The *Trypanosoma cruzi* Genome Project: Nuclear Karyotype and Gene Mapping of Clone CL Brener

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By using improved pulsed field gel electrophoresis conditions, the molecular karyotype of the reference clone CL Brener selected for Trypanosoma cruzi *genome project was established. A total of 20 uniform chromosomal bands ranging in size from 0.45 to 3.5 Megabase pairs (Mbp) were resolved in a single run. The weighted sum of the chromosomal bands was approximately 87 Mbp. Chromoblots were hybridized with 39 different homologous probes, 13 of which identified single chromosomes. Several markers showed linkage and four different linkage groups were identified, each comprising two markers. Densitometric analysis suggests that most of the chromosomal bands contain two or more chromosomes representing either homologous chromosomes and/or heterologous chromosomes with similar sizes.*

Key words: *Trypanosoma cruzi* - clone CL Brener - genome project - molecular karyotype - chromosome mapping - genetic map

The establishment of the molecular karyotype of *Trypanosoma cruzi* is an essential part of the genome project of this parasite, particulary in the construction of detailed physical and genetic maps. This review focuses on the nuclear karyotype analysis and gene mapping of the reference clone CL Brener selected for the *T. cruzi* genome project. We have also included in this article some of the noteworthy features that can be found in the genome of this parasite.

SIZE AND ORGANIZATION OF THE *T. CRUZI* GENOME

The genome size of *T. cruzi* has been estimated by different procedures (renaturation kinetic analysis, microfluorometry, flow cytometry, chemical analysis, molecular karyotyping). A striking feature of *T. cruzi* is that the total DNA content per cell varies among different strains, isolates and among clones derived from the same strain (Castro et al. 1981, Lanar et al. 1981, Dvorak et al. 1982, Kooy et al. 1989, Thompson & Dvorak 1989, McDaniel & Dvorak 1993). In the renaturation studies, the complexity of the *T. cruzi* genome was estimated to be 1.8-2.5 x 10^8 base pairs (bp) (Castro et al. 1981, Lanar et al. 1981). The absolute amount of total DNA (nuclear+kinetoplast) varies from 0.12 to 0.33 pg per cell among different strains and among clones isolated from the same strain (Lanar et al. 1981, Kooy et al. 1989, Thompson & Dvorak 1989, McDaniel & Dvorak 1993).

REPETITIVE DNA SEQUENCES

The genome of *T. cruzi*, like that of many other eukaryotic organisms, is composed by three major classes of DNA sequences: (a) protein-coding DNA (single copy genes; duplicated and diverged gene families); (b) tandemly repeated DNA (genes encoding RNAs and proteins, e.g., rRNA, medRNA, histone, trans-sialidase, tubulin, amastin, etc); (c) repetitious DNA (micro and minisatellites, short and long interspersed elements, SINE-like DNA sequences, retroposons, retrotransposons, etc). In addition to these sequences, spacer or connecting DNA sequences can be found between genes encoding proteins or RNAs.

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Reassociation kinetics studies of the *T. cruzi* DNA have shown that the highly and middle repetitive sequences account for about 44% of the genome of this parasite (Castro et al. 1981, Lanar et al. 1981). For instance, a highly repetitive, tandemly organized minisatellite DNA sequence (195 base pairs long, 120 000 copies/genome) and an interspersed repetitive element, named E13 (1 025 bp long, 10 000 copies/genome), account for 9% and 7% of the total DNA of the parasite (Sloof et al. 1983, Gonzalez et al. 1984, Requena et al. 1992). In addition to these sequences, two other highly repeated interspersed DNA sequences, called E12 (1 123 bp long, 5 000 copies/genome) and E22 (7 000 copies/genome) were isolated from the nuclear DNA (Requena et al. 1993, 1994, 1996).

Several short interspersed middle repeated DNA sequences have been found in the *T. cruzi* genome: SIRE (428 bp long, 2 000 copies/genome), SRE (40-120 bp long, 400-900 copies/genome) and RLE (317 bp long). These sequences show several features of retroposon-like elements such as the insertion in different loci and flanked by short direct repeats, and the presence of a short A-rich tail at the end of the repeat (Kendall et al. 1990, Novak et al. 1993, Vazquez et al. 1994).

Retrotransposon like-sequences have been also described in the *T. cruzi* genome. These sequences are about 6 kilobase pairs (Kbp) long, share gene homologies and structural features with retrovirus and contain open reading frames encoding enzymes which could be involved in their own transposition. For example, a site-specific retrotransposon (siteposon) called CZAR (6.0 Kbp long), was identified in the miniexon gene repeat region of *T. cruzi* (Aksoy 1991). Another retrotransposon, called L1Tc or B11 element (5.0 Kbp long, 2 000 copies/genome), was found to be distributed in the *T. cruzi* genome. Sequences homologous to reverse transcriptase have been also detected in the L1Tc retrotransposon (Martin et al. 1995, Gruber 1995).

GENE ORGANIZATION: SINGLE COPY GENES, MULTICOPY GENE FAMILIES

Many trypanosome genes encoding housekeeping proteins, antigens, enzymes and structural proteins are arranged as allelic tandem repeats. Tandem arrangement has been described for genes encoding RNA polymerases, actin, Hsp70, glycolytic enzymes, histones, cysteine proteases, tubulin, ribosomal proteins, ubiquitin and repetitive antigens (Michels et al. 1990). There is some correlation between the copy number of a gene and the amount of its product in the cell. Highly abundant proteins and RNAs (e.g., tubulin, Hsp70, cysteine protease, histones, medRNA, rRNA) are encoded by multiple copy genes.

The infective forms of *T. cruzi* express stage-specific surface antigens which are involved in the parasite entry into the host cell. Several genes for surface antigens display homology among them and can be grouped in a superfamily designated as the gp85/sialidase multigene family (Takle & Cross 1991, Campetella et al. 1992, Cross & Takle 1993, Colli 1993, Franco et al. 1993, Araya et al. 1994).

There is a tendency for the clustering of unrelated genes in certain parts of the genome of trypanosomes. Evidences indicate that the array of genes are multi-cistronic transcription units whose transcripts are processed by trans-splicing (Michels et al. 1990). It has been suggested that gene duplication and the clustering of various genes in multicistronic transcription units could be a common means by which trypanosomes regulate protein levels.

SEPARATION OF CHROMOSOMES BY PULSED FIELD GEL ELECTROPHORESIS

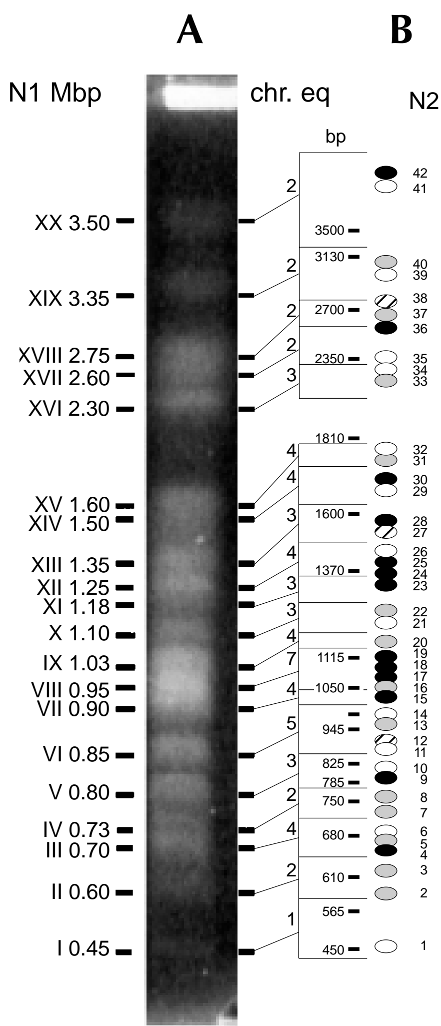
The genetic material of *T. cruzi* is organized in small chromosomes which poorly condensate during the cell division; therefore its analysis by conventional cytogenetic techniques is not possible. The development of pulsed field gel electrophoresis (PFGE) techniques has allowed the separation of intact chromosomes of lower eukaryotes, including *T. cruzi*.

Size fractionation of chromosomal bands by PFGE and hybridization to different DNA probes have been used to establish the molecular karyotype of several strains and clones of *T. cruzi*. The nuclear genome of the *T. cruzi* is of a highly plastic nature, as observed by PFGE. The sizes and number of chromosomal bands vary among strains and clones of this parasite (Gibson & Miles 1986, Engman et al. 1987, Aymerich & Goldenberg 1989, Henriksson et al. 1990, 1993, 1995, 1996, Wagner & So 1990, Cano et al. 1995).

ELECTROKARYOTYPE OF CLONE CL BRENER

In our laboratory, we have established a protocol that allows a good separation of the chromosomal bands ranging from 0.45 Mbp to 4.0 Mbp. The running, which takes 132 hr, is done in a Gene Navigator apparatus (Pharmacia) in 1.2% agarose gel with 0.5x (times) TBE (45 mM Tris/45 mM boric acid/1 mM EDTA, pH 8.3) (Cano et al. 1995). We have used five phases of homogeneous pulses with interpolation for 132 hr at 80 volts at 13 degrees Celsius.

The molecular karyotype obtained with this procedure is shown in Figure. The chromosomal profile is very complex with 20 chromosomal bands ranging from 0.45 Mbp to 3.5 Mbp: 12 megabase



Molecular karyotype of clone CL Brener. Comparison of the data from Cano et al. (1995) (Panel A) and Henriksson et al. (1995) (Panel B). Panel A: ethidium bromide-stained pattern (Et Br) of chromosomal bands after separation by PFGE. The sizes (Mbp) and the numbers of the chromosomal bands are indicated in roman and arabic numerals on the left of the figure. Chromosome equivalents of each band are indicated on the right. Panel B: schematic karyotype based on several experiments using different separation conditions and hybridization with chromosomal markers (Henriksson et al. 1995, 1996). The sizes (bp) and the numbers of the chromosomal bands are indicated on the left and the right of the figure, respectively. Some of the bands were not resolved after EtBr staining and were identified only by hybridization with specific probes (Henriksson et al. 1995).

bands ranging from 3.5 to 1.0 Mbp and eight intermediate chromosomal bands ranging from 0.45 Mbp to 1.0 Mbp. The distribution of the ethidium bromide fluorescence is not the same for all chromosomal bands indicating the existence in a same band of two or more homologous chromosomes or the existence of heterologous chromosomes with the same size (aneuplody).

In order to estimate the number of chromosomes per band, we have hybridized the chromoblot with a probe containing the telomeric repetition of *T. brucei* (Van der Ploeg et al. 1984). There is a good correlation between the fluorescence pattern and the hybridization profile obtained with the telomeric probe. We have scanned the autoradiogram and the relative area of the smaller band (band I) was chosen arbitrarily as standard for minimal ploidy. The number of chromosomes per band, defined here as chromosome equivalent, was estimated dividing the densitometric value obtained of the area of each band by the area of band I.

With this approximation, we estimated that there are 64 chromosomes per cell (epimastigote). It is important to note that this is a rough estimation and it cannot be taken as definitive, since (i) the exact number of chromosomes in band I (our standard for minimal ploidy) is not known; (ii) the exact distribution of the sequences homologous to *T. brucei* telomeric sequences within the chromosomes is not known. Thus, our results suggest that most of the chromosomal bands contain two or more chromosomes representing homologous chromosomes and/or heterologous chromosomes with similar sizes.

To estimate the genome complexity of clone CL Brener, the chromosome equivalent of each band was multiplied by its corresponding molecular size. By summing up these values, the total nuclear DNA content was estimated to be 87 Mbp, whereas the genome size determined by microfluorometry was estimated at approximately 100 Mbp. This result corroborates the hypothesis that *T. cruzi* is an aneuploid organism.

LOCATION OF *T. CRUZI* CLONED SEQUENCES ON CHROMOSOMAL BANDS OF CLONE CL BRENER

We have hybridized the chromoblots of clone CL Brener with a panel of cloned sequences (repetitive sequences, polymorphic sequences, genes encoding proteins and structural RNAs, anonymous sequences) (Table I). Table II compiles the chromosomal location of 39 *T. cruzi* homologous sequences.

Several repetitive sequences were mapped on all chromosomal bands such as SIRE and C6 (middle repetitive sequences which resemble retroposon-like sequences). Other repetitive sequences can be found in most of the chromosomal bands (for instance, B11, 196 bp satellite, SRE). It is interesting to analyse the chromosomal distribution of the genes encoding surface antigens of the parasite's infective forms. For instance, genes encoding trypomastigote surface antigens (gp90, gp82 and gp85) could be mapped on the majority

TABLE I

Chromosomal markers[a]

Locus	Genbank Accession Number	Function	Reference
C6	U16295	Interspersed repetitive element	Araya et al. in press
SIRE	X75033	Short interspersed repetitive element	Vazquez et al. (1994)
Minisatellite	K00393	Satellite repetitive element	Gonzalez et al. (1984)
Gp 82	L14824	82 kDa surface antigen	Araya et al. (1994)
Gp 85	J04667	85 kDa surface antigen	Takle & Cross (1991)
Gp 90	L11287	90 kDa surface antigen	Franco et al. (1993)
TcP2β	X52323	Ribosomal protein P2β	Schijman & Levin (1992)
B11	U15615	Retrotransposon-like element	Gruber (1995)
1F8	X02838	Calcium-binding protein	Gonzalez et al. (1990)
H49	L09564	Flagellar repetitive antigen	Cotrim et al. (1995)
CRA	J04016	Cytoplasmic repetitive antigen	Lafaille et al. (1989)
SZ5	X83599	Non Repetitive SIRE Associated Sequence	Vazquez et al. (1996)
SL-RNA	K02632	Spliced leader sequence	Gonzalez et al. (1990)
Hsp 60	X67473	60 kDa heat shock protein	Rondinelli et al. unpubl.
Hsp 70	X07083	70 kDa heat shock protein	Rondinelli et al. unpubl.
B12	L07759	230 kDa repetitive antigen	Gruber & Zingales (1993)
B13	U15616	140/116 kDa repetitive antigen	Gruber & Zingales (1993)
α and β Tubulins		α and β Tubulins	Rondinelli et al. (1986)
Ubiquitin	J03945	Ubiquitin	Slezynger et al. unpubl.
Actin	U20234	Actin	Rondinelli et al. unpubl.
MIP	X69655	Peptidyl-prolyl *cis-trans* isomerase	Moro et al. (1995)
KAP	M25364	Kinetoplast-associated protein	Gonzalez et al. (1990)
24SαrDNA	M28885	24Sα rRNA	Arruda et al. (1990)
24Sβ rDNA		24Sβ rDNA	Novak et al. (1993)
Amastin	U04337 a 04341	Amastigote stage-specific antigen	Teixeira et al. (1994)
Tc40	U24190	90 kDa cytoplasmic antigen	Lenesechal et al. (1997)
Cruzipain	M90067	Cysteine protease	Lima et al. (1994)
C11		90 kDa surface antigen	Cummings (1989)
SRE	M63895	Spacer Repetitive Element	Novak et al. (1993)
cDNA 68		Unknown Open Reading Frame (ORF)	Santos et al. unpubl.
cDNA 78		γ elongation factor	Santos et al. unpubl.
cDNA 40		Unknown ORF	Santos et al. unpubl.
cDNA 51		Unknown ORF	Santos et al. unpubl.
SZ 23-14		Short interspersed repetitive element	Vazquez et al. (1996)
SZ 7-39		Short interspersed repetitive element	Vazquez et al. (1996)
SZ 39		Short interspersed repetitive element	Vazquez et al. (1996)
SZ 32		Short interspersed repetitive element	Vazquez et al. (1996)
SZ 7-32		Short interspersed repetitive element	Vazquez et al. (1996)

a: an additional list of chromosomal markers used in *Trypanosoma cruzi* genome project can be found in Henriksson et al. (1995).

of the chromosomal bands. On the other hand, genes encoding trans-sialidase or SAPA, which displays homology with these genes and therefore belongs to the superfamily of trans-sialidases, were mapped on two chromosomal bands (Egima et al. 1996).

Some genes have an unique chromosomal location and can be used as specific markers for these chromosomes. Several markers, such as a repetitive flagellar antigen (H49), a cytoplasmic repetitive antigen (CRA), Hsp70 and KAP were mapped on neighboring chromosomal bands, which may represent size-polymorphic homologous chromosomes.

As discussed above, the non-stoichiometric staining of chromosomal bands by ethidium bromide suggests the existence of size-identical heterologous chromosomes. Gene mapping data corroborate the evidences of aneuploidy for the chromosomal bands which are poorly resolved in PFGE.

Evidences obtained in our laboratory suggest that bands XVI and XVII could carry a pair of size-polymorphic homologous chromosomes which are defined by the genes encoding the repetitive antigens H49 and CRA (Santos MRM, unpublished results). It is noteworthy that several genetic markers (spliced leader sequence, gp90, gp82, cDNA

TABLE II

Localization of repetitive sequences, genes encoding proteins and structural RNAs and anonymous markers on chromosomal bands of clone CL Brener

Band No.	Size Mbp	Repetitive sequence	Multigenic family	Protein, unknown ORF Structural RNA	Anonymous marker
XX	3.50	C6, Sire, Sat.		Hsp6O	
XIX	3.35	C6, Sire, Sat., SRE, B11		Amastin, cDNA68	SZ23-14
XVIII	2.75	C6, Sire, Sat., SRE	gp82, gp90, gp85	C11, Ubiquitin, cDNA78	SZ23-14
XVII	2.60	C6, Sire, Sat., SRE	gp85	CRA, H49, cDNA68, cDNA78	SZ23-14
XVI	2.30	C6, Sire, Sat., SRE, B11	gp82, gp90, gp85	CRA, H49, cDNA40, cDNA78, SL	Z23-14
XV	1.60	C6, Sire, Sat., SRE, B11	gp82, gp90, gp85, P2β	Amastin, αtubulin	SZ7-39
XIV	1.50	C6, Sire, Sat., B11	P2β	18/24S rDNA	
XIII	1.35	C6, Sire, SRE		B12, B13, cDNA51, cDNA57	SZ23-14
XII	1.25	C6, Sire, B11	gp82, gp90, gp85, P2β	cDNA51	SZ39
XI	1.18	C6, Sire			
X	1.10	C6, Sire, SRE, B11	gp82, gp90, gp85, P2β	Hsp70	SZ39
IX	1.03	C6, Sire, B11	gp82, gp90, gp85, P2β	MIP, Hsp70	
VIII	0.95	C6, Sire, Sat.	gp82, gp90, gp85	KAP	SZ5
VII	0.90	C6, Sire, SRE			SZ7-32
VI	0.85	C6, Sire	gp82, gp90	KAP, Actin, Cruzipain	SZ32
V	0.80	C6, Sire, Sat., SRE	gp82, gp90, gp85	1F8, SL, Tc40, cDNA40	
IV	0.73	C6, Sire	gp85		
III	0.70	C6, Sire, SRE	gp82, gp90	Tc40	SZ5
II	0.60	C6, Sire	gp85	1F8, Cruzipain	SZ39
I	0.45	C6, Sire	gp85		

40, B11) mapped on band XVI are absent in band XVII. On the other hand, cDNA 68 which was mapped on band XVII is absent in band XVI. We could explain these results by the existence of more than two different homologous chromosomes in bands XVI and XVII. In fact, the chromosome equivalent data suggest the existence of two or three chromosomes in these bands.

IDENTIFICATION OF GENETIC LINKAGE GROUPS

Several markers were mapped on the same chromosomal bands suggesting that they could be linked. For example, genes encoding antigens B12 and B13 were mapped on the chromosomal band XIII and the hybridization patterns of gp82 and gp90 genes are very similar. The physical linkage between these genes, B12 and B13 or gp82 and gp90, was demonstrated by isolation of genomic recombinant clones carrying linked copies of B12 and B13 or gp90 and gp82 (Gruber 1995, Cano 1995).

Henriksson et al. (1995) identified nine different linkage groups, each comprising 2-4 markers. The linkage between the markers was maintained in eight of the nine linkage groups when a panel comprising 26 different *T. cruzi* strains were analyzed. This information will be very useful for the construction of physical chromosomal maps required for the *T. cruzi* genome project.

COMPARISON THE MOLECULAR KARYOTYPE OF CL BRENER CLONE OBTAINED BY DIFFERENT GROUPS

The molecular karyotype of clone CL Brener was also determined using several different run conditions (Henriksson et al. 1995, 1996) (see Fig.). General speaking there is a reasonable correlation between the results of Cano et al. (1995) and those from the Henriksson and co-workers. Several genes were mapped on the same chromosomal bands by both groups. For instance, the genes encoding the antigens H49 (also designed as JL7, FRA, Tc1), CRA (Tc30, JL8, Tc27), B13 (Tc2) and ubiquitin. Four linkage groups found in our laboratory were also reported by Henriksson and co-workers.

Both groups mapped genes Hsp70, SAPA, cruzipain and 1F8 on two chromosomal bands. But, there are small differences in the sizes of the chromosomes recognized by these probes. For instance, we have mapped the 1F8 on two bands of 0.80 and 0.60 Mbp whereas Henriksson et al. (1995) mapped this marker on two bands of 1.15 and 0.875 Mbp.

There is a discrepancy between the number of chromosomes estimated by PFGE and the number of kinetochores. Data obtained in the electrokaryotypes suggest that at least most of the chromosomal bands are diploid and the maximal number of chromosomes was estimated as 64 (Cano et al. 1995) or 80 (Henriksson et al. 1995). Three-dimensional

reconstructions from electron micrographs of serial sections of mitotic nuclei in trypanosomes showed the existence of ten dense plaques which are putative kinetochores (Solari 1980a,b, 1995). Among the trypanosomatids the number of dense plaques is lower than the number of chromosomal bands visualized by PFGE. In contrast, other parasite protozoans and yeast present a good correlation between the number of dense plaques and the chromosome-sized DNA molecules. The reason for the difference in the number of dense plaques and the bands in the electrokaryotypes of trypanosomes is not known.

IDENTIFICATION OF ESTs (EXPRESSED SITE TAG) USING ISOLATED CHROMOSOMES

Chromosomal bands were isolated from preparative gels, labeled by random priming and used as probes in the screening of an epimastigote cDNA library (Urmenyi et al. 1996). Several cDNA clones were isolated with this procedure (Table II). For instance, we have isolated more than ten cDNA clones using DNA from band XVI as a probe. Clone 78 (Elongation Factor 1g) hybridizes with several chromosomal bands including the XVI and XVII bands. Specific chromosome markers were also isolated by this procedure such as clone 67 which is specific for chromosome XIII. This procedure can thus be very useful to identify ESTs specific for chromosomes.

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