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Molecular Cloning of mtp70, a Mitochondrial Member of the hsp70 Family

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We have isolated a gene from the protozoan parasite *Trypanosoma cruzi* that encodes a previously unidentified member of the 70-kilodalton heat shock protein (hsp70) family. Among all the eucaryotic hsp70 proteins described to date, this trypanosome protein, mtp70, is uniquely related in sequence and structure to the hsp70 of *Escherichia coli*, DnaK, which functions in the initiation of DNA replication. This relationship to DnaK is especially relevant in view of the intracellular location of the protein. Within the trypanosome, mtp70 is located in the mitochondrion, where it associates with kinetoplast DNA (kDNA), the unusual mitochondrial DNA that distinguishes this order of protozoa. Moreover, mtp70 is located in the specific region of the kinetoplast in which kDNA replication occurs. In view of the known functions of DnaK, the localization of mtp70 to the site of kDNA replication suggests that mtp70 may participate in eucaryotic mitochondrial DNA replication in a manner analogous to that of DnaK in *E. coli*.

The mammalian hsp70 family (for recent reviews, see references 22, 28, and 29) is composed, at least in part, of the 78-kilodalton (kDa) glucose-regulated protein (grp78), the 70-kDa heat shock cognate protein (hsc70), and the 70-kDa major stress protein (hsp70). Although these three proteins share certain properties, such as the ability to bind ATP (3, 39), and likely have a common mechanism of action, they have different intracellular locations and proposed functions.

grp78 is located in the lumen of the endoplasmic reticulum, where it has been proposed to have a general role in the assembly of protein complexes (26). hsc70 is a cytoplasmic protein that appears to have at least two distinct functions. It releases clathrin from coated vesicles in vitro (3, 37) and is a mediator of the translocation of proteins across membranes in vivo (4, 7). In addition, hsp70 and hsc70 migrate to the nucleus upon heat shock, where they are presumed to participate in the recovery of nucleoli from stress-induced damage (40). All three eucaryotic hsp70 proteins appear to function through interactions with other proteins.

The *Escherichia coli* hsp70, DnaK, also functions through protein-protein interactions. It plays an essential role in the initiation of bacteriophage λ DNA replication (42), where it acts in an ATP-dependent fashion with the DnaJ protein to release λ O and λ P proteins from the preprimosomal complex (21). Furthermore, although the exact mechanism has not yet been elucidated, DnaK is involved in *E. coli* chromosomal DNA replication, possibly through an analogous interaction with the DnaA protein (32).

Here we report the characterization of a new member of the hsp70 family. This 70-kDa mitochondrial protein (mtp70) has a specific location within the trypanosomal mitochondrion, which coincides with a known site of DNA synthesis. Given the known role for the *E. coli* hsp70 in procaryotic DNA replication, these results suggest an analogous function for this DnaK-like protein in the eucaryotic mitochondrion.

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MATERIALS AND METHODS

Cloning and analysis of nucleic acids. Methods used in the isolation of *T. cruzi* RNA, the construction and immunological screening of cDNA expression libraries in bacteriophage λgt11, and Northern (RNA) analysis have been previously described (16). The two tandemly linked *MTP70* genes were cloned directly from agarose gels and from a genomic library by standard techniques (23). A detailed comparison of the *T. cruzi MTP70* and *HSP70* genes will be presented elsewhere. DNA sequences were determined by chemical (24) and enzymatic (33) methods.

Production of recombinant proteins. For the generation of β-galactosidase fusion proteins, *E. coli* Y1090 was infected with approximately 50,000 PFU of λgt11, λgt11:chicken ovalbumin (Clontech), or λTC1, plated on 150-mm-diameter L broth (LB)-ampicillin plates in LB top agar containing 2 mM isopropyl-β-D-thiogalactopyranoside, and incubated at 42°C for 6 h. Fusion proteins were eluted from the plate lysate into 10 ml of 10 mM Tris hydrochloride (pH 8.0)–2 mM $MgSO_4$ for 2 h at room temperature. The solution was removed from the plate, combined with one-fourth of its volume of 5× sodium dodecyl sulfate (SDS) sample buffer, and boiled before electrophoresis. For the production of other fusion proteins in *E. coli*, pUC19 was modified so that *Eco*RI inserts would be expressed in the same reading frame as they are in λgt11. The *Hin*cII-to-*Sma*I heptadecanucleotide of pUC19 was excised, resulting in a *lacZ* reading frameshift of −2. In this modified vector, the *Eco*RI cloning site is expressed as Glu-Phe (GAA-TTC), as it is in λgt11. The TC1 cDNA insert was cloned into this vector (pUC19Δ17), and recombinant and control plasmids were introduced into *E. coli* DH5α (Bethesda Research Laboratories, Inc.), which constitutively expresses *lacZ* fusions. For lysate preparation, stationary (24 h) cultures (1 ml) of bacteria harboring each of the two plasmids described above were centrifuged in a microfuge for 20 s, and the cells were suspended in 0.5 ml of 1× SDS sample buffer and boiled extensively before electrophoresis. For Western blot (immunoblot) analysis, a trypanosome lysate was similarly prepared from 1 ml of a culture (15) of logarithmic-phase epimastigotes.

Antisera. Anti-gp72 rabbit serum NIH α-GP72 has been previously described (15). To produce mtp70 antiserum, a bacterial lysate (see above) containing recombinant p47 (see Fig. 2B) was subjected to preparative gel electrophoresis on a SDS polyacrylamide slab gel (see below). After staining the edges of the gel with Coomassie blue, a gel strip containing the fusion protein was excised. Antisera were raised in mice by a series of intraperitoneal injections of this gel strip homogenized first in Freund complete adjuvant, then in Freund incomplete adjuvant, and finally in phosphate-buffered saline (PBS).

Protein analysis. SDS-polyacrylamide gel electrophoresis was performed on 10% polyacrylamide slab gels by the method of Laemmli (19). Approximately 50 μg of protein (40 μl of the bacterial or trypanosomal lysate) was applied to each lane. After electrophoresis, proteins were detected by Coomassie blue staining. Alternatively, for immunochemical analysis, proteins were transferred to nitrocellulose (36) and the resulting blot was probed sequentially with mtp70 mouse antiserum and horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G. Total *T. cruzi* epimastigote RNA was translated in a rabbit reticulocyte lysate in the presence of [^{35}S]methionine, and labeled proteins were immunoprecipitated and analyzed by fluorography.

Immunofluorescence assay. *T. cruzi* epimastigotes were spotted on printed slides, allowed to dry, and fixed with methanol. The fixed parasites were incubated for 20 min in a moist chamber at room temperature with blocking buffer (1% bovine serum albumin, 0.5% casein, 0.005% thimerosal, 0.0005% phenol red, 2% normal goat serum in PBS), followed by a 1-h incubation with mouse serum diluted 1:100 in blocking buffer. The parasites were then washed extensively with PBS and incubated for 30 min with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G diluted 1:200 in blocking buffer. After more PBS washes, the parasites were washed again in PBS and mounted in glycerol for microscopy. For DNA staining, fixed parasites were stained with 0.1 μg of 4′,6-diamidino-2-phenylindole (DAPI) per ml (12) for 30 min, washed, and mounted as described above.

RESULTS

Molecular cloning and sequence analysis of mtp70. We originally set out to clone the gene for a highly immunogenic surface glycoprotein of *T. cruzi*, gp72 (for a recent review, see reference 35). To this end, we constructed a *T. cruzi* cDNA expression library in λgt11 (16) and screened it with NIH α-GP72 (15), a rabbit serum raised against gp72 purified by monoclonal antibody affinity chromatography. Of the 12 positive cDNA clones, 4 encode a 40-kDa protein (p40), which will be described elsewhere and 8 encode part of a 71-kDa polypeptide, which is described here. Antisera raised against recombinant p40 and antisera raised against recombinant mtp70 did not immunoprecipitate a radioiodinated surface protein (data not shown), suggesting that the two proteins are internal antigens. Thus, the NIH α-GP72 serum appears to recognize at least three different proteins, one surface antigen (gp72 [35]) and two internal antigens (p40 and mtp70).

We used one cDNA from the group of eight, TC1 (1.6 kilobases [kb]), to isolate clones from a *T. cruzi* genomic DNA library and determined the complete sequences of the TC1 cDNA and its corresponding gene. The gene encodes a polypeptide of 656 amino acids with a molecular mass of about 71 kDa. A computer-assisted analysis of the deduced amino acid sequence revealed that this protein (henceforth referred to as mtp70) is a member of the 70-kDa family of heat shock proteins and glucose-regulated proteins (Fig. 1; Table 1). The five proteins shown are identical at 201 (~30%) of the approximately 650 amino acid positions (solid squares), most of which are clustered in domains in the N-terminal three-fourths of the protein.

mtp70 is about 45% identical to the eucaryotic hsp70 proteins, and about 55% identical to DnaK (Table 1). The 10% difference is largely due to the 86 residues (Fig. 1, open circles) that are the same in, and unique to, mtp70 and DnaK. This close relationship is further demonstrated by the four small relative deletions and one insertion (mtp70 positions 143, 311, 377, and 405) that distinguish mtp70 and DnaK from the other hsp70 proteins. Two additional nonidiosyncratic deletions are found in mtp70, DnaK, and grp78 (mtp70 positions 216 and 405). Considered together, these findings reveal a special phylogenetic relationship between mtp70 and DnaK (see below).

Among these hsp70 proteins, mtp70 possesses several unusual structural features. Like grp78, mtp70 has an N-terminal extension compared with the other hsp70 proteins. However, unlike the grp78 leader, which is a nearly perfect consensus secretory leader peptide, the mtp70 leader is rich in basic and hydroxylated amino acids and lacks acidic amino acids and extensive hydrophobic stretches. These are properties of mitochondrial leader sequences (for a recent review, see reference 38). Other notable features of mtp70 are the C-terminal stretches of polyserine and polyglutamine, and the absence of the Glu-Glu-Val that is found in most hsp70 proteins.

Generation and characterization of mtp70 antisera. To determine whether the leader present in mtp70 directs the protein to the mitochondrion, we raised antibodies against a recombinant mtp70 polypeptide for use in an immunofluorescence assay. For this work we chose recombinant phage clone λTC1, which contains a partial mtp70 cDNA and encodes a β-galactosidase fusion protein of 159 kDa (Fig. 2A). We subcloned the cDNA insert, which encodes a 45-kDa C-terminal polypeptide beginning at Leu-247 (arrowhead in Fig. 1), into a plasmid vector in which it was expressed as a fusion protein with a small (2-kDa) polylinker-encoded N-terminal peptide (Fig. 2B). We then immunized several mice with this 47-kDa protein and determined the specificity of the resulting antisera (Fig. 2C and D).

The antisera precipitated a *T. cruzi* in vitro translation product of 71 kDa (Fig. 2C). The synthesis of this protein was completely inhibited when RNA was translated in the presence of an mtp70-specific antisense oligonucleotide and ribonuclease H (results not shown), demonstrating not only that the antisera specifically bind mtp70 but also, and importantly, that they do not bind hsp70. Western blot analysis shows native mtp70 to be about 68 kDa, 3 kDa smaller than the primary translation product (Fig. 2D). Although this result does not prove a precursor-product relationship for the 71- and 68-kDa proteins, it is consistent with the notion that the leader is cleaved, as occurs in many other mitochondrial proteins (38).

Localization of mtp70 within the trypanosome. The trypanosome possesses a single mitochondrion that has a complex tubular structure, so we did not expect the mtp70 antiserum to provide an easily interpretable immunofluorescence staining pattern. However, to our initial surprise, the antiserum stained only the kinetoplast (Fig. 3A), an intramitochondrial structure that lies within the matrix and appears as an oval depression or crater by phase-contrast

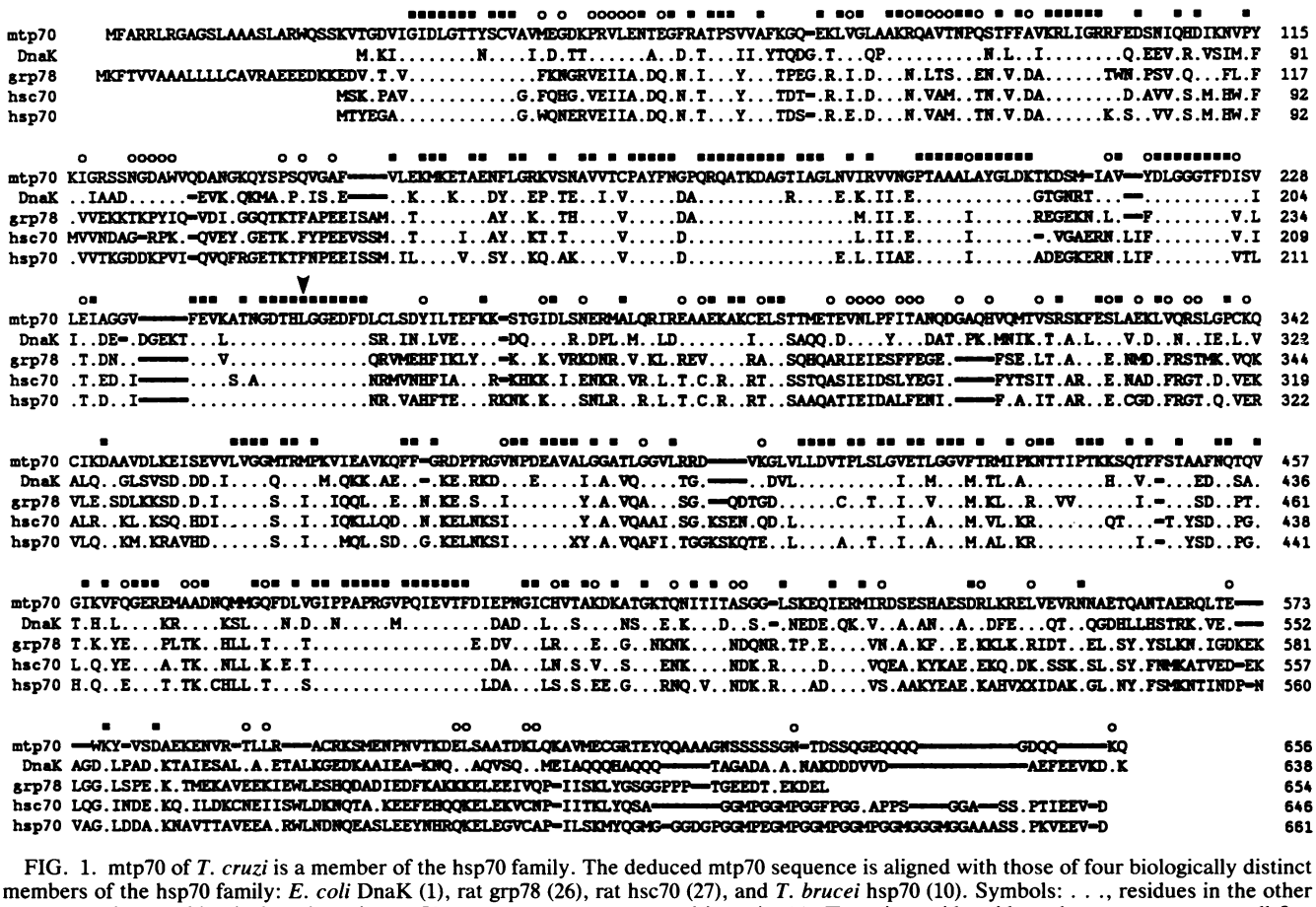

FIG. 1. mtp70 of *T. cruzi* is a member of the hsp70 family. The deduced mtp70 sequence is aligned with those of four biologically distinct members of the hsp70 family: *E. coli* DnaK (1), rat grp78 (26), rat hsc70 (27), and *T. brucei* hsp70 (10). Symbols: . . ., residues in the other sequences that are identical to those in mtp70; —, computer-generated insertion(s); ■, amino acid residues that are common to all five proteins; ○, residues that are shared exclusively by mtp70 and DnaK. The arrowhead marks the N-terminal boundary of the protein encoded by the TC1 cDNA.

microscopy (top). The kinetoplast, which characterizes members of the order Kinetoplastida, harbors the kinetoplast DNA (kDNA) (for a recent review, see reference 31). Each kinetoplast contains a concatenated network of 25 to 50 maxicircles of about 20 kb, which are analogous to higher eucaryotic mitochondrial DNA, and several thousand heterogeneous minicircles of about 1.5 kb, whose functions are unknown.

We confirmed the location of the kinetoplast by staining the A+T-rich *T. cruzi* kDNA with DAPI, a stain that binds preferentially to A+T-rich DNA (Fig. 3C). One of the two nuclei of this dividing cell also could be seen as a faint circle adjacent to the kinetoplast. On close inspection however, there was a difference between the locations of kDNA and mtp70. Whereas DAPI stained throughout the kinetoplast, the mtp70 antibody stained at the periphery. The kDNA network is organized as a tightly packed disk in which the concatenated minicircles are stretched out and lie parallel to the axis (31). Since the kDNA disk axis is parallel to the cell axis, mtp70 appears to be located at each face of the disk.

Response of *MTP70* mRNA to heat shock. Like the *HSP70* genes of *T. cruzi*, the (two tandemly linked) *MTP70* genes are located in the nucleus, as determined by Southern hybridization of *T. cruzi* chromosomes separated by pulsed-

TABLE 1. Identity matrix of hsp70 family members[a]

Protein	% Identity of protein with:					
	T. cruzi mtp70	*E. coli* DnaK	Rat grp78	Rat hsc70	*T. brucei* hsp70	*T. cruzi* hsp70
T. cruzi mtp70	100					
E. coli DnaK	55	100				
Rat grp78	46	50	100			
Rat hsc70	45	48	64	100		
T. brucei hsp70	44	46	63	73	100	
T. cruzi hsp70	44	47	62	74	91	100

[a] Proteins are those shown in Fig. 1 and *T. cruzi* hsp70 (30; D. M. Engman, S. R. Sias, J. D. Gabe, J. E. Donelson, and E. A. Dragon, Mol. Biochem. Parasitol., in press).

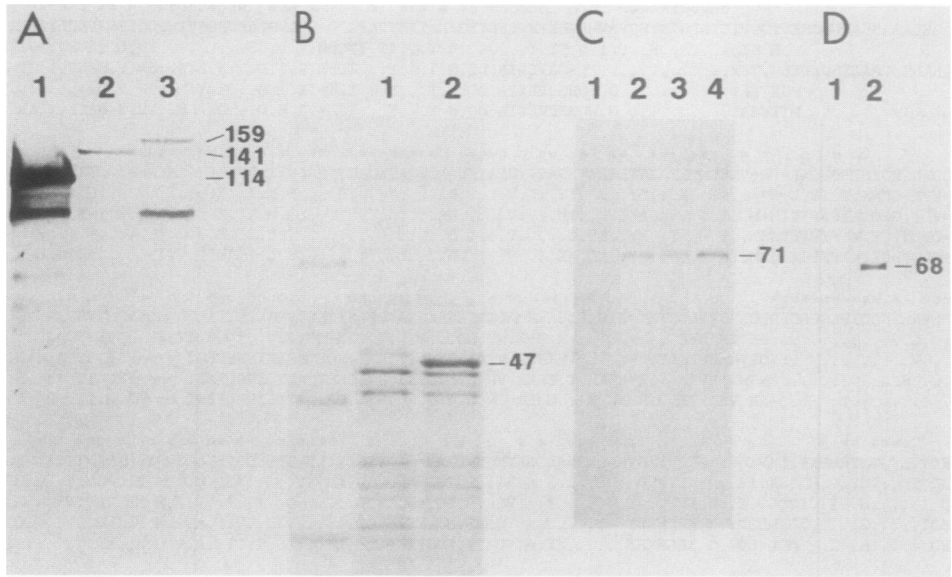

FIG. 2. Generation and characterization of mtp70-specific antisera. (A) Western blot analysis of β-galactosidase fusion proteins. Phage lysates containing β-galactosidase (lane 1), β-galactosidase-ovalbumin (C-terminal 248 amino acids of chicken ovalbumin [lane 2]) and β-galactosidase-TC1 (mtp70) (C-terminal 410 amino acids [lane 3]), were electrophoresed on an 8% SDS-polyacrylamide gel, electroblotted to nitrocellulose paper, and probed with antiserum against *E. coli* β-galactosidase. (B) Production of an mtp70 fusion protein containing a 19-amino-acid N-terminal peptide. The *Eco*RI cDNA insert of λTC1 was subcloned into a derivative of pUC19 in both orientations (incorrect orientation [lane 1] and correct orientation [lane 2]). Proteins from stationary-phase bacterial cultures were visualized by Coomassie blue staining after SDS-polyacrylamide gel electrophoresis. Size standards (leftmost lane) are (from bottom to top) 26, 43, 68, and 97-kDa. (C) Immunoprecipitation of *T. cruzi* in vitro translation products with sera from a nonimmune mouse (lane 1) or from three different mice immunized with the 47-kDa fusion protein shown in panel B (lanes 2 to 4). (D) Western blot analysis of a *T. cruzi* lysate probed with nonimmune mouse serum (lane 1) or with one of the anti-mtp70 mouse sera (lane 2).

field gradient gel electrophoresis (results not shown). The transcriptional regulation of *MTP70* however, as reflected by the steady-state mRNA level, differs from that of *HSP70*. The levels of the two transcripts to which the *MTP70* cDNA hybridizes (2.5 and 3.0 kb) decreased by a factor of 1.4 upon heat shock (Fig. 4A). When we probed the same blot with the *T. cruzi HSP70* gene, we observed a 2.2-fold increase in the 2.4-kb *HSP70* mRNA, as expected (Fig. 4B). Thus, the steady-state levels of the *MTP70* mRNAs decreased in response to heat shock, as do the levels of most non-heat shock mRNAs. Although little is known about transcriptional regulatory elements in trypanosomes, conserved sequences occur upstream of the *T. cruzi, Trypanosoma brucei*, and *Leishmania major HSP70* and *T. cruzi HSP83* genes that are not found upstream of the *MTP70* genes (20; also unpublished data). These sequence differences could be responsible for the observed differential regulation of *MTP70* and *HSP70* genes.

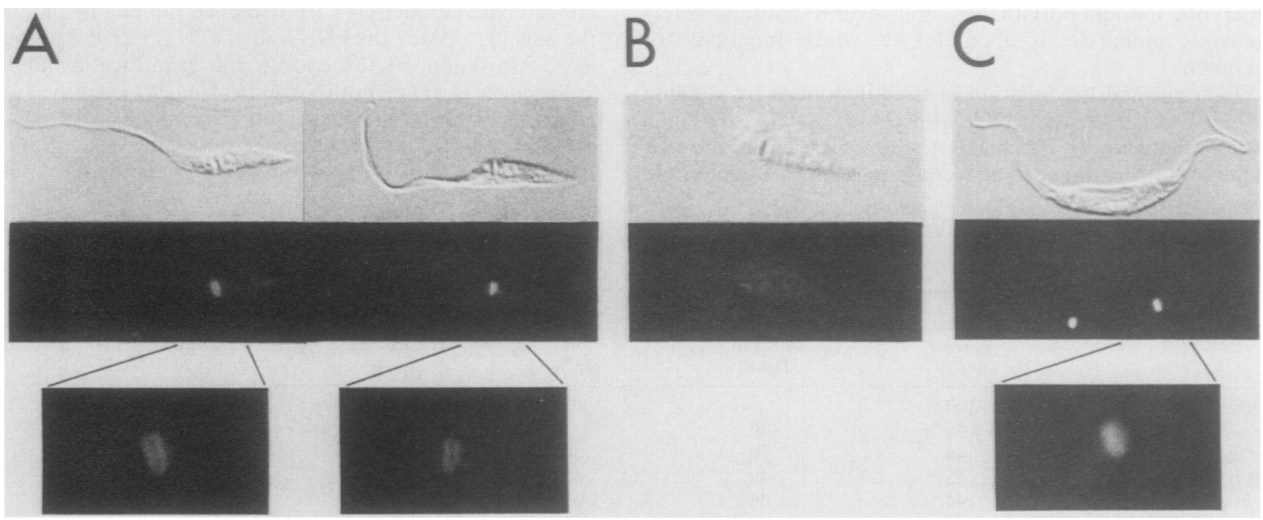

FIG. 3. Native mtp70 is located in the kinetoplast. Parasites were stained with anti-mtp70 mouse serum (A), nonimmune mouse serum (B), or DAPI (C) and photographed during direct interference contrast microscopy (top), fluorescence microscopy (bottom, A and B) or UV microscopy (bottom, C).

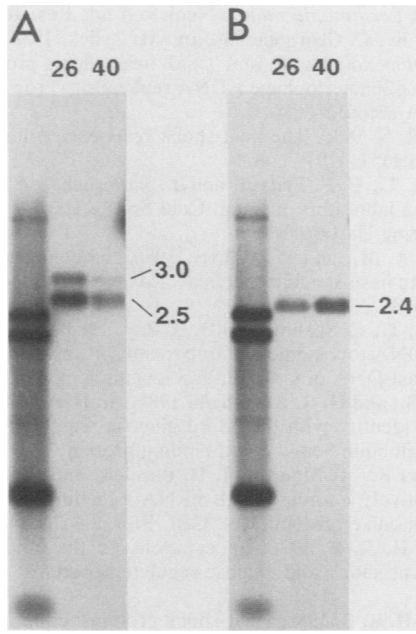

FIG. 4. The steady-state *MTP70* mRNA level decreases upon heat shock. A Northern blot was prepared with total RNA from *T. cruzi* epimastigotes grown at 26 or 40°C for 3 h. The blot was probed with a plasmid containing the TC1 cDNA insert (A), washed, and reprobed with a plasmid containing the *T. cruzi HSP70* gene (B). Size standards (leftmost lanes) are λ DNA digested with *Hin*dIII (from bottom to top) 0.13, 0.56, 2.0, 2.3, and 4.4 kb.

DISCUSSION

The events leading to the molecular cloning of mtp70 resemble those reported by O'Malley et al., who cloned the rat hsc70 (27). They inadvertently isolated a partial hsc70 cDNA while immunologically screening a λgt11 cDNA expression library with antiserum against catecholamine-synthesizing enzymes. In our case, the NIH α-GP72 antiserum is specific for at least three proteins, gp72, which is on the surface, and mtp70 and p40, which are internal. Since mtp70 and p40 share only four tetrapeptides (unpublished data), it is likely that the gp72 preparation used to produce the NIH α-GP72 antiserum was contaminated with mtp70 and p40. Alternatively, all three proteins may share a cross-reacting epitope that may not be linear and, as a result, not identifiable by amino acid sequence alignment.

The latter possibility is supported by the finding that when antiserum against recombinant grp78 of the malaria parasite, *Plasmodium falciparum*, was used to precipitate native *P. falciparum* antigens, a 230-kDa surface protein was coprecipitated (18). Thus, for reasons not yet clear, parasite surface antigens and hsp70 proteins appear to be coprecipitated by antisera directed against either antigen. The hsp70 family member involved, however, seems to be specific, since we detected no hsp70 cDNAs using the gp72 antiserum, even though they are 5 to 10 times more abundant in the library than mtp70 cDNAs (unpublished data).

A special evolutionary relationship between mitochondrial mtp70 and its bacterial homolog can be inferred from the comparative sequence analysis (Fig. 1). The most likely explanation for this close similarity is that the nuclear *MTP70* gene was acquired from an ancient proto-organellar procaryote, in accordance with the endosymbiont hypothesis for the origin of mitochondria (11, 41). This does not imply, however, that ancestral mtp70 was the predecessor to the more modern hsp70 proteins, since it is possible—indeed likely, considering the important physiologic functions of hsp70 proteins—that the proto-eucaryotic host possessed hsp70 before the symbiosis occurred. Whatever the phylogeny of the hsp70 family, the maintenance of a mitochondrial form of the protein suggests that it fulfills a fundamental biologic function in the mitochondrion, such as in DNA replication.

In the trypanosome mitochondrion during S phase, individual kDNA minicircles detach from the center of the kDNA network and are replicated as free DNA molecules near its surface (2, 9). Newly synthesized progeny minicircles then reattach at the network periphery (8, 34). Therefore, mtp70 is located in the region where kDNA replication occurs. The colocalization of mtp70 and the site of kDNA replication, coupled with the known functions of its bacterial homolog, DnaK, suggests that mtp70 may participate in this replication. In fact, the regions of exclusive sequence identity between mtp70 and DnaK are precisely those previously proposed to confer functional specificity to DnaK and eucaryotic hsp70 (14).

A different location within the kinetoplast is found for the trypanosome mitochondrial type II DNA topoisomerase (25). This protein appears to be located at the circumference of the kDNA disk, rather than at the two faces. As a result, immunofluorescence staining of the topoisomerase II yields two dots that flank the kDNA disk, rather than the oval pattern produced by the mtp70 antibody. The distinct locations of mtp70 and topoisomerase II within the kinetoplast suggest distinct functions for these proteins and may be due to their participation in different steps of the kDNA replication process.

A similar relationship between procaryotic and nucleus-encoded eucaryotic organellar heat shock proteins has recently been described. The 60-kDa GroEL protein is another *E. coli* heat shock protein with homologs in eucaryotic organelles. GroEL and its mitochondrial and chloroplast homologs all participate in the assembly of oligomeric protein complexes (13). Therefore, by analogy, it is not surprising to find a DnaK homolog having a similar function in the eucaryotic mitochondrion. It will be interesting to determine whether DnaK, mtp70, and the recently identified (17) chloroplast hsp70 do indeed, like members of the GroEL family, have analogous functions.

Although this is the first description of an hsp70-like protein associated with mitochondrial DNA, it is not the first account of a mitochondrial hsp70. Scc1p, a member of the yeast hsp70 family, has been localized to the mitochondrion (5, 6), and accordingly, we initially thought that mtp70 was the trypanosomal ssc1p homolog. However, the *T. cruzi* mtp70 cDNA hybridizes to yeast restriction fragments distinct from those published for the other known *HSP70* family members (including *SSC1*), indicating that there may be more than one mitochondrial *HSP70* gene in yeast cells (R. Hallberg, and G. Schatz, unpublished results). Therefore, our proposition that mtp70 serves a function in DNA replication may be best tested in yeast cells, where genetic manipulations not yet possible for trypanosomes can be employed.

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