Hyperosmotic Stress Induces Aquaporin-dependent Cell Shrinkage, Polyphosphate Synthesis, Amino Acid Accumulation, and Global Gene Expression Changes in *Trypanosoma cruzi*[^1]

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**Background:** *Trypanosoma cruzi* is subjected to hyperosmotic stress during its life cycle.

**Results:** The recovery from hyperosmotic stress involves the function of an aquaporin, amino acid accumulation, polyphosphate synthesis, and global gene regulation.

**Conclusion:** The response to hyperosmotic stress is different from that observed in mammalian cells or yeasts.

**Significance:** Learning the mechanism of osmoregulation is important for finding new drug targets.

The protist parasite *Trypanosoma cruzi* has evolved the ability to transit between completely different hosts and to replicate in adverse environments. In particular, the epimastigote form, the replicative stage inside the vector, is subjected to nutritional and osmotic stresses during its development. In this work, we describe the biochemical and global gene expression changes of epimastigotes under hyperosmotic conditions. Hyperosmotic stress resulted in cell shrinking within a few minutes. Depending on the medium osmolarity, this was followed by lack of volume recovery for at least 2 h or by slow recovery. Experiments with inhibitors, or with cells in which an aquaporin gene (*TcAQPI*) was knocked down or overexpressed, revealed its importance for the cellular response to hyperosmotic stress. Furthermore, the adaptation to this new environment was shown to involve the regulation of the polyphosphate polymerization state as well as changes in amino acid catabolism to generate compatible osmolytes. A genome-wide transcriptional analysis of stressed parasites revealed down-regulation of genes belonging to diverse functional categories and up-regulation of genes encoding trans-sialidase-like and ribosomal proteins. Several of these changes were confirmed by Northern blot analyses. Sequence analysis of the 3’UTRs of up- and down-regulated genes allowed the identification of conserved structural RNA motifs enriched in each group, suggesting that specific ribonucleoprotein complexes could be of great importance in the adaptation of this parasite to different environments through regulation of transcript abundance.

The life cycle of *Trypanosoma cruzi*, the etiologic agent of Chagas disease, involves four major developmental stages that alternate between an insect vector and a mammalian host. The parasite enters the mammalian host when the insect vector defecates in the vicinity of the bite, and the natural infective stage, the metacyclic trypomastigote, is carried into the wound by scratching and then penetrates and infects nearby cells. Once inside the host cells, metacyclic trypomastigotes differentiate into amastigotes. These replicative forms multiply in the cytoplasm and, after several rounds of replication, differentiate back into trypomastigotes, which gain access into the bloodstream and eventually invade new cells, thus perpetuating the infection. When the insect bites an infected mammal, the trypomastigotes carried over with the blood meal differentiate into epimastigotes, which are a free replicative form living in the insect intestine. In the rectum, where the insect’s urine is discharged, the epimastigotes differentiate to metacyclic trypomastigotes by a process termed metacyclogenesis, and these forms are able to start a new round of infection.

* T. cruzi has evolved the ability to transit between completely different hosts and to replicate in adverse environments. Inside the vector, after entering the stomach with the blood meal and during their migration through the intestine of the vector, *T. cruzi* is subjected to dramatic changes in environmental conditions. Of particular importance are the changes in nutrient supply and osmolarity that occur when epimastigotes reach the rectum to undergo metacyclogenesis. There is a very considerable increase in osmolarity in the lower digestive tract of the insect. Osmolarity increases slightly from the feces to the urine,
Transcriptome of T. cruzi under Hyperosmotic Stress

from 320 to 410 mosmol/kg, but there is a very strong increase in the yellow rectal content, up to 1,000 mosmol/kg (1). Starvation conditions are also predominant in the rectum, and simulation of these conditions (transfer of epimastigotes into triatome artificial urine supplemented with amino acids, bicarbonate, and glucose, TAU3AAG) has been used to increase metacyclogenesis in vitro (2). Therefore, the parasites are subjected to both nutritional and hyperosmotic stresses at the final stage of their cycle in the vector intestine.

Starvation of epimastigotes in PBS in vitro has been shown to result in changes in cell morphology and degradation of the abundant dispersed gene family-1 protein (3), formation of stress granules (4), and appearance of autophagic vacuoles (5), although starvation in TAU3AAG was shown to increase epimastigote endocytic activity, promote their adhesion to surfaces, and trigger metacyclogenesis (6). Starvation causes global genomic expression responses in yeast (7), as well as physiological responses in a variety of eukaryotic cells (8). Hyperosmotic stress, at variance with hyposmotic stress (9–11) has not been studied so far in epimastigotes. Adaptation to hyperosmotic stress has been examined in several cells, although most of what is known is in part due to studies in yeast (12). Upon exposure to an elevation in external osmolality, cells shrink and then regain normal volume by a process called regulatory volume increase. However, this regulatory volume increase response seems not to be a general process; a number of cell types from mammals have been shown not to regain the original volume after hyperosmotic stress, at least within an hour (for review see Ref. 13). High osmolarity has also been shown to elicit global genomic expression responses in yeast (7). Another form of environmental stress produced by an increase in temperature (heat stress) has been reported to reduce the steady-state levels of most mRNAs in Trypanosoma brucei procyclic stages, although a number of mRNAs were increased (14).

In this study, we report biochemical and global gene expression changes occurring in epimastigotes under hyperosmotic stress. Our results suggest that epimastigotes are able to cope with elevated osmoraly by regulating polyphosphate (polyP)⁶ polymerization state and amino acid metabolism. Moreover, microarray analysis suggests that there is an extensive regulation of mRNA abundance associated with exposure of epimastigotes to different environments.

EXPERIMENTAL PROCEDURES

Cell Culture—T. cruzi epimastigotes (CL Brener and Y strains) were grown at 28 °C in liver infusion tryptose medium (15) supplemented with 5% newborn calf serum. The epimastigotes transformed with pTEX-TcAQP-GFP constructs (16) were maintained in liver infusion tryptose medium supplemented with 5% heat-inactivated fetal bovine serum and 0.1–1 mg/ml geneticin (G418).

Chemicals—Fetal bovine serum, newborn calf serum, Dulbecco’s phosphate-buffered saline (PBS), 4’,6-diamidino-2-phenylindole (DAPI), paraformaldehyde, bovine serum albumin, and TRI Reagent⁶ were purchased from Sigma. Restriction enzymes, T4 DNA ligase, and goat serum were from New England Biolabs (Ipswich, MA). pCR2.1-TOPO cloning kit, superscript reverse transcriptase, 1 kb plus DNA ladder, and 5–6-carboxyfluorescein diacetate succinimidyl ester were from Invitrogen. RQ1 RNase-free DNase I was from Promega. RNeasy kit was from Qiagen (Germantown, MD). Hybond-N nylon membrane and [32P]dCTP (3,000 mCi/mmole) were obtained from PerkinElmer Life Sciences. Tag polymerase was purchased from Denville Scientific Inc. (Metuchen, NJ). All other reagents were analytical grade. The oligonucleotides were ordered from Sigma or Integrated DNA Technologies (Corvallis, IA). The oligonucleotides used in this study and their target genes are listed in supplemental Table S1.

Construction of TcAQP1 Knock-out Cassettes—A recombinant PCR approach was used to generate the knock-out cassettes. The 5’- and 3’-flanking sequences of the TcAQP1 gene were amplified by PCR. The open reading frames (ORFs) of the neomycin- and hygromycin-resistant genes were also amplified by PCR using primers containing ~20 extra nucleotides at the 5’ end overlapping with the 5’- and 3’-flanking sequences of TcAQP1, respectively. The three PCR fragments (5’-TcAQP1 flanking sequence, hygromycin- or neomycin-resistant genes, and the 3’-TcAQP1 flanking sequence) were linked together by sequential PCR. The drug resistance genes were therefore flanked with the exact 5’ and 3’UTR of TcAQP1. The final PCR products were cloned into pCR 2.1 Topo vector. Several clones were sequenced to confirm that the drug resistance gene ORF was correct. The EcoRI-linearized fragments with the drug-resistant gene cassette was then used to transfect Y strain epimastigotes. After obtaining drug-resistant parasites, the genomic DNA was extracted for PCR and Southern blot analysis.

Cell Volume Measurements—Cells were washed and resuspended in isosmotic buffer (20 mm Hepes, 137 mm NaCl, 4 mm KCl, 1.5 mm KH₂PO₄, 8.5 mm Na₂HPO₄, 1 mm CaCl₂, 0.8 mm MgSO₄ and 11 mm glucose, adjusted to pH 7.4 with NaOH, and to an osmolarity of 300 ± 5 mosmol, as determined using an Advanced Instruments 3D3 osmometer). Hyposmotic stress was induced by dilution of the isosmotic cell suspension with deionized water to a final osmolarity of 150 mosmol at time 0. Hyperosmotic stress was induced by dilution of the cells suspended as above with a 1,000 or 1,300 mosM sorbitol solution (final osmolarity: 650 or 800 mosM, respectively). Relative changes in cell volume were followed monitoring absorbance at 550 nm in a plate reader with continuous agitation. To eliminate changes in absorbance due to decantation of the parasites, readings were normalized against changes in isosmotic buffer. Isosmotic control experiments consisted of dilution of cell suspensions with appropriate volumes of isosmotic buffer. Alternatively, epimastigotes (1 × 10⁶ cells/ml) were loaded with 50 μg/ml 5–6-carboxyfluorescein diacetate for 1 h to obtain uniform cytosolic fluorescence and then treated as described above. Relative changes in volume were followed by monitoring fluorescence (excitation, 491 nm; emission, 530 nm). At the end of each experiment, cells were lysed using 0.1% Triton X-100, and total fluorescence was recorded. Fluorescence was normalized by dividing the fluorescence at each time point by the total fluorescence after detergent addition.

6The abbreviations used are: polyP, polyphosphate; nt, nucleotide.
Membrane Integrity—Following the induction of hypometric or hyperosmotic stress, membrane integrity was determined by ethidium bromide exclusion (17). Briefly, aliquots of cells were placed in isosmotic buffer containing 50 μM ethidium bromide and hypometric or hyperosmotic stress was induced as described above. Ethidium bromide fluorescence was monitored (excitation, 365 nm; emission, 580 nm) during 2 h in a plate reader (Molecular Devices).

RNA, Protein, and PolyP Determination after Hyperosmotic Stress—For total RNA, a 1-ml aliquot was taken at different times, and RNA was extracted with TRI Reagent®. Total RNA was determined by spectrophotometry. Proteins were measured using the bicinchoninic acid assay (BCA, Pierce). Extractions of long-chain and short-chain polyP and polyP measurements were performed as described before (18).

Amino Acid Quantification—Cells were incubated in isosmotic buffer or submitted to hyperosmotic stress at a final concentration of ~1 × 10^9 cells/ml. At various time points, 50-μl aliquots were withdrawn, and the cell suspensions were centrifuged for 10 min at 1,000 × g. Total amino acid content of resulting supernatants was assayed using the fluorescamine micromethod. To determine total intracellular amino acid content, the resulting cell pellets were resuspended in 0.5 M perchloric acid and extracted for 30 min on ice. After centrifugation at 14,000 rpm in an Eppendorf centrifuge during 10 min at 4 °C, the resulting cell-free extracts were neutralized with 5 M KOH. Precipitated KClO₄ was removed by centrifugation, and the extracts were used for amino acid determination using the fluorescamine micromethod. For the fluorescamine micromethod, 50 μl of sample (supernatants or cell-free extracts) were mixed in a 96-well plate with 150 μl of 0.2 M sodium borate, pH 8.5, and 25 μl of 0.3 mg/ml fluorescamine (dissolved in acetone). Plates were shaken for 1 min, and then the fluorescence was recorded (λ excitation = 390 nm and λ emission = 465 nm). To correlate the fluorescence intensity of the reaction product with the amino acid concentration, a standard curve was prepared using 0–50 nmol of histidine.

Ammonium Determinations—Cells were incubated in isosmotic buffer or submitted to hyperosmotic stress at a final concentration of ~1 × 10^5 cells/ml. At various time points, aliquots of 110 μl were withdrawn and centrifuged for 10 min at 4,500 × g. Pellets were resuspended in 0.6 ml of PBS containing 0.1% Triton X-100, frozen overnight, and then boiled for 5 min. Supernatants (110 μl) were withdrawn and centrifuged for 1 min at 5,000 rpm in a microfuge. Total amino acid concentrations were determined as follows using an adaptation of a method described previously (10). Six hundred microliters of sample (pellets, 500 μl of sample + 100 μl of water; supernatants, 110 μl of sample + 490 μl of water) were used for the ammonium determination. Three hundred microliters of sample was mixed with 36 μl of phenol solution and then vortexed. Thirty six microliters of sodium nitroprusside solution were then added, and the mixture was vortexed again. Finally, 90 μl of oxidizing solution were added, and the mixture was vortexed one final time. Samples were placed in the dark for 1 h and then 200 μl were transferred to a microtiter plate, and absorbance was recorded at 630 nm. To correlate the absorbance of the reaction product with the ammonium concentration, a standard curve was obtained using 0–0.004 μg of NH₄Cl. The order of reagent addition, vortexing after each addition, and incubation in the dark were all essential for proper functioning of the assay. Phenol solution was made by adding 1 part 90% liquid phenol to 9 parts 95% ethanol. Sodium nitroprusside solution consisted of 5 mg/ml recrystallized sodium nitroprusside in distilled H₂O (reagent-grade sodium nitroprusside was recrystallized by dissolving 10 g in 10 ml of distilled H₂O and then adding 20 ml of 95% ethanol and freezing overnight; the resulting crystals were washed with ice-cold 95% ethanol and dried by vacuum filtration). Phenol solution and sodium nitroprusside solution were stable when protected from light and stored at 4 °C. Oxidizing solution (4 parts 20% (w/v) sodium citrate, 1% (w/v) NaOH solution, and 1 part 5% NaOCl) was freshly prepared before each experiment.

Electron Microscopy—About 1 × 10⁸ epimastigotes were harvested and washed twice with cold PBS. The parasites were fixed with freshly prepared 2.5% glutaraldehyde, 4% paraformaldehyde, 0.1 M sodium cacodylate buffer, pH 7.3, on ice for 1 h and then embedded in epoxy resin, sectioned, and stained using standard methods. Images were acquired on a Phillips CM-200 transmission electron microscope operating at 120 kV.

Microarrays—The T. cruzi microarrays were obtained from the Pathogen Functional Genomics Resource Center. The microarray description is available at J. Craig Venter Institute web site. Epimastigotes in the exponential phase of growth in liver infusion tryptose medium were collected by centrifugation, washed twice with isosmotic buffer, resuspended in the same buffer, and incubated in the same buffer or mixed with sorbitol solution to reach a final osmolarity of 800 ± 5 mosmol, as verified with an Advanced Instruments 3D3 osmometer (Norwood, MA). Cells were collected at time 0 or after 3, 6 and 20 h of incubation. At least three biological replicates of all hybridizations were performed. This is to account for sample heterogeneity, variations due to hybridizations, and variation between slides. To prevent bias by preferential label incorporation into particular sequences, dyes were swapped between the two cDNA probes each time. Each slide used has two spots corresponding to each gene, and we used at least six slides for each time point. We therefore had at least 12 spots for each gene at each time point. For each individual time point, S.D. and p values were calculated using the MultiExperiment View Program from the TM4 suite. We include the p values for the most abundantly up-regulated and down-regulated genes.

Total RNA Extraction and cDNA Labeling—We strictly followed Pathogen Functional Genomics Resource Center protocol for all steps of the microarray experiments. Total RNA was isolated from different samples of T. cruzi using TRI Reagent® following the manufacturer’s instructions. The extracted total RNAs were further treated with RQ1 RNase-free DNase I for 30 min at 37 °C to remove genomic DNA contamination. The purified total RNAs were then cleaned up by RNasey kit.

Aminallyl-labeled cDNA Synthesis for Hybridization—We strictly followed the TIGR protocol to synthesize cDNA probes for hybridization. Briefly, 2 μg of total RNA were mixed with 2 μl of random hexamers (3 mg/ml) and 1 μl of RNaseOUT in diethyl pyrocarbonate water (final volume 18.5 μl) and incubated at 70 °C for 10 min. The samples were then snap-frozen.
Transcriptome of *T. cruzi* under Hypersonotic Stress

on wet ice for 30 s, centrifuged briefly at 10,000 × g, and then mixed with 6 μl of 5× First Strand buffer, 3 μl of 0.1 M dithiothreitol (DTT), 0.6 μl of 25 mM dNTP/aa-UTP labeling mix, and 2 μl of Superscript II RT. After mixing, the samples were incubated in a 42°C water bath overnight. The reaction was stopped by adding 10 μl of 0.5 M EDTA and 10 μl of NaOH and incubated at 65°C for 15 min to hydrolyze RNA. The solution was brought to neutrality by adding 25 μl of 1 M Tris-HCl, pH 7.0. The unincorporated amino acid-dUTP was removed using a MiniElute column. The purified cDNA was then labeled with Cy3 or Cy5 dye at room temperature for at least 1 h. The uncoupled Cy dye was also removed by the MiniElute column.

**Microarray Hybridization**—Slides were prehybridized in 50 ml of prehybridization buffer (5× SSC, 0.1% SDS, 1% BSA) at 42°C for at least 1 h. The slides were then washed with MilliQ water extensively. The slides were dried by centrifugation at 1,000 × g for 10 min. Equal amounts of Cy3 cDNA and Cy5 cDNA were mixed and vacuum-dried. The probes were then resuspended in 50 μl of hybridization buffer (40% formamide, 5× SSC, 0.1% SDS, 0.6 μg/μl salmon sperm DNA). The mixture was heated at 95°C for 5 min, vortexed, and heated again at 95°C for another 5 min. The hybridization solution was then applied to the pre-hybridized slides. The slides were sealed in a hybridization chamber and hybridized at 42°C overnight. The slides were then washed in pre-heat low stringency buffer (2× SSC, 0.1% SDS) at 55°C for 5 min twice and then transferred to medium stringency buffer (0.1× SSC, 0.1% SDS) to wash at room temperature for 5 min twice. The slides were then washed in high stringency buffer (0.1× SSC) twice before drying after centrifuging.

**Scanning and Data Analysis**—The slides were scanned by using ProScanArray microarray scanner (PerkinElmer Life Sciences). The data analyses were performed as described previously (19) by using TM4 microarray software suite. We used the Gingko program (Pathogen Functional Genomics Resource Center website) instead of the Microarray Data Analysis System module to filter and normalize the spots identified by Spot Finder module of TM4.

**Northern Blot Analysis**—For the Northern blot analysis, total RNA was isolated from epimastigotes using TRI Reagent®. RNA samples were subjected to electrophoreses in 1% agarose gels containing 2.2 M formaldehyde, 20 mM Mops, pH 7.0, 1 mM EDTA, and 8 mM sodium acetate, transferred to nylon membranes, and hybridized with radiolabeled probes. All probes used in this study were generated by PCR (supplemental Table S1). The PCRs were performed in a PTC-100 Programmable Thermal Controller (Mf Research, Inc., Watertown, MA) at 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min/cycle (25 cycles) using Taq DNA polymerase. DNA probes were labeled with [α-32P]dCTP using random hexanucleotide primers and the Klenow fragment of DNA polymerase 1 (Prime-a-Gene labeling system). The rRNA gene was used as a loading control assuming a similar level of expression of this gene under all conditions (20). The results of Northern blots were quantified by PhosphorImager analysis.

**Databases**—Trypanosome database (*T. cruzi* CL Brener Genomic Sequence Release 5.1) utilized in this work was obtained from TcruziDB server. 5′ upstream genomic sequences and 3′ downstream genomic sequences were obtained using TcruziDB sequence retrieval tool. A length of 50 nt upstream to the coding sequence was used to obtain sequences resembling the 5′UTR, and 350 nt downstream to the coding sequences were used for 3′UTR, in agreement with previously reported data from trypanosomes (21–23).

**Computational Analysis**—Programs used to carry out computational analysis were free internet downloads and compiled in the LINUX environment (Ubuntu 9.10 distribution). Consensus motifs were predicted from each of the 3′UTR groups (up- or down-regulated genes at 3, 6, and 20 h and constantly regulated during all time courses) using Infernal (24). Candidate motifs obtained were used to build the stochastic context-free grammar model. The stochastic context-free grammar model for each candidate motif was used to search against different datasets to obtain the number of hits for each motif (CMSEARCH program). The motif with the highest enrichment in the specific dataset over the literature database was considered to be the best candidate motif. The motif logo was constructed using WebLogo 2.8.2. Finally, RNAfold server (25) was used to plot the secondary structure of the representative RNA motifs. DAVID Functional Annotation Chart tool (26) was used to categorize and compare the differently regulated microarray gene list against a *T. cruzi* background (using Fisher’s exact test and Benjami-Hochberg correction). Hierarchical clustering was carried out using R software.

**RESULTS**

*T. cruzi* Epimastigote Stage Is Able to Tolerate a Hypersonotic Environment—When epimastigotes were subjected to hypersonotic stress (from 300 to 800 mosM by addition of sorbitol) and then observed microscopically, they shrank dramatically within a few minutes. In contrast to what has been described for the hypersonotic stress (9), the parasites seem not to regain their normal volume at least during the following 2 h (data not shown). However, they adapted perfectly well to these conditions because they are indistinguishable, in terms of motility, from control cells maintained in isosmotic buffer (data not shown). These initial observations were confirmed by light-scattering experiments. Because cell shrinking leads to an increase in their absorbance (9), we were able to follow the process by monitoring the absorbance of cell suspensions at 550 nm (Fig. 1A). The progression of shrinking is extremely rapid, being maximal in a few seconds after the shock, and persistent for the whole experimental period of observation (up to 2 h, data not shown). To ensure that other light-scattering artifacts not related to volume changes did not contribute to the observed signal, we also monitored cell volume changes using a fluorescent method. The fluorophore used was an ester form of 5–6-carboxyfluorescein diacetate succinimidyl ester, which is membrane-permeable but with low fluorescence until it is cleaved intracellularly by nonspecific esterases to produce the impermeable fluorescent form (27). As the cell shrinks or swells in response to osmotic changes, the concentration of the entrapped fluorophore will rise or fall with a consequent change in fluorescence output; the concentration-dependent self-quenching properties of the fluorophore enable cell volume changes to be recorded as changes in fluorescence. We
observed again that shrinking occurs almost immediately after treatment without any evidence of significant volume recovery (Fig. 1, bottom curve). As a control, hyposmotic stress was also induced, showing that the process of volume recovery is extremely rapid and is essentially complete by 6 min (Fig. 1, upper curve). We confirmed that the plasma membrane remained intact during the hyperosmotic stress by performing the same experiment in the presence of extracellular ethidium bromide (EtBr). Because EtBr exhibits a fluorescence enhancement upon binding DNA, a compromise in membrane integrity would result in increased fluorescence (17). No significant increase in EtBr fluorescence could be observed during the induction of hyperosmotic stress (Fig. 1C). When epimastigotes were incubated at lower osmolarities (<650 mosM) a slight drop of absorbance was detected after a long incubation period, suggesting partial recovery of their volume (see Figs. 2A and 3E). However, the recovery was not significant, and the slight decrease in absorbance could be attributed to shape changes under stress conditions.

**Aquaporin Mediates Water Efflux through Contractile Vacuole during Hyperosmotic Stress**

In a previous study (16), we identified an aquaporin (TcAQP1) that is located in acidocalcisomes and the contractile vacuole complex of *T. cruzi* epimastigotes. In contrast to other aquaporins that are inhibited by high micromolar concentrations of either HgCl2 or AgNO3, TcAQP1 was shown to be exquisitely sensitive to inhibition by very low concentrations of these compounds (16). To determine the importance of this aquaporin in water permeability during the hyperosmotic stress response, we treated cells with 1, 3, 5, and 10 μM HgCl2, concentrations shown previously to inhibit osmotic swelling in *Xenopus laevis* oocytes transfected with TcAQP1 (16), and we then monitored volume changes during hyperosmotic stress using the light-scattering technique. As seen in Fig. 2A, HgCl2 reduced the intensity of shrinking as well as increased the time needed to achieve the maximal level of shrinking. Epimastigotes under isosmotic conditions treated with these concentrations of HgCl2 for 5 min showed neither morphological nor motility alterations and did not increase their permeability to ethidium bromide (data not shown) suggesting the absence of toxic side effects. We next...
Transcriptome of T. cruzi under Hyperosmotic Stress

FIGURE 3. TcAQP1 is involved in the hyposmotic and hyperosmotic stress response of T. cruzi epimastigotes. A, schematic diagram of the knock-out strategy used. TcAQP1 gene knock-out cassettes were constructed by replacing TcAQP1 ORF with the ORFs of the hygromycin or neomycin resistance genes. PstI cuts both hygromycin (Hyg) and neomycin (Neo) ORFs once but not TcAQP1 ORF. B, Southern blot analysis indicates that Y-HNR (Y strain that is both hygromycin- and neomycin-resistant) line is a single knock-out with a second Neo gene knock-out cassette somewhere else. The genomic DNA was cut with both EcoRI and PstI and separated on a 0.8% agarose gel. A probe against the TcAQP1 upstream sequence (outside the knock-out cassette as depicted in A, 5′ probe) was used for the first hybridization (left panels); the wild type TcAQP1 locus produces a 2.5-kb band specific for the 5′ probe after cutting with EcoRI and PstI. The 1.4-kb band indicates a hygromycin gene knock-out cassette has successfully replaced TcAQP1. The same membrane was then hybridized with Neo gene ORF (5′ + Neo, right panels). The arrows indicate the hybridization signals specific to Neo gene. C, Northern blot analysis indicates that TcAQP1 mRNA is significantly down-regulated in Y-HNR line. The membrane was first hybridized to TcAQP1 coding sequence and then washed and hybridized to the β-tubulin gene as a loading control. All experiments were performed in triplicate. D, TcAQP1 knockdown epimastigotes fail to recover their volume after hyposmotic stress. Epimastigotes were diluted 1:1 with water (final osmolality = 150 mosM). E, TcAQP1 knockdown epimastigotes shrink less after hyperosmotic stress (final osmolality = 650 mosM). Data shown in D and E indicate relative volume as compared with the initial volume taken as 100 and are expressed as means ± S.D. of n = 3.

evaluated the response of stable transfectant parasites expressing different levels of TcAQP1 (16). We found a direct correlation between levels of shrinking and aquaporin overexpression, which together with the previous results suggests that TcAQP1 could be mediating water efflux during osmotic challenge (Fig. 2B). When epimastigotes under hyperosmotic stress were observed under the microscope, the appearance of a huge contractile vacuole was already evident by bright field microscopy. This observation was further confirmed by electron microscopy. There was a great increase in the size of the contractile vacuole compared with epimastigotes maintained in isosmotic conditions (supplemental Fig. S1). Taken together, these results indicate that epimastigotes shrink after they are submitted to hyperosmotic stress and adapt to this new environment without changes in viability, and these results suggest that these changes are mediated by water extrusion facilitated by an aquaporin.

Generation of a TcAQP1 Knockdown Strain—To further establish the role of TcAQP1 in the response to osmotic stress, we attempted the knock-out of its gene. Two knock-out cassettes with drug-resistant genes were successfully constructed by recombinant PCR (Fig. 3A). Although attempts to generate a TcAQP1 knock-out strain failed (see supplemental material), we obtained strains in which the TcAQP1 expression was knocked down. Southern blot analysis of a typical Y-HNR strain (Y strain that is both hygromycin- and neomycin-resistant) of parasites is shown in Fig. 3B as assayed using a 5′ probe (Fig. 3B, left lanes) outside the knock-out cassette or the 5′ probe plus a Neo probe (Fig. 3B, right lanes). The results indicate that there is still one wild type TcAQP1 allele left in this strain (as in the single knock-out parasites). The neomycin-resistant gene knock-out cassette seems to be present at different loci either episomally or integrated into other regions, because Southern blot analysis with a probe against the Neo ORF produced strong and multiple bands different from the upstream sequence of TcAQP1 (Fig. 3B, right lanes, arrows). Northern blot analysis indicated that the steady-state TcAQP1 mRNA level was decreased by 71% in Y-HNR parasites compared with wild type Y strain epimastigotes (Fig. 3C). We then concluded that the Y-HNR parasite was not a double knock-out strain but rather a TcAQP1 knockdown mutant. Epimastigotes in which TcAQP1 expression was down-regulated were less able to recover their volume after hyposmotic stress (Fig. 3D, TcAQP1-KD) or to shrink under hyperosmotic stress (Fig. 3E, TcAQP1-KD).

Acidocalcisomes Counteract High Osmolarity by Raising Long-chain PolyP Levels—Acidocalcisomes are acidic organelles rich in calcium and other cations bound to long-chain and short-chain polyP. The high osmotic potential of polyP as well...
Global Changes on Gene Expression of Epimastigotes under Hyperosmotic Stress

As its abundance in the parasite suggests that the regulation of its levels as well as the degree of polymerization could be very important as part of the hyperosmotic stress response. We measured both long-chain and short-chain polyP levels and found that long-chain polyP increased 3- and 2.35-fold after 3 and 6 h of hyperosmotic stress, respectively, suggesting that phosphate polymers could play an important role at the early stages by sequestering ions in the acidocalcisomes to reduce the ionic strength of the cells (Fig. 4A). Conversely, 2 h after the stress, long-chain polyP reached lower values than those at the beginning of the experiment suggesting that an increase in the number of free osmolytes (because of the lower amount of polyP) could be balancing the internal osmolarity. In contrast, short chain polyP did not change with time (Fig. 4B).

Generation of Compatible Osmolytes—A common strategy to reduce water loss during the hyperosmotic stress is the generation of compatible osmolytes. In particular, the amino acid levels have been reported to increase after hyperosmotic stress. We evaluated the amino acid content in cell-free extracts of epimastigotes maintained under isosmotic conditions or under hyperosmotic stress and found that it remained constant in the isosmotic control, although it increased suddenly within 10 min after hyperosmotic stress (Fig. 5A). Interestingly, total protein levels decreased during the initial hours of hyperosmotic stress (Fig. 5B), and the ammonium levels declined in cell-free extracts (Fig. 5C) and supernatants (Fig. 5D) of stressed parasites. These findings suggest that inhibition of protein synthesis and amino acid catabolism could both contribute to elevated amino acid levels.

Global Changes on Gene Expression of Epimastigotes under Hyperosmotic Stress—To investigate the transcriptional response of T. cruzi epimastigotes to a sudden increase in medium osmolarity, we carried out a time course microarray analysis of parasites transferred from isosmotic buffer to the same buffer with the addition of sorbitol (final osmolarity 800 ± 5 mosM). The time points 3, 6, and 20 h post-treatment were chosen to study both early and late responses. RNA was extracted from the cells, and labeled cDNA was hybridized to gene arrays provided by the Pathogen Functional Genomics Resource Center. Using a cutoff value of ±50%, we were able to detect 691 differentially expressed genes at least at one time point during the time course analysis (supplemental Table S2). Three hours after treatment and at a threshold or 1.5-fold, 268 genes were identified (Fig. 6A). At 6 h 248 genes were affected, and for the 20-h time point 175 differentially expressed genes were found. For all the time points, a similar proportion of genes was up- and down-regulated (Fig. 6B). To assess the likelihood of falsely reported and differentially regulated genes, we performed a transcriptional profile of epimastigotes incubated in isosmotic buffer. Interestingly, no significant overlapping with the osmotic response gene list was observed, suggesting that the majority of genes affected by the hyperosmotic stress are indeed responding to the osmotic shock rather than to the nutritional limitation (supplemental Table S3).

We verified some of the microarray results by Northern blot analyses. As shown in Fig. 7A, calcium-binding protein and flagellar calcium-binding protein are down-regulated after 3 h of hyperosmotic stress, but they gradually recovered after continued incubation. The results of the Northern blot analyses of HSP10 and flagellar calcium-binding protein after 3, 6, and 20 h of hyperosmotic stress were quantified and plotted together with the microarray results and are shown in Fig. 7, B and C, respectively. The transcript levels of HSP10 increased and those of flagellar calcium-binding protein decreased as detected using both methods.

GO Annotation Shows Down-regulation of ATP Synthesis Pathway—A nonredundant set composed of 444 genes was filtered from the 691 regulated genes of the complete time course analysis, from which 236 were up-regulated and 209 down-regulated. Conspicuously, one gene coding for a hypothetical protein (Tc00.1047053511807.180) was simultaneously down-regulated at 3 h and up-regulated at 6 h. From this nonredundant list, 38 were consistently down-regulated at all time points, and 18 were always up-regulated (see Tables 1 and 2).

Next, the expression profiles of the differentially expressed genes were used for a cluster analysis to identify groups of similarly regulated genes using the nonredundant set. Eight major clusters of genes could be identified (Fig. 8 and supplemental material). Clusters 2 and 3 accounted for 40% of the 444 genes and corresponded to up-regulated genes with the highest expression levels at 3 h (cluster 3) or between 3 and 6 h (cluster 2). Analysis of these clusters with the DAVID Functional Annotation Chart tool (NIAID, National Institutes of Health) revealed the enrichment of genes encoding for trans-sialidase-like and ribosomal proteins. Cluster 5 also contained up-regulated genes, but in this case these genes reached maximum expression levels at 20 h. This cluster, enriched in genes encoding ribosomal proteins, as cluster 2 and 3, also included genes encoding flagellar proteins. Cluster 4 represents the early down-regulated genes (3 h) that remained at low transcript levels during the course of the experiments. This cluster 4 was
mainly enriched in genes encoding proteins involved in protein and amino acid catabolism, carbohydrate and intermediate metabolism, and ATP synthesis. Clusters 1, 6, and 7 also grouped down-regulated genes but in this case genes whose expression levels were minimum at 3, 6, and 20 h, respectively, and then seem to recover (clusters 1 and 6) or remained down-regulated (cluster 7). Cluster 1 was enriched in genes encoding chaperones, flagellar proteins, lipid, and ATP biosynthesis enzymes, although clusters 6 and 7 were mainly enriched in genes encoding fatty acid metabolism and glycolysis enzymes, respectively. Finally, cluster 8 contained genes exhibiting a variable response, increasing and decreasing gene expression over time, and it was not enriched in any particular functional group. The supplemental Fig. S2 shows the top differentially regulated genes by hyperosmotic stress.

Identification of cis-Elements in the 3′/H11032 UTR of Genes Affected by Hyperosmotic Stress

— From the nonredundant list, 38 genes were consistently down-regulated at all time points, and 18 were always up-regulated (see Tables 1 and 2).

There is little transcriptional control in trypanosomes, and the mRNA steady-state levels are mostly determined by posttranscriptional mechanisms. In general cis-elements in the 3′UTR and their binding proteins are key factors in maintaining mRNA stability/steady-state transcript level in trypanosomes. Accordingly, sequence analysis of these transcripts was utilized to search for conserved RNA motifs. For each group, a length of 300 nt downstream to the coding sequences were downloaded using TcruziDB to obtain sequences resembling the 3′UTR, in agreement with previously reported data from trypanosomes.
(23). Using CMfinder software, we found conserved RNA elements in the 3′UTRs for both datasets analyzed (see Table 3). Covariance model searches using Infernal software (24) allowed us to identify transcripts encompassing these motifs in all of the 1.5-fold regulated genes to determine statistical significance.

More than 83% (15/18) of the consistently up-regulated transcripts have a common structural RNA motif, termed up-m (Table 1), whereas the coverage in down-regulated mRNAs is less than 11% ($\chi^2, p < 0.001$). Moreover, 16 of 26 2-fold up-regulated mRNAs at least at one time point encompass the up-m motif, whereas the motif abundance in the 60 most down-regulated genes at least at one time point were less than 9% ($\chi^2, p < 0.001$) (Table 3).

An analysis of the down-regulated groups exhibited one significant RNA element, down-m, that was conserved in 30 3′UTR sequences of the 38 (78.4%) down-regulated transcripts (Tables 2 and 3), when the abundance of this motif in the up-regulated list were lower than 39% ($\chi^2, p < 0.05$). WebLogo representation indicated a GC-rich sequence composition for the up-m, whereas down-m has an AU content (Fig. 9A). Predicted secondary structures revealed that these three RNA motifs have a stem-loop of 30–40 nt (Fig. 9B). When the relative frequencies of motifs within 5′ and 3′UTR of the experimental datasets were compared, a higher enrichment in the 3′ downstream genomic sequence was observed, denoting a preferred 3′UTR localization for both motifs (data not shown). Our results showed that the identified motifs are enriched in each corresponding experimental dataset; RNA motif up-m has a high coverage in up-regulated sequences and the down-m element is essentially found in down-regulated genes (Table 3).

Clusters 2, 4, and 8 have a significantly different representation of up and down RNA motifs in their 3′UTRs that matched their gene expression profiles ($\chi^2, p < 0.05$). Accordingly, the structural elements identified in this work could be signature marks for these clusters of differentially regulated genes (supplemental Fig. S3).

### TABLE 1

Up-regulated genes at the three time points using a cutoff of +50%

| GeneID           | Description                                    | $p$ value$^a$ | Motif location |
|------------------|------------------------------------------------|---------------|----------------|
| Tc00.1047053504229.100 | trans-Sialidase (pseudogene), putative haploid, non-Emesmeraldolike | $<0.00001$ | 123–147         |
| Tc00.1047053507275.40 | Hypothetical protein, conserved                 | $<0.00001$ | 23–46           |
| Tc00.1047053506129.89 | Hypothetical protein, conserved$^b$             | $0.00002$  |                 |
| Tc00.1047053508827.79 | 40 S ribosomal protein S17, putative            | $<0.00001$ | 191–212         |
| Tc00.1047053511277.200 | RNA-binding protein, putative                   | $0.00078$  | 15–40           |
| Tc00.1047053507515.20 | Hypothetical protein, conserved                 | $0.00004$  | 40–63           |
| Tc00.1047053506227.200 | Hypothetical protein$^c$                        | $<0.00001$ | 136–158         |
| Tc00.1047053511061.40 | trans-Sialidase, putative$^d$                   | $<0.00001$ | 3–28            |
| Tc00.1047053510879.120 | Ribosomal protein L3, putative$^e$              | $<0.00001$ |                 |
| Tc00.1047053507509.40 | Hypothetical protein, conserved; Pfam PF00069 (E-value 1.50E-02) phosphokinas                   | $<0.00001$ | 1–23            |
| Tc00.1047053508823.21 | 40 S ribosomal protein S12, putative$^e$       | $<0.00001$ | 206–231         |
| Tc00.1047053511133.10 | Hypothetical protein, conserved                 | $<0.00001$ | 44–65           |
| Tc00.1047053506559.470 | Ribosomal protein L1,5S, putative              | $<0.00001$ | 224–247         |
| Tc00.1047053506399.80 | Hypothetical protein, conserved; Pfam PF10568 (E-value 6.60E-04) Tom37, outer mitochondrial membrane transport complex protein | $<0.00001$ |                 |
| Tc00.1047053510363.130 | Hypothetical protein$^f$                        | $0.0004$   | 47–73           |
| Tc00.104705350471.110 | Amal1 protein, putative$^g$                     | $0.00374$  | 18–41           |
| Tc00.1047053504205.20 | trans-Sialidase (pseudogene), putative gene with more than two copies in the genome that does not belong to a merged region and that could not be assigned to a particular haplotype allele of Tc00.1047053510491.60 | $0.00007$  | 3–28            |

$^a$ The $p$ value of each gene was calculated from all slides at 3-, 6-, and 20-h time points.
$^b$ Sequences that do not contain the Up-m.
$^c$ This T. cruzi sequence has no orthologue in kinetoplastids.
In this study, we have characterized physiological, morphological, and gene expression changes occurring in epimastigotes of *T. cruzi* when submitted to a hyperosmotic stress equivalent to that present in the vector’s intestine.

In most mammalian cells, hyperosmotic stress causes cell shrinkage because of osmotic efflux of water leading to increases in intracellular ionic strength (29). This rapid reduction in cell volume is corrected by the regulatory volume increase (29). Cells counteract the additional increase in ionic strength produced by the further uptake of inorganic ions by substituting them by either the synthesis or uptake and cellular accumulation of compatible osmolytes, such as neutral amino acids or their derivatives, polyols such as sorbitol and myo-inositol, and methylamines such as betaine (29). Compatible osmolytes replace the inorganic ions without impairing normal biochemical functions such as protein synthesis (29). For example, in a cell-free protein synthesis system, both initiation and elongation are inhibited by high concentrations of inorganic ions but not by compatible osmolytes (32). Compatible osmolytes protect the cells from apoptosis and modulate their adaptive responses (33, 34).

In contrast to what happens with mammalian cells, when epimastigotes are subjected to hyperosmotic stress they shrink dramatically within a few seconds but do not significantly regain their normal volume, suggesting that there is no immediate inorganic ion and water uptake. Despite the initial increase in intracellular ionic strength, they adapt well to these conditions, being virtually indistinguishable in terms of motility from control cells maintained in isosmotic buffer. However, within minutes of hyperosmotic stress, there is a decrease in ammonium production and accumulation of amino acids, which then stabilize at concentrations higher than those under isosmotic conditions. Protein content also decreased within 3 h suggesting protein degradation to increase the amino acid pool.
The polyP synthesis is also stimulated within minutes of hyperosmotic stress (35), and we now show that this stimulation results in a 3- and 2.35-fold increase in long-chain polyP content at 3 and 6 h after hyperosmotic stress. Taken together, these results are in agreement with a model in which reduced amino acid catabolism and increased protein degradation result in amino acid accumulation. These amino acids are the compatible osmolytes that replace the inorganic ions (Na\(^+\) and K\(^+\)) that are sequestered together with newly formed polyP in the acidocalcisomes, thus reducing the cytosolic ionic strength increased after water elimination and preventing cell damage.

Treatment of the epimastigotes with low concentrations of HgCl\(_2\), a known inhibitor of T. cruzi aquaporin 1 (TcAQP1), or knockdown of TcAQP1 expression reduces the intensity of shrinking after hyperosmotic stress, whereas overexpression of TcAQP1 increased shrinking, suggesting that aquaporin mediates water efflux during hyperosmotic challenge. Hyperosmotic stress also resulted in a great increase in the size of the contractile vacuole. In previous work, we demonstrated that the TcAQP1 localized to the contractile vacuole was involved in the regulatory volume decrease after hyposmotic stress (36). Our present results suggest that TcAQP1 and the contractile vacuole are also involved in water efflux during hyperosmotic stress.

A genome-wide transcriptional analysis of T. cruzi epimastigotes submitted to hyperosmotic stress (800 mosM) resulted in up-regulation and down-regulation of the expression of a number of genes. In contrast to what occurs in mammalian cells after hyperosmotic stress (29), there was no increase in the expression of genes encoding for heat shock proteins (except for Hsp10). In mammalian cells, molecular chaperones are induced during the 1st h of hyperosmotic treatment to counteract the detrimental effects of elevated intracellular ionic strength and to protect intracellular macromolecules against unfolding and aggregation (37, 38). This difference suggests that the early synthesis of polyP and sequestration of inorganic ions in acidocalcisomes of epimastigotes and the simultaneous increase in compatible osmolytes prevents the deleterious effects of a cellular increase in ionic strength.

The second phase of recovery after hyperosmotic stress is characterized in mammalian cells by induction of amino acid transporters, and this is also seen in epimastigotes. The higher expression of genes encoding for amino acid transporters (Tc00.1047053510507.40, Tc00.1047053511545.80, and Tc00.1047053511543.30) is evident, suggesting again that amino acids are the compatible osmolytes needed to replace the inorganic ions sequestered by the stimulated synthesis of polyP in the acidocalcisomes. Interestingly, Tc00.1047053511545.80 and Tc00.1047053511543.30 have high similarity to the sodium-dependent neutral amino acid transporter-2 (SNAT2) known as system A, which is known to increase upon exposure...
Transcriptome of T. cruzi under Hyperosmotic Stress

![Sequence logo (A) and conserved secondary structures (B) of RNA motifs identified in transcripts affected by hyperosmotic stress. Predicted secondary structures of consensus RNA element (B) and WebLogo graphic (A) indicating the bit representation of each residue at each position within the motif are shown. WebLogo representation indicated a GC-rich sequence composition for the up-m, whereas down-m has an AU content. B, predicted secondary structures revealed that these three RNA motifs have a stem-loop of 30–40 nt.](image)

of mammalian cells to hyperosmotic stress or amino acid deprivation (33, 39–42).

Previous studies in yeast (43, 44) have shown that hyperosmotic stress results in up-regulation of genes encoding enzymes involved in glycerol production and active glycerol uptake and down-regulation of genes encoding proteins related to growth and biomass formation, such as ribosomal proteins, translation factors, and glycolytic enzymes (45). Only some of these genes followed similar changes in epimastigotes submitted to hyperosmotic stress. For example, the gene encoding for glycerol-3-phosphate dehydrogenase (Tc00.1047053511151.90) slightly decreased expression, whereas genes encoding ribosomal proteins had increased expression after hyperosmotic stress (clusters 2, 3 and 5). Conversely, genes encoding glycolytic enzymes such as glyceraldehyde 3-phosphate dehydrogenase (Tc00.1047053506943.50 and Tc00.1047053509065.70), translation factors (translation elongation factor 1β, Tc00.1047053509733.100, and eukaryotic translation initiation factor 5, Tc00.1047053504119.10), and metabolic enzymes (clusters 4, 6, and 7) decreased their expression levels. In addition, hyperosmotic stress in epimastigotes resulted in up-regulation of genes encoding flagellar proteins (cluster 5), as well as trans-sialidase-like proteins (clusters 2 and 3). These observations are compatible with changes that occur during the metacyclogenesis process. For example, trans-sialidases are enzymes expressed during the late stationary phase of epimastigotes before their transformation into metacyclic trypanosomes (28). The results suggest that the hyperosmotic conditions present in the intestine of the vector (1) could be conducive for metacyclogenesis.

In conclusion, the response of epimastigotes to hyperosmotic stress is different from that observed in mammalian cells or yeasts. An aquaporin and the contractile vacuole are involved in water efflux leading to cell shrinkage, and there is little regulatory volume increase. The results suggest that the increase in ionic strength is counteracted by the early synthesis of polyP and sequestration of inorganic ions in acidocalcisomes. Amino acids are the compatible osmolytes that replace the inorganic ions sequestered in acidocalcisomes, and they initially accumulate by a reduction in their catabolism and then later by protein degradation and uptake through induced amino acid transporters. The results also indicate that despite the lack of gene regulation at the level of transcription initiation, there is an extensive regulation of mRNA abundance associated with exposure of epimastigotes to different environments. T. cruzi reveals a notable plasticity in its adaptation to different environments, and transcript abundance appears to be an important level of gene expression regulation.

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Transcriptome of T. cruzi under Hyperosmotic Stress

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