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Genome Res. 2000 10: 1996-2005

Access the most recent version at doi:[10.1101/gr.146300](https://doi.org/10.1101/gr.146300)

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Cold Spring Harbor Laboratory Press

A Random Sequencing Approach for the Analysis of the *Trypanosoma cruzi* Genome: General Structure, Large Gene and Repetitive DNA Families, and Gene Discovery

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A random sequence survey of the genome of *Trypanosoma cruzi*, the agent of Chagas disease, was performed and 11,459 genomic sequences were obtained, resulting in ~4.3 Mb of readable sequences or ~10% of the parasite haploid genome. The estimated total GC content was 50.9%, with a high representation of A and T di- and trinucleotide repeats. Out of the estimated 5000 parasite genes, 947 putative new genes were identified. Another 1723 sequences corresponded to genes detected previously in *T. cruzi* through expression sequence tag analysis. 7735 sequences had no matches in the database, but the presence of open reading frames that passed Fickett's test suggests that some might contain coding DNA. The survey was highly redundant, with ~35% of the sequences included in a few large sequence families. Some of them code for protein families present in dozens of copies, including proteins essential for parasite survival and retrotransposons. Other sequence families include repetitive DNA present in thousands of copies per haploid genome. Some families in the latter group are new, parasite-specific, repetitive DNAs. These results suggest that *T. cruzi* could constitute an interesting model to analyze gene and genome evolution due to its plasticity in terms of sequence amplification and divergence. Additional information can be found at <http://www.iib.unsam.edu.ar/tcruzi.gss.html>.

[The sequence data described in this paper have been submitted to the dbGSS database under the following GenBank accession nos.: AQ443439–AQ443513, AQ443743–AQ445667, AQ902981–AQ911366, AZ049857–AZ051184, and AZ302116–AZ302563.]

Protozoan parasites from the order Kinetoplastida are the causative agents of widespread diseases in humans as well as of considerable economic loss through infection of domestic animals and wildlife. Among them are *Trypanosoma cruzi* and *Trypanosoma brucei*, the agents of Chagas disease in the Americas and sleeping sickness in Africa, respectively, and *Leishmania* spp., causing a variety of pathologies in humans. In addition to their medical importance, these parasites were the source of discoveries of fundamental cellular and molecular phenomena such as RNA editing (Stuart et al. 1997; Estevez and Simpson 1999), mRNA trans-splicing (Nilsen 1995; Lee and Van der Ploeg 1997), glycosylphosphatidylinositol anchoring of proteins (Krakow et al. 1986), and antigenic variation (Rudenko et al. 1998), among others. Kinetoplastids are also interesting evolutionarily because they represent one of the earliest eukary-

otic organisms that diverged from the ancestor of the main eukaryotic branch.

Initial studies on the genome structure of these organisms were hampered by the difficulty of applying classical genetic tools. As is the case with yeast, chromosomes do not condense during mitosis and cannot be visualized directly with color stains. In spite of these drawbacks several recent studies have revealed a highly plastic genome with an unusual gene organization (Zingales et al. 1997; Network 1998; Ersfeld et al. 1999). In the case of *T. cruzi* it has been shown that there is a large chromosomal size variation between strains that can also be observed between pairs of homologous chromosomes (Henriksson et al. 1995).

Many genes in kinetoplastids, including housekeeping genes, are present in multiple copies, either clustered in tandems or distributed in different chromosomes (Campetella et al. 1992b; El-Sayed and Donelson 1997). Recently, the complete sequence of chromosome 1 from *L. major* Friedlin (Myler et al. 1999) and a partial sequence of chromosome 3 from *T. cruzi* (Andersson et al. 1998) were obtained, showing in both cases a similar organization with two clusters of

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Article published online before print: *Genome Res.*, 10.1101/gr.146300.
Article and publication are at www.genome.org/cgi/doi/10.1101/gr.146300.

genes per chromosome transcribed in opposite directions. These studies also showed that the gene density is high in the regions where genes are clustered, with ~1 gene every 3.6 Kb in both cases. These and other unusual characteristics in genome structure and organization make these parasites an interesting field of study to further understand eukaryote gene and genome evolution.

As part of the parasite genome projects launched by the Tropical Disease Program of the World Health Organization, we and others have performed expressed sequence tag (EST) analysis of the *T. cruzi* genome as a means for rapid gene finding (Verdún et al. 1998; Porcel et al. 2000). As a first step toward obtaining the complete sequence of the clone selected for the *T. cruzi* genome project (CL-Brener clone) we have now made a random genome survey of ~10% (4.3 Mb) of the haploid genome of the parasite. The results obtained allowed us to make a general characterization of the *T. cruzi* genome, identify putative new genes, and define large gene families and repetitive sequence families present in the parasite genome, including a novel repetitive element and uncharacterized abundant sequences.

RESULTS AND DISCUSSION

Overall Structure of the *T. cruzi* Genome

A random genomic library of *T. cruzi* DNA was constructed and used to produce 11,459 reads with an average length of 374 bp after vector removal and quality clipping. These GSS (Genomic Sequence Survey) sequences represent 4.3 Mb of readable sequences or ~10% of the parasite haploid genome, which is ~40 Mb (Frohme et al. 1998). The total GC content of the sequence produced was 50.9%, a value that is slightly larger than the one obtained from estimations made on 8796 *T. cruzi* ESTs (49.6%), and the one obtained for 93.4 Kb from chromosome 3 (48.5%) (Andersson et al. 1998). The fractional GC content for all the GSSs varied from 0.18–0.71, with ~69% of the sequences falling in the 0.47–0.57 range.

The frequency of di- and trinucleotide repeats calculated for all the GSS sequences (11,459 GSS) is shown in Figure 1. *T. cruzi* has levels of CpG, TpG, and CpA dinucleotides which are near their expected values, unlike mammalian or vertebrate genomes that show a suppression of the CpG dinucleotide and increased levels of the TpG and CpA dinucleotides (Regev et al. 1998). The TpA dinucleotide, however, is suppressed in *T. cruzi*, with an observed/expected ratio of 0.54. This dinucleotide is also suppressed in mammalian genomes (Smith and Waterman 1983), and in *Fugu* the pufferfish (Elgar et al. 1999), although the reason for this is not known. Also, the abundance of (A)n and (T)n di- and trinucleotides is remarkable. The fre-

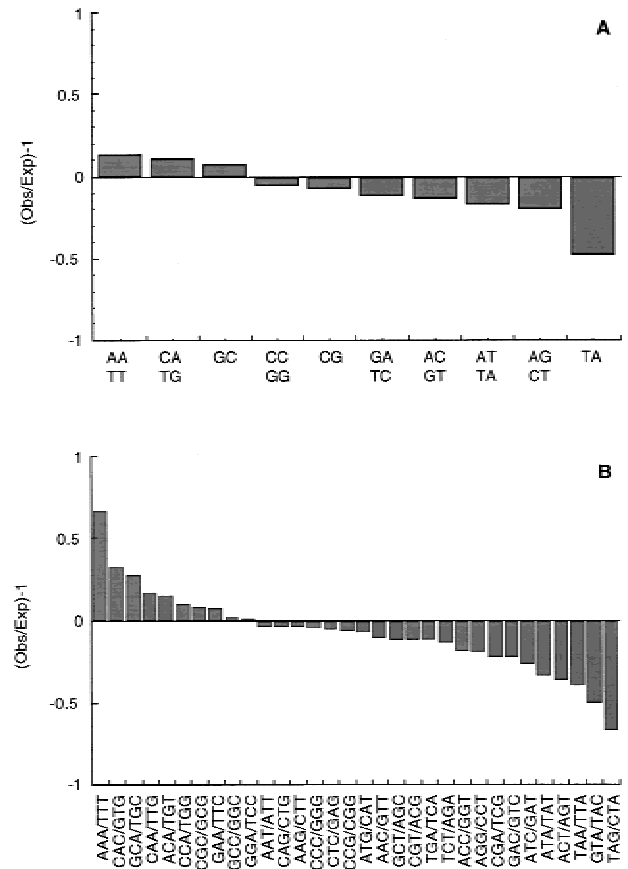


Figure 1 Frequency of di- and tri-nucleotide repeats in the *Trypanosoma cruzi* genome. The total of 11,459 sequences were used to search for the occurrence of all possible words of length 2 (A) and 3 (B) on both strands of the sequences using COMPSEQ. The expected frequency of each word is based on the assumption that all words have the same probability of occurrence. Di- and tri-nucleotide frequencies are expressed as Observed (Obs)/Expected (Exp) - 1, so that negative values correspond to suppressed di- and tri-nucleotides and positive values correspond to di- and tri-nucleotides with frequencies over that expected. Because the search is done on both strands, only one reverse complementary di- or tri-nucleotide of a pair is shown.

quency of these di- and trinucleotides is greater than the observed frequency for the CpA dinucleotide, the most common dinucleotide in vertebrates. When the same analysis was done for the partial sequence of chromosome 3, we obtained similar values, except for the (A)n and (T)n di- and tri-nucleotides. These also represent the most frequent species, but their observed/expected ratios are higher. In our survey the (A)n dinucleotide had a 0.13 obs/exp ratio, whereas the partial sequence of chromosome 3 had a 0.4 ratio. For the (A)n trinucleotide these values are higher: a 0.67 ratio in our survey and a 1.43 ratio in the partial sequence of chromosome 3.

Coding Content of the GSS Sequences

We compared the sequences in our data set to a protein

nonredundant database using BLASTX (Altschul et al. 1997). From this analysis, 3724 GSS (32.5%) showed significant ($E < 1e-5$) similarities to sequences present in the public database (GenBank release 115) (Table 1); 2778 GSS (24.2%) matched *T. cruzi* sequences while 947 GSS (8.3%) matched non-*T. cruzi* sequences. 67.5% did not match any sequence in the database. When we compared each GSS to the dbEST database, only 1723 (15%) of the GSSs gave significant matches and 96% of these were matches to *T. cruzi* ESTs. The low hit rate against ESTs could be explained in part by the low sequence coverage attained in the EST sequencing, which only covered one of the four main life cycle stages of the parasite. Thus, the coding content of our survey could be considered to be 15% based on the number of matches against dbEST or 32.5% based on the number of matches against proteins in nonredundant databases. However, this does not include sequences absent from the databases.

To detect putative coding sequences using a different criteria, we first searched our sequences for open reading frames (ORFs) > 300 bp (defined as sequences without a stop codon and no requirement for start codons). We then evaluated which of these resulting sequences could be regarded as coding using the testcode algorithm developed by Fickett (1982), which measures the positional randomness of a sequence, and is independent of the reading frame. About 83% of the sequences (9520 GSSs) had ORFs with the mentioned requirements, and 23% of these (2222 GSSs) were found to be potentially coding by the testcode algorithm. Using this figure as the minimum number of coding sequences in our survey, we can estimate the number of genes for *T. cruzi* to be about 5000 per haploid genome (considering an average gene size of 1500 bp and a haploid genome size of 40 Mbp).

Identification of Putative New Genes

Based on the BLASTX analysis, which is summarized in Table 1, we detected 947 putative new genes, which are

Table 1. BLASTX Matches to Nonredundant Databases

	No. of GSS	% of GSS	No. ESTs	% of ESTs
Total Database matches:	11,459	100	8,796	100
Total	3,724	32.5	2,552	29
<i>T. cruzi</i>	2,778	24.2	861	9.8
Other trypanosomatids	331	2.9	262	3
Other organisms	616	5.4	1,429	16.2
No database match	7,735	67.5	6,244	71

All of the GSSs presented in this paper and all the *T. cruzi* ESTs present in the dbEST division of GenBank were used to search the NCBI nonredundant database (GenBank Release 115). Matches with an E value $\leq 1e-5$ were considered positive.

those positive matches against a non-*T. cruzi* protein in the nonredundant database. The best 50 matches are shown in Table 2 (detailed information on *T. cruzi* GSS sequences can be found at <http://www.iib.unsam.edu.ar/tcruzi/gss.html>).

Among the new genes found there is a GSS (GSSTc12036) with similarity to N-myristoyl transferases (Nmt). Nmt is only found in eukaryotic cells and transfers fatty acid myristate from myristoyl-CoA to the amino-terminal glycine of substrate proteins (Russell Johnson et al. 1994). Genetic and biochemical studies have established Nmt as a target for the development of a new class of fungicidal drugs, and the structure of Nmt from two lower eukaryotes, namely *Saccharomyces* and *Candida* has been solved (Weston et al. 1998; Bhatnagar et al. 1999). In trypanosomes protein N-myristoylation has not yet been demonstrated, whereas it has been shown that these parasites can do S-myristoylation of proteins (Armah and Mensa-Wilmot 1999). Other interesting findings were two GSS homologous to proteins involved in chromatin remodeling. GSSTc788 is homologous to the *Drosophila* ISWI protein, which is part of several ATP-dependent chromatin remodeling complexes such as NURF (Nucleosome remodeling factor), CHRAC (chromatin accessibility complex) and ACF (ATP-utilizing chromatin assembly and remodeling factor) (Mucharadt and Yaniv 1999), whereas GSSTc11568 is homologous to several histone deacetylases. Chromatin remodeling is a mechanism of transcriptional regulation that has been demonstrated in many eukaryotes including yeast, which as trypanosomes does not show chromosome condensation during its cell cycle. Another GSS (GSSTc12012) had homology with a *T. brucei* VSG expression site-associated protein precursor (ESAG-2), which is a member of a large gene family that includes nonfunctional genes (Kooter et al. 1988). Also, several GSSs with homology to proteins having RNA binding domains were identified, including one clone (GSSTc11533) that showed homology to the RNA binding domain present in the developmentally regulated proteins p37 and p34 from *T. brucei* (Zhang et al. 1998).

Identification of Large Gene Families

To identify large gene families, GSS sequences were clustered using the PHRAP program to assemble contigs. Using this method we were able to group 7883 reads in 2091 contigs; the other 3576 were singlets (reads having no nonvector match to any other read). This means that our survey contains 5667 unique sequences, according to our clustering method, and thus a redundancy of at least ~50%. Further clustering is possible, however, because the results from BLASTX contained several GSS that belong to different contigs showing matches to the same sequences in the database. To estimate the total number of GSS that belong

Table 2. Identification of New *T. cruzi* Genes

dbGSS ^a	Description ^b	Score	Expect
11462	ref-NP_011059.1-GLC7-protein phosphatase type I [<i>Saccharomyces cerevisiae</i>]	474	0.00E+00
10993	sp-P22679-elongation factor TU (EF-TU) [<i>Mycoplasma hominis</i>]	312	0.00E+00
00184	sp-P46794-cystathionine beta-synthase [<i>Dictyostelium discoideum</i>]	303	0.00E+00
11438	sp-O76767-lumen protein retaining receptor [<i>Drosophila melanogaster</i>]	208	0.00E+00
11472	emb-CAB56598.1-alpha dynein heavy chain [<i>Chlamydomonas reinhardtii</i>]	202	0.00E+00
11026	gi-2425121-Spalten [<i>Dictyostelium discoideum</i>]	116	0.00E+00
12036	gb-AAF19802.1-N-myristoyl transferase [<i>Brassica oleracea</i>]	107	0.00E+00
01761	gl-3004644-trypanothione synthetase [<i>Crithidia fasciculata</i>]	428	5.00E-42
11137	gb-AAB67249.1-T-complex protein 1, Beta subunit [<i>Homo sapiens</i>]	424	9.00E-42
11193	ref-NP_014850.1-RET1-second-largest subunit of RNA polymerase III [<i>Saccharomyces cerevisiae</i>]	422	1.00E-41
11590	gb-AAF08387.1-26S proteasome regulatory complex subunit p48A [<i>Drosophila melanogaster</i>]	419	2.00E-41
11120	emb-CAA65384-malate dehydrogenase [<i>Mesembryanthemum crystallinum</i>]	371	2.00E-35
11285	gi-1931649-DNA helicase isolog [<i>Arabidopsis thaliana</i>]	353	2.00E-33
11563	ref-NP_013458.1-transaldolase [<i>Saccharomyces cerevisiae</i>]	348	7.00E-33
09938	gi-2246458-S-adenosyl-methionine-sterol-C-methyltransferase [<i>Ricinus communis</i>]	348	8.00E-33
0705	pir-T1017324-sterol C-methyltransferase-castor bean [<i>Ricinus communis</i>]	348	9.00E-33
11338	gb-AAC67249.1-3-hydroxyisobutyryl-coenzyme A hydrolase [<i>Arabidopsis thaliana</i>]	341	5.00E-32
11467	gb-AAF04493.1-acetyl-CoA carboxylase 1 [<i>Toxoplasma gondii</i>]	330	7.00E-31
10956	dbj-BAA84364.1-DEIH-box RNA/DNA helicase [<i>Arabidopsis thaliana</i>]	328	2.00E-30
11575	gb-AAC73040.1-putative AAA-type ATPase [<i>Arabidopsis thaliana</i>]	323	5.00E-30
11516	sp-P05439-ATP synthase alpha chain [<i>Rhodobacter blasticus</i>]	319	2.00E-29
11606	sp-P51044-citrate synthase, mitochondrial precursor [<i>Aspergillus niger</i>]	321	2.00E-28
11417	gb-AAF21464.1-proline oxidase 2 [<i>Homo sapiens</i>]	310	2.00E-28
11502	gi-2654103-MAPKK kinase [<i>Neurospora crassa</i>]	304	9.00E-28
11523	gb-AAD26855.1-phenylalanyl tRNA synthetase beta subunit [<i>Mus musculus</i>]	301	2.00E-27
11568	gi-4101722-histone deacetylase mHDA1 [<i>Mus musculus</i>]	301	2.00E-27
11480	gi-2462752-phosphatidylinositol 3-kinase [<i>Arabidopsis thaliana</i>]	299	4.00E-27
11463	sp-P32826-serine carboxypeptidase precursor [<i>Arabidopsis thaliana</i>]	299	4.00E-27
10965	gi-687208-dynein heavy chain isotype 5C [<i>Tripneustes gratilla</i>]	289	5.00E-26
11328	pir-A56220-protein kinase aurora-fruit fly [<i>Drosophila melanogaster</i>]	287	1.00E-25
11446	sp-O15228-dihydroxyacetone phosphate acyltransferase (DAP-AT) [<i>Homo sapiens</i>]	280	7.00E-25
11433	gb-AAF11511.1-acetyl-CoA acetyltransferase [<i>Deinococcus radiodurans</i>]	279	9.00E-25
11601	sp-P30575-enolase 1 (2-phosphoglycerate dehydratase) [<i>Candida albicans</i>]	278	1.00E-24
01810	sp-O94476-eukaryotic translation initiation factor 6 (EIF-6) [<i>Schizosaccharomyces pombe</i>]	275	3.00E-24
0740	gb-AAF62506.1-ribosomal protein LS [<i>Trypanoplasma borreli</i>]	273	5.00E-24
11274	pir-S70896-aminomethyltransferase [<i>Saccharomyces cerevisiae</i>]	271	5.00E-24
11279	gi-780410-helicase [African swine fever virus]	272	6.00E-24
11882	sp-Q07405-ATP synthase alpha chain [<i>Myxococcus xanthus</i>]	269	1.00E-23
11432	emb-CAB40791.1-centrin [<i>Euplotes octocarinatus</i>]	265	3.00E-23
11654	gi-1872473-delta-24-sterol methyltransferase [<i>Triticum aestivum</i>]	258	7.00E-23
01015	pir-A56492-protein kinase ERK2 [<i>Dictyostelium discoideum</i>]	280	8.00E-23
11584	ref-NP_005678.1-phenylalanyl-tRNA synthetase beta-subunit [<i>Homo sapiens</i>]	259	1.00E-22
11434	sp-O05593-methionyl-tma synthetase [<i>Mycobacterium tuberculosis</i>]	254	6.00E-22
11038	gi-1354084-axonemal dynein light chain p33 (<i>Strongylocentrotus purpuratus</i>)	251	2.00E-22
11440	gi-2665637-mismatch repair protein MSH6 [<i>Mus musculus</i>]	248	4.00E-21
11248	gb-AAF22155.1-ARD-1 N-acetyltransferase homologue [<i>Mus musculus</i>]	244	1.00E-20
11852	gb-AAC32590.1-sperm flagellar protein Repro-SA-1 [<i>Homo sapiens</i>]	239	5.00E-20
01825	dbj-BAA20996-kinesin-like protein [<i>Caenorhabditis elegans</i>]	239	6.00E-20
11210	pir-A35630-regulatory protein aelR3 [<i>Pseudomonas aeruginosa</i>]	237	7.00E-20

GSS sequences were used to search NCBI's non-redundant database using BLASTX. The first 50 GSSs out of the 947 GSSs showing matches against non-*T. cruzi* sequences are listed. Detailed information about the homologies found for GSSs can be found at <http://www.iib.unsam.edu.ar/genomelab/tcruzi/gss.html>.

^aGSS names in dbGSS are the numbers given here preceded by GSSTc (e.g., GSSTc11210).

^bDescriptions are taken directly from the BLAST reports.

to a given gene family, we used the consensus sequence from each contig to search a local *T. cruzi* GSS database using BLASTN. Based on this analysis we were able to delineate abundant sequences in our survey, which are the main contributors to the observed redundancy. The most abundant gene families in the *T. cruzi* genome are summarized in Table 3.

Among the largest families identified is the superfamily of *T. cruzi* antigens, also known as transsialidase-like molecules (632 copies per haploid genome). Their members have a number of different activities, most of them involved in the host-parasite interaction (Frasch 2000). Other sequences already known to conform large gene families in *T. cruzi* iden-

Table 3. Large Gene Families in *T. cruzi*

A Gene Families	No. of GSSs	Estimated No. of Copies	Relative %	No. of ESTs	Reference
dgf-1	494	154	4.3	6	(Wincker et al. 1992)
trans-sialidase	427	632	3.7	10	(Parodi et al. 1992)
L1 non-LTR retrotransposon	214	149	1.9	5	(Martín et al. 1995)
Mucin	122	710	1.1	5	(Di Noia et al. 1995)
Cysteine proteinase (Cruzipain)	39	91	0.3	7	(Campetella et al. 1992a)
predicted ORF (gi3053534), chromosome 3	38	103	0.3	4	(Andersson et al. 1998)
gp63	34	70	0.3	9	AF110951, unpubl.
Histone H4	29	337	0.2	10	(Soto et al. 1997)
Casein kinase homolog	23	81	0.2	7	AF089709, unpubl.
Adenylyl cyclase	19	18	0.2	0	(Taylor et al. 1999)
Hsp70	18	25	0.2	48	(Requena et al. 1988)
Histone H2A	17	145	0.1	106	(Puerta et al. 1994)
Helicase	14	24	0.1	15	
Hsp90	11	18	0.1	7	(Mottram et al. 1989)
Total	1499		14.5		

B Repetitive DNA Families	No. of GSSs	Estimated No. of Copies	Relative %	No. of ESTs	Reference
minichromosomal 195 bp repeat	854	15287	7.45	ND	(Gonzalez et al. 1984)
TcIRE (I)	266	1664	2.3	8	this work
VIPER	174	257	1.5	ND	(Vázquez et al. 2000)
C6 interspersed element	230	560	2.0	ND	(Araya et al. 1997)
SIRE	201	3011	1.8	ND	(Vázquez et al. 2000)
telomere associated sequences	131	1963	1.1	ND	
TcIRE (II)	47	2310	0.4	2	this work
TRBSEQA	31	105	0.3	ND	(Requena et al. 1992)
HCR6	10	57	0.1	ND	(de Mendonça-Lima and Traub-Cseko 1991)
Spliced Leader gene	12	69	0.1	ND	
Total	2133		18.6		

C Unknown Families	No. of GSSs	Estimated No. of Copies	Relative %	No. of ESTs	Consensus Size (bp)
Cluster 2009	19	54	0.1	0	1220
Cluster 2047	85	136	0.7	1	2170
Cluster 2015	53	96	0.5	0	1917
Cluster 1994	25	82	0.2	1	1056
Cluster 2056	22	30	0.2	0	2571
Cluster 2019	21	102	0.2	3	1051
Cluster 2027	12	58	0.1	0	718
Cluster 1986	10	48	0.1	0	728
Total	247		2.1		

GSS sequences were clustered and their similarities against sequences in nonredundant databases were determined. Total number of GSS for each family was determined as described in the text. Note, however, that this approach can lead to a GSS belonging to more than one family. To calculate the number of copies, the value of the gene size (GS) used was the length of the coding sequence (excluding UTRs) of a representative member from each family; in the case of genes with different sizes, an average was used. When only partial sequences were available, copy numbers were not determined as this could lead to overestimation of the figures. To determine the number of ESTs for a given gene family, the consensus sequence or a sequence of a representative member was used to do a BLASTN search against the 8796 *T. cruzi* ESTs. Matches with $E < 1e-40$ were considered positive. In the case of unpublished sequences that are available from nucleotide databases, the GenBank accession number is given. (A) Gene families (protein coding). (B) Repetitive DNA families (likely to be noncoding). (C) Uncharacterized sequences described in this work. Information about these unknown families (consensus sequence and individual GSSs included in the contig) can be found at <http://www.iib.unsam.edu.ar/genomelab/tcruzi/gss.html>.

tified through this screening were the cysteine proteinase cruzipain (Campetella et al. 1992a), dgf-1, a large protein of unknown function (Wincker et al. 1992),

and the parasite mucins (Di Noia et al. 1998). In all of these cases, the estimated number of copies agreed well with experimental data, showing that our sampling

can be considered a good representation of the whole genome. A considerable number of GSS gave matches to proteins known to be part of retrotransposons: reverse transcriptase, apurinic/aprimidinic endonuclease (AP-nuclease) and homologs of the *gag* protein present in retroviral and LTR-retrotransposons. These proteins most probably correspond to the L1Tc non-LTR retrotransposon (Martín et al. 1995; Olivares et al. 1997), the most abundant retrotransposable element found in our survey (250 GSS). However, they could also come from other elements since 40 GSS with homology to the *T. brucei* INGI retrotransposon and 11 GSS with homology to an *Anopheles* retrotransposon were detected when we performed a search in a database of repetitive elements (see below).

Some GSS contigs failed to show similarity to any known protein in the nonredundant databases (BLASTX), and also failed to show homology to any known DNA sequence (BLASTN) in the nonredundant database. To see if these unknown sequences were part of uncharacterized large gene families, we used the consensus sequence from each of these contigs to search a database made from all the 2091 consensus sequences of the contigs. We then grouped together contigs showing significant ($E < 1e-10$) homology as reported by BLASTN. None of the homologous contigs found, nor any GSS that formed those contigs, had significant matches in the databases. Out of 1050 contigs analyzed (2562 GSSs, 22.3% of the survey), the first 10 representing the most abundant unknown sequences in the *T. cruzi* genome are included in Table 3B.

Identification of Repetitive DNA Families

The program REPEATMASKER was used to search for simple repeats (1–6 bp), low-complexity regions, and known repetitive DNA. Using the database of simple repeats from RepBase (Genetic Information Research Institute, <http://www.girinst.org/>, Release 5.02) the program found 1556 GSS (13.5%) containing 1091 matches to 62 different simple repeats comprising 46,965 bp (0.74%) and 1001 matches to low-complexity regions comprising 56,455 bp (0.89%). The most abundant simple repeats are shown in Figure 2A. As was observed in the analysis of di- and trinucleotide repeats, the most abundant simple repeat in *T. cruzi* is (T)_n and its complement. Interestingly, (TA)_n and (TAA)_n appear to be the second two most abundant repeats in our survey. In agreement, the most abundant low-complexity regions, as reported by REPEATMASKER are AT-rich, T-rich and A-rich regions, which together represent >85% of the low complexity regions in Figure 2B. Taken together, this analysis and the data on frequencies of di- and tri-nucleotide repeats suggest that, although suppressed in the whole survey, (TA)_n and (TAA)_n are abundant in a subset of the GSS. This

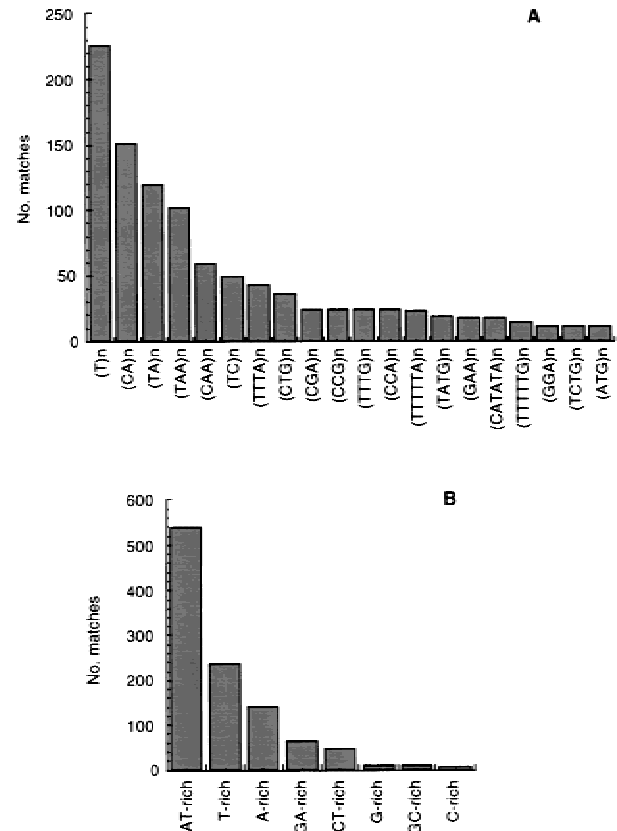


Figure 2 Microsatellite repeats and low-complexity regions in the *Trypanosoma cruzi* genome. Simple repeats and low-complexity regions were searched for in the *T. cruzi* GSS database using REPEATMASKER as described in the text. (A) The 20 most abundant microsatellite repeats in the survey are shown. The minimum value of n is the one that gives a Smith-Waterman (SW) score ≥ 180 , which is the cutoff to consider a match as positive. This value varied from 18 for a single nucleotide repeat to 3 for a hexanucleotide repeat. Each named microsatellite in the graph includes all combinations thereof; so (A)_n also includes its complement (T)_n, and (ATG)_n also includes (CAT)_n, (ATC)_n, (TCA)_n, (TGA)_n and (GAT)_n. (B) Low-complexity regions were searched for as described in the text. The length of the regions detected varied from 16 bp to 308 bp.

subset most probably includes GSS corresponding to intergenic regions that are rich in A and T in *T. cruzi*.

To search for repetitive elements, we used the invertebrate database of RepBase that, although not specific for trypanosomatids, contains repetitive elements from these organisms. The program found 3523 matches to 13 different elements comprising 629,831 bp (14.7%) in 3290 GSS (29%). The great majority of these elements were from *T. cruzi* or *T. brucei* with the exception of a repetition from *T. borreli* (TBRP1) which had five matches and a retrotransposon from *Anopheles gambiae* (RT1) which had 11 matches. As shown in Table 3C, the most abundant element in the *T. cruzi* genome is the 195-bp minichromosomal repetitive element (González et al. 1984). Also included in Table 3C are repetitive elements that were described in *T.*

cruzi but were absent from RepBase. This is the case of telomere-associated sequences, TRBSEA (Requena et al. 1992), HCR6 (de Mendonça-Lima and Traub-Cseko 1991), and the spliced leader gene for which we detected 27, 31, 9, and 12 GSS, respectively, using BLASTN ($E < 1e-40$). We did not find any match to primate or mammalian repetitive elements when we searched the respective RepBase libraries.

TcIRE, A New *T. cruzi* Repetitive Element

A new repetitive element apparently dispersed in the *T. cruzi* genome was found during the analysis of the GSS contigs. This element, which was named TcIRE (for *T. cruzi* Interspersed Repeated Element) was found in 44 contigs (266 GSS) representing almost 3% of the genome with 1664 copies per haploid genome. The structure of TcIRE, depicted in Figure 3, shows a central conserved core flanked by less conserved regions. The BLASTN analysis showed that the 3' region of TcIRE is similar in some cases to the last 70 bp of the 3'-UTRs of some mucin genes. This results in two different groups of TcIRE elements that differ in their 3' regions. As can be seen in the alignment shown in Figure 3B, the two groups start to differ after the conserved region. The 5' region, although less conserved, appears to be shared by both groups.

One copy of TcIRE is present in an intergenic region of a recently sequenced *T. cruzi* cosmid containing enzymes for the synthesis of pyrimidines (Gao et al. 1999). TcIRE is located between the OMPDCase-OPRTase gene and a surface antigen gene from the transsialidase superfamily. This intergenic region is ~4.5 Kb long, and the TcIRE copy is located past the center (~13,480–13,960 bp) of this region. For means of comparison, we found only three GSS showing significant homology with the OMPDCase-OPRTase gene. Southern blots from several strains and clones of *T. cruzi* and from *Leishmania* were probed with a 300-bp fragment amplified from clone GSSTc11311 corresponding to the most conserved region of TcIRE. This analysis showed that TcIRE is present in high copy numbers in all the strains and clones tested (Fig. 4). Conversely, it seems to be absent in *Leishmania*, as we did not get any hybridization signal even under low-stringency conditions (data not shown). Hybridization of chromosomes separated by PFGE using the same probe showed that TcIRE might be present in several chromosomes, although the region of hybridization corresponds to poorly resolved chromosomes of high molecular weight. When the TcIRE consensus sequence was used to search the *T. cruzi* EST database using BLASTN, it showed significant ($E < 1e-40$) homology with eight ESTs. None of these ESTs, however, had homologs in nonredundant databases. Furthermore, no significant matches were obtained when these ESTs

or the TcIRE consensus sequence were used to search the Pfam database of multiple alignments using HMMER.

When the same fragment from clone GSSTc11311 was used to probe a Northern blot made from epimastigote total RNA, no signal was found (data not shown), which suggests that the ESTs detected might come from a genomic contamination of the original library used to sequence the ESTs. This data suggest that TcIRE does not code for a protein product and can be considered part of the *T. cruzi* repetitive DNA.

METHODS

Genomic Library

A random genomic library from *T. cruzi* strain CL Brener was constructed in the plasmid vector pBS(-) (Stratagene). DNA was prepared by using the proteinase K-phenol extraction method (Sambrook et al. 1989) and mechanically sheared by using a nebulizer. After treatment with Bal31 nuclease, phenol extraction, and ethanol precipitation, the DNA was blunt-ended with T4 DNA polymerase. Fragments were size-fractionated by agarose gel electrophoresis and the range between 1.4–2.1 Kb were recovered and cloned into the dephosphorylated *HincII* site of the vector.

Nucleotide Sequencing

Fresh plated colonies were grown at 37°C in Terrific broth containing 100 µg/mL ampicillin in 96 deep-well plates at 350 rpm. The bacterial cultures were grown in two steps, first in 0.2 mL for 14–16 h; afterward 0.8 mL of medium was added and grown overnight. The template DNA for the sequencing reaction was prepared by a modified alkaline lysis method (Sambrook et al. 1989) using a 96-well format, followed by a purification step with Wizard Midipreps DNA Purification Resin (Promega) and 96-well MultiScreen plates with glass fiber membrane (Millipore).

The amount of isolated DNA template was estimated on 1.0% agarose gel by comparison to serial dilutions of pBlue-script II KS(+) (Stratagene). Sequencing reactions were performed in a Genius (Techne) or an Uno II (Biometra) thermal cycler by using BigDye Terminator Cycle Sequencing Ready Reaction kits, with AmpliTaq DNA Polymerase (FS enzyme) (PE Biosystems) following the protocols supplied by the manufacturer, and analyzed in an ABI prism 377 (PE Biosystems). Single-pass sequencing was performed on each template using T7 or T3 primer. Bases were called by PHRED, and edited to remove vector sequence (as detected by CROSS-MATCH) from the 5' end and unreliable data from the 3' end of sequences. Sequences longer than 100 bases were further analyzed.

Sequence Analysis

The sequences were compared against the NCBI nonredundant protein or nucleotide databases by using the program BLASTX or BLASTN respectively (Altschul et al. 1997) on the BLAST network service at the National Center for Biotechnology Information (NCBI). Blast searches against in-house databases were run locally using the BLAST suite of programs as distributed by the NCBI in a PC computer running Linux. Sequences used to search the databases were either consensus sequences or individual GSS sequences as explained in the text.

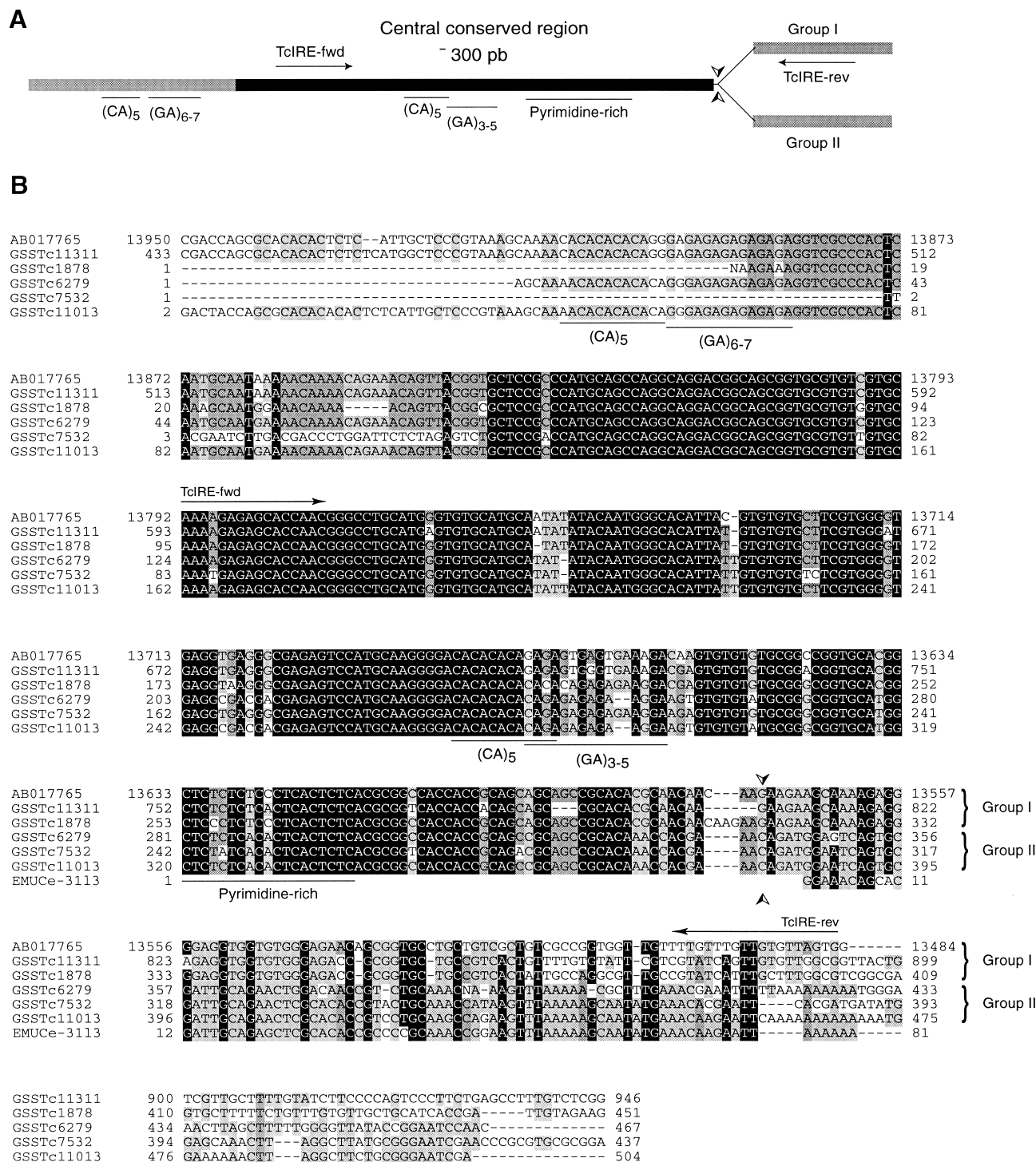


Figure 3 Structure of TcIRE. (A) General scheme of the structure of TcIRE. (B) Five GSS containing a copy of TcIRE were aligned using CLUSTALW with the corresponding region of the 25-Kb cosmid sequenced by Gao et al. 1999 (GenBank accession no. AB017765) and the last portion of the 3' UTR from the Emuce-3113 mucin gene. The rest of the mucin gene does not show any homology with the sequences aligned and was cut off for the sake of clarity. Coloring is based on BLOSUM 62 scores: 3.0, black; 1.5, gray; 0.5, light gray. Similar residues are colored as the most conserved one. Arrows indicate the two oligonucleotides used to generate the probe for the Southern blot analysis. Arrowheads indicate the site of divergence between the two groups of TcIRE sequences. The top three sequences, including AB017765, are representative of one group of sequences, denoted Group I, whereas the other sequences, including the last portion of the 3'-UTR region from the Emuce-3113 mucin gene are representative of another group, denoted Group II.

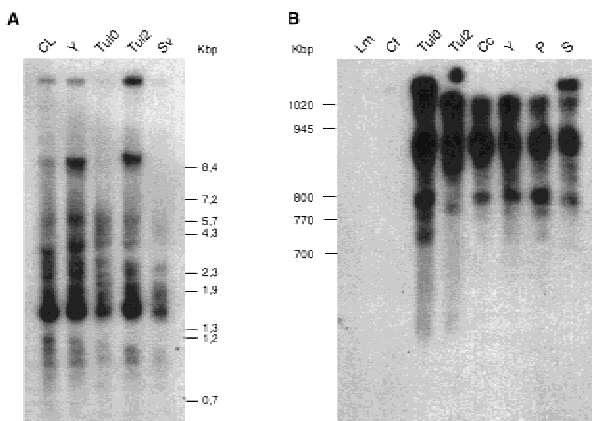


Figure 4 (A) Genomic DNA was prepared as described in Methods, digested with PstI and run in a 0.7% agarose gel at 3 V/cm. and transferred to a nylon membrane. (B) PFGE blots were prepared and processed as described (Henriksson et al. 1995). Chromosomal DNA markers were the CHEF DNA size markers, 0.2–2.2 Mbp (BioRad). Both nylon membranes (A,B) were hybridized with a radioactively labeled 300-bp fragment amplified by PCR using the oligonucleotides TcIRE-fwd and TcIRE-rev as shown in Figure 3 and described in Methods. Parasites, strains, and clones used in A or B are: *Leishmania mexicana* (Lm); *Crithidia fasciculata* (Cf); *Trypanosoma cruzi* strains and clones Tu0, Tu2, Corpus christi (Cc), Y, Perú (P), Sonya (S), CL-Brener (CL), and Sylvio (Sv).

For contig assembly, sequences were assembled with PHRAP; the assemblies were visually inspected with CONSED. PHRED, PHRAP, CROSS-MATCH, and CONSED are courtesy of B. Ewing, P. Green, and D. Gordon (University of Washington, Seattle). The *T. cruzi* GSS database used to run local BLAST searches was compiled from the 11,459 GSS after base-calling and vector masking with the mentioned programs. The frequency of di- and trinucleotides was calculated using COMSEQ (EMBOSS, European Molecular Biology Open Software Suite; The Sanger Centre, UK) and searching for all possible words of length 2 and 3, respectively. The expected values are calculated on the assumption that all words have the same probability of occurrence. The fractional GC content of sequences was calculated with the program GEECEE (EMBOSS). The number of ORFs was calculated using GETORF (EMBOSS). Fickett's test (testcode) was implemented as a Perl program, based on the original algorithm (Fickett 1982), and used to calculate a testcode value for each sequence. Microsatellite repeats, low-complexity regions and repetitive elements were searched for using the program REPEATMASKER (courtesy of A. Smit and P. Green, University of Washington, Seattle) over the entire GSS database using different repeat libraries that are part of RepBase (release 5.02). The Pfam database (Bateman et al. 2000) was searched using HMMPFAM, from the HMMER package (courtesy of S. Eddy, Washington University, St. Louis).

Estimation of the Copy Number and the Relative Abundance

Copy number (CN) was estimated directly from the number of GSS (GSS) belonging to a given gene family. The estimation also included the size (GS) of the gene/sequence element to account for the fact that bigger sequences should be more represented in a random sequence survey than smaller ones at the same copy number. For this calculation the haploid ge-

nome size (HGS) used was 4×10^7 bp (Frohme et al. 1998) and the total number of GSS (TGSS) was 11,459:

$$CN = \frac{GSS}{TGSS \times \left(\frac{GS}{HGS} \right)}$$

The estimation of the relative abundance (R) was done using the estimated number of copies as follows:

$$R = \frac{CN \times GS}{HGS} \times 100$$

Southern Blot and Pulse Field Gel Electrophoresis

DNA was purified using the conventional proteinase K-phenol extraction method. 4 μ g from each sample was digested with PstI (New England BioLabs) and separated on a 0.7% agarose gel. Southern blot transfer and analysis was performed following standard procedures (Sambrook et al. 1989). For the PFGE blots, DNA was prepared and processed as described (Henriksson et al. 1995). The probe was radioactively labeled with [α - 32 P]dCTP (NEN Life Science Products, Inc.) by a PCR-based method using DNA from clone GSSTc11311 as template and the following oligonucleotides: TcIRE-rev, 5'-cgccaacacaactgatacg-3', and TcIRE-fwd, 5'-gcaaaagagagcacaac-3'. After hybridization the nylon filters were washed under stringent conditions (0.1 \times SSC, 0.1 % SDS, at 62°C).

ACKNOWLEDGMENTS

We thank J.J. Cazzulo for critical reading of the manuscript. We thank Diego Rey Serantes, Fernanda Peri and Rodrigo Pavón for technical assistance. This work was funded by grants from the UNDP/World Bank/WHO Special Program for Research and Training in Tropical Diseases; SAREC, the Research Department of the Swedish International Development Agency (SIDA); Agencia Nacional de Promoción Científica y Tecnológica, Argentina and Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina. The work of A.C.C.F. was partially supported by an International Research Scholar award from the Howard Hughes Medical Institute.

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Received May 2, 2000; accepted in revised form September 20, 2000.