Available online at www.sciencedirect.com
SCIENCE DIRECT®
Molecular & Biochemical Parasitology 138 (2004) 183–194
MOLECULAR & BIOCHEMICAL PARASITOLOGY

DNA microarrays for comparative genomics and analysis of gene expression in *Trypanosoma cruzi*☆

Cassio Silva Baptista[a], Ricardo Z.N. Vêncio[a,b], Sarah Abdala[a], Maria Paula Valadares[a], Camila Martins[a], Carlos Alberto de Bragança Pereira[b], Bianca Zingales[a,*]

[a] *Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, Avenida Professor Lineu Prestes 748, CEP 05508-000 São Paulo, SP, Brazil*
[b] *Departamento de Estatística, Instituto de Matemática e Estatística, Universidade de São Paulo, São Paulo, SP, Brazil*

Received 23 September 2003; received in revised form 25 May 2004; accepted 9 June 2004
Available online 21 September 2004

Abstract

Trypanosoma cruzi presents high genetic diversity and parasite isolates show remarkable differences in biological parameters. In this study, we evaluated whether DNA microarrays containing CL Brener cDNAs can be used for comparative genomics and for the analysis of gene expression in *T. cruzi*. We constructed a prototype microarray with 710 expression sequence tags of CL Brener and 20 sequences of *T. cruzi* strains. These probes represent 665 unique genes. Results from four hybridisations with genomic DNA of Silvio (*T. cruzi* I) and CL Brener (hybrid genotype) identified 9.3% of the probes (68/730) differentially represented in the two genomes. Data from eight hybridisations with cDNA obtained from three independent parasite harvests of Silvio and CL Brener disclosed 84 sequences of 730 (11.5%) that showed statistical significant ($P \leq 0.01$) changes in expression (1.6–6.5-fold). Some of the array-identified sequences were confirmed by Southern and Northern blot analysis. Only 20% of the probes with increased expression in Silvio or CL Brener presented higher hybridisation with genomic DNA of either strain. Approximately 2.5% (18/730) and 9.0% (65/730) of the probes were differentially expressed ($P \leq 0.01$), respectively, in epimastigotes and metacyclic trypomastigotes of two *T. cruzi* II strains isolated from chronic chagasic patients. Microarrays identified several sequences for which differences in gene copy number and/or in the levels of RNA transcripts were previously demonstrated by different approaches. The data indicate that DNA microarrays are a useful tool for comparative studies between strains and provide further evidence for a high level of post-transcriptional regulation of RNA abundance in *T. cruzi*.
© 2004 Elsevier B.V. All rights reserved.

Keywords: *Trypanosoma cruzi*; Stocks; DNA microarrays; Comparative genomics; Gene expression; Transcript levels

1. Introduction

The protozoon *Trypanosoma cruzi* is the etiological agent of Chagas disease, which affects 16–18 million people in South and Central America. *T. cruzi* life cycle alternates between vertebrates and triatomine insects, with different developmental stages in each host: epimastigotes and metacyclic trypomastigotes in the insect vector and intracellular amastigotes and bloodstream trypomastigotes in the mammalian host. *T. cruzi* is diploid, its mode of replication is predominantly asexual and, therefore, parasite strains represent independent clonal lineages [1]. The strains, also named as stocks or isolates, were shown to be divergent for various

Abbreviations: EST, expressed sequence tag; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; rRNA, ribosomal RNA; SIRE, short interspersed repetitive element; TEUF, TENF, TEUQ and TENQ, EST from a non-normalised (TEUF, TEUQ) and normalised (TENF, TENQ) cDNA library of CL Brener epimastigotes sequenced at FIOCRUZ (TEUF, TENF) and Instituto de Química (TEUQ, TENQ)

☆ *Note:* Nucleotide sequence of 134 ESTs reported in this paper have been submitted to the GenBank™ database with the accession numbers CF134346–CF134365 and CF243279–CF243392.

* Corresponding author. Tel.: +55 11 3091 3810x217; fax: +55 11 3815 5579.
E-mail address: zingales@iq.usp.br (B. Zingales).

0166-6851/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.molbiopara.2004.06.017

biological characteristics such as in vitro growth and differentiation, course of experimental infection, tissue tropism, and susceptibility to drugs. In addition, the diversity of symptoms of Chagas disease: indeterminate, cardiac and digestive forms have been attributed to the interplay between the genetic characteristics of the parasite and the human host [2]. In the last years, evidence provided by independent DNA markers such as ribosomal RNA (rRNA) and mini-exon genes [3], isoenzyme phenotypes [4] and phylogenies of the 18S rRNA gene [5] indicate that *T. cruzi* clonal genotypes cluster into two major lineages, that were named as *T. cruzi* I and II [6]. Epidemiological studies showed that *T. cruzi* I strains circulate in the sylvatic cycle of the parasite transmission, whereas *T. cruzi* II strains predominate in the domestic cycle where Chagas disease is more severe [7,8]. Recently, genetic analysis points to the presence of hybrid genotypes in natural populations of *T. cruzi* [9–12]. CL Brener, the reference organism of the *T. cruzi* genome project [13], is one example of these putative hybrid strains.

Although DNA microarrays have proven to be a useful tool to discover new genes and to identify coordinated expression of families of genes in apicomplexan parasites, few laboratories have employed this technology for expression profiling of genes in trypanosomatids (revised in [14]). These studies revealed stage-specific gene expression in bloodstream and procyclic forms of *Trypanosoma brucei* [15] and changes in RNA abundance between different stages of *Leishmania major* [16,17]. At present, a single paper has been published that describes the application of a microarray containing genomic clones and open reading frames of *T. cruzi* to identify genes up-regulated during trypomastigote to amastigote transformation in Brazil strain [18]. Because *T. cruzi* isolates present high genetic diversity, in the present study we aimed at evaluating whether DNA microarrays bearing CL Brener expressed sequence tags (ESTs) can be used for comparative genomics and to investigate differential gene expression in parasite strains. For this purpose, we have produced a *T. cruzi* prototype DNA microarray slide containing 710 ESTs and 20 well-characterised sequences of various *T. cruzi* isolates. The microarray was hybridised with DNA or cDNA from two pairs of isolates: CL Brener (hybrid) and Silvio X10 cl1 (*T. cruzi* I) and two *T. cruzi* II strains isolated, respectively, from an asymptomatic individual and a patient with cardiac and digestive disorders. Some differentially recognised DNA probes were confirmed by Southern and Northern blot experiments. The data indicate that DNA microarrays can be used for comparative studies of parasite strains and provide evidence for a high level of post-transcriptional regulation of RNA abundance in *T. cruzi*.

2. Materials and methods

2.1. Parasite stocks and cultivation

Four Brazilian strains were used in most of the studies: CL Brener (hybrid genotype), isolated from *Triatoma infestans*; Silvio X10 cl1 (*T. cruzi* I), isolated from a patient in the acute phase of Chagas disease; Famema (*T. cruzi* II), obtained from a chronic asymptomatic individual; and Hem 179 (*T. cruzi* II), isolated from a chronic chagasic patient presenting cardiac and digestive disorders. The characteristics of other stocks used for confirmation of the microarray data can be found elsewhere [3]. Epimastigote forms were cultured in liver infusion tryptose (LIT) medium with 10% fetal calf serum, at 28 °C. Metacyclic trypomastigotes were obtained by differentiation of epimastigotes in LIT medium plus 20% (v/v) Grace's Insect medium (Sigma) for 7–10 days and purified by chromatography on DEAE-cellulose (DE-52, Whatman) columns as described [11].

2.2. Construction of DNA microarrays

DNA microarrays were constructed with 710 ESTs obtained from non-normalised and normalised cDNA libraries of CL Brener epimastigotes [19]. Polymerase chain reaction (PCR) amplification of the ESTs (average length 800 bp) was obtained with T3 and T7 primers using DNA polymerase (Biolase). The amplification products were purified with Multiscreen plates (Millipore). Inserts representing 20 cloned *T. cruzi* coding and non-coding genes, control sheared DNAs and oligonucleotides were also immobilised on the glass slides with Generation III Microarray System spotter (Molecular Dynamics) according to manufacturer's instructions. The DNA probes were distributed in 10 sub-arrays, containing 288 spots each. Each sub-array was spotted in duplicate (left and right side of the slide) resulting in at least 6 and at most 60 replicates of each probe. Detailed spotting map and relevant information about the probes are available in supplementary data at http://www.vision.ime.usp.br/∼rvencio/Tcruzi/ (User name: guest password: mbp). After immobilisation, DNA was UV-irradiated at 254 nm (Stratagene lamp) with 50 mJ total energy.

2.3. Sample preparation, labelling and hybridisation

2.3.1. DNA targets

Whole genomic DNA of CL Brener and Silvio strains was purified as previously described [20]. Targets for hybridisation were generated from DNA templates by incorporation of fluorophor-labelled dCTP in a random primer polymerisation reaction. In brief, 50 μl labelling reaction contained 4 μg DNA, 60 μM of either Cy3- or Cy5-dCTP (Amersham Biosciences), 50 mM Tris–HCl pH 6.8, 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 0.12 mM each dATP, dGTP, dTTP and 0.06 mM dCTP, 1 μl of random nonamers (Amersham) and 80 U of Klenow DNA polymerase (GIBCO-BRL). Incubation was at 37 °C for 2 h. The reaction was stopped by addition of 5 μl of 0.5 M EDTA and the products were purified with Multiscreen plates (Millipore). The incorporation was determined by photometric measurement (550 nm for Cy3 and 650 nm for Cy5). The samples were dried in vacuum and dissolved in H_2O to reach 25% of the solution plus

25% hybridisation buffer (Amersham Pharmacia) and 50% formamide. DNA was denatured at 98 °C for 2 min. Hybridisation to the array was performed under a glass cover-slide (24 mm × 60 mm). The slides were kept in a waterproof hybridisation chamber in a 42 °C water bath for at least 16 h. After hybridisation, slides were washed with 1× SSC, 0.2% SDS, followed by two washes in 0.1× SSC, 0.2% SDS. All washings were performed at 55 °C for 10 min. An additional wash was performed at RT in 0.1× SSC for 1 min. Slides were dried and subjected to fluorescence detection.

2.3.2. RNA targets

The parasite cells (5×10^8) were lysed in 1 ml Trizol (Invitrogen) and total RNA was extracted according to the manufacturer's protocol. RNA was DNase-treated and approximately 20 μg was labelled with fluorophor-labelled dCTP in the first-strand cDNA synthesis. The reaction mix contained 0.05 mM of either Cy3- or Cy5-dCTP, 1 μl oligo-$dT_{(15)}$ primer (Amersham Biosciences), 4 μl of random nonamers (Amersham), 0.1 mM each of dATP, dCTP, dTTP and 0.05 mM dCTP, 10 mM dithiothreitol and 400 U of Superscript II Reverse Transcriptase (Invitrogen) in the buffer provided by the manufacturer. Incubation was performed at 42 °C for 2.5 h. Subsequently, RNA was hydrolysed by addition of 2 μl of 2.5 M NaOH and an incubation at 37 °C for 15 min. The solution was neutralised by addition of 10 μl of 2 M HEPES. The samples were purified with Multiscreen plates (Millipore). Incorporation measurement, hybridisation with the slides and washing conditions were as described above.

2.4. Image acquisition and data analysis

The slides were scanned with a laser scanner (Molecular Dynamics) resulting in 16-bit images. Intensities were extracted using ArrayVision 6.0 (Image Research) software. To verify the influence of the image extraction method, the data were also analysed using Scanalyse 2.5 (http://www.rana.lbl.gov/EisenSoftware.htm) software resulting in no qualitative differences. For comparative genomic analysis, four hybridisation experiments were performed. This means that a particular probe was measured at least 24 times and at most 240 times, depending on the number of spotted replicates. For differential gene expression, RNA preparations were obtained from three independent parasite harvests for each strain (biological replicates). Different combinations of cDNAs of a given pair of strains (CL Brener × Silvio and Famema × Hem 179) were used for hybridisation with the microarray, resulting in a total of eight microarray experiments for each pair of isolates. Normalisation of the hybridisation data was achieved by LOWESS fitting (included in the R package, available at http://www.r-project.org) on M versus A-plot, where $M = \log_2(\text{normalised Cy5/Cy3})$ and $A = 1/2 \log_2(\text{Cy5} \times \text{Cy3})$, as described [21,22]. Local background was subtracted from the intensity value of each spot on the array. Spots were manually examined to assess their quality and those that exhibited poor quality, low-intensity measurements ($A \leq 2$) or saturated intensity ($A \geq 9.5$) were excluded from the analysis. For each probe the mean ($\bar{M}$) of the M values of all biological and technical replicates was obtained. The results presented for each probe refer to the hybridisation ratio ($R = 2^{\bar{M}}$). To identify differentially expressed probes we used the well-known t-test. The null hypothesis tested was "$\bar{M} \geq -0.58$" and "$\bar{M} \leq 0.58$" for down- and up-regulated probes, respectively. Probes with P-values ≤ 0.01 in the t-test were considered differentially expressed. The cutoff criterion of ± 0.58 for description of differential expression (i.e., 1.5-fold up- or down-regulation) was defined in homotypical hybridisations where the microarray slide was hybridised simultaneously to CL Brener cDNA labelled with Cy3 and Cy5 (see Section 3). Further details of data analysis can be found elsewhere [23]. Raw data, normalised data, LOWESS graphics and additional information can be found in the above-mentioned webpage.

2.5. Sequence analysis and search of similarity in database

DNA was sequenced with the ABI 377 automatic sequencing equipment. DNA sequences were compared with others through searches in protein and DNA databases at the National Center for Biotechnology Information (NCBI, NIH, Bethesda, USA) using the BLASTN and BLASTX programs. Sequence similarities identified by BLASTN and BLASTX programs were considered statistically significant with $E \leq 10^{-5}$. Clustering of the ESTs was performed with Cap3 [24].

2.6. Confirmation of hybridisation variations

2.6.1. Southern blot

Total parasite DNA (2.0 μg) was digested with *Pst*I (New England Biolabs), run-on 0.8% agarose gels, and blotted to nylon membranes (Hybond-N, Amersham Pharmacia) using standard protocols. DNA probes were labelled with [α-^{32}P] dCTP with the random primer DNA labelling kit (Invitrogen). Probes were hybridised at 60 °C in 0.1% Ficoll, 0.05% PVP, 1 mM EDTA, 3× SSC, 0.1% SDS and 100 μg ml^{-1} salmon sperm DNA. Blots were washed to a final stringency of 0.1× SSC, 0.1% SDS at 60 °C. The radioactive images were collected in phosphor screens (Kodak) exposed for different times and scanned with the Storm System (Molecular Dynamics). Densitometric analysis of the radioactive signal was obtained with the ImageQuant Molecular Dynamics Program. Prior to further probing, the probes were stripped by washing at 0.1× SSC, 0.5% SDS at 90 °C for 60 min. Membranes were exposed to phosphor screens to verify the efficiency of the process.

2.6.2. Northern blot

Total parasite RNA (10 μg) was separated on formaldehyde-containing 1% agarose gels in MOPS

buffer according to standard procedures and blotted to nitrocellulose membranes (Hybond-C, Amersham Pharmacia). DNA probes (100 ng) were labelled by random primer extension with [α-^{32}P] dCTP as described above. Hybridisation was carried out in 5× SSPE, 50% formamide, 5× Denhardt, 0.5% SDS at 42 °C, overnight. Blots were washed to final stringency of 0.1× SSPE, 0.1% SDS at 42 °C and exposed to phosphor screen (Kodak). The hybridisation signals were quantified as indicated above. Probes were removed by washing of the blot at 95 °C in 0.1× SSPE, 0.1% SDS.

3. Results

3.1. Characterisation of DNA probes for the microarray

In this article, the term probe refers to the DNA sequences spotted on the array and the term target refers to the sample hybridised to the array [25]. To construct *T. cruzi* microarray, 710 clones from normalised and non-normalised cDNA libraries of CL Brener epimastigotes were randomly selected. The nucleotide sequence was available for 576 ESTs, which are named as TENF (normalised library) and TEUF (non-normalised library). We have sequenced 134 additional cDNA clones and the sequences have been deposited in dbEST of GenBank and named as TENQ (normalised library) and TEUQ (non-normalised library). Search for similarity with BLASTN and BLASTX programs indicated that 75% of the ESTs (532/710) have no matches in protein and DNA databases. Twenty characterised *T. cruzi* sequences, sheared CL Brener genomic DNA and negative control probes were also immobilised in the array. Considering the sequences with matches in database (178 ESTs and 20 cloned genes), 77.7% (154/198) of the probes showed similarity with previously identified *T. cruzi* or trypanosomatid genes, whereas 22.3% (44/198) had significant matches to genes of other organisms. The most represented functional categories were genes related to protein synthesis (24%); cell metabolism (16%); cell surface proteins (14%) and structural or cytoskeletal elements (12%). Clustering of the sequences was performed with Cap3 program, indicating 51 clusters containing 2–5 sequences, 614 singletons and 665 (91%) unique sequences.

3.2. Image acquisition and data analysis

A well-known source of noise that occurs in every microarray experiment arises from intrinsic experimental variation. In this study, the noise estimate was assessed in experiments where two identical mRNA samples of CL Brener were labelled with Cy3 and Cy5 and hybridised to the same slide. This theoretically should give a log expression ratio (Cy5/Cy3) of 0 for all the elements arrayed on the slide. A scatter plot comparing the hybridisation of Cy3- and Cy5-labelled cDNA is shown in Fig. 1A. The line on the top of the figure indicates signal saturation. The data can also be visualised in a MA-plot [22] (Fig. 1B). From the analysis of the data we defined the expression log ratio $M = \log_2$(normalised Cy5/Cy3) cutoff as ±0.58. This corresponds to a ratio cutoff = $2^{0.58} = 1.5$ (i.e., 1.5-fold up or down-regulation).

3.3. Comparative genomic analysis of CL Brener and Silvio X10 cl1 isolates

Aiming at validating DNA microarrays for comparative genomic analysis, we chose two "polar" strains: CL Brener, considered a hybrid genotype and Silvio X10, typed as *T. cruzi* I. Parasite DNAs were labelled with Cy3 and Cy5, mixed and hybridised with the slide. In four independent experiments, we observed that 9.3% (68/730) of the probes showed statistical significant ($P \leq 0.01$) differential hybridisation: 31 probes gave higher hybridisation signal with CL Brener DNA, whereas 37 probes hybridised more intensely with Silvio DNA. This suggests differences in the abundance of the genes in the genomes of the strains and/or variation in sequence similarity (the identity of many of these sequences is shown in Tables 1 and 2).

Some of these probes were confirmed by Southern blot analysis with DNA of CL Brener and Silvio strains. In Fig. 1C, the patterns obtained for three sequences that gave, respectively, CL Brener/Silvio dye ratios of 2.1 and 2.7 and Silvio/CL Brener ratio of 5.1 are shown. A control gene with a hybridisation ratio of 1 was also analysed. Variation in the genomic arrangement of the sequences was also observed (Fig. 1C). The genomic distribution of TENF0426, which showed hybridisation ratio CL Brener/Silvio = 2.7 was analysed in nine *T. cruzi* isolates (Fig. 1D). The Southern blot confirmed great variation in the copy number or sequence similarity of this probe between the strains and suggests that it may correspond to a repetitive element.

3.4. Differential gene expression in CL Brener and Silvio isolates

Differential gene expression in CL Brener and Silvio was investigated by hybridisation of the microarray with cDNA obtained from total RNA of epimastigotes in mid-log growth phase. The doubling time of CL Brener and Silvio is 44 and 45 h, respectively. A total of eight hybridisation experiments were performed with RNA preparations obtained from three parasite harvests of the strains. We identified 84 probes of 730 (11.5%) that showed statistically significant ($P \leq 0.01$) changes in expression: 35 probes up-regulated in Silvio and 49 probes up-regulated in CL Brener. The list describing these probes is available in supplementary data.

To confirm differential expression, nine sequences were used as probes in Northern blots of total RNA of CL Brener and Silvio, and the patterns obtained for five of these probes are shown in Fig. 2A. The hybridisation signals on the blots were quantified by densitometric analysis using the ImageQuant Molecular Dynamics Program. Hybridisation

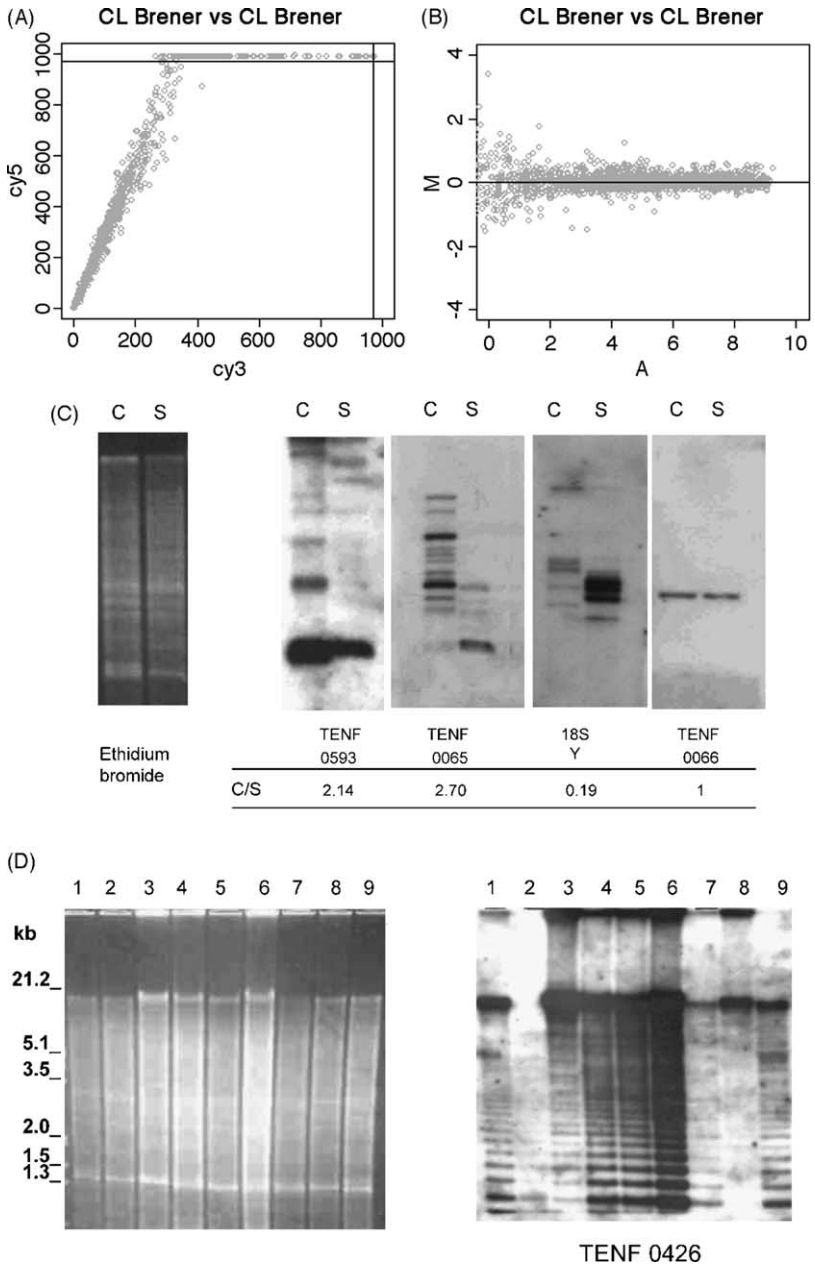

Fig. 1. Scatter plot (A) and MA-plot (B) of the hybridisation of the microarray with cDNA of CL Brener epimastigotes labelled with Cy3 and Cy5. (C) Southern blot of genomic DNA of CL Brener (C) and Silvio (S) digested with *Pst*I. Ethidium bromide stained gel and hybridisation with ^{32}P-labelled probes. Below each panel the designation of the probe and the dye ratio (C/S) of the hybridisation in the microarray are indicated. (D) Genomic DNA of *T. cruzi* isolates digested with *Pst*I. Ethidium bromide stained gel and hybridisation with TENF0426 probe. Lanes: CL Brener (1), Silvio X10 cl1 (2), Y (3), NR cl3 (4), SO3 cl5 (5), SC43 cl1 (6), Dm 28c (7), Esmeraldo cl3 (8) and YuYu (9) strains.

with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probe was used for normalisation (data not shown). The CL Brener/Silvio hybridisation ratios obtained in the Northern blots were compared with the ratios obtained in the microarray experiments (Fig. 2A). The microarray ratios were always lower than those obtained by densitometric analysis of the Northern blots. This discrepancy, as reported before [15], might be caused by competition between the two fluorescent targets used in the microarray, as well as by non-linearity in the detection systems.

Then we asked whether the differential gene expression was related to differences in the gene copy number and/or sequence similarity between the strains. Accordingly, we compared the data of the two sets of experiments where microarrays were hybridised with DNA and cDNA targets. Among the 35 probes with higher expression in Silvio, only 7 (7/35; 20%) showed higher hybridisation with Silvio DNA. Among the 49 probes up-regulated in CL Brener, 11 probes (11/49; 22.4%) presented higher hybridisation with DNA of this strain. An interesting result was obtained for three

Table 1
Sequences with higher hybridisation with CL Brener or Silvio X10 DNA and more expressed in CL Brener or Silvio X10

Sequences ($n = 11$ of 730; 1.5%) with higher hybridisation with CL Brener DNA and more expressed in CL Brener			
Identity	Similarity[a]	DNA hybridisation ratio[b]	cDNA hybridisation ratio[b]
CL65[d]	195 bp repetitive DNA	1.68	4.67
G3[d]	195 bp repetitive DNA	6.07	6.47
TENF0385	Tc mRNA with SIRE sequence	1.88	1.88
TENF0396[e]	Tc one repeat of 35.2 gene	2.06	2.95
TENF0421	Intergenic region between Tc genes for OMPDCase-OPRTase and surface protein	1.61	2.69
TENF0593	No match	2.14	3.01
TENF0599	No match	2.22	1.61
TENF0693[e]	Tc one repeat of 35.2 gene	2.00	3.05
TENF0775	No match	1.90	2.74
TENF0855	Tc histone H2A	2.00	2.00
TENQ0711	Tc strain Y genomic DNA	1.90	2.10

Sequences ($n = 7$ of 730; 0.96%) with higher hybridisation with Silvio X10 DNA and more expressed in Silvio			
Identity	Similarity[a]	DNA hybridisation ratio[c]	cDNA hybridisation ratio[c]
B11[f]	Retrotransposon (similar to reverse transcriptase)	1.75	4.29
Pact	Actin 2	1.83	2.48
Telomere	Telomeric region	1.61	2.95
TENF0453	*T. cruzi* sterile alpha motif	1.70	1.70
TENQ0659	No match	2.00	1.77
TENQ0889	Tc SIRE repeat region	1.73	1.65
TEUF0049[f]	Tc non-LTR retrotransposon	1.72	3.46

Sequences ($n = 3$ of 730; 0.41%) with higher hybridisation with Silvio X10 DNA and more expressed in CL Brener			
Identity	Similarity[a]	DNA hybridisation ratio[c]	cDNA hybridisation ratio[b]
TENF0310	No match	2.05	2.10
TENQ0584[g]	Tc Esmeraldo histone H3 gene	1.80	1.90
TENF0516[g]	Tc Tulahuen histone H3 mRNA	2.04	1.99

[a] The best hit obtained by BLASTN and/or BLASTX programs ($E \leq 10^{-5}$).
[b] CL Brener/Silvio X10 (P-value ≤ 0.01 in t-test).
[c] Silvio X10/CL Brener (P-value ≤ 0.01 in t-test).
[d–g] ESTs belonging to defined gene clusters.

probes (3/49; 6.1%) that showed higher hybridisation with Silvio DNA and were more expressed in CL Brener. The data describing the identity of the probes and the DNA and cDNA hybridisation ratios are described in Tables 1 and 2.

We also investigated the variation of RNA levels in epimastigotes of CL Brener harvested at mid-log phase (48 h) and stationary phase (336 h) of culturing in LIT medium. We found that 6 genes of 730 were more expressed in mid-log phase, presenting 1.6–1.9-fold variation ($P \leq 0.01$). On the other hand, we found five probes more expressed in the stationary phase, presenting dye ratios between 1.6 and 2.0. The confirmation of four of these probes by Northern blot is shown in Fig. 2B. The identity of these sequences is presented in supplementary data.

3.5. Differential gene expression in T. cruzi strains isolated from chagasic patients

In an initial attempt to identify genes differentially expressed in human *T. cruzi* isolates, total RNA was extracted on three occasions from mid-log phase epimastigotes of Famema and Hem 179 strains, both typed as *T. cruzi* II and obtained, respectively, from an asymptomatic individual and from a patient with severe cardiomyopathy and digestive disorders. Analysis of the data indicated a significant ($P \leq 0.01$) differential expression of 18 probes (18/730; 2.46%) (identity of the probes and hybridisation ratios are presented in supplementary data). The transcripts of six of these probes were confirmed by Northern blot. In Fig. 3A and B, some patterns are presented.

We have also compared gene expression between metacyclic trypomastigotes of the two strains. Sixty-six probes (66/730; 9.0%) were identified as up-regulated (supplementary data) and the RNA transcript corresponding to TEUF 0045 probe is shown in Fig. 3D.

We verified that four coincident probes were up-regulated in both epimastigotes and metacyclic trypomastigotes of Famema, and that eight coincident probes were up-regulated in the two stages of Hem 179. These sequences are identified in Table 3, and the signal ratios obtained for each developmental stage are indicated.

Table 2
Sequences with higher hybridisation with CL Brener or Silvio X10 DNA and non-differentially expressed

Sequences ($n = 20$ of 730; 2.7%) with higher hybridisation with CL Brener DNA

Identity	Similarity[a]	Hybridisation ratio[b]
TENF0018	No match	1.58
TENF0065	CL Brener cosmid 1m17 chromosome 3	2.71
TENF0146	No match	1.83
TENF0198	No match	1.90
TENF0267	No match	2.06
TENF0296	No match	2.02
TENF0303	No match	1.94
TENF0306	No match	1.83
TENF0333	No match	1.97
TENF0409	No match	1.77
TENF0426	No match	2.75
TENF0479	Tc RNA polymerase II largest subunit gene	1.97
TENF0588	No match	2.27
TENF0598	Tc histone H1 (H1.M6.1) gene, complete cds	1.80
TENF0714	SIRE sequence of putative translation factor (eIF6) gene	1.75
TENF0787	No match	1.68
TENF0912	No match	2.10
TENQ0900	No match	1.63
TENU1969	Tc adenylyl cyclase (TczAC) gene	1.87
TEUF0051	No match	1.69

Sequences ($n = 25$ of 730; 3.42%) with higher hybridisation with Silvio X10 DNA

Identity	Similarity[a]	Hybridisation ratio[c]
18SG[d]	Tc 18S rRNA	5.80
18SY[d]	Tc 18S rRNA	5.10
24S	Tc 24S rRNA	2.86
B12	230 kDa antigen	1.87
EF[e]	Elongation factor subunit α	2.07
Mini-exon	Spliced leader RNA and intergenic region	21.52
TENF0105	*L. major* strain BT010 ribosomal protein S2 mRNA	2.18
TENF0163	No match	2.10
TENF0233	*T. brucei* RNA-editing complex protein MP42	1.94
TENF0388	No match	1.85
TENF0452	Tc clone 21 SIRE repeat region	2.14
TENF0536	No match	2.33
TENF0625	Tc partial G2RP gene for trans-sialidase superfamily group 2 related protein	2.16
TENF0702	No match	1.59
TENF0718	24S β ribosomal RNA	4.18
TENQ0583	Tc trypomastigote surface glycoprotein (TSA-1)	1.66
TENQ0828	Tc mRNA for elongation factor 1 alpha	1.89
TENQ0861	No match	2.40
TENQ0901[d]	Tc strain Y 18S ribosomal RNA gene	3.91
TEUF0050	Tc mitochondrial HSP70 (MTP70) gene	1.60
TEUF0096[f]	Tc alpha tubulin mRNA	1.69
TEUF0102	*L. major* antigen	1.67
TEUF0113	No match	1.65
TEUF0253[f]	Tc alpha tubulin mRNA	2.20
TEUF0259[e]	Tc mRNA for elongation factor 1 alpha	1.88

[a] The best hit obtained by BLASTN and/or BLASTX programs ($E \leq 10^{-5}$).
[b] CL Brener/Silvio X10 (P-value ≤ 0.01 in t-test).
[c] Silvio X10/CL Brener (P-value ≤ 0.01 in t-test).
[d–f] ESTs belonging to defined gene clusters.

Northern blot experiments attested that the transcript of TEUF0045, which has high identity with a *T. cruzi* gene encoding subunit 7 of NADH dehydrogenase (E-value = 0.0 by BLASTN), was 25-fold more abundant in metacyclic trypomastigotes of Hem 179, as compared with Famema (Fig. 3B). The expression of this mRNA in epimastigotes of different isolates was investigated by Northern blot (Fig. 3D). We also observed that the abundance of this transcript was very low in metacyclic trypomastigotes of Famema (Fig. 3B) and CL Brener (Fig. 3D, lane 3) as compared with epimastigotes of this isolate (Fig. 3D, lanes 1 and 2).

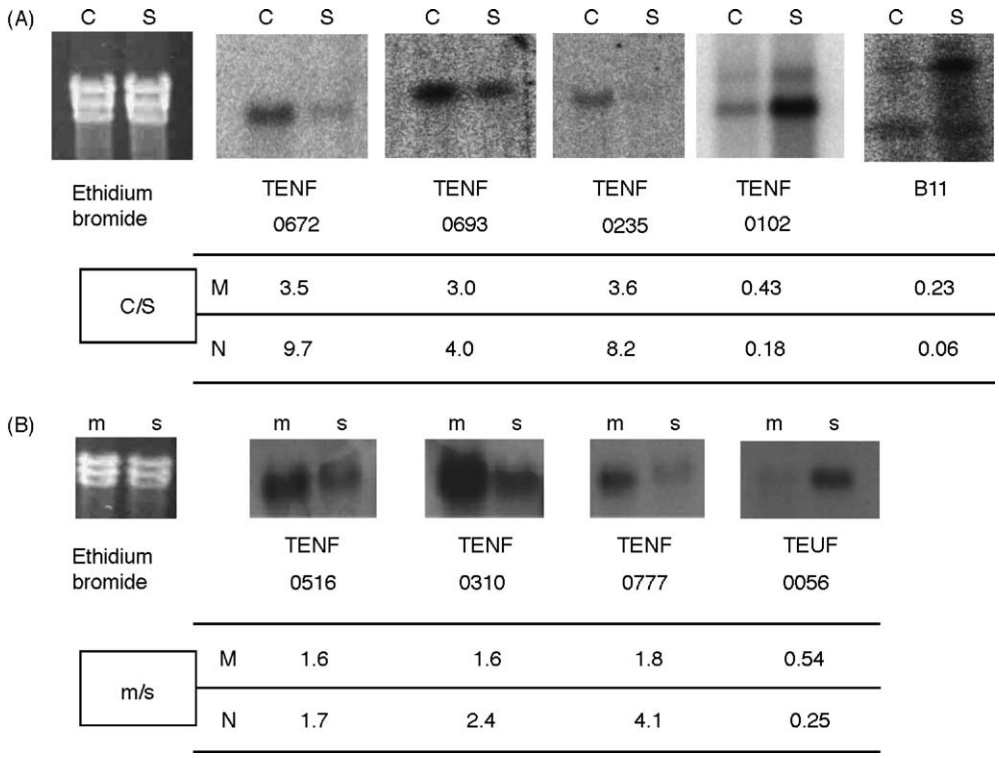

Fig. 2. Northern blot of total RNA of CL Brener (C) and Silvio (S) epimastigotes (A) and CL Brener mid-log (m) and stationary phase (s) epimastigotes (B) hybridised with ^{32}P-labelled probes. Below each panel the designation of the probe, the hybridisation ratio in the microarray (M) and the Northern blot (N) are indicated. In each panel, the ethidium bromide stained region of the gel where 18S, 24S α and 24S β rRNAs migrate is shown.

4. Discussion

Given the diverse biological characteristics of *T. cruzi* strains, it is expected that whole genomic comparisons among such isolates may provide important data for epidemiological studies. However, whole genomic comparisons based on nucleotide sequences of many *T. cruzi* isolates are not feasible. Alternatively, genomic comparisons among microorganisms have been proposed with the support of microarray hybridisations (see references cited in [23,26]). In this

Table 3
Coincident sequences differentially expressed in epimastigotes and metacyclic trypomastigotes of Famema (F) and Hem 179 (H) strains

More expressed in Famema ($n = 4$ of 730; 0.54%)

Identity	Accession number	Similarity[a]	Epimastigote ratio[b] F/H	Metacyclic ratio[b] F/H
B13	U15616	Tc 140/116 kDa antigen	1.90	1.92
TENF0209	AA556049	No match	2.06	1.88
TENF0753[c]	AI717881	Tc RNA binding protein (RBP) mRNA	2.96	6.24
TENQ0689[c]	CF243287	Tc RNA binding protein (RBP) mRNA	2.84	5.66

More expressed in Hem 179 ($n = 8$ of 730; 1.09%)

Identity	Accession number	Similarity[a]	Epimastigote ratio[b] H/F	Metacyclic ratio[b] H/F
CL56[d]	AY188964	195-bp repetitive DNA	2.61	3.11
CL65[d]	AY327543	195-bp repetitive DNA	3.31	3.31
G3[d]	K01771	195-bp satellite DNA	3.07	3.07
TENF0236	AA532144	No match	2.60	2.34
TENF0857[e]	AI717801	Tc kinetoplast maxicircle DNA-NADH dehydrogenase subunit 7 (ND7)	51.22	15.70
TEUF0045[e]	AA676164	Tc kinetoplast maxicircle DNA-NADH dehydrogenase subunit 7 (ND7)	4.15	25.29
TEUF0105	AA399706	*L. amazonensis* 40S ribosomal protein S24e mRNA	2.24	4.12
TEUF0146[e]	AA676184	Tc kinetoplast maxicircle DNA-NADH dehydrogenase subunit 7 (ND7)	3.81	15.87

[a] The best hit obtained by BLASTN and/or BLASTX programs ($E \leq 10^{-5}$).
[b] Statistically significant (P-value ≤ 0.01 in t-test).
[c–e] ESTs belonging to defined gene clusters.

context, pioneer studies evaluated genomic differences between *Mycobacterium tuberculosis*, *M. bovis* and BCG vaccines and identified several chromosome regions and open reading frames that were deleted in attenuated strains [27]. More recently, chromosomal anomalies resulting in amplification or deletions of genomic regions were identified in the myelogenous tumor line HL-60 relative to normal leukocytes using human DNA microarrays [28].

The sequencing of CL Brener genome is essentially complete with 19× whole genome shotgun coverage. Current efforts are now focused on optimising the assembly. At present, about 11,000 ESTs of CL Brener have been sequenced. In this study, we evaluated whether a DNA microarray containing predominantly CL Brener ESTs can be used for genomic comparisons and gene profiling in *T. cruzi* isolates. The use of rigorous statistical methods, rather than simple fold-changes is now becoming the standard for analysis of microarray experiments. Recently, the use of *P*-values estimates has allowed the identification, with high confidence, of many genes that are regulated by less than 2-fold during differentiation of *L. major* procyclics to metacyclics [17]. In the present study we used stringent cutoff criteria ($P \leq 0.01$) in the statistical analysis of the data to define changes in the hybridisation of DNA or cDNA targets with the microarray.

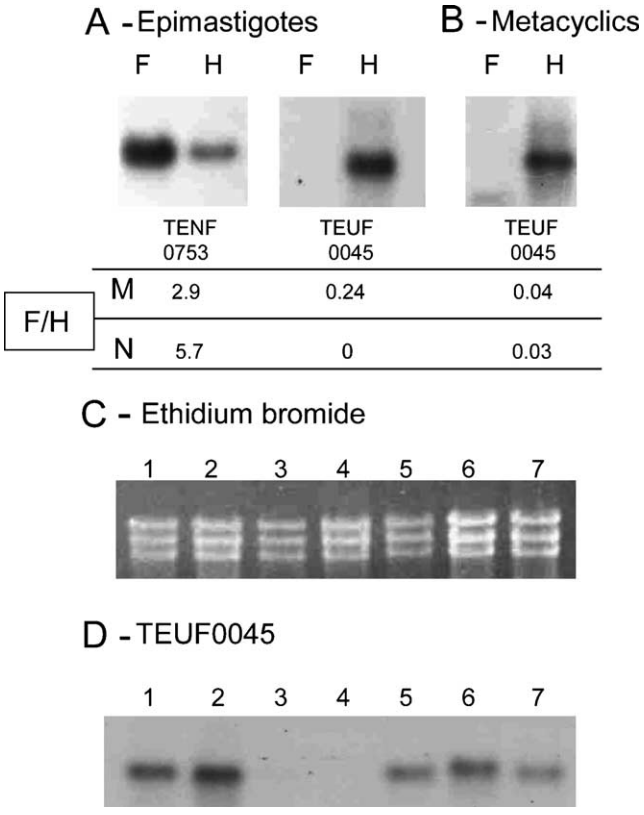

Fig. 3. Northern blot of total RNA of epimastigotes (A) and metacyclic trypomastigotes (B) of Famema (F) and Hem 179 (H) strains hybridised with ^{32}P-labelled probes. Below each panel the designation of the probe, the hybridisation ratio (F/H) in the microarray (M) and in the Northern blot (N) are indicated. (C) Electrophoresis of total RNA of parasites and staining with ethidium bromide. The region of the gel where 18S, 24S α and 24S β rRNAs migrate is shown. (D) Northern blot hybridised with TEUF0045 ^{32}P-labelled probe. Lanes: CL Brener epimastigotes in mid-log (1) and stationary phase (2), metacyclic trypomastigotes (3), epimastigotes of Y (4), Esmeraldo cl3 (5), Silvio X10 cl1 (6) and Dm 28c (7) strains.

The hybridisation of the slide with genomic DNA of CL Brener and Silvio strains showed that all the probes hybridised with both DNAs. However, 37 probes (37/730; 5%) gave stronger signal with Silvio DNA and 31 probes (31/730; 4.2%), with CL Brener DNA, suggesting differences in the abundance or sequence similarity of the corresponding genes. The hybridisation of the DNA of the two strains with the sheared CL Brener genomic DNA probe showed CL Brener/Silvio ratio of 1.4. This result could be due to a higher abundance of repetitive DNA sequences in CL Brener and/or differences in sequence similarity. In favour to the first hypothesis, we observed that the 195 bp satellite DNA probe, the most abundant reiterated sequence that accounts for about 10% of *T. cruzi* genome [29], showed CL Brener/Silvio ratio of 6.0. Coincidentally, we have recently demonstrated, *via* chromosome hybridisation and copy number quantification, that this satellite DNA is 6-fold more abundant in CL Brener genome as compared to Silvio [30].

Data from the microarray suggested a 3–5-fold increase of the rDNA units in Silvio genome as compared to CL Brener. In fact, we noticed DNA hybridisation ratios Silvio/CL Brener of 2.8–5.8 for the probes representing 18S (three probes), 24S α (one probe) and 24S β (one probe) rRNA genes (Table 2). Because 99% sequence similarity exists between the 18S rRNA gene of Silvio and CL Brener, the concomitant increase in the DNA hybridisation ratios Silvio/CL Brener with rRNA genes supports the conclusion of a higher copy number of the rDNA units in Silvio and indicates the reliability of the microarray data. Nevertheless, we have not observed an increase in the rRNA transcripts in Silvio, as compared to CL Brener. This could be due to differences in the strength of the rRNA promoters and efficiency of transcription factors in the two strains. In fact, divergences in the activity of rDNA promoter regions of *T. cruzi* strains have been reported [11,31,32]. Accordingly, the increase in the rDNA copy number in Silvio could reflect a compensatory response phenomenon.

On the other hand, for the probe designated as mini-exon (Table 2), which represents the spliced leader RNA gene (exon and intron) and the intergenic non-transcribed region, we observed a Silvio/CL Brener DNA hybridisation ratio of 21.52 ($P \leq 0.01$) (Table 2). In this case, the differential hybridisation most certainly is due to differences in sequence similarity. In fact, comparative alignment of the sequences of the 39 bp exons and 73 bp introns of the mini-exon gene of four strains indicated approximately 98% identity, whereas only 57–59% identity was observed between the intergenic regions (range 484–494 bp) [3]. More specifically, we observed that the identity between CL and Silvio strains inter-

genic regions was 57.9% [3]. It should be pointed out that the mini-exon probe did not show variation in the expression levels in Silvio and CL Brener.

Some probes with differential hybridisation to genomic DNA were confirmed in Southern blot experiments with DNA of the two isolates (Fig. 1C), and one probe, with DNA of nine isolates (Fig. 1D). Because the microarray slide contains predominantly CL Brener probes, the comparative genomic analysis here reported is unidirectional and represents only a subset of the differences between the isolates. That is, sequences only found in Silvio X10 genome would not be evidenced in our experiments. Nevertheless, this approach allows genomic comparisons between CL Brener and other isolates, and between different strains. This information is valuable for the sequencing project and for further analysis of genetic diversity in *T. cruzi*.

Hybridisation of cDNA obtained from CL Brener and Silvio epimastigotes with CL Brener DNA spotted in the microarray showed a CL Brener/Silvio ratio of 1.1. We found that 11.5% of the probes (84 of 730) were differentially expressed in the two strains as defined by signal ratios from 1.6 to 6.5 (Table 1 and supplementary data). When we compared the data of the hybridisation of the microarray with genomic DNA and cDNA of CL Brener and Silvio, we verified no correlation between the putative abundance of a particular gene and its expression level. In fact, only 20% of the probes with increased expression levels in Silvio or CL Brener presented differential hybridisation with genomic DNA. In a few cases, we noticed that sequences with higher hybridisation with genomic DNA of one strain were more expressed in the other. These observations suggest control of gene expression and/or differences in sequence similarity. Previous data based on nuclear run-on assays and experiments in detergent-treated cells showed that transcription activity in *T. cruzi* is not directly proportional to the gene copy number [33]. In fact, it was observed that the 195 bp satellite sequence, represented in about 10^5 copies in the Y strain genome [29], is transcribed only 10^3 times more than single copy genes. An analogous situation was found for the transcription of multicopy genes encoding mucins and the 85 kDa glycoproteins [33].

Differential expression of 2.5% (18/730) and 9.0% (65/730) of the probes was observed, respectively, in epimastigotes and metacyclic trypomastigotes of two strains isolated from chronic chagasic patients. We have not compared gene expression between epimastigotes and metacyclic trypomastigotes of the same strain because 97% of the probes of the microarray were cDNAs from epimastigotes. The analysis of the identities of the probes differentially expressed indicated that sequences that belong to the same gene cluster show similar up-regulation (Table 3 and supplementary data). This was also observed for the up-regulated genes in CL Brener and Silvio. The internal consistency of the microarray data indicates that the results are reliable.

A further indication of the control of gene expression is obtained when the transcripts of B12 and B13 genes, that encode two surface antigens [20], is analysed (Table 3 and supplementary data). Unpublished observations of our group indicate that the B12 and B13 genes are contiguous, separated by 1.5 kb and transcribed in the same direction (Gruber and Zingales, unpublished data). This suggests that the two genes most probably are co-transcribed into a polycistronic RNA. Data from the microarray indicated that only B13 gene is up-regulated in Famema in both epimastigotes and metacyclic trypomastigotes (cDNA hybridisation ratio Famema/Hem 179 = 1.90, Table 3), whereas B12 gene is up-regulated only in epimastigotes of Hem 179 (Hem 179/Famema = 1.89; data not shown). This suggests different stabilities of the two transcripts and/or a developmentally regulated gene expression. The differential expression of amastin and tuzin genes, two contiguous *T. cruzi* genes transcribed into the same polycistronic pre-mRNA has been reported [17,34,35].

Among the probes more expressed in epimastigotes and metacyclic trypomastigotes of Hem 179, three ESTs presenting high similarity with Silvio X10 gene encoding subunit 7 of the NADH dehydrogenase (ND7) and belonging to the same gene cluster were identified (Table 3). Northern blots hybridised with one of these probes (TEUF0045) indicated absence or very low level of this mRNA transcript in epimastigotes of the Y strain (Fig. 3D, lane 4), as compared with epimastigotes of four isolates where a 1.1 kb mRNA was detected (Fig. 3D, lanes 1, 2, and 5–7). In addition, ND7 RNA transcripts were observed in CL Brener epimastigotes (Fig. 3D, lanes 1 and 2) and were absent or much less abundant in metacyclic trypomastigotes (Fig. 3D, lane 3). The ND7 gene is localised in maxicircle kDNA and has been sequenced in several trypanosomatids [36]. In *T. brucei*, the expression of ND7 is developmentally regulated and fully edited transcripts of this subunit are only found in bloodstream forms [37]. The differential expression of ND7 transcripts in *T. cruzi* strains and developmental stages of the two trypanosomes suggests that this sequence may have an important role that deserves further investigation.

In this study, we observed that two ESTs (TENF0753 and TENQ0689) with similarity to a cDNA encoding an RNA binding protein previously described in *T. cruzi* [38] are more intensely expressed in Famema in relation to Hem 179, either in epimastigotes (cDNA hybridisation ratio, 2.9) or metacyclic trypomastigotes (cDNA hybridisation ratio, 5.6–6.2) (Table 3). The functional role of these proteins in the stability of Famema RNA populations remains to be elucidated.

The short interspersed repetitive element of *T. cruzi* named SIRE is found in the 3′ end of several mRNAs and participates in the polyadenylation process [39]. SIRE was also found in the 5′ untranslated region of some transcripts acting as a trans-splicing signal donor [39]. The presence of SIRE in several ESTs spotted in the microarray could determine cross-hybridisation between the probes. Nevertheless, this was not observed since SIRE-containing probes showed differential expression in the analysed strains (see tables and supplementary data).

The data here presented allow us to conclude that microarrays are a powerful tool for comparative analysis of gene expression in *T. cruzi* isolates and gene discovery. By this technology we have identified several sequences for which differences in gene copy number and/or levels of RNA transcripts have been demonstrated by other approaches. Having validated microarrays for gene profiling in *T. cruzi*, we intend to construct slides with a greater number of DNA probes aiming to compare isolates from patients with different manifestations of Chagas disease.

Acknowledgements

EST clones were kindly provided by Dr. Wim Degrave and Dr. Adeilton Brandão (FIOCRUZ—Rio de Janeiro). We thank Michel Tibayrenc, Luciamáre P.A. Martins, Eliane Lages-Silva and Luis E. Ramirez for providing *T. cruzi* isolates. We thank Marcelo N. Silva and Adriana Matsukuma for technical assistance, Mara C. Mioto for helpful image treatment and Nancy Vargas for the donation of RNA preparations of some *T. cruzi* strains. We are indebted to Dr. D. Campbell for critical reading of the manuscript and insightful suggestions. This work was supported by grants of Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). C.S.B. and R.Z.N.V. are graduate fellows of FAPESP. M.P.V., S.A. and C.M. are fellows from CNPq.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molbiopara.2004.06.017.

References

[1] Tibayrenc M, Ayala F. Isoenzyme variability in *Trypanosoma cruzi*, the agent of Chagas' disease genetical, taxonomical and epidemiological significance. Evolution 1988;42:277–92.
[2] WHO Expert Committee on the Control of Chagas Disease. Second report. Geneva: World Health Organization; 2002.
[3] Souto RP, Fernandes O, Macedo AM, Campbell DA, Zingales B. DNA markers define two major phylogenetic lineages of *Trypanosoma cruzi*. Mol Biochem Parasitol 1996;83:141–52.
[4] Tibayrenc M. Population genetics of parasitic protozoa and other microorganisms. Adv Parasitol 1995;36:47–115.
[5] Kawashita SY, Sanson GFO, Fernandes O, Zingales B, Briones MRS. Maximum likelihood divergence date estimates based on rRNA gene sequences suggest two scenarios of *Trypanosoma cruzi* intraspecific evolution. Mol Biol Evol 2001;18:2250–9.
[6] Anonymous recommendations from a satellite meeting. Mem Inst Oswaldo Cruz 1999;94:429–432.
[7] Zingales B, Souto RP, Mangia RH, et al. Molecular epidemiology of American trypanosomiasis in Brazil based on dimorphisms of rRNA and mini-exon gene sequences. Int J Parasitol 1998;28:105–12.
[8] Breniere SF, Bosseno MF, Telleria J, et al. Different behavior of two *Trypanosoma cruzi* major clones: transmission and circulation in young Bolivian patients. Exp Parasitol 1998;89:285–95.
[9] Machado CA, Ayala FJ. Nucleotide sequences provide evidence of genetic exchange among distantly related lineages of *Trypanosoma cruzi*. Proc Natl Acad Sci USA 2001;98:7396–401.
[10] Sturm NR, Vargas NS, Westenberger SJ, Zingales B, Campbell DA. Evidence for multiple hybrid groups in *Trypanosoma cruzi*. Int J Parasitol 2003;33:269–79.
[11] Stolf BS, Souto RP, Pedroso A, Zingales B. Two types of Ribosomal RNA genes in hybrid *Trypanosoma cruzi* strains. Mol Biochem Parasitol 2002;126:73–80.
[12] Pedroso A, Cupolillo E, Zingales B. Evaluation of *Trypanosoma cruzi* hybrid stocks based on chromosomal size variation. Mol Biochem Parasitol 2003;129:79–90.
[13] Zingales B, Pereira ES, Oliveira RP, et al. *Trypanosoma cruzi* genome project: biological characteristics and molecular typing of clone CL Brener. Acta Trop 1997;68:159–73.
[14] Duncan D. DNA microarray analysis of protozoan parasite gene expression: outcomes correlate with mechanisms of regulation. Trends Parasitol 2004;20:211–5.
[15] Diehl S, Diehl F, El-Sayed NM, Clayton C, Hoheisel JD. Analysis of stage-specific gene expression in the bloodstream and the procyclic form of *Trypanosoma brucei* using a genomic DNA-microarray. Mol Biochem Parasitol 2002;123:115–23.
[16] Almeida R, Norrish A, Levick M, et al. From genomes to vaccines: *Leishmania* as a model. Phyl Trans: Biol Sci 2002;357:5–11.
[17] Saxena A, Worthey EA, Yan S, Leland A, Stuart KD, Myler PJ. Evaluation of differential gene expression in *Leishmania major* Friedlin procyclics and metacyclics using DNA microarray analysis. Mol Biochem Parasitol 2003;129:103–14.
[18] Minning TA, Bua J, Garcia GA, McGaw RA, Tarleton RL. Microarray profiling of gene expression during trypomastigote to amastigote transition in *Trypanosoma cruzi*. Mol Biochem Parasitol 2003;131:55–64.
[19] Ürmenyi TP, Bonaldo MF, Soares MB, Rondinelli E. Construction of a normalized cDNA library for the *Trypanosoma cruzi* genome project. J Eukaryot Microbiol 1999;46:542–4.
[20] Gruber A, Zingales B. *Trypanosoma cruzi*: characterization of two recombinant antigens with potential application in the diagnosis of Chagas' disease. Exp Parasitol 1993;76:1–12.
[21] Yang YH, Dudoit S, Luu P, et al. Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. Nucleic Acids Res 2002;30:e15.
[22] Dudoit S, Yang YH, Callow MJ, Speed TP. Statistical methods for identifying genes with differential expression in replicated cDNA microarray experiments. Stat Sin 2002;12:111–39.
[23] Koide T, Zaini PA, Moreira LM, et al. DNA microarray-based genome comparison of a pathogenic and a non-pathogenic strain of *Xylella fastidiosa* delineates genes important for bacterial virulence. J Bacteriol 2004;186:5442–9.
[24] Huang X, Madan A. CAP3: a DNA sequence assembly program. Gen Res 1999;9:868–77.
[25] The Chipping forecast. Nat Genet 1999;21(Suppl).
[26] Oliveira RC, Yanai GM, Muto NH, et al. Competitive hybridization on spotted microarrays as a tool to conduct comparative genomic analysis of *Xylella fastidiosa* strains. FEMS Microbiol Lett 2002;10677:1–7.
[27] Behr MA, Wilson MA, Gill WP, et al. Comparative genomics of BCG vaccines by whole-genome DNA microarray. Science 1999;284:1520–3.
[28] Ulger C, Toruner GA, Alkan M, et al. Comprehensive genome-wide comparison of DNA and RNA level scan using microarray technology for identification of candidate cancer-related genes in HL-60 cell line. Cancer Genet Cytogenet 2003;147:28–35.
[29] Gonzalez A, Prediger E, Huecas ME, Nogueira N, Lizardi PM. Minichromosomal repetitive DNA in *Trypanosoma cruzi*: its use in

a high-sensitivity parasite detection assay. Proc Natl Acad Sci USA 1984;81:3356–60.
[30] Elias MCQB, Vargas N, Zingales B, Schenkman S. Organization of satellite DNA in the genome of *Trypanosoma cruzi*. Mol Biochem Parasitol 2003;129:1–9.
[31] Floeter-Winter LM, Souto RP, Stolf BS, Zingales B, Buck GA. Can activity of the rRNA gene promoter be used as a marker of speciation? Exp Parasitol 1997;86:232–4.
[32] Nunes LR, Carvalho MRC, Buck GA. *Trypanosoma cruzi* strains partition into two groups based on the structure and function of the spliced leader and rRNA gene promoters. Mol Biochem Parasitol 1997;86:211–24.
[33] Elias MC, Marques-Porto R, Freymuller E, Schenkman S. Transcription rate modulation through the *Trypanosoma cruzi* life cycle occurs in parallel with changes in nuclear organisation. Mol Biochem Parasitol 2001;112:79–90.
[34] Teixeira SMR, Kirchhoff LV, Donelson JE. Post-transcriptional elements regulating expression of mRNAs from the amastin/tuzin gene cluster of *Trypanosoma cruzi*. J Biol Chem 1995;270:22586–94.
[35] Teixeira SMR, Russell DG, Kirchhoff LV, Donelson JE. A differentially expressed gene family encoding “amastin”, a surface glycoprotein of *Trypanosoma cruzi* amastigotes. J Biol Chem 1994;269:20509–16.
[36] Maslov D, Avila HA, Lake JA, Simpson L. Evolution of RNA editing in kinetoplastid protozoa. Nature 1994;368:345–8.
[37] Koslowsky DJ, Bhat GJ, Perrollaz AL, Feagin JE, Stuart K. The MURF3 gene of *T. brucei* contains multiple domains of extensive editing and is homologous to a subunit of NADH dehydrogenase. Cell 1990;62:901–11.
[38] DaRocha WD, Bartholomeu DC, Macêdo CDS, et al. Characterization of cDNA clones encoding ribonucleoprotein antigens expressed in *Trypanosoma cruzi* amastigotes. Parasitol Res 2002;88:292–300.
[39] Vázquez M, Ben-Dov C, Lorenzi H, Moore T, Schijman A, Levin MJ. The short interspersed repetitive element of *Trypanosoma cruzi*, SIRE, is part of VIPER, an unusual retroelement related to long terminal repeat retrotransposons. Proc Natl Acad Sci USA 2000;97:2128–33.