MOLECULAR AND CELLULAR BIOLOGY, Mar. 1987, p. 1271–1275
0270-7306/87/031271-05$02.00/0

Vol. 7, No. 3

The Genome of *Trypanosoma cruzi* Contains a Constitutively Expressed, Tandemly Arranged Multicopy Gene Homologous to a Major Heat Shock Protein

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Received 5 August 1986/Accepted 2 December 1986

cDNA libraries have been constructed in the plasmid vector pUC18 with mRNA isolated from both epimastigotes and trypomastigotes of the Peru strain of *Trypanosoma cruzi*. Pools of randomly selected clones were analyzed by hybridization-selection-translation. Translation products were immunoprecipitated either with normal human sera or with sera from patients with Chagas' disease (chagasic sera), and the immunoprecipitates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. With this approach, a cDNA clone (pEC5) was identified which encodes a portion of an 85,000-M_r polypeptide. A genomic clone was subsequently isolated (FG1) by using oligonucleotide probes derived from the DNA sequence of this cDNA clone. A portion of this clone was isolated and sequenced, and the coding region for the protein was identified. Computer analysis of the predicted protein sequence indicates that this protein is closely related to the 83,000-M_r heat shock protein (hsp83) of *Drosophila melanogaster*, the hsp90 of *Saccharomyces cerevisiae*, and the hsp90 of chicken. This gene is tandemly organized in the *T. cruzi* genome as a cluster of 6 to 10 copies.

Trypanosoma cruzi, a protozoan parasite, causes Chagas' disease, a potentially debilitating disease to which millions of people in Central and South America are constantly exposed. The life cycle of the parasite is complex, with multiple developmental stages in both the insect vector and the mammalian host. In the insect vector, *T. cruzi* replicates in the midgut as an epimastigote and transforms to an infectious metacyclic trypomastigote in the insect hindgut. After its transmission to the mammalian host, the parasite replicates intracellularly as the amastigote and transforms into the infectious trypomastigote before its release into the bloodstream. The immune system of the infected mammalian host mounts a strong response to the parasite. Tests of sera isolated from individuals infected with *T. cruzi* could help identify the gene products of the parasite which are recognized by the immune system of the host.

T. cruzi Peru was used in all experiments. Epimastigotes and trypomastigotes were grown as previously described (6). Parasites were harvested from culture by centrifugation and washed several times. Epimastigotes were suspended at a concentration of 10^9 ml in PEB-EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid] buffer (20 mM Tris hydrochloride [pH 7.6], 25 mM EGTA, 50 mM $MgCl_2$, 25 mM NaCl, 1.0% Triton X-100, and 4 mM dithiothreitol) plus 250 U of RNasin (Promega Biotec, Madison, Wis.) per ml, incubated on ice for 20 min, and centrifuged at 8,000 × *g* for 15 min at 4°C. The supernatant containing the RNA was extracted with phenol three times, extracted once with chloroform-isoamyl alcohol (24:1), and precipitated with ethanol. The pellet (nuclei and kinetoplasts) was suspended at a concentration of 10^9 parasite equivalents per ml in 10 mM Tris hydrochloride (pH 8.0), 500 mM EDTA, 0.1% sodium dodecyl sulfate (SDS), and 150 μg of proteinase K per ml, and total DNA was prepared by the method of Maniatis et al. (16). Trypomastigote DNA and RNA were prepared in an identical manner, except that the parasites were suspended at a concentration of 5 × 10^9/ml. Poly(A)$^+$ RNA was isolated by oligo(dT)-cellulose chromatography (2). cDNA was prepared as previously described (9, 25).

From an epimastigote cDNA library cloned into the vector pUC18 (27) and transformed into *Escherichia coli* JM83, 96 random cDNA clones were grown in individual wells of a microtiter dish. Plasmid DNA from eight pools containing 12 clones each was purified, linearized, and immobilized on nitrocellulose filters. Hybridization-selection-translation (HST) experiments (20) were performed in a rabbit reticulocyte lysate (Promega) with epimastigote total RNA, and a sample of the HST products was analyzed by SDS-polyacrylamide gel electrophoresis. In addition, each of the translation reactions was divided and immunoprecipitated with either normal human serum (NHS) or chagasic serum (CHA); the immunoprecipitated polypeptides were analyzed by SDS-polyacrylamide gel electrophoresis. The immunoprecipitation reactions were performed as previously described (6). Approximately five specific polypeptides were recognized by CHA (Fig. 1A and B). Previous work from our laboratory (6) and others (1, 3, 14, 17, 18, 22, 23) has demonstrated that the major surface glycoproteins of *T. cruzi* have M_rs of approximately 80,000 to 90,000. Therefore, we originally set out to examine clones corresponding to HST polypeptides with M_rs of 70,000 to 90,000, hoping to find a gene for a major surface antigen. Pool C was designated a good candidate for the isolation of clones encoding polypeptides in this size range, and DNA was isolated from each of the 12 constituent clones making up the pool. Results from the HST and immunoprecipitation analyses described above indicated that clone C5 (pEC5) contained sequences complementary to an mRNA that encodes an 85,000-M_r polypeptide. The DNA sequence was determined for the insert of the cDNA clone (pEC5), and this information was used to design specific oligonucleotide probes for subsequent use in the isolation of a genomic clone and as sequencing primers.

An epimastigote genomic clone, FG1, was isolated by screening a *Sau*3A (partial) epimastigote genomic library with the oligonucleotide probes COD 203 (5'-GCCCGTT

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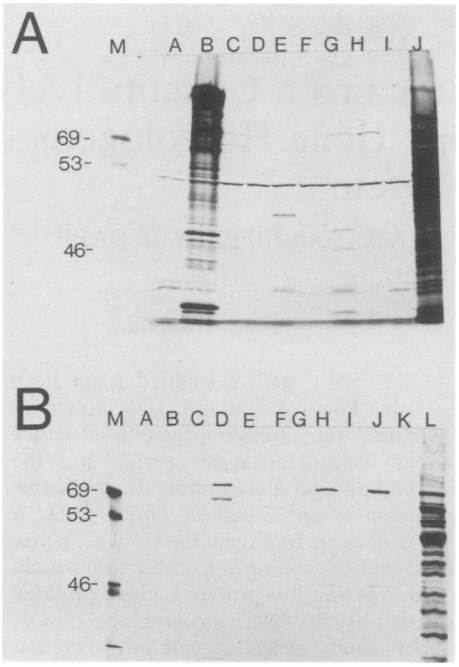

FIG. 1. Fluorogram of a 10% SDS-polyacrylamide gel of the HST experiment with mixed plasmid pools of DNA. Lane M, Molecular weight standards (in kilodaltons) for bovine serum albumin (M_r, 69,000), immunoglobin G (M_r, 53,000), and ovalbumin (M_r, 46,000). Lanes A through I, Translation products of RNA control (lane A), brome mosaic virus mRNA control (lane B), pool C DNA (lane C), pool D DNA (lane D), pool E DNA (lane E), pool F DNA (lane F), pool G DNA (lane G), pool H DNA (lane H), and pUC19 DNA (lane I). Lane J, Translation products of epimastigote total RNA. (B) Fluorogram of a 10% SDS-polyacrylamide gel of the immunoprecipitation products of the experiment described for panel A. Lane M, Molecular weight standards; lane A, polypeptides from the pUC19 HST immunoprecipitated with NHS; lane B, polypeptides from the pUC19 HST immunoprecipitated with CHA; lane C, pool C HST immunoprecipitated with NHS; lane D, pool C HST immunoprecipitated with CHA; lane E, pool E HST immunoprecipitated with NHS; lane F, pool E HST immunoprecipitated with CHA; lane G, pool G HST immunoprecipitated with NHS; lane H, pool G HST immunoprecipitated with CHA; lane I, pool H HST immunoprecipitated with NHS; lane J, pool H HST immunoprecipitated with CHA; lane K, total translated epimastigote RNA immunoprecipitated with NHS; lane L, total translated epimastigote RNA immunoprecipitated with CHA. CHA used in this experiment was a pool of sera from patients with Chagas' disease who were residing in Argentina.

GCTGGCAC) and COD 213 (5′-GCGTTCGGCGT ACGACG) derived from pEC5 (Fig. 2). Partial restriction endonuclease mapping of FG1 (data not shown) revealed that there were four intact tandem copies of an approximately 2.7-kilobase (kb) *Sal*I fragment and a fifth truncated repeat located presumably where the *T. cruzi* DNA had been cut at a *Sau*3A site and inserted into the phage vector. Southern blot analysis (data not shown) of FG1 DNA digested with *Sal*I, *Pst*I, or *Pvu*II and probed with either COD 203 or COD 213 revealed two hybridizing bands for each restriction digest. A 2.7-kb band was common to all three digests; the second band of each digest differed in size. The signal corresponding to the 2.7-kb hybridizing band was much stronger than the other bands, as is expected for multiple copies of a tandemly arranged sequence. The additional band reflects the remaining piece of *T. cruzi* DNA joined to vector DNA. Partial restriction mapping of both epimastigote and trypomastigote genomic DNA has shown that the 2.7-kb fragment is tandemly repeated 6 to 10 times in the genome (data not shown). One of these repeat units was subcloned (pEG1) by digesting FG1 with *Sal*I and inserting the fragment into pUC18, and the complete DNA sequence of the insert was determined (11, 21).

The complete nucleotide sequence of the gene is shown in Fig. 2. Since this gene is tandemly arranged in the genome, this sequence was generated by linking two copies of the 2.7-kb *Sal*I clone DNA sequence. The limits of one gene unit are defined by the poly(A) addition site and the end of the cDNA clone. It is impossible to determine specifically which of the repeat units was cloned and sequenced.

The open reading frame in this sequence encodes 704 amino acids, corresponding to a protein of approximately 80,000 M_r. The cDNA clone, pEC5, begins 225 base pairs upstream from the TGA stop codon and runs for 428 base pairs. A 50,000-M_r fusion protein was encoded for by the *Sal*I subclone (pEG1). This polypeptide consists of the first 16 amino acids of β-galactosidase coded for by pUC18 fused in frame to 405 amino acids of the *T. cruzi* protein.

Northern blot analysis, with either a nick-translated pEC5 insert or pEG1, demonstrated hybridization to a single RNA species of approximately 2.7 kb (data not shown). This RNA was identical in size in both epimastigotes and trypomastigotes. The size of the hybridizing RNA molecule corresponded to the size of the repeat unit.

To confirm that the gene for the 85,000-M_r protein was, in fact, expressed in *T. cruzi* epimastigotes and trypomastigotes, the 50,000-M_r fusion protein encoded by pEG1 was gel purified and used to raise antiserum in mice. Western blot analysis (4, 24) demonstrated that the antiserum specifically reacted with a 50,000-M_r polypeptide in pEG1 containing *E. coli* and an 85,000-M_r polypeptide in both trypomastigotes and epimastigotes (Fig. 3). This evidence strongly suggests that the *T. cruzi* gene we have cloned is, indeed, expressed in both the insect and mammalian forms of the parasite.

We compared the DNA sequence encoding the 85,000-M_r protein with known DNA sequences by using the dfastn and dfastp computer programs (15). Figure 4 illustrates the striking homology between the predicted amino acid sequence of the *T. cruzi* 85,000-M_r protein and the sequences of the 90,000-M_r heat shock protein (hsp90) of *Saccharomyces cerevisiae* (7), the hsp83 of *Drosophila melanogaster* (10), and the hsp90 of the chicken (5).

We have described the isolation and characterization of a tandemly linked, multicopy gene of *T. cruzi*. Computer analysis of the DNA and putative protein sequence of this gene has demonstrated that the *T. cruzi* protein has a 62% amino acid homology with the hsp90 of *S. cerevisiae* and a 56% homology in a 2,511-nucleotide overlap with the yeast gene. There is a 64% homology in a 371-amino-acid overlap with the hsp83 of *D. melanogaster* and a 58.4% homology in a 1,274-nucleotide overlap. This gene of *T. cruzi* was not isolated by using heterologous probes of heat shock genes. Rather, this gene was isolated because its protein was recognized immunologically by sera from patients with Chagas' disease. Although no conclusive evidence has been generated to date, it is interesting to speculate on this observation, given the autoimmune consequences of Chagas' disease (12). For example, given the homology that exists between the *T. cruzi* 85,000-M_r protein and the other known 83,000- to 90,000-M_r heat shock proteins (HSPs), one would suspect that CHA might also recognize the human HSP. If the human HSP were recognized by CHA, this

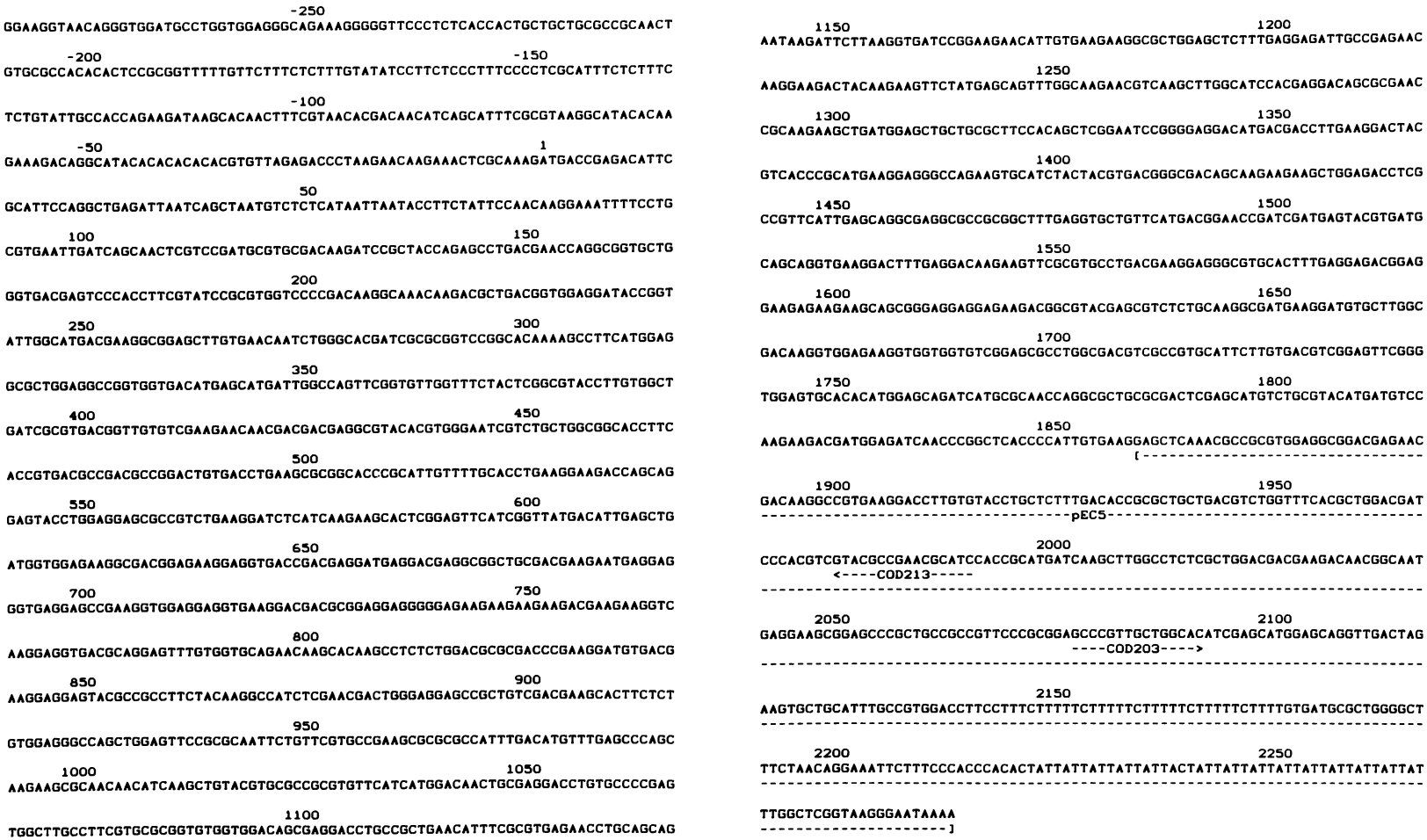

FIG. 2. Complete nucleotide sequence of the 85,000-M_r polypeptide gene. The sequence has been illustrated by combining two pEG1 2.7-kb inserts at the *Sal*I site in head-to-tail fashion, as it is tandemly linked in the genome, and aligning the sequence so that the first base shown in the sequence is the first base following the end of the cDNA transcript isolated (pEC5). Nucleotide 1 is the A in the putative initiator ATG. The sequence and location of the initially isolated cDNA clone, pEC5, are indicated, as are the locations of the two oligonucleotide probes (COD 203 and COD 213) used to identify the genomic clone.

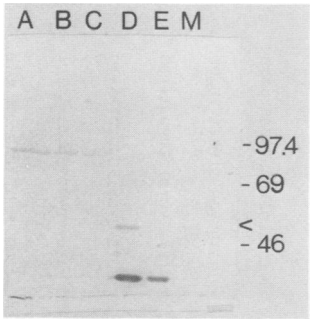

FIG. 3. Western blot showing the reaction of mouse serum raised against the 50,000-M_r fusion protein coded for by pEG1. Lane A, Trypomastigote lysate; lanes B and C, epimastigote lysates; lane D, Triton X-100 insoluble (cement) fraction from pEG1-containing cells; lane E, cement fraction from pUC18-containing cells; lane M, molecular weight markers (phosphorylase *b* [M_r, 97,400], bovine serum albumin [M_r, 69,000], and ovalbumin [M_r, 46,000]).

protein might be a target of the autoimmune response. Uninfected control sera do not recognize these proteins. Ullrich et al. (26) recently described a murine tumor-specific transplantation antigen that shares extensive protein sequence homology with the yeast hsp90 and the *Drosophila* hsp83. Although HSPs are cytosolic, the Meth A tumor-specific transplantation antigen was expressed on the surface of the expressing tumor cells, as well as in the cytosol. This placement allows for the recognitiion of this protein by the T cells of the host. Perhaps a similar mechanism is in effect during the chronic phase of Chagas' disease, allowing for immunorecognition of the HSP.

It should be stated here that the 85,000-M_r polypeptide described in this paper is not the same as the 85,000-M_r surface antigen of *T. cruzi* trypomastigotes that has been described by others (1, 3). In immunoprecipitation studies with rabbit antisera directed against *T. cruzi* trypomastigote surface antigens, the sera did not recognize this 85,000-M_r polypeptide (unpublished observations).

The evidence presented here shows that the 85,000-M_r protein of *T. cruzi* is constitutively expressed in both epimastigotes and trypomastigotes. Both the RNA used for cDNA cloning and HST experiments and the protein lysates used for Western blot experiments were isolated from parasites grown at normal physiological temperatures. The gene organization and level of transcription are quite similar in both epimastigotes and trypomastigotes. In addition, the amount of posttranslational modification of this protein appears to be minimal, since the migrations in SDS-polyacrylamide gels of the in vitro-synthesized polypeptide and the in vivo polypeptide appear identical.

The gene organization of the *T. cruzi* 85,000-M_r polypeptide is quite different from that of the *S. cerevisiae* hsp90 and the *D. melanogaster* hsp83. In the fruit fly, the hsp83 gene is a single-copy gene. In yeast cells, there are two unlinked copies of the 90 kilodalton gene, while the *T. cruzi* gene is a tandemly linked multicopy gene. The expression of one of the hsp90 genes of *S. cerevisiae* appears to be under the control of heat shock regulation, while the other is constitutive (D. Finkelstein, personal communication). Although the hsp83 gene of *D. melanogaster* is under heat shock control, the temperature of its maximum induction is much lower than those of the other HSPs. These observations are in contrast to the apparent constitutive expression of the 85,000-M_r protein of *T. cruzi*. Neither the *T. cruzi* gene nor the yeast gene contains an intron, while the hsp83 gene of *D. melanogaster* contains one intron.

We have assigned the translation start of the hsp85 gene to the first ATG in the open reading frame; however, there is an additional ATG in the same reading frame 15 codons downstream in the sequence. Although we cannot unequivocally state which ATG is the initiator of translation, the strong sequence homology that exists between the proposed *T. cruzi* amino acid sequence and the yeast and *D. melanogaster* HSPs for 10 of the 12 amino acids preceeding the second ATG codon suggests that our assignment of the first ATG as the initiator is correct.

Gonzalez et al. (8) described the isolation from *T. cruzi* of a tandemly linked gene family (IF8) coding for a polypeptide whose predicted amino acid sequence shows strong homology to a number of calcium-binding proteins such as troponin and myosin. Interestingly, this polypeptide also is recognized by CHA. We have described here the tandem organization of the 85,000-M_r polypeptide gene of *T. cruzi*. In addition, we have identified the tandem gene organization for a 70,000-M_r protein of *T. cruzi* (manuscript in preparation). Manning and his colleagues (personal communication) have identified two genes in *T. cruzi*, one of which is also a multicopy gene. The observations of only multicopy gene families agree with the observation of Lanar et al. (13) concerning the complexity of the *T. cruzi* genome. They describe three kinetic components of the *T. cruzi* genome: (i) a moderately repetitive component with a frequency of several thousand, present in 9% of the DNA; (ii) a slightly

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MT---ETFAFQAEINQLMSLIINTFYSNKEIFLRELISNSSDACDKIRYQSLTNQAVLGD   TC
MAS--...E.....T.........V.............A...L.....K..ADPKQ.ET   SC
MPEEA.........A.........................A...L.....E...DPSK.DS   DM

ESHLRIRVVPDKANKTLTVEDTGIGMTKAELVNNLGTIARSGTKAFMEALEAGGDMSMIG   TC
.PD.F..IT.KPEQ.V.EIR.S.........I.......K..........S..A.V....   SC
GKE.Y.KLI.N.TAG...II........SD.........K..........Q..A.I....   DM
                                          ..........A.I....   CK


QFGVGFYSAYLVADRVTVVSKNNDDEAYTWESSAGGTFTVTPTPDCDLK-RGTRIVLHLK   TC
........LF......Q.I..S....Q.I...N...S....LDEVNERIG...ILR.F..   SC
..............K...T........Q.V.......S...RAD-NSEPL....K...YI.   DM
.....G.......EK...ITST                                         CK

EDQQEYLEERRLKDLIKKHSEFIGYDIELMVEKATEKEV----TDEDEDEAAAT----KN   TC
D..L.....K.I.EV..R....VA.P.Q.V.T.EV....PI--PE.EKKDEKKKDEKKK.   SC
...TD....SK..EIVN...Q....P.K.L...ER....SDDEA.DEKK.GD----EK.E   DM

EEGEEPKVEEVKDDAEEGEKKKKTKKVKEVTQEFVVQ---NKHKPLWTRDPKDVTKEEYA   TC
.DDKK..L...---D..E...P.......EV..IE---EL..T......N.S.ITQ...N   SC
M.TD...I.D.GE.EDADK.D.DA..K.TIKEKYTEDEEL..T..I...N.D.ISQ...G   DM

AFYKAISNDWEEPLSTKHFSVEGQLEFRAILFVPKRAPFDMFEPSKKRNNIKLYVRRVFI   TC
....S.......D..YV..................I.......L..SK..K.........   SC
E....LT....DH.A.....................I.R.T...L..NQ.............   DM

MDNCEDLCPEWLAFVRGVVDSEDLPLNISRENLQQNKILKVIRKNIVKKALELFEEIAEN   TC
T.EA...I....S..K...........L...M......M..........LI.A.N....D   SC
.......I..Y.N.MK....                                          DM

KEDYKKFYEQFGKNVKLGIHEDSANRKKLMELLRFHSSESGEDMTTLKDYVTRMKEGQKCI   TC
S.QFE...SA.S..I...V...TQ..AA.AK...YN.TK.VDEL.S.T......P.H..N.   SC

YYVTGDSKKKLETSPFIEQARRRGFEVLFMTEPIDEYVMQQVKDFEDKKFACLTKEGVHFE   TC
..I..E.L.AV.K...LDALKAKN.....L.D.....AFT.L.E..G.TLVDI..DFELE.   SC


ETEEEKKQREEEKTAYERLCKAMKDVLGDKVEKVVVSERLATSPCILVTSEFGWSAHMEQI   TC
TD..KAEREK.I.E-..P.T..LKEI...Q.......YK.LDA.AAIR.GQ.....N..R.   SC

MRNQALRDSSMSAYMMSKKTMEINPAHPIVKELKRRVE-ADENDKAVKDLVYLLFDTALLT   TC
.KA.........S..S....F..S.KS..I....K..DEGGAQ..T....TK..YE.....   SC

SGFTLDDPTSYAERIHRMIKLGLSLDDEDNGNEEAEPAAAVPAEPVAGTSSMENVD       TC
...S..E...F.S..N.L.S...NI.EDEETETAP.AST.APV.E.PADTE..E..       SC
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FIG. 4. Alignment of protein sequences of the hsp90 family. TC, Single-letter amino acid sequence for the 85,000-M_r *T. cruzi* polypeptide. SC, DM, and CK, Published sequences for yeast hsp90 (7), *D. melanogaster* hsp83 (10), and chicken hsp90 (5), respectively, in a best-fit alignment. Capital letters indicate where the amino acid sequences differ, a period indicates where all sequences match, and a hyphen indicates spaces in the sequence to allow for best fit.

repetitive component with a frequency of 10 to 50, present in 51% of the DNA and; (iii) a single-copy component present in 23% of the DNA. The multicopy genes described to date fit into the low-repetition DNA class.

There are no consensus TATA boxes present upstream from the putative initiator ATG in the gene for the 85,000-M_r polypeptide. To date, there have been no published examples of a consensus promoter sequence for *T. cruzi* or for any of the trypanosomes. Comparison of the upstream sequences for the IF8 gene (8) and the 85,000-M_r polypeptide gene has not revealed any apparent common sequences. In fact, the $(GT)_n$ sequence found upstream from the initiator ATG of the IF8 gene is noticeably lacking in the 85,000-M_r polypeptide gene upstream sequence. Neither the consensus heat shock promoter sequence CTGGAATNTTCTAGA (19) nor anything closely related to it is present within the 286 base pairs of DNA upstream of the initiator ATG. The lack of a heat shock promoter might explain the constitutive expression of the 85,000-M_r polypeptide in both epimastigotes and trypomastigotes.

We thank Jerry Manning, Joel Kirschbaum, and Joe Hegdpeth for critical reading of this manuscript and Daniel Wong and Lenny Lebin for performing the DNA sequencing.

This work was supported by National Institutes of Health Small Business Innovation Research grant AI20839.

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