4856–4863 *Nucleic Acids Research, 2003, Vol. 31, No. 16*
DOI: 10.1093/nar/gkg673

The sequence and analysis of *Trypanosoma brucei* chromosome II

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Received April 15, 2003; Revised May 29, 2003; Accepted June 9, 2003 DDBJ/EMBL/GenBank accession nos+

ABSTRACT

We report here the sequence of chromosome II from *Trypanosoma brucei*, the causative agent of African sleeping sickness. The 1.2-Mb pairs encode about 470 predicted genes organised in 17 directional clusters on either strand, the largest cluster of which has 92 genes lined up over a 284-kb region. An analysis of the GC skew reveals strand compositional asymmetries that coincide with the distribution of protein-coding genes, suggesting these asymmetries may be the result of transcription-coupled repair on coding versus non-coding strand. A 5-cM genetic map of the chromosome reveals recombinational 'hot' and 'cold' regions, the latter of which is predicted to include the putative centromere. One end of the chromosome consists of a 250-kb region almost exclusively composed of RHS (pseudo)genes that belong to a newly characterised multigene family containing a hot spot of insertion for retroelements. Interspersed with the RHS genes are a few copies of truncated RNA polymerase pseudogenes as well as expression site associated (pseudo)genes (ESAGs) 3 and 4, and 76 bp repeats. These features are reminiscent of a vestigial variant surface glycoprotein (VSG) gene expression site. The other end of the chromosome contains a 30-kb array of VSG genes, the majority of which are pseudogenes, suggesting that this region may be a site for modular *de novo* construction of VSG gene diversity during transposition/gene conversion events.

INTRODUCTION

The protozoan parasite *Trypanosoma brucei* is the causative agent of African sleeping sickness. More than a half a billion people live in areas inhabited by the tsetse fly that transmits the parasite, and hundreds of thousands are newly infected each year (1). *Trypanosoma brucei* exhibits unusual and complex mechanisms in the control of gene expression

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ranging from polycistronic transcription (2), trans-splicing of precursor RNAs (3,4) and mitochondrial RNA editing (5) to gene rearrangements during antigenic variation (6,7). Several of these phenomena have become the focus of intense research in this organism as well as in higher eukaryotes. In addition, the many elegant mechanisms used by trypanosomes and related parasites to evade the immune responses of their mammalian hosts have led to a better understanding of the diversity and complexity of host–parasite interactions. The sequences of the first two chromosomes (chromosomes I and II) of *T.brucei* are reported in this issue (8). Here we present an analysis of the 1.2-Mb DNA sequence of *T.brucei* chromosome II (*chr*II) fully annotated into one contig.

MATERIALS AND METHODS

Parasite DNA and chromosome sequencing

Trypanosomoa brucei TREU (Trypanosomiasis Research Edinburgh University) 927/4 (GPAL/KE/70/EATRO 1534) single VAT derivative GUTat 10.1 (9) (Tb927) was selected as the reference stock for the genome sequencing project. A bacterial artificial chromosome (BAC) library, RPCI93 (http://www.chori.org/bacpac/tbrucei93.htm), was the main substrate used for sequencing. Sheared DNA from selected BAC clones (1.6–2 kb) was cloned into a modified pUC18 vector via BstXI linkers. Sequences were assembled and gaps were closed using a combination of BAC walking, directed PCR or transposon insertion. The final assembly of the chromosome was verified by comparison with an XbaI optical restriction map. The restriction maps predicted from the sequence of all the internal BACs agreed with the optical map. Quality alignments could not be obtained in the subtelomeric regions. This can be explained by homologous chromosome pair polymorphisms in *T.brucei* that are not represented or are not resolved in the current optical map. We have used multiple combinations of unique primers from BACs RPCI93-3B10 ('left' end of the *chr*II) and RPCI93-36E18 ('right' end) and a known telomeric hexamer repeat to amplify and clone the telomeres from *chr*II. PCR products ranging from 5 to 12 kb in size have resisted repeated cloning attempts.

Annotation

A custom-modified version of Glimmer (10) was trained on 305 *T.brucei* genes and cDNAs taken from GenBank and then used to generated gene predictions on *chr*II. Following manual evaluation and model curation, genes on finished BACs were assigned systematic names based on the RPCI93 BAC from which they originated (e.g. 10C8.440, 10C8.445, etc.). Genes on the assembled chr*II* pseudomolecule were assigned systematic names according to a scheme agreed upon with the Sanger Institute (e.g. Tb927.2.3280, Tb927.2.3290, etc.) and reflecting organism (Tb), strain (927) and chromosome (2). Predicted proteins were searched against a non-redundant amino-acid database using BLASTP; other features were identified by specialised searches using the following programs and databases: InterPro (11), Pfam (12), Gene Ontology (GO) (13); transmembrane domains, TMHMM (14); signal peptides and signal anchors, SignalP-2.0 (15). The results of all analyses were reviewed using Manatee, a tool created at TIGR that interfaces with a relational database of all the information produced by the annotation software. Predicted gene products were manually assigned GO (13) terms. The annotation discussed in this report is also on the *T.brucei* Genome Annotation Database at TIGR (http://www.tigr.org/tdb/e2k1/tba1/tba1.shtml) and in GeneDB (http://www.genedb.org).

Genetic analysis

Mini- and microsatellite sequences were identified by analysing the *chr*II sequence with Tandem repeats finder (16) and a subset of these sequences were used to construct the genetic map. Primers were designed to unique sequences flanking three minisatellites and 16 microsatellites and these sequences are available on request. F1 progeny clones from a cross of *T.brucei* stocks Tb927 × Tb247 were used in this analysis (17). Further progeny clones were generated from cryopreserved uncloned populations that included products of mating as described (18). Genetically independent progeny were defined on the basis of either being derived from different tsetse flies or differing in genotype after screening with five unlinked mini- and microsatellite markers. Thirty-eight progeny were used to construct the map. Each clone was amplified by infection of mice, the trypanosomes purified from blood and DNA prepared. Each progeny clone was genotyped by PCR amplification of each locus and separation of the products by agarose gel electrophoresis, typically 3% Nusieve agarose gels.

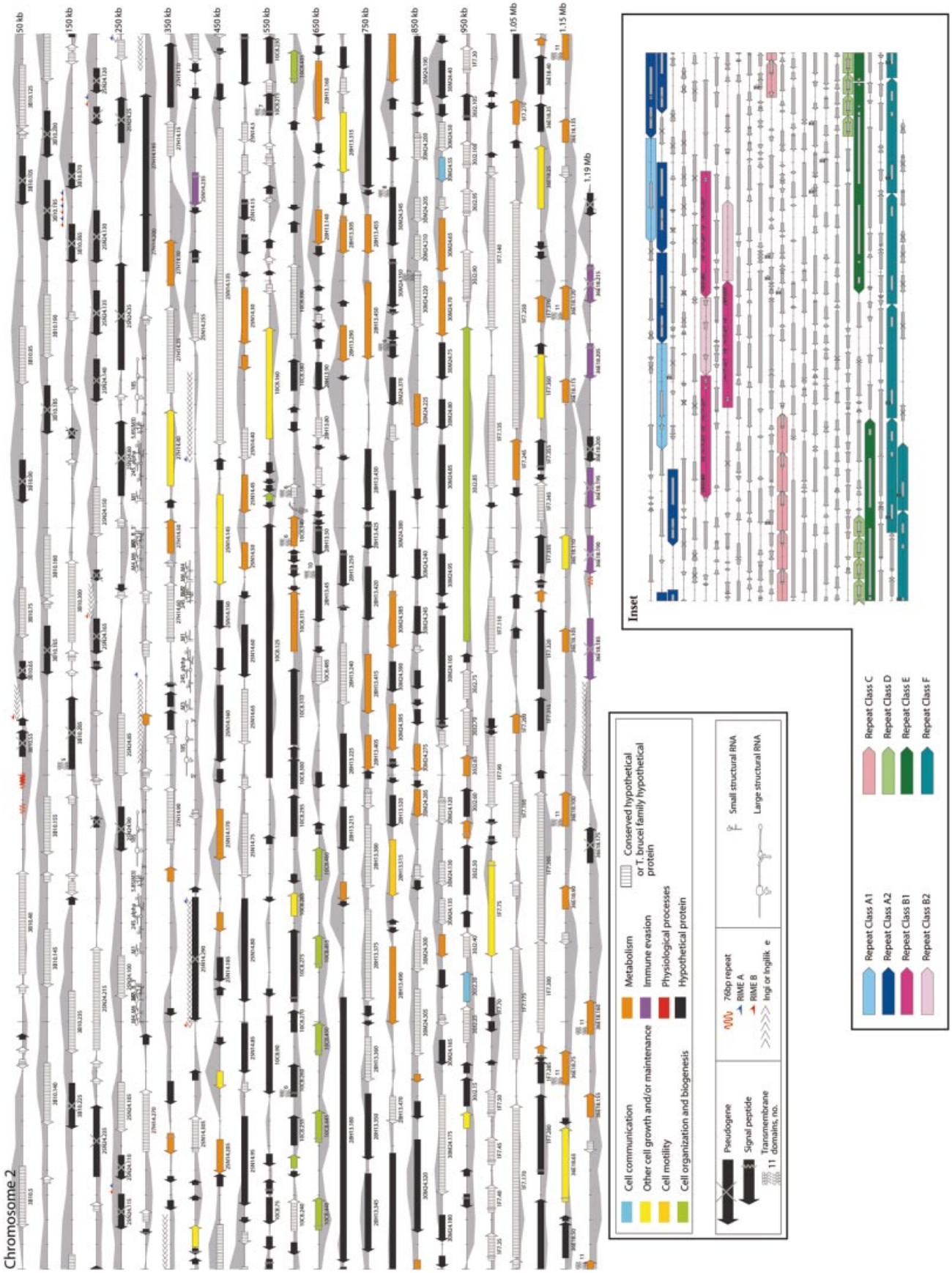
RESULTS AND DISCUSSION

Gene content and structure of *chr*II

Sequencing was initiated using BACs that we mapped to *chr*II of *T.brucei* reference stock TREU927/4 GUTat10.1 (Tb927) by hybridisation to specific genetic markers (19). Subsequently, BAC end sequences and BAC fingerprint data allowed extension from three initial seed points and completion of the chromosome using a map-as-you-go approach (20). Ten BAC clones were sequenced and assembled into one contig representing 1 193 931 bp of non-redundant sequence terminating ~5–20 kb from each of the telomeres.

Using a combination of gene prediction programs and database searches, the chromosome was manually annotated (Fig. 1). Four hundred and seventy-three putative coding sequences (CDSs) >200 bp were predicted on *chr*II (see Table S1 available as Supplementary Material at NAR Online). We have excluded from our discussion in this paper all hypothetical CDSs that are smaller than 450 bp and do not encode matches to previously characterised proteins or functional domains. The remaining 318 coding sequences occur at an average density of one gene per 3741 bp (Fig. 1 and Table 1). Of the 284 protein-encoding genes, 167 were designated as hypothetical. In addition, 34 pseudogenes, most of which resemble genes for variant surface glycoproteins (VSGs) and retrotransposon hot spot (*RHS*) genes, occur in the subtelomeric regions of the chromosome. Table 1 summarises the statistically salient features of *chr*II.

A remarkable feature of *chr*II is its gene organisation. All the genes are densely packed within a total of 17 directional clusters, the longest of which spans ~284 kb and contains 92 protein-coding genes (Fig. 1). This unusual distribution of



genes is reminiscent of bacterial operons and has been observed in *Leishmania* (21), albeit at a smaller scale; similar observations are reported for *T.brucei chr*I (8). Several lines of evidence from this and previous studies of trypanosomatid genes suggest, however, that the directional gene clusters are not regulated like conventional bacterial operons. First, unlike most prokaryotic and eukaryotic organisms, where gene expression is regulated primarily at the level of transcription, in trypanosomatids gene regulation is largely post-transcriptional (22). Expression data from a microarray containing 450 CDSs from *chr*II and probed with RNA from two developmental stages of the parasite (bloodstream forms circulating in the mammalian host versus procyclic form in the tsetse fly vector) reveal many instances of putative genes within the same directional cluster that exhibit a differential expression (data not shown). Second, the gene clusters do not appear to encode proteins that share a metabolic pathway as do genes of many bacterial operons. Instead, the gene products have apparently unrelated functions as illustrated by their assignments to different 'biological processes' within the GO classification system (Fig. 1). It is worth noting that no recognisable features of either eukaryotic or prokaryotic promoters were detected in the nucleotide sequences between the gene clusters where bidirectional transcription would presumably begin.

A statistical analysis of the nucleotide distribution of *chr*II reveals a remarkable correlation between the direction of the gene clusters and the sign of the GC-skew (Fig. 1). This (C–G)/(C+G) value, typically calculated in overlapping sliding windows along a sequence (23), has been used to detect strand bias or asymmetry in the base composition of almost all completely sequenced bacterial genomes (24–26). In most cases, a deviation switch at the origin and terminus of replication has strongly suggested a link with replication. On *T.brucei chr*II (and *chr*I, data not shown), the sign of the GC-skew correlates with the direction of the gene clusters, changing precisely and consistently at the boundaries of their convergence or divergence. This strongly suggests that the observed base composition asymmetry between the coding and non-coding strand is linked to transcription or transcription-coupled repair. Similar potential sources of strand asymmetry in other organisms were recently described (27–29). In order to rule out the possibility that polypyrimidine tracts found in *Trypanosoma* intergenic regions could be contributing to the GC-skew, we performed the same analysis on the concatenated coding and non-coding sequences and found no significant differences (data not shown). While consistent with nucleotide composition analyses of bacteria, our findings are in contrast with similar analyses of *Leishmania major* chromosome 1 (30). With so little known about nuclear DNA replication and RNA polymerase II transcription in trypanosmatids, the biological significance of the observed nucleotide bias remains to be determined.

Segmental duplications

We observe a high degree of gene redundancy in *chr*II with the presence of local gene duplications. These paralogous genes are present in tandem arrays. For example, the gene for the 65 kDa invariant surface glycoprotein is present as an array of six copies (10C8.435–10C8.460 in Fig. 1 inset, repeat class C); the genes for a nucleoside transporter and the iron/ascorbate oxidoreductase appear as a pair five times, ending with an extra copy of the nucleoside transporter gene (36E18.75–36E18.160, Fig. 1 inset, repeat class F); and a gene encoding a hypothetical protein is repeated eight times (30J2.95–30J2.105, 1F7.30–1F7.50, Fig. 1 inset, repeat class D). The nucleoside transporter-iron/ascorbate oxidoreductase units also contain intergene duplications but these segments have conserved very low nucleotide identity.

Another type of duplication observed in *chr*II involves segment duplications rather than single-gene copies. We have found three regions where 6–19 kb segmental copies are juxtaposed and have >95% identity (Fig. 1 inset, repeat classes A, B, E). Within these duplicated blocks, the homologous genes appear in the same order along the distinct segments. This type of duplicated blocks of genes was previously reported in other organisms such as *Arabidopsis thaliana*, *Saccharomyces cerevisae* and *Caenorhabditis elegans* (31–34). However, while in the case of *A.thaliana* and *S.cerevisae* most of the blocks are duplicated between chromosomes, in *C.elegans* they occur intra-chromosomally (35). Another interesting feature of most block duplications is that there is very little conservation between non-coding duplicated regions (32). In *T.brucei chr*II, we observe very high conservation of non-coding regions.

The duplicated ribosomal clusters (repeat class B1) and the 19-kb segments (repeat class E) contain inverted copies. In the case of the ribosomal RNA cluster, retroposon-like elements (Ingis) can be found at a certain distance on either side of a complete unit (Fig. 1) suggesting that these elements may have been involved in the duplication process. As for the 19-kb inverted segment, both units are an exact mirror image of each other, separated by 100 nucleotides of unique sequence. Only when the genome is completed will we be able to evaluate the existence of extensive duplication of blocks between chromosomes, and the extent to which mechanisms responsible for gene duplication are a mixture of ancient polyploidisation events versus unequal crossing over events.

Figure 1. (Opposite) Schematic representation of *T.brucei* chromosome II. Each predicted CDS >200 bp in length is represented by an arrow. The labels refer to the systematic name for each gene (see Table S1). The colours of the arrows represent the corresponding *T.brucei* GO assignment for each gene according to the Biological Process ontology (12). A set of high level GO categories were selected to represent the most informative high-level terms without overlapping paths in the GO hierarchy. Grey peaks reflect the GC skew scores calculated for a 2-kb window sliding in 1-kb increments. 'Hypothetical proteins' are proteins with no similarity to characterised proteins or with similarity to a hypothetical protein over less than 70% of the protein length. 'Conserved hypothetical proteins' are proteins with similarity to one or more hypothetical protein from an organism other than *T.brucei* over at least 70% of the protein length. '*Trypanosoma brucei* family hypothetical proteins' are proteins with similarity with two or more *T.brucei* hypothetical proteins over at least 70% of the protein length. The predicted transmembrane domains are shown for proteins containing at least five domains. (Inset) Map of duplicated blocks on *chr*II. The copies of each putative duplicated block are classified by colour. Direction of arrowheads indicate the relative orientation of duplicated blocks. An enlarged version of this figure is included with the Supplementary Material (available at NAR Online).

Table 1. Chromosome II summary statistics

Chromosome II content	
Size (bp)	1 193 931
G+C content (%)	44
G+C content in protein coding region (%)	49.8
Gene density (bp per gene)	3755
Mean CDS length (bp)	1831
Number of predicted CDSs	318
Percent of protein coding region	43.6
Organisation features	
Number of directional gene clusters	17
Maximum length of gene cluster (bp)	283 564
Number of VSG clusters	1
Number of rRNA clusters	3
Structural RNA	
Number of rRNA genes	24
Number of tRNA genes	0
Proteome	
Number of predicted protein-coding genes	284
Number of pseudogenes	34
Hypothetical protein (total)	167
Hypothetical protein[a]	109
Conserved hypothetical protein[b]	5
T.brucei family hypothetical protein[c]	49
Conserved/*T.brucei* hypothetical protein[d]	4
Number of putative *VSG*s (total)	8
Number of VSG pseudogenes	6
Number of genes with InterPro match	179
Number of genes with Pfam match	157
Structural features	
Transmembrane domain(s)[e]	80
Signal peptide	50
Signal anchor	26
Non-secretory protein	211

The analyses were performed on CDSs greater than 450 nucleotides. Using this criterion allows the exclusion of 155 hypothetical genes, the majority of which are not likely to code for a protein. It results, however, in the exclusion of genes for two small nuclear ribonucleoproteins, ribosomal protein L44 and a putative histone H4. Specialised searches used the following programs and databases: InterPro (11), Pfam (12), Gene Ontology (13); transmembrane domains, TMHMM (14); signal peptides and signal anchors SignalP-2.0 (15).

[a]Hypothetical proteins are proteins with no similarity to characterised proteins or with similarity with a hypothetical protein over less than 70% of the protein length.

[b]Conserved hypothetical proteins are proteins with similarity to one or more hypothetical protein from an organism other than *T.brucei* over at least 70% of the protein length.

[c]*Trypanosoma brucei* family hypothetical proteins are proteins with similarity to two or more *T.brucei* hypothetical proteins over at least 70% of the protein length.

[d]Conserved/*T.brucei* hypothetical protein are proteins with similarity, over at least 70% of the protein length, to two or more *T.brucei* hypothetical proteins plus one or more matches with hypothetical proteins from other organism than *T.brucei*.

[e]Number of predicted proteins with one or more transmembrane domains.

Genetic map

Trypanosoma brucei parasites undergo genetic exchange when two stocks are co-transmitted through tsetse flies in the laboratory (18,35). The role of genetic exchange in field populations is controversial, however (36). Marker analysis of laboratory trypanosome crosses shows that the progeny are products of a diploid Mendelian genetic system and partial genetic maps have been constructed using anonymous amplified fragment length polymorphism (AFLP) markers (17).

To identify polymorphic markers with which to construct a genetic map of *chr*II, we analysed the sequence for micro- and minisatellites. The former were defined as sequences containing >10 copies of a repeat motif of 2–5 nucleotides with >70% sequence identity and the latter were defined by the same criteria but with a repeat motif of >6 nucleotides. A total of 73 microsatellites and nine minisatellites were identified on the chromosome. Primers were designed to generate PCR products spanning a selection of these distributed across the chromosome and 17 were selected for map construction because they showed sufficient size variation, and were heterozygous for Tb927 and homozygous for stock Tb247. The inheritance of Tb927 alleles into a panel of 38 F1 progeny of a genetic cross between Tb927 × Tb247 enabled a genetic map to be constructed (Fig. 2). To do this, the parental haplotypes were determined on the basis of the most frequently inherited haplotypes and the positions of cross-over events were then identified in recombinant haplotypes. The genetic map of *chr*II is 83.1 cM in length, which gives an average density of approximately one marker per 4.9 cM. This map covers ~1007 kb of the chromosome, giving an average physical size for a recombination unit of 12.1 kb/cM. This value is approximately half of that for *chr*I (8) but is within the range observed for single-celled eukaryotes (37). A genetic marker occurs approximately every 59 kb along *chr*II but comparison of the genetic and physical maps indicates that recombination events are unevenly distributed along its length ($\chi^2 = 50.7$, df = 11, $P < 0.01$; taking as our null hypothesis that there is an equal probability of crossovers in all regions of the chromosome). There is one recombination 'hot spot' (between markers 2 and A9) where crossovers occur readily (3.3 kb/cM). In contrast, between markers A22 and A30, in the 'right hand' half of the chromosome, resides a major recombination 'cold spot' where cross-overs are absent within a 269-kb region. In classical genetics a centromere may be defined by the absence of cross-over events in its vicinity; therefore, this cold spot may correspond to a candidate centromere.

Antigenic diversity by the recombination of pseudogenes

A distinctive feature near the 'right' end of *chr*II is the presence of a small subtelomeric array of putative *VSG*s, the majority of which are highly degenerate pseudo-*VSG*s disrupted by in-frame stop codons and frameshift mutations. The region spans ~30 kb and contains seven putative *VSG*s, including six pseudo-*VSG*s (Fig. 1). African trypanosomes evade the immune response of their mammalian hosts by sequentially expressing different *VSG*s from telomere-linked expression sites. Although hundreds of transcriptionally silent *VSG*s are present in the genome, only one is usually expressed at a time in a given bloodstream parasite (38). The activation of a new *VSG* is often associated with one of three types of gene rearrangements. The best characterised rearrangement is the duplicative transposition of a silent, donor *VSG* from either an interior chromosomal location or a telomeric location to a telomeric-linked expression site, displacing the *VSG* already at that site. In many cases, this gene conversion is mediated on the 5′ side by homologous recombination between a few copies of a 70–76 bp repeat upstream of the donor gene and hundreds of copies of this same repeat in the expression site. On the 3′ side of the duplication, homologuous recombination often occurs between the segments of sequence similarities in the C-terminal coding regions or the 3′-untranslated regions.

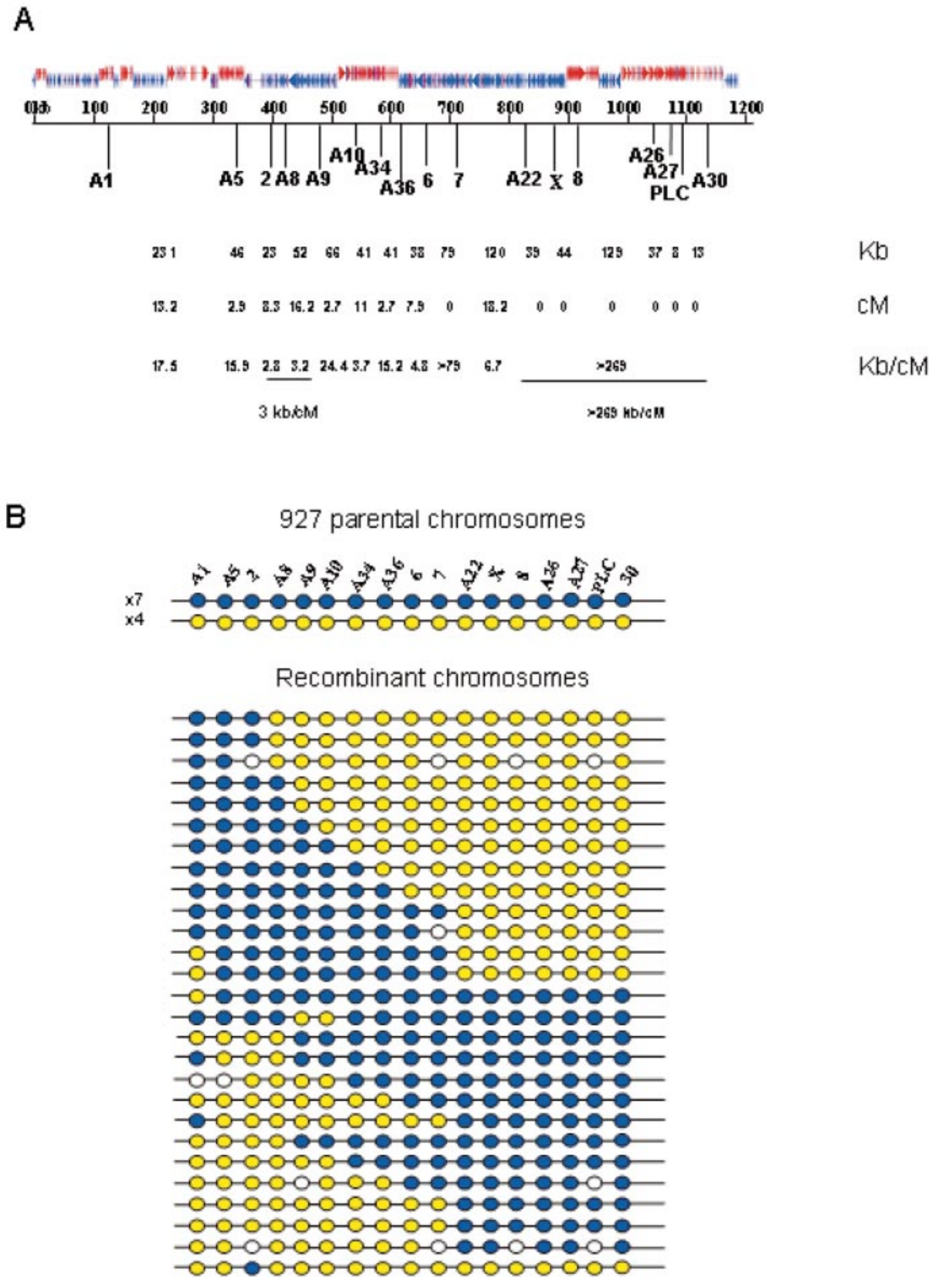


Figure 2. (**A**) The physical map of chromosome II with the directional gene clusters and position of polymorphic mini- and microsatellite markers indicated. The physical distances between markers are shown (kb) and the genetic distances (cM) were calculated from recombination frequencies. Genetic distances shown are uncorrected. The relationship of physical and genetic maps to show hot and cold spots of recombination is also indicated (kb/cM). (**B**) Diagrams of the haplotypes of stock Tb927 showing 11 parental haplotypes shown as blue or yellow, and 27 recombinant types. White circles are unscored markers.

We postulate that this region of *chr*II is a prototype of other similar storage areas in the genome that provide a repertoire of *VSG*s. Although previously speculated to exist (39), this is the first sequence of an array of *VSG*s that could potentially serve as gene conversion donors. A recent report by Robinson *et al.* (40) highlights the predominance of *VSG* conversion events in

antigenic variation and Borst recently proposed an elegant *VSG* transposition model favouring a DNA synthesis-dependent, strand-annealing model over a break-induced, DNA-replication one (39). Unexpectedly, the *VSG* repertoire in this region consists mostly of pseudogenes. The six putative pseudo-*VSG*s contain 11 stop codons and five frameshift mutations evenly spread along the length of the sequences with a slight preference for the region encoding the C-terminal domain. The existence of expressed composite *VSG*s, derived from combinations of several donor genes or pseudogenes, has already been demonstrated (41–45). These recombinational processes have been proposed as a way for African trypanosomes to expand their repertoire of antigenic diversity by reassorting *VSG* sequences. The existence of a relatively large proportion of pseudo-*VSG*s shows there is no strong selection for the maintenance of intact *VSG*s in *T.brucei*. These data also predict that a modular *de novo* construction of *VSG* diversity during segmental conversion events may be more frequent than originally believed.

Retroposon hot spot (RHS) genes

A comparison of *chr*II and *chr*I reveals that about one-fifth of each chromosome (250 kb in *chr*II and 175 kb in *chr*I) is composed of a complex, repetitive and novel subtelomeric region that includes the *ingi* and RIME non-LTR retrotransposons (Fig. S1, see Supplementary Material). This 'left' subtelomeric region of *chr*II and its *chr*I counterpart each contain about half of their chromosome's complement of RIME and *ingi* non-LTR retrotransposons (seven of 15 for *chr*II and eight of 13 for *chr*I). This region also contains genes and pseudogenes of two recently identified multigene families called *LRRP1* (leucine rich repeat protein) and *RHS* (retrotransposon hot spot) (46,47). The *chr*II region has 4 *LRRP1* and 29 *RHS* copies (including very degenerated *RHS* pseudogenes), whereas the *chr*I region has three *LRRP1* and 15 *RHS* copies (Fig. S2) (8). The *RHS* multigene family is itself composed of six subfamilies called *RHS1–RHS6* (47). Furthermore, all of the *RHS4* repeat units in these regions contain a highly degenerated DNA-dependent RNA polymerase III pseudogene, and the *LRRP1* repeat units contain *ESAG4* (adenylate cyclase) pseudogenes (Fig. S2). The general organisation of this subtelomeric region in both chromosomes consists of large clusters of tandemly arranged *RHS* repeat units (*chr*II has two large *RHS* clusters composed of 13 and 10 units), separated by a single *LRRP1* repeat unit (Fig. S2). In *chr*II, all 25 full-length *RHS* copies (with the exception of a *RHS6* copy at the right extremity of the region) are on the same DNA strand, whereas all the *LRRP1* copies are on the other strand (Fig. S2), indicating that the *RHS* clusters and *LRRP1* repeat units are flanked by strand switches. Finally, all the *ingi*/RIME retroelements in these two regions are inserted inside the RHS (pseudo)genes, which contain a hot spot for retroelement insertions (47), demonstrating that the high prevalence of *ingi*/RIME previously observed in this region of *chr*Ia (48) is associated with the presence of the *RHS* (pseudo)genes.

Interspersed with the RHS genes are six copies of truncated DNA-directed RNA polymerase pseudogenes, three expression site associated (pseudo)genes (*ESAG*s) 3 and one *ESAG 4* (Fig. 1 and Table S1). Immediately upstream of one of the *ESAG3* pseudogenes (3B10.55), there is a small cluster of 70–76 bp repeats. These data suggest that this subtelomeric region may be the vestige of a highly degenerate *VSG* expression site.

In summary, the 1.2-Mb *chr*II has three distinct regions. The 250-kb 'left' region (21% of the chromosome) is composed of a complex group of repeats, also found in *chr*I, whose biological significance is unclear. The 915-kb central region (76% of *chr*II) has 10 directional clusters of genes encoding many housekeeping and parasite-specific proteins and/or RNAs. No *VSG*s or pseudo-*VSG*s occur in these two regions. The remaining 30-kb 'right' region (3%) contains a small cluster of *VSG*s and pseudo-*VSG*s that could potentially serve as either or both a 'salvage yard' for retaining previously used *VSG*s or *VSG* segments and a repertoire for assembling new *VSG*s. Unlike at least some of the other megachromosomes of this organism, the *chr*II homolog sequenced here does not seem to possess telomere-linked *VSG* expression sites, so potential donor *VSG*s/partial *VSG*s on this chromosome need to undergo inter-chromosomal gene conversion to reach an expression site.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

ACKNOWLEDGEMENTS

We wish to acknowledge our colleagues at the Sanger Institute for useful discussions and close coordination of efforts, and Sarah McLellan for technical assistance. We are also grateful to the *T.brucei* research community worldwide for its continuous support of this project. In addition, we would like to thank the TIGR faculty, system administrators, sequencing facility, bioinformatics department and administrative staff. The work at the Institute for Genomic Research (TIGR) was supported by a grant from the National Institute for Allergy and Infectious Diseases, National Institutes of Health to N.E.S. (U01 AI43062). The genetic mapping studies carried out at University of Glasgow were supported by grants from The Wellcome Trust and The Sir Halley Stewart Trust.

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