–XI) of *T.brucei*. All mini- and microsatellite markers (to the right in each map) have been physically assigned to chromosomes except three. Their positions on each chromosome are identified in Supplementary Tables I–XI. The genetic distance between each marker is given in centimorgans, Haldane corrected. The genetic size of the linkage groups is given below each linkage group.
The average map unit distance is 15.6 kb/cM, based on the physical distances between genetic markers, but there is considerable variation in the physical size of the centiMorgan within and between chromosomes (Supplementary Figures 1–11). Previously hot and cold spots of recombination had been identified on Chromosome I and II (22, 23) and the data presented here show variation in crossover frequency to be a common feature of each of the remaining 9 chromosomes. For example, there is a region of chromosome VII where the physical size of the recombination unit is 1.58 kb/cM and conversely, on chromosome XI, where the recombination unit is 95.64 kb/cM. Hot and cold spots have been observed in many eukaryotes (26), but surprisingly not in P. falciparum which has an apparently uniform meiotic crossover activity per physical distance (25). Although local variations in the physical size of a recombination unit contradict the correlation between physical and genetic length, this appears to average out over the full length of each chromosome (Figure 3).

The T. brucei genome contains many copies of VSG sequences, which have been previously associated with mitotic recombination resulting in antigenic variation (27). The genome sequence of T. brucei had revealed several regions of tandemly repeated VSG sequences, which mainly consist of pseudogenes (19). It has been suggested that the vast number of these pseudogenes have arisen through hybrid VSG gene formation via mitotic recombination, but the same result could also theoretically arise from meiotic recombination. In order to investigate if these arrays of VSG sequences are associated with meiotic recombination, we examined one of the largest VSG pseudogene arrays in the genome, on chromosome IX, (position 2476002–3055206 bp), by comparing the crossover frequencies in the region of the genetic map covering the VSG array with the map for the rest of the chromosome. The physical size of a recombination unit in the VSG array region is 50.2 kb/cM (measured between positions 2455704 and 2927791 bp), which is markedly larger than the average for the rest of chromosome IX of 15 kb/cM indicating that, surprisingly, this is a region of low meiotic recombination activity, with 3 crossovers identified whereas the average for this chromosome, would predict 12 crossovers ($\chi^2 = 9.3, P < 0.05$) the null hypothesis being equivalent crossover frequency in both regions of the chromosome. Whether low meiotic recombination frequency is a feature of all VSG arrays has not been determined as yet due to the inherent difficulties in identifying unique sequences/markers within these regions.

The vast majority of markers demonstrate the inheritance of equal numbers of parental alleles in the progeny, in agreement with Mendelian predictions (13). However, there are two regions of the genetic map (on chromosomes III and IX) where there is segregation distortion, i.e. where one haplotype is inherited more frequently in the progeny than predicted (Figure 4). The origin of this distortion is unknown at the present time but could reflect selection acting on the uncloned progeny populations before cloning for alleles at loci in these regions of the chromosomes.
From the segregation analysis of markers, one progeny clone, F532/53 mcl 1, appears to be trisomic for chromosome I, having inherited both alleles from parental stock TREU 927 for all chromosome I markers analysed. Analysis of the inheritance of markers on other chromosomes, however, indicates that this hybrid has inherited only one homologue from each parental stock, clearly demonstrating that this progeny clone is trisomic for chromosome I, but not triploid. This is the first case of trisomy as opposed to triploidy reported in T. brucei and probably arose due to chromosomal non-disjunction at meiosis. The frequency of trisomy in this genetic cross is 2.5% and, while this clone cannot be used for mapping purposes for chromosome I, it is informative for all other chromosomes and so was included in the panel of informative hybrids.

Thirty-nine progeny clones typed for the 182 markers typed has generated a dataset of 6797 scored alleles (Supplementary Tables 1–11), and has identified two spontaneous mutations (10), with the frequency of 0.0003 mutants/alleles genotyped. These finding raises the overall mutation frequency for all markers combined of 0.027 mutants/alleles genotyped. This generates an average mutation rate of 1.8 x 10^-6 and a mutation rate per nucleotide of 1.5 x 10^-9. The mutation rate for chromosome I is 0.0016 mutants/alleles genotyped and for chromosome IX is 0.0003 mutants/alleles genotyped. These results are consistent with previous reports of mutation rates in T. brucei (2). The large sample size and the relatively high mutation frequency are consistent with the hypothesis that T. brucei is a rapidly evolving species.

The high resolution genetic map we have generated for T. brucei for this pathogen has opened up the possibility of identifying genes that determine traits of importance by genetic analysis and positional cloning (1). The next challenge is to exploit this new toolset to understand better traits such as human infectivity (28), drug resistance or virulence (1) that all contribute to the severity of sleeping sickness and Nagana.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

We thank IAEA, Vienna for supply of tsetse flies for cyclical transmission of trypanosomes. We are grateful for financial support from the Wellcome Trust to A.T., C.M.R.T. and A.M.L., The Royal Society of Edinburgh to A.M.L., Tenovus Scotland to A.M.L. and from the Sir Halley Stewart Trust to S.T. We are grateful to an anonymous referee for constructive criticism of this paper and to Alex MacKay for help with the website. Funding to pay the Open Access publication charges for this article was provided by the Wellcome Trust.

Conflict of interest statement. None declared.

REFERENCES

1. Tait,A., Masiga,D., Ouma,J., MacLeod,A., Sasse,J., Melville,S., Lindegard,G., McIntosh,A. and Turner,C.M.R. (2002) Genetic analysis of phenotype in Trypanosoma brucei: a classical approach to potentially complex traits. Philos. Trans. R. Soc. Lond., B, 357, 89–99.
2. Su,X., Kirkman,L.A., Fujioka,H. and Wellem,T.E. (1997) Complex polymorphisms in an approximately 330 kDa protein are linked to chloroquine-resistant P. falciparum in Southeast Asia and Africa. Cell, 91, 593–603.
3. Fidock,D.T., Cooper,R.A., Mu,J., Deng,B., Joy,D.A., Su,X.Z. and Wellem,T.E. (2004) Dissecting the loci of low-level quinine resistance in malaria parasites. Mol. Microbiol., 52, 985–997.
4. Culleton,R., Martinelli,A., Hunt,P. and Carter,R. (2005) Linkage group selection: rapid gene discovery in malaria parasites. Genome Res., 15, 92–97.
5. Fidock,D.A., Nomura,T., Talley,A.K., Cooper,R., Dzekunov,S.M., Fidock,M.T., Usos,L., Sidhu,A.B., Naude,B., Deitsch,K.W. et al. (2000) Mutations in the P. falciparum digestive vacuole transmembrane protein PFCRT and evidence for their role in chloroquine resistance. Mol. Cell., 6, 861–871.
6. Sidhu,A.B., Verdier-Pinard,D. and Fidock,D.A. (2002) Chloroquine resistance in Plasmodium falciparum malaria parasites conferred by pfcrt mutations. Science, 298, 210–213.
7. Su,C., Howe,D.K., Dubey,J.P., Ajieka,J.W. and Sibley,L.D. (2002) Identification of quantitative trait loci controlling acridine virulence in Toxoplasma gondii. Proc. Natl Acad. Sci. USA, 99, 10753.
8. Martinelli,A., Chessman,S., Hunt,P., Culleton,R., Raza,A., Mackinnon,M. and Carter,R. (2005) A genetic approach to the de novo identification of targets of strain-specific immunity in malaria parasites. Proc. Natl Acad. Sci. USA, 102, 814–819.
9. Jenni,L., Mami,S., Schweizer,J., Betschart,B., Le Page,R.W., Wells,J.M., Tait,A., Painadavine,P., Pays,E. and Steiner,M. (1986) Hybrid formation between African trypanosomes during cyclical transmission. Nature, 322, 173–175.
10. Sternberg,J., Turner,C.M., Wells,J.M., Ranford-Cartwright,L.C., Le Page,R.W. and Tait,A. (1989) Gene exchange in African trypanosomes: frequency and allelic segregation. Mol. Biochem. Parasitol., 34, 269–279.
11. Gibson,W.C. (1989) Analysis of a genetic cross between Trypanosoma brucei rhodesiense and T. b. brucei. Parasitology, 99, 391–402.
12. Turner,C.M., Sternberg,J., Buchanan,N., Smith,E., Hide,G. and Tait,A. (1990) Evidence that the mechanism of gene exchange in Trypanosoma brucei involves meiosis and syngamy. Parasitology, 101, 377–386.
13. MacLeod, A., Tweedie, A., McLellan, S., Taylor, S., Cooper, A., Sweeney, L., Turner, C.M.R. and Tait, A. (2005) Allelic segregation and independent assortment in *Trypanosoma brucei* crosses: proof that the genetic system is Mendelian and involves meiosis. *Mol. Biochem. Parasitol.*, **143**, 12–19.

14. Gibson, W. (1995) The significance of genetic exchange in trypanosomes. *Parasitol. Today*, **11**, 465–468.

15. Gibson, W. and Stevens, J. (1999) Genetic exchange in the trypanosomatidae. *Adv. Parasitol.*, **43**, 1–46.

16. Hope, M., MacLeod, A., Leech, V., Melville, S., Sassa, J., Tait, A. and Turner, C.M.R. (1999) Maintenance of diploidy (in megabase chromosomes) in *Trypanosoma brucei* after genetic exchange. *Mol. Biochem. Parasitol.*, **104**, 1–9.

17. Gaunt, M.W., Yeo, M., Frame, I.A., Stothard, J.R., Carrasco, H.J., Taylor, M.C., Mena, S.S., Veazey, P., Miles, G.A., Acosta, N. et al. (2003) Mechanism of genetic exchange in American trypanosomes. *Nature*, **421**, 936–939.

18. Benson, G. (1999) Tandem repeats finder: a program to analyze DNA sequences. *Nucleic. Acids Res.*, **27**, 573–580.

19. Berriman, M., Ghedin, E., Hertz-Fowler, C., Blandin, G., Renaud, H., Bartholomeu, D.C., Lennard, N.J., Caler, E., Hamlin, N.E., Haas, B. et al. (2005) The genome of the African trypanosome *Trypanosoma brucei*. *Science*, **309**, 416–422.

20. MacLeod, A., Turner, C.M.R. and Tait, A. (1999) A high level of mixed *Trypanosoma brucei* infections in tsetse flies detected by three hypervariable minisatellites. *Mol. Biochem. Parasitol.*, **102**, 237–248.

21. Manly, K.F., Cudmore, J.R.H. and Meer, I.M. (2001) Map Manager QTX, cross-platform software for genetic mapping. *Mamm. Genome*, **12**, 930–932.

22. Hall, N., Berriman, M., Lennard, N.J., Harris, B.R., Bart-Delabesse, E.N., Hertz-Fowler, C., Gerrard, C.S., Atkin, R.J., Barron, A.J., Bowman, S. et al. (2003) The DNA sequence of chromosome I of an African trypanosome: gene content, chromosome organisation, recombination and polymorphism. *Nucleic. Acids Res.*, **31**, 4864–4873.

23. El-Sayed, N.M.A., Ghedin, E., Song, J., MacLeod, A., Bringaud, F., Larkin, C., Wanless, D., Peterson, J., Hou, L., Taylor, S. et al. (2003) The sequence and analysis of *Trypanosoma brucei* chromosome II. *Nucleic. Acids Res.*, **31**, 4856–4863.

24. El-Sayed, N.M., Hegde, P., Quackenbush, J., Melville, S.E. and Donelson, J.E. (2000) The African trypanosome genome. *Int. J. Parasitol.*, **30**, 329–345.

25. Su, X.-Z., Ferdig, M.T., Huang, Y., Huynh, C.Q., Lui, A., You, J., Wootton, J.C. and Wellems, T.E. (1999) A genetic map and recombination parameters of the human malaria parasite *Plasmodium falciparum*. *Science*, **286**, 1351–1353.

26. Petes, T.D. (2001) Meiotic recombination hot spots and cold spots. *Nat. Rev. Genet.*, **2**, 360–369.

27. Barry, J.D. and McCulloch, R. (2001) Antigenic variation in trypanosomes: enhanced phenotypic variation in a eukaryotic parasite. *Adv. Parasitol.*, **49**, 1–70.

28. Turner, C.M., McLellan, S., Lindergard, L.A., Bioni, L., Tait, A. and MacLeod, A. (2004) Human infectivity trait in *Trypanosoma brucei*: stability, heritability and relationship to sra expression. *Parasitology*, **129**, 445–454.