A DnaJ-like Protein Homologous to the Yeast Co-chaperone Sis1 (TcJ6p) Is Involved in Initiation of Translation in Trypanosoma cruzi*

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In eukaryotes, proteins homologous to the bacterial DnaJ protein are involved in regulation of the Hsp70 molecular chaperones, which are implicated in a variety of protein biogenesis pathways. We report here the molecular characterization of a T. cruzi DnaJ gene, termed TcJ6, encoding a protein that displays high sequence homology with the Saccharomyces cerevisiae Sis1 co-chaperone required for the initiation of translation. TcJ6 protein was expressed as a polypeptide of 36.5 kDa at a constant level during parasite differentiation and was associated to the cytoplasmic fraction. We showed that overexpression of TcJ6 complemented a temperature-sensitive yeast sis1 mutant. In addition, sucrose gradient sedimentation analysis of polysomes from T. cruzi and a yeast mutant overexpressing TcJ6p showed that the trypanosomal co-chaperone was closely associated with ribosomal subunits, 80 S monosomes and the smaller polysomes, as observed for Sis1p. Furthermore, in T. cruzi TcJ6p was also found to be preferentially concentrated around the nucleus, giving a speckled staining pattern. This suggests that TcJ6p is associated with the endoplasmic reticulum. Taken together, these data suggest that the trypanosomal DnaJ is involved in initiation of translation.

In Escherichia coli the DnaK, DnaJ, and GrpE genes encode heat shock proteins that are essential for growth at temperatures above 42 °C (reviewed in Ref. 1). DnaJ stimulates the weak intrinsic ATPase activity of DnaK (2) and can also directly interact with specific substrates of the chaperone machinery (3). Various DnaJ homologs have been discovered in eukaryotic cells. Eukaryotic DnaJ-like proteins belong to the conserved heat shock protein 40 (Hsp40) family and are involved in regulation of the Hsp70 molecular chaperones (DnaK-like proteins), mediating the biogenesis of proteins. Some Hsp40s may be considered to be true molecular chaperones in that they prevent aggregation by binding directly to unfolded polypeptide substrates (4, 5). All DnaJ-like proteins contain a J domain of about 70 amino acids, generally N-terminal, which represents the signature of the family (6) and is required for interaction with the ATPase domain of Hsp70 (7). The basic mechanism of action of the Hsp70 proteins is sequential binding and release in an ATP-dependent manner of polypeptides in non-native conformations (protein folding). The subcellular compartmentalization of different Hsp70 members and their specific interactions with various DnaJ-like proteins allows these chaperones to be involved in a variety of protein biogenesis pathways including the assembly and disassembly of protein complexes, proteolysis, the translocation of proteins into organelles and translation initiation (reviewed in Ref. 8). The Saccharomyces cerevisiae genome data base (genome-www.stanford.edu/Saccharomyces) contains 20 putative Hsp40s homologous to E. coli DnaJ, but only half have been characterized and localized to major cellular compartments (4, 8, 9). The Hsp40 family has been divided into three distinct subgroups based on the presence of conserved functional domains in addition to the J domain (7, 9). Class I Hsp40s have a glycine-phenylalanine-rich (G-F) region followed by a cysteine-rich region (CRR domain), which forms a zinc finger motif with four repeats of a CXXCXXGXG motif and a weakly conserved C-terminal domain. The archetypal E. coli DnaJ and the S. cerevisiae Ydj1 protein are class I Hsp40s. Class II Hsp40s include the S. cerevisiae Sis1 protein and its mammalian homolog, the co-chaperone Hdj1, and lack the CRR domain. Finally, in class III Hsp40s, only the conserved J domain is present. The J domain is thought to be involved in interactions with Hsp70s via its HPD tripeptide loop (10, 11), whereas the G-F region is a critical determinant required for the specificity of S. cerevisiae Sis1p (9). In class I Hsp40s, the zinc finger motif and the poorly conserved C-terminal domain have been shown to act as binding sites for proteins in denatured state (12, 13). It has also been suggested that the J domain and G-F region may be sufficient for the basic functions of class I and class II Hsp40s in vivo, whereas the distal C-terminal regions are important in yeast in suboptimal growth conditions (9).

The unicellular protozoan, Trypanosoma cruzi, the etiological agent of Chagas’ disease, has a complex life cycle where the parasite passes through three differentiation forms and two hosts, a reduviid insect vector (triatomine) and a mammalian host. A host-specific phase of differentiation has been described and involves the expression of a parasite-specific chaperone, the DnaJ homolog TcJ6p, which is involved in initiation of translation. In this study, we describe the molecular characterization of the TcJ6p gene and its expression during the parasite differentiation. The TcJ6p protein was shown to be associated with the endoplasmic reticulum and to be involved in initiation of translation.
Like family. The yeast Sis1p is the only class II Hsp40 protein that has been functionally characterized. This protein is required for the initiation of translation in *S. cerevisiae* (25). These observations suggested that the Sis1 and TcJ6 proteins might have similar functions. In this study, we used heterologous functional complementation and polysome sucrose gradient sedimentation to demonstrate that TcJ6p has a function similar to that of the Sis1 co-chaperone, which is essential for translation initiation in yeast.

**EXPERIMENTAL PROCEDURES**

**Growth and Differentiation of Parasites and Isolation of Nucleic Acids**

*T. cruzi* clone Dm28c (26) epimastigotes were cultured in liver infusion tryptose medium (27). In *vitro* metacyclogenesis of *T. cruzi* (Dm28c) was performed under chemically defined conditions as previously described (15). Recovering of parasites at different stages of *in vitro* differentiation was carried out as previously described (28). Total parasite DNA was extracted as described elsewhere (29). Total parasite RNA was prepared after the LiCl-urea method as previously described (30).

**Strains**

*E. coli* strains XL1-Blue and TOP10F* were used for plasmid construction. *S. cerevisiae* strain FY883 (MATa, his3Δ200, ura3-52, leu2Δ213, lys2Δ202, trp1Δ63, GAL2*+) was used. Complementation of Sis1 function by TcJ6 protein was carried out on the control strain CY376 expressing wild type Sis1p and on the temperature-sensitive strain CY372, carrying a *sis1*-85 allele. The genotypes of the strains are CY736 (MATa, ura3-1, leu2-3, 112, his3-11, 15, trp1-1 ade-1 ade2-1 ssd1-d2 can1-100 ∆sis1::HIS3 (SIS1 on LEU2/CEN plasmid)) and CY732 (MATa, ura3-1, leu2-3, 112, his3-11, 15, trp1-1 ade-1 ade2-1 ssd1-d2 can1-100 ∆sis1::HIS3 (NH₃-hemagglutinin-tagged *sis1*-85 on LEU2/CEN plasmid)). These strains were kindly donated by Dr. Kim T. Arndt (31).

**Media**

Rich media contained 1% yeast extract, 2% bacto-peptone, and 2% glucose or 2% galactose. Synthetic media contained 0.67% yeast nitrogen base without amino acids, 2% glucose, or 2% galactose and the supplements to satisfy auxotrophic requirements.

**Gene Cloning and Genomic Mapping**

A densely arrayed *T. cruzi* Dm28c AEMBL3 Sau3A genomic library (29) was screened at high stringency with a probe corresponding to the 5′ conserved region of a gene related to the sialidase family. The cDNA probe was obtained by reverse transcriptase polymerase chain reaction (RT-PCR). A reverse oligonucleotide, 5′-CCCTAAAGCATTCTCAGC-3′, corresponding to the 5′-conserved region of a sialidase-like gene was used as a primer using the Superscript II enzyme (Life Technologies, Inc.) as indicated by the manufacturer, on total RNA from the parasites pretreated by RQ1 RNase-free DNase (Promega). The first-strand cDNA was subsequently amplified by PCR according to the manufacturer’s instructions (Life Technologies) using a forward miniexon oligonucleotide BamH1 (5′-GGGCGGATCCGACAGTTTGATCTGATCG-3′) (BamH1 site is underlined) and the reverse oligonucleotide with the following cycle parameters: 94°C, 30 s; 52°C, 30 s; 72°C, 1 min for 3 cycles and 94°C, 30 s; 52°C, 30 s; 72°C, 1 min for 30 cycles. The purified PCR product was cloned and sequenced (GenBank24 data base accession number YA017371). All PCR reactions were performed on a PerkinElmer DNA Thermal Cycler (GenAmp PCR System 9600), and PCR products were separated in an agarose gel, purified on glass beads (Gene-clean, Bio 101) or directly purified on QIAquick column (Qiagen, Inc.), and subcloned into PCR2.1 or PCR-Blunt vectors (Invitrogen). Sequencing was performed manually using the Thermosequenase sequencing kit (Amersham Pharmacia Biotech) with [α-32P]dNTPs (1500 Ci/mmol) as terminators, according to the manufacturer’s instructions. Sequences were determined by sequencing on both strands of DNA. From 200,000 recombinant phages hybridized with the cDNA probe, 14 phages were isolated after three consecutive screenings. Analysis of DNA inserts of each phage were characterized by restriction enzyme mapping and by Southern blot hybridization with the same probe. Two types of recombinants were found based on SsuI digestion pattern, and two of them, designed, respectively, cI263g (14.3 kb) and cI381 g (19.8 kb) were chosen for hybridization analysis. Positive bands of different sizes were subcloned from phages cI263g and...
c3831g in bacteriophage M13 (mp18 and mp19) or in plasmid pBlue-Script SK+ (Stratagene, La Jolla, CA) to be sequenced. The Gen-Bank™ data base accession number of a 4.3-kb SalI cl263g genomic subclone containing the sialidase pseudogene, TcJ6, and 3′-coding region of Rob1 homolog is AF254536. The relative position of DNA fragments in the phage subclone 4.3-kb cl263g genomic DNA was confirmed by sequencing extremities of each cloned fragment by PCR. The complete genetic map of cl263g and the relative positions of adenylate cyclase and small G-protein YPT7 homolog genes were determined with probes derived from the corresponding genes contained in the CosTer1 cosmid of the CL strain, kindly donated by Dr. F. Bringuier and T. Baltz (18). The ends of these clones were identified by SDFI digestion, transfection, and hybridization. The ORFs were confirmed by sequencing the 5′- and 3′-flanking regions of the 4.3-kb SalI fragment of cl263g. The 3′-splice site of TcJ6 transcript was determined by RT-PCR using the forward mini-exon oligonucleotide BamHI and a reverse oligonucleotide specific to the 5′-end of TcJ6 gene, 5′-CCGGAGTATACTTATCAAGGCGC-3′. The polymerase addition site of TcJ6 transcript was determined by RT-PCR using a forward oligonucleotide BamHI and a reverse oligo-GATCC(T)18-3′ oligonucleotide, 5′-GGCGGGC-GATCC(T)18-3′ (the BamHI site is underlined). The cycle conditions of both RT-PCRs were identical as described above.

**DNA and RNA Analysis**

The procedures employed for Southern and Northern blot hybridizations are described elsewhere (30, 32). Nucleic acids were blotted onto Hybond-N Extra membranes (Amersham Pharmacia Biotech) and fixed following the manufacturer’s instructions. Probes were labeled by nick translation (Amersham Pharmacia Biotech) or random priming (Roche Molecular Biochemicals) using [α-32P]dCTP (3000 Ci/mmol) (Amersham Pharmacia Biotech) and purified through a Sephadex A-25 column. A small subunit ribosomal RNA probe used as a loading control in Southern analysis was obtained using the forward oligonucleotide, 5′-GGCAACAGGAGCCATCCGCAAGAG-3′, and the reverse oligonucleotide, 5′-CCCTATCTTTCATTTTTTGTCACTGTTG-3′, as primers for RT-PCR on 5 µg of T. cruzi total RNA (33). The conditions for RT-PCR reaction were identical, as described above but using the following cycle parameters: 94 °C, 30 s; 55 °C, 30 s; 72 °C, 1 min for 30 cycles. The purified PCR fragment of 168 base pairs was cloned and sequenced. The α-tubulin probe was obtained from an incomplete cDNA of 1.3 kb sub-cloned in M13, kindly provided by Dr. Mónica Carreira (Instituto Oswaldo Cruz, Departamento de Bioquímica e Biologia Molecular). Sequence analysis was performed with the GCG/Wisconsin software (34).

**Pulsed-field Gel Electrophoresis**

Chromosome separation (2 × 10^7 parasites/slot) was performed using the Pulsaphor system (Amersham Pharmacia Biotech) with 1.2% agarose/0.25% EDTA electrophoresis buffer. The electrophoretic conditions were as follows: pulses of 90 s at 50 V for 1 h followed by pulses of 90 s at 100 V for 1 h and at 150 V with a pulse time of 90 s ramped to 150 s at 4 °C for 50 h.

**Expression of a Truncated TcJ6 Protein in E. coli and Production of Polyclonal Antibsera**

To obtain a truncated form of TcJ6 E. coli recombinant protein (TcJ6Jp), a BamHI site was created downstream of the N-terminal J domain to insert the TcJ6 ORF lacking the first 74 amino acids into the E. coli expression vector PQE8 (Qiagen, Inc.). The truncated ORF was amplified by PCR using a Vent DNA polymerase (New England Biolabs) on a genomic template using the forward oligonucleotide, 5′-AGG-GATCCAAAGGGCCGCTTCCCG-3′ (where the BamHI site, underlined, corresponds to position 217 of TcJ6 gene), and the reverse oligonucleotide, 5′-GGATCCAGCGCGCTTATCGACATCTGCG-3′, where the HindIII site was created immediately upstream of stop codon (the HindIII site and the stop codon are underlined). After PCR amplification and treatment by a Tq2 polymerase to add 3′ A-overhangs, the PCR fragment of 827 base pairs was subcloned into PCR2.1. The BamHI/HindIII fragment was subcloned into BamHI/HindIII-digested pQEs. The fusion construct was sequenced using flanking vector sequences to ensure that the sequence derived from TcJ6 gene was in the correct orientation and in-frame with the 6×His tag. Expression of the recombinant protein was induced in E. coli strain M15, and cell lysates were prepared and analyzed by SDS-PAGE. The fusion product was purified on a Ni²⁺-nitrilotriacetic acid affinity column according to the manufacturer’s instructions (Qiagen, Inc.). Polyclonal anti-TcJ6Jp antibodies were raised in rabbits immunized three times at 4-week intervals with 150 µg of pure recombinant protein. Antibody depletion on yeast lysate was performed by serial incubations with yeast acetone powder. The IgGs were purified on protein A-Sepharose CL-4B (Amersham Pharmacia Biotech) and used for indirect immunofluorescence.

**Preparation of Cellular Extracts and Immunoblotting**

Yeast protein extracts were prepared as previously described (35). Total protein extracts of different stages of T. cruzi were prepared by washing the cells twice with PBS and resuspending them in 1× Laemmli buffer (36) to a final concentration of 1 × 10⁷ cells/ml. Proteins were resolved by electrophoresis using 10% SDS-PAGE (37) and blotted onto Hybond-C Extra membranes (Amersham Pharmacia Biotech) following standard procedures. Cytosolic extracts of T. cruzi were prepared as described (37). Blots were probed with anti-TcJ6Jp polyclonal antibody (1/10,000 dilution) and a secondary alkaline phosphatase-conjugated antibody (1/7,500 dilution; Promega) following the manufacturer’s instructions (Promega).

**Conditional Expression of TcJ6**

Two different plasmids containing the GAL1-inducible promoter were used. A centromeric plasmid pRN93, termed YCP for yeast centromeric plasmid, a gift from C. W. Slayman from Yale University and a high-copy vector pYES2, termed YEP for yeast episomal plasmid, from Invitrogen. Complete TcJ6 ORF was amplified by PCR on genomic template using a forward primer with a HindIII and BamHI restriction sites immediately upstream of TcJ6 ATG, 5′-GGGAAGCTTGGATCTTGTTGAAAACATGGGC-3′ (the HindIII and BamHI sites and TcJ6 ATG are underlined), and a reverse primer with a ScaI restriction site at position +11 of TcJ6 stop codon, 5′-GGAAGCTTGGTTTAAACAAGTTTACCC-3′ (ScaI site and stop codon are underlined). 1-kb HindIII/SacI and BamHI/SacI DNA fragments were subcloned, respectively, into HindIII/SacI-digested pYES2 and BamHI/SacI-digested pRN93 vectors. Yeast were transformed with the resulting plasmids by the lithium acetate method (38). Total yeast RNA was isolated as described elsewhere (39).

**Polysome Analysis**

In Yeast—Cells overexpressing TcJ6p were grown to mid-logarithmic phase in minimal medium. 15 min before harvesting the cells, cycloheximide was added to a final concentration of 60 µg/ml, and the polysome profile was analyzed in a 15–45% sucrose gradient as reported (40). In T. cruzi—10⁷ cells were incubated for 5 min at 28 °C in liver infusion tryptose medium containing 100 µg/ml cycloheximide. After centrifugation, the parasites were chilled and washed twice with ice-cold washing buffer (140 mM NaCl, 5 mM KCl, 2 mM MgCl₂, 10 mM Hepes, pH 7.4, 100 µg/ml cycloheximide, and 10 units/ml heparin) (41). The cells were lysed according to Brecht and Parsons (37) using the buffer system A and the preparation of cytoplasmic extracts was carried out as reported. The polysome profile was analyzed in a linear 15–50% sucrose gradient. After centrifugation, the gradients were harvested from the bottom using a pump system coupled to a fraction collector (Amersham Pharmacia Biotech) with a flow rate of 1.1 ml/min and a fraction size of 400 µl. RNA isolation was carried out as reported (37). Protein fractions were precipitated by 12.5% trichloroacetic acid at 20 °C, and washed twice with acetone, resuspended in Laemmli buffer (36), and analyzed after SDS-PAGE by immunoblotting.

**Immunofluorescence Microscopy**—Parasites were harvested by centrifugation and washed twice in PBS. Cells (5 × 10⁷ ml⁻¹) were fixed by the addition of an equal volume of formaldehyde in PBS/0.1% IV and incubated at room temperature for 30 min. The fixed cells were washed twice with PBS and allowed to adhere to poly-L-lysine-coated glass coverslips. After blocking the cells with 3% bovine serum albumin, 5% milk, and methylamine 0.1% in PBS for 1 h at room temperature, the parasites were incubated with 4 µg/ml polyclonal anti-TcJ6Jp IgG in PBS containing 1% milk and 0.05% Tween 20 for 2 h. The cells were washed with PBS and incubated with fluorescein isothiocyanate-labeled anti-rabbit IgG secondary antibody (1/250 dilution, Sigma) and propidium iodide (10 µg/ml) in the same buffer as used for primary antibodies. The coverslips were washed extensively, resuspended in anti-quench solution, and viewed and photographed using a laser confocal microscope equipped with a digital in situ imaging system.

**RESULTS**

**Genomic Characterization of TcJ6**—A sequence derived from a 291-bp RT-PCR clone corresponding to the 5′-conserved re
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FIG. 1. Southern analysis of the TcJ6 gene. Panel A, restriction map and genomic organization of the genes flanking the gene TcJ6 in a 14.3-kb Sall fragment cloned from a genomic Sau3A λEMBL3 T. cruzi library (cl263g). B, BamHI; E, EcoRI; HI; HindIII; HI; HincII; H, HaeIII; P, PstI; Sac, SacII; Sal, SalI; S, Smal. Panel B, TcJ6 gene organization. T. cruzi (Dm28c) genomic DNA was digested with BamHI (B), EcoRI (E), HaeIII (Hae), HindIII (HII), HindIII (HI), PstI (P), SalI (S), Stul (Stu), Stul + HindIII (Stu + Hae), Stul + HindIII (Stu + HII), and XbaI (Xba) and analyzed by Southern blot hybridization with a sialidase pseudogene cDNA probe (probe A) and a TcJ6 genomic probe (Stu1-HindIII, 0.89 kb, probe B), represented by a solid bar under the map in panel A. Panel C, chromosomal location of the TcJ6 gene and sialidase pseudogene (ψ-sialidase) in three different T. cruzi strains. T. cruzi chromosomes from the strain CL, Y, and Dm28c (Dm) were separated by pulsed-field gradient gel electrophoresis and analyzed by Southern blot hybridization with a sialidase pseudogene cDNA probe (SacII-Smal, 0.94 kb, probe C) and a TcJ6 genomic probe (Stu1-HindIII, 0.89 kb, probe B), represented by a solid bar under the map in panel A. S. cerevisiae chromosomes were included as size standards (molecular mass in megabases).

TABLE I

Percentage of amino acid sequence similarity among DnaJ-like proteins from T. cruzi

<table>
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<tr>
<th></th>
<th>TcJ1</th>
<th>TcJ2</th>
<th>TcJ3</th>
<th>TcJ4</th>
<th>TcD1</th>
<th>Tc6</th>
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<td></td>
<td>45.7</td>
<td>21.3</td>
<td>39.6</td>
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<tr>
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<td>56.6</td>
<td>43.7</td>
<td>45.7</td>
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<tr>
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<td>45.7</td>
<td>25.3</td>
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<td>21.2</td>
</tr>
<tr>
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<td>24.1</td>
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<td>25.3</td>
<td>35.2</td>
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<td>39.6</td>
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The highest DnaJ pair homologies are in boldface (percentage similarity ≥40%).

gion of a gene related to the sialidase family (24) was used as a probe to screen a phage genomic library of T. cruzi. Two types of recombinant phage were found based on the Sall digestion pattern and contained 14.3- and 19.8-kb inserts; two of them designed, respectively, cl263g and cl381g, were selected for further analysis. The genomic clone cl263g was partially sequenced (Fig. 1A). Analysis of the complete nucleotide sequence of the sialidase-like gene, with sequence similarity extending over about 1 kb (boxed in Fig. 1A), revealed that this gene was interrupted by multiple stop codons in each frame and contained a small ORF (196 nucleotides). This ORF encodes a putative 66-amino acid polypeptide presenting similarities to yeast YPT7 (Fig. 1A). Thus, the 14.3-kb genomic fragment is highly homologous to the duplicated region flanking the glucose transporter gene cluster and may therefore be an alternative genomic rearrangement of the downstream-flanking region of the THT gene cluster in T. cruzi. The nucleotide sequence of the TcJ6 gene predicts an open reading frame of 1014 base pairs encoding a putative protein of 338 amino acids with a molecular mass of 36.5 kDa. The 3’ splice site and the poly(A) addition site were determined by RT-PCR using a 5’ reverse internal primer with a mini-exon-derived primer and a 3’ forward internal primer with a oligo-dT primer, respectively. This analysis predicted a small 5’-untranslated region (UTR) of 20 nucleotides and a 3’-UTR of 191 nucleotides. Southern blotting with a restriction enzyme cutting outside the gene (PstI (P) in Fig. 1B) was used to determine the number of copies of the TcJ6 gene in the genome. Several copies were detected using a
probe spanning the entire ORF. We investigated whether these members of the new DnaJ-like subfamily of genes were, like other T. cruzi genes encoding Hsps, organized as direct tandem repeats by digestion with two restriction enzymes, one with a single site in the 5′ extremity of the ORF (StuI) and one with a single site in the 3′ extremity of the ORF (HincII). Low stringency hybridization of T. cruzi genomic DNA digested with StuI alone or with HincII alone to a probe covering the entire ORF gave no hybridizing fragment common to both digests. This suggests that the various copies of the gene encoding the TcJ6 protein are not organized in tandem repeats but are instead spread throughout the genome of T. cruzi. Southern blot hybridization of a pulse-field gel of three strains of T. cruzi showed that the gene copies encoding TcJ6p are located on a chromosome or a pair of homologous chromosomes of ~1,300 kb (Fig. 1C). In addition, probes for the sialidase pseudogene (Fig. 1C), Rab1, and TcrHT1 (data not shown), labeled the same set of chromosomes. These results suggest that, despite the difference in chromosome size between the various strains of T. cruzi, the TcJ6 gene copies are present on a subset of chromosomes of the megabase range that is conserved between various strains of T. cruzi. Altogether these data are consistent with the study by Bringaud et al. (18), showing the existence of various duplications of a large area characteristic of the 3′-end of the TcrHT1 gene cluster.

TcJ6 Co-chaperone Homologous to Yeast Sis1p—Data base searches showed similarity between TcJ6 and E. coli DnaJ proteins (60% similarity for the first 76 amino acids, which define the J domain), and only 21−40% similarity sequence to the other five members of the T. cruzi family (tcj1-4 and TcDJ1) (Table I) (16, 17). TcJ6p was most similar (~40% similarity) to the putative cytosolic DnaJ protein tcj3. Similar characteristics can be observed for tcj1 and the putative mitochondrial TcDJ1, two class III Hsp40 members from the T. cruzi family lacking the GF domain and the zinc finger region. Surprisingly, the TcJ6 protein displays its highest score for sequence similarity with two eukaryotic class II Hsp40s, 54% similarity to a C. elegans hypothetical DnaJ-like protein (F54D5.8) and 50% similarity to the yeast Sis1p protein, which is required for normal initiation of translation in S. cerevisiae (Fig. 2) (25, 31). Particularly well conserved were the N-terminal sequence containing the highly conserved 70-amino acid J domain and its HPD tripeptide motif (boxed in Fig. 2) and the glycine-phenylalanine-rich “spacer” region of about 50 amino acids (G-F region), which is not followed by the zinc finger motif and is therefore a typical class II Hsp40. In the middle third of the sequence, Sis1p contains a striking glycine/methionine-rich sequence (G-M region), which in the case of TcJ6p, is replaced by a glycine-rich sequence (with a conservation of almost all the glycine residues of Sis1p). In a previous study (9), chimeric fusion proteins in which the various functional domains (J, G-F, G-M, C terminus) had been exchanged between the two yeast cytosolic proteins Sis1p and Ydj1p were used in experiments to rescue the Δsis1 yeast mutant. The G-F region was found to be essential for the function of Sis1p and redundant to the function of the G-M and C-terminal regions. These structural features suggest that the trypanosomal DnaJ may have a function similar to that of yeast Sis1p. In addition, analysis of the predicted amino acid sequence of TcJ6p suggests that, like Sis1p, the trypanosomal co-chaperone is cytosolic. Indeed, its hydrophilic character and the absence of both a detectable N-terminal putative peptide signal and a C-terminal CAAX motif (substrate for prenyl modification of some DnaJ-like proteins, which allows association with cell membranes) suggest that this protein is probably cytosolic.

TcJ6 Is Constitutively Expressed during the Parasite Development—The expression pattern of TcJ6 was determined during the in vitro metacyclogenesis of T. cruzi by both Northern blot analysis and immunoblotting (Fig. 3). Total RNA was extracted at various stages of the in vitro differentiation of T. cruzi epimastigotes and analyzed with probes for the TcJ6 gene and the nuclear gene encoding the small subunit ribosomal RNA, as a loading control for constitutively expressed genes (33). Analysis of the relative intensities of the bands corre-
sponding to the TcJ6 transcript and small subunit ribosomal RNA (ssrRNA) showed that the amount of the 1.4-kb mRNA increased during in vitro metacyclogenesis, reaching a maximum in metacyclic forms (Met in Fig. 3A and arrow). Similar results were obtained by semi-quantitative RT-PCR (data not shown). A larger RNA species (the asterisk in Fig. 3A) was also detected (sensitive to RNaseA-DNase-free, data not shown), reaching a maximum 24 h after the start of the differentiation.

Fig. 3. Analysis of the expression pattern of TcJ6. T. cruzi epimastigotes exponentially growing in complete medium (Epi) were subjected to a nutritional stress for 1 h (Str) and then allowed to differentiate into infectious metacyclic cells (Met). 6 and 24 h after the start of in vitro differentiation (6H, 24H), most of the parasites had adhered to the plastic culture flask, and the process was complete within 96 h (28). Panel A, regulated expression of TcJ6 mRNA during metacyclogenesis in vitro and during a heat shock. 10 μg of total RNA from each differentiation stage of T. cruzi were analyzed by Northern blotting with a TcJ6 genomic probe (StuI-HincII, 0.89 kb, probe B in Fig. 1, panel A) or a nuclear small subunit ribosomal RNA (ssrRNA) cDNA fragment. A similar Northern blot analysis was carried out using 10 μg of total RNA from epimastigotes subjected to a heat shock at 37 °C for 6 and 24 h (6H and 24 H). The arrow indicates the TcJ6 mRNA, and the asterisk indicates the putative polycistronic transcript of high molecular mass. Panel B, Western blot analysis of the TcJ6 protein expression during metacyclogenesis in vitro and heat shock at 37 °C and comparison with the recombinant expression of TcJ6ΔJp in E. coli (ΔJ represents the difference of molecular mass due to the J domain between the truncated recombinant protein and the trypanosomal DnaJ). Total protein was extracted from T. cruzi during metacyclogenesis in vitro (5 × 10⁶ cells/lane), separated by SDS-PAGE in a 13% acrylamide gel, electroblotted, and probed with an anti-TcJ6ΔJp rabbit polyclonal antibody (anti-TcJ6p) or a preimmune serum (PI). Panel C, cross-reactivity of the anti-TcJ6ΔJp antibody with other species. Cell extracts from L. major promastigotes (Lm, 10⁷ cells, CC1 strain), T. b. brucei bloodstream forms (Tbb, 10⁷ cells, AnTat1.1 clone), T. cruzi epimastigotes (Tc, 10⁷ cells, Dm28c clone), and cytoplasmic (Cyt) and membrane (Mb) fractions of T. cruzi epimastigotes (equivalent to 5 × 10⁶ cells) were separated by SDS-PAGE in a 13% acrylamide gel and immunoblotted with anti-TcJ6ΔJp antibody (anti-TcJ6p) or preimmune serum (PI).
DNA probes corresponding to the flanking genes (adenylate cyclase, *Rab1*) recognized apart from their individual mRNAs the same high molecular weight transcripts, hence suggesting that this RNA is polycistronic (data not shown). The unusual detection of polygenic transcripts might result from the inhibition of processing of primary transcripts due to the stress treatment (acidic and nutritional) required for *in vitro* differentiation of *T. cruzi*, as already described for the tubulin unit of heat-shocked *T. brucei* cells in which were accumulated high molecular weight mRNA precursors containing both the α- and β-tubulin-coding region (43). Epimastigotes subjected to a heat shock at 37 °C for 6 h (6F in Fig. 3A) accumulated about 2–3 times more mRNA than did untreated epimastigotes (28 °C). This probably reflects an increase in stability of the co-chaperone mRNA during heat shock, as reported for other trypanosomids (44).

To analyze protein expression during parasite differentiation, polyclonal rabbit antibodies were raised against a recombinant His-tagged TcJ6p lacking its J domain (ΔJ in Fig. 3B) to prevent the cross-reactivity with other members of the TcDnaJ family. In Western blot analysis of T. cruzi cell extracts, this antiserum recognized a polypeptide with an apparent molecular mass of 36.5 kDa, which corresponds to the molecular mass predicted from analysis of the primary polypeptide sequence (Fig. 3B). This suggests that this protein is unmodified (not prenylated nor glycosylated). No major changes in protein level were observed during differentiation *in vitro*, and only a modest reduction was observed in metacyclic forms. Thus, the increase in abundance of steady-state RNA observed in metacyclic cells was not concomitant with an increase in the amount of protein. Heat shock at 37 °C for 6–24 h increased the amount of TcJ6 protein by about 2-fold, possibly due to a delay in translation of the mRNA that accumulated during the first 6 h of heat shock.

The antiserum detected a protein of similar electrophoretic mobility in other trypanosomatid species (*T. b. brucei, Leishmania major*) (Fig. 3C). The increase in molecular weight as observed in *L. donovani* against 338 and 336 amino acids, respectively, in *T. cruzi* and *T. b. brucei*, (18). Therefore, the cross-reactivity of the anti-TcJ6ΔJp antiserum with the DnaJ of other trypanosomatids species (*T. b. brucei, L. major*) corroborates a high epitope conservation of the DnaJ-like protein among these species, which share with TcJ6p more than 81% sequence similarity (18). In contrast, in yeast, although some short stretches of peptide sequence are found conserved between Sis1p and TcJ6p (Fig. 2), the anti-TcJ6ΔJp antiserum did not react with the protein immunoprecipitated Sis1p (data not shown). Cytoplasmic and membrane fractions of *T. cruzi* epimastigotes were probed with antiserum against TcJ6p, and it was found that the protein was associated exclusively to the cytosolic fraction (Cyt in Fig. 3C). Taken together these results demonstrate that the highly conserved trypanosomosal cytosolic co-chaperone is constitutively expressed during metacyclogenesis *in vitro*.

**Fig. 5. Functional complementation in *S. cerevisiae*.** Panels A and B, growth phenotypes of wild-type (wt) strain CY736, *sis1-85* strain (CY732), and CY732 transformed with insert-less vectors (CY732-YCP, CY732-YEP) or with vectors carrying the TcJ6 gene (CY732-YCPTcJ6, CY732-YEPtCJ6). The strains were streaked onto YPgal (1% yeast extract, 2% bacto-peptone, and 2% galactose) plates and grown for 4 days at 30 °C or 39 °C. Panel C and D, growth curves of the strains used in panels A and B. YPgal medium was inoculated with the various strains, which were grown at 30 °C (solid line and closed symbols) or 38 °C (dotted line and open symbols); growth was recorded by measurement of the absorbance at 600 nm. Strains in panel C were CY736 (open and closed circles), CY732-YEP (open and closed triangles), CY732-YEPtCJ6 (open and closed squares) and in panel D were CY732-YCP (open and closed circles), CY732-YCPTcJ6 (open and closed inverted triangles). The asterisk indicates that the cells stop growing.
A T. cruzi DnaJ-like Protein Involved in Initiation of Translation

but not in the presence of glucose (Fig. 4B). The fact that heterologous protein migrated in SDS-PAGE with an apparent molecular mass similar to the endogenous T. cruzi DnaJ (Tc in Fig. 4B) suggested that TcJ6 protein is properly expressed in S. cerevisiae. We have therefore investigated whether the expression of TcJ6p could abolish the temperature-sensitive phenotype of the sis1-85 mutant (CY732 strain). As shown in Fig. 5A, at the permissive temperature (30 °C) in the presence of galactose, the growth phenotype was the same for all strains, except for the sis1-85 strain transformed with YEPTcJ6, which grew more slowly (small colonies). At the nonpermissive temperature (39 °C, Fig. 6B) in the presence of galactose, sis1-85 cells did not grow, but when they expressed TcJ6p, they displayed the same slow-growth phenotype as the control strain expressing the wild-type SIS1. Thus, the heterologous expression of the trypanosomal DnaJ gene complemented the sis1-85 strain at nonpermissive temperatures. The accentuated slow-growth phenotype of the mutant strain overproducing the TcJ6 protein (CY732-YEPTcJ6) grown in galactose reached a plateau at a level about half that of the wild-type strain (CY736), expressing SIS1 or the sis1-85 mutant (CY732-YEP) transformed with the insert-less vector (Fig. 5C). This confirms that overexpression of TcJ6 protein limits yeast growth. As expected, at the nonpermissive temperature, sis1-85 mutant transformed with the insert-less vector did not grow (the asterisk in Fig. 5C). The growth of CY732 strain transformed with a low copy number plasmid carrying TcJ6 (CY732-YCPTcJ6 in Fig. 5D) was absolutely normal at 30 °C and similar to that of strain CY732 transformed with the insert-less plasmid (comparable with the wild-type, CY736, in Fig. 5C). At a restrictive temperature, CY732-YCPTcJ6 grew slowly. The adaptation period (lag phase) was longer in these cells expressing low levels of TcJ6p than in cells overexpressing the co-chaperone from a multicopy plasmid. This suggests that complementation depends on the number of copies of TcJ6, with excessive expression limiting growth.

Close Association of TcJ6p with Ribosomal Subunits, 80 S Monosomes, and, the Smaller Polysomes—In S. cerevisiae, a large fraction of Sis1p is associated with 40 S ribosomal subunits, 80 S initiation complexes, and smaller polysomes. At nonpermissive temperatures, the sis1-85 mutant rapidly accumulates high levels of 80 S ribosomes, and the amount of polysomes decreases. These data indicate that the Sis1 co-chaperone is required for the normal initiation of translation (25). The complementation by TcJ6 of a SIS1 mutant suggests that the trypanosomal protein may also be associated with ribosomes. We investigated this possibility by sedimentation on a sucrose density gradient of cytoplasmic extracts of yeast cells overproducing the heterologous protein. Western blot analysis showed that, according to our fractionation procedure, the cytoplasmic trypanosomal protein sedimented throughout most of the gradient, including positions corresponding to ribosomal subunits, 80 S monosomes, and the smaller polysomes (Fig. 6A). However, a great proportion of the protein remained at the top of the sucrose gradient (T in Fig. 6A), which contains free cytosolic proteins. This may be due either to the presence of a large excess of overexpressed protein competing for binding factors required by the translation machinery (e.g., certain mammalian-like Hsp70 proteins containing RNA-binding sites that might be involved in the regulation of translation (45)) or to the trypanosomal co-chaperone having other physiological functions in the cytosol, such as a role in the regulation of protein degradation catalyzed by proteases through its chaperone activity, as has been demonstrated for Sis1p (46).

In T. cruzi, the cosedimentation of TcJ6 protein with ribosomes/small polysomes depended on the in vitro growth conditions. Indeed, the polysome profile of trypanosomes is developmentally regulated. In proliferating cells (epimastigotes in logarithmic growth phase) the secondary polysome peak was more pronounced in the gradient because of the dense loading of mRNA with ribosomes (Fig. 6C). In stationary cells, this peak decreased, whereas the peak of ribosomal subunits and monosomes increased because the cells were arrested in G2 phase (Fig. 6B). The sedimentation profile of the trypanosomal...
co-chaperone followed similar pattern changes. In epimastigotes in stationary phase (Fig. 6B), the sedimentation profile of the trypanosomal chaperone showed peaks corresponding to ribosomal subunits/80 S monosomes, whereas in exponentially growing parasites (Fig. 6C) it was spread over the smaller polysomes. As for the transformed yeast, a large excess of the protein was found associated with the top of the sucrose gradient (T in Fig. 6, B and C). Similarly, in yeast, the most important fraction of Sis1p and Ssap, which functions as the Hsp70 partner for translational function of Sis1p, is found associated to the cytoplasm (47). Whether this result confirmed the possibility that a cytosolic pool of TcJ6p might be involved in other biogenesis pathways, we cannot rule out that this cytosolic TcJ6p was stripped off the ribosomes during the harvest procedure. We noted that two additional upper bands with apparent molecular masses of around 40 and 45 kDa were associated with 80 S ribosomes (Fig. 6B, open arrow). The nature of these bands is still unknown, but they may correspond to hyperphosphorylated forms of the protein (see “Discussion”).

Localization of Trypanosomal DnaJ by Indirect Immunofluorescence—The subcellular distribution of TcJ6p was determined by confocal microscopy at two stages of parasite differentiation, dividing epimastigotes and quiescent metacyclic trypanomastigotes. In the case of epimastigotes (Fig. 7A), the staining was distributed throughout the cytoplasm of the cell and was preferentially concentrated in speckles in the peri-nuclear region (Fig. 7B). The overall distribution of the trypanosomal protein was similar in metacyclic cells, but more speckles were observed close to the kinetoplast and in the peri-nuclear region (Fig. 7C). The preferential TcJ6p location in the peri-nuclear region suggests a protein association with the endoplasmic reticulum.

**DISCUSSION**

The TcJ6 Gene Is Highly Conserved in Trypanosomatids—A novel member of the T. cruzi DnaJ-like gene family was found together with a sialidase pseudogene and genes encoding proteins homologous to an adenylate cyclase and two small G-proteins in a 14.3-kb genomic DNA fragment highly homologous to the 3′ region of the glucose transporter gene cluster (18). Southern blot analysis detected at least four copies of TcJ6, which, unlike the Hsp60, Hsp70, and Hsp90 genes of T. cruzi (48–50), were not arranged in tandem repeats but were instead dispersed on a single chromosome in the megabase range. The situation was different in T. b. brucei and L. donovani, in which two allelic copies of this gene were present (18). This may be accounted for by intrachromosomal amplification of large genomic fragments, typical in T. cruzi, even in the absence of drug selection, and resulting in extensive variations in the genome size of T. cruzi strains and clones (51). It has been postulated that the organization of the genes flanking the glucose transporter gene cluster has been highly conserved during evolution, because these genes may have related functions (18). In this respect, analysis of expression of the genes detected in the c2623g clone has shown that the transcript level of adenylate cyclase and TcJ6 genes increases in metacyclic cells (Fig. 3A and data not shown). This increase in the mRNA levels of adenylate cyclase and TcJ6 genes observed in metacyclic cells might be a result of processing of the primary transcripts into individual steady state mRNAs, occurring by trans-splicing and polyadenylation, which have been accumulated during the differentiation process. However, at the protein level, the amount of adenylate cyclase increases during metacyclogenesis, but this is clearly not the case for TcJ6p (Fig. 3B and data not shown). These observations reveal no obvious functional coupling between the various genes of the unit.

**TcJ6p, a New Member of the Class II Hsp40 Subfamily—**TcJ6p belongs to the class II Hsp40s, since it has the highly conserved N-terminal J domain and lacks the cysteine-rich region but displays an extensive G-F region. Functional complementation of a class II Hsp40, the yeast Sis1p, and association with ribosomal subunits and translating ribosomes demonstrated that the physiological role of TcJ6p is similar to that of Sis1p. Do all the class II subfamily members have functions similar to Sis1p? A very important piece of evidence was provided by gene swapping experiments with the building domains (J, G-F, G-M, C terminus) of two yeast cytosolic proteins, Ydj1p (class I) and Sis1p (class II), in experiments of rescue of Sis1 yeast mutant. This work showed that although J domains are interchangeable, G-F regions, which are essential for the function of Sis1p, are not (9). Thus, the G-F region is specifically required to discriminate between the function of Ydj1p and Sis1p and not the J domain, as was previously thought. Analysis of the sequence of the trypanosomal DnaJ of L. donovani reveals a very interesting feature; the presence of a G-M-rich extension (GMGMPGP, which is absent from the other trypanosomatids analyzed (18) and is very similar to the yeast G-M motif, is repeated twice in Sis1p (GMGMHGGMGMPGP) (31). In this respect, it is noteworthy that the molecular mass of the L. major chaperone is close to the 37.5-kDa of Sis1p (see Fig. 3C).

**Does TcJ6p Behaves as a Heat Shock Protein?—**Although the amount of Sis1p has been shown to double after a heat shock from 23 to 39 °C (31), our data provide no clear evidence that heat shock from 28 to 37 °C results in significant overproduction of the trypanosomal co-chaperone. After 6 h of heat shock, we detected a 2-fold enrichment of TcJ6 transcript, which was restored to normal after 24 h. This probably reflects an increase in the stability of co-chaperone mRNA during heat shock, as already observed for Hsp83 from Leishmania amazonensis (44). Because the presence of wild-type Sis1p in the sis1-85 mutant was found to repress the overexpression of Sis1-85 protein (52), we are currently investigating whether TcJ6p could work as a transcriptional regulator in yeast by down-regulating expres-
In the trypanosomal DnaJ, indeed, the most likely scenario to explain the detection in Western analysis of two slower-migrating forms (around 40 and 45 kDa) is that they represent differentially phosphorylated forms. In yeast, a band shift of similar forms is consistent with a direct role for the trypanosomal DnaJ.

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