Identification and Characterization of a Novel Deoxyhypusine Synthase in *Leishmania donovani*[*][S]

Bhavna Chawla[‡1], **Anupam Jhingran**[‡1], **Sushma Singh**[‡], **Nidhi Tyagi**[§1], **Myung Hee Park**[¶], **N. Srinivasan**[§],
Sigrid C. Roberts[‖], **and Rentala Madhubala**[‡2]

From the ‡*School of Life Sciences, Jawaharlal Nehru University, New Delhi 110067, India, the* §*Molecular Biophysics Unit, Indian
Institute of Science, Bangalore 560012, India, the* ¶*Oral and Pharyngeal Cancer Branch, NIDCR, National Institutes of Health,
Bethesda, Maryland 20892-4340, and the* ‖*School of Pharmacy, Pacific University, Portland, Oregon 97123*

Deoxyhypusine synthase, an NAD$^+$-dependent enzyme, catalyzes the first step in the post-translational synthesis of an unusual amino acid, hypusine (N^ϵ-(4-amino-2-hydroxybutyl)lysine), in the eukaryotic initiation factor 5A precursor protein. Two putative deoxyhypusine synthase (DHS) sequences have been identified in the *Leishmania donovani* genome, which are present on chromosomes 20: *DHSL20* (DHS-like gene from chromosome 20) and *DHS34* (DHS from chromosome 34). Although both sequences exhibit an overall conservation of key residues, DHSL20 protein lacks a critical lysine residue, and the recombinant protein showed no DHS activity *in vitro*. However, DHS34 contains the critical lysine residue, and the recombinant DHS34 effectively catalyzed deoxyhypusine synthesis. Furthermore, *in vivo* labeling confirmed that hypusination of eukaryotic initiation factor 5A occurs in intact *Leishmania* parasites. Interestingly, the DHS34 is much longer, with 601 amino acids, compared with the human DHS enzyme (369 amino acids) and contains several unique insertions. To study the physiological role of DHS34 in *Leishmania*, gene deletion mutations were attempted via targeted gene replacement. However, chromosomal null mutants of *DHS34* could only be obtained in the presence of a *DHS34*-containing episome. The present data provide evidence that DHS34 is essential for *L. donovani* and that structural differences in the human and leishmanial DHS enzyme may be exploited for designing selective inhibitors against the parasite.

Leishmania is a protozoan parasite that results in a wide spectrum of diseases ranging from simple cutaneous lesions to the usually fatal visceral form. *Leishmania donovani* is the major causative agent of the visceral leishmaniasis. Pentavalent antimonials are the standard first line of treatment for leishmaniasis, but due to toxicity and increasing resistance of the present repertoire of drugs (1–3), an urgent need exists for newer drugs and drug targets.

The polyamine pathway is one such pathway that has been exploited successfully for antiparasitic chemotherapy (4). The enzymes ornithine decarboxylase, adenosylmethionine decarboxylase, and spermidine synthase (SPDS) have been identified as important drug targets (4). Spermidine, the final product of the polyamine biosynthetic pathway in kinetoplastids, acts as a precursor for trypanothione (a key molecule in defense against oxidative stress) (5) and hypusine (6). Hypusine (N^ϵ-(4-amino-2-hydroxybutyl)lysine) is an unusual amino acid named after its two structural components, hydroxyputrescine and lysine (7). Hypusine synthesis is the result of a post-translational modification occurring exclusively in one cellular protein, eukaryotic initiation factor 5A (eIF5A),[3] by the transfer of the 4-aminobutyl moiety from spermidine to its lysine side chain (8). Hypusine formation occurs mainly in two enzymatic steps (9). The first step is catalyzed by the enzyme deoxyhypusine synthase (DHS), which catalyzes the NAD$^+$-dependent transfer of the 4-aminobutyl moiety of spermidine to a specific lysine residue of the eIF5A precursor protein to form an intermediate, deoxyhypusine (10, 11). This intermediate is subsequently hydroxylated by the enzyme deoxyhypusine hydroxylase (12), which completes the synthesis of hypusine and maturation of eIF5A.

Biosynthesis of hypusine represents a novel, unique, and the most specific post-translational modification known in literature. In mammalian cells, inhibitors of spermidine biosynthesis (13) and hypusine biosynthetic enzymes, DHS and deoxyhypusine hydroxylase, exert antiproliferative effects (14–16), including in cancer cell lines, and cause arrest of cell cycle progression (17). DHS has also been suggested as the potential target for antiretroviral therapy (18). Direct evidence showing the essential nature of eIF5A and DHS comes from gene inactivation studies in *Saccharomyces cerevisiae*. Disruption of the two eIF5A genes (*TIF51A* and *TIF51B*) (19) and the deoxyhypusine synthase gene (20) produces a lethal phenotype.

Deoxyhypusine synthase is found in archaea and all eukaryotes. It is a highly conserved protein and exists as a single gene product of 300–400 amino acids (33–48 kDa) in most eukaryotes. The native enzymes of human and yeast recombinant proteins exist as a ~160-kDa molecule, a tetramer of four identical subunits of ~40 kDa (21, 22). Crystal structures of the human DHS tetrameric enzyme in complex with a cofactor NAD and its inhibitor have been reported (23, 24).

In the present study, we, for the first time, report the identification, molecular cloning, expression, and characterization of

---

[3] The abbreviations used are: eIF5A, eukaryotic initiation factor 5A; DHS, deoxyhypusine synthase; ORF, open reading frame.

L. donovani Deoxyhypusine Synthase

the enzyme deoxyhypusine synthase from *L. donovani.* Two DHS-like genes have been identified in *L. donovani*, which show low homology with the human DHS. Both genes have been cloned and expressed, but only one, *DHS34*, exhibits deoxyhypusine synthase activity. Gene replacement studies for *DHS34* indicate that the enzyme deoxyhypusine synthase and eIF5A modification play an essential role in cell viability of this pathogenic organism.

EXPERIMENTAL PROCEDURES

Materials—Radiolabeled spermidine trihydrochloride [1,8-^{3}H]spermidine (16.6 Ci/mmol) and radiolabeled [1,4-^{14}C]putrescine (110 mCi/mmol) were purchased from PerkinElmer Life Sciences. [α-^{32}P]dCTP (3000 Ci/mmol) was acquired from Amersham Biosciences. All restriction enzymes and DNA-modifying enzymes were obtained from MBI Fermentas (Germany). Neomycin, phleomycin, and hygromycin were obtained from Sigma. N^1-guanyl-1,7-diaminoheptane was synthesized as described earlier (14). Recombinant human DHS was purified from *Escherichia coli* cells as reported earlier (21). The other materials used in this study were of analytical grade and were commercially available.

Parasite and Culture Conditions—*L. donovani* AG83 (MHOM/IN/1983/AG83) promastigotes were cultured at 22 °C in modified M199 medium (Sigma) supplemented with 100 units/ml penicillin (Sigma), 100 μg/ml streptomycin (Sigma), and 10% heat-inactivated fetal bovine serum (Hyclone). The genetically manipulated parasites were derived from the wild type *LdBob* strain of *L. donovani* (25) that was originally obtained from Dr. Stephen Beverley (Washington University, St. Louis, MO). *LdBob* promastigotes and axenic amastigotes were routinely cultivated at 28 °C, pH 7.4, and 37 °C, pH 5.5, respectively, in the culture media previously reported (25, 26). All parasite strains were cycled back and forth between the promastigote and axenic amastigote forms. Wild type parasites were routinely cultured in media with no drug supplementations, whereas the DHS heterozygotes were maintained in either 50 μg/ml hygromycin (*DHS34/HYG::dhs*) or 50 μg/ml phleomycin (*DHS34/PHLEO::dhs*). The *DHS34/HYG::dhs* strain containing the pX63PAC-*DHS* episome was grown in 50 μg/ml hygromycin and 20 μM puromycin, and the putative Δ*dhs34*[pX63PAC-*DHS34*] clones were kept in 50 μg/ml hygromycin, 50 μg/ml phleomycin, and 20 μM puromycin.

Cloning of Putative DHS Genes from L. donovani—A 1.14-kb DNA fragment encompassing the whole open reading frame (ORF) of the *L. donovani* DHS-like-gene *DHSL20* was amplified from genomic DNA using a sense primer with a flanking BamHI site, 5′-CGCGGATCCATGCTTGCCTCTGTCCCAG-3′ and an antisense primer with a flanking HindIII site, 5′-CCCAAGCTTGCGGCGCTCTGCGCTAC-3′. A 1.8-kb DNA fragment encompassing the whole ORF of the *L. donovani DHS34* gene was also amplified from genomic DNA using the sense primer with a flanking BamHI site, 5′-CGGGATCC-ATGGCGAATATTGCGGAATC-3′, and the antisense primer with the flanking HindIII site, 5′-CCCAAGCTTGAGTGCCC-GGAGACGTCTTG-3′. PCR was performed in 50-μl reaction volumes containing 100 ng of genomic DNA, 25 pmol each of the gene-specific forward and reverse primers, 200 μM each of

dNTPs, 2 mM MgCl$_2$, and 5 units of Pfu DNA polymerase (MBI Fermentas). The conditions of the PCR were as follows: 94 °C for 10 min, 30 cycles of 94 °C for 1 min, 60 °C for 30 s, and 72 °C for 1.5 min for *DHSL20* and 72 °C for 3 min for *DHS34*. Final extension was carried out for 10 min at 72 °C. A single ~1140-bp (*DHSL20*) and 1806-bp (*DHS34*) PCR products were obtained. Each gene was then cloned into the pTZ57R/T (MBI Fermentas) vector and sequenced.

Sequence and Structural Analysis—Sequence comparison of *DHSL20* and *DHS34* with other sequences of the data base was done using the search algorithm BLAST (available on the NCBI Web site). Multiple alignment of amino acid sequences was performed using the program ClustalW (available on the World Wide Web) The bootstrap analysis was done and the phylogenetic tree was constructed using the software MEGA version 4.0 (27). DHSL20 and DHS34 protein sequences were searched against the multiple position-specific substitution matrix data base (MulPSSM) (28) of Pfam protein domain families (29) using an RPS-BLAST approach (30). Local alignments of DHS34 and DHSL20 protein were compared with human DHS to analyze the conservation of important amino acid residues. Subsequently, DHS34 was searched against the Protein Data Bank (31) using the BLAST program (32) for closely related DHS homologs with known three-dimensional structure. The DHS34 protein sequence was modeled on the basis of the tertiary structure of the human DHS template (Protein Data Bank code 1dhs) (23) using MODELLER version 8.0 (available on the World Wide Web) (33). The generated model was energy-minimized using Kollman united atom forced field in SYBYL (Tripos Inc., St. Louis, MO) to relieve short contacts if any. The overall fit of the sequence to the modeled structure was checked using PROSA (34, 35). The stereochemistry of the models was evaluated using PROCHECK (36, 37).

Construction of DHSL20 and DHS34 Expression Vectors and Purification of the Proteins—The coding regions of the *DHSL20* gene and *DHS34* gene were subcloned into the BamHI-HindIII site of pET30a vector (Novagen). The fidelity of the PCR amplification of *L. donovani DHSL20* and *DHS34* was confirmed by automated DNA sequencing. The recombinant constructs were transformed into the BL21 (DE3) strain of *E. coli*. Expression from the constructs *DHSL20*-pET30a and *DHS34*-pET30a was induced at an A_{600} of 0.6 with 0.25 mM isopropyl-1-β-D-galactopyranoside at 16 °C for 12 h. Bacteria were then harvested by centrifugation at 5000 × *g* for 10 min, and the cell pellet was resuspended in binding buffer (50 mM Tris-Cl, pH 8.0, 10 mM imidazole, 300 mM sodium chloride, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture). The resulting cell suspension was sonicated six times for 30 s with 1-min intervals. The lysate was centrifuged at 10,000 × *g* for 30 min at 4 °C. The resulting supernatant, which contained the protein, was loaded onto pre-equilibrated Ni^{2+}-nitrilotriacetic-agarose resin (Qiagen). The mixture was kept on a rocking platform for 2 h at 4 °C. It was centrifuged at 400 × *g* for 1 min at 4 °C. The supernatant was removed, and the resin was washed three times with wash buffer (50 mM Tris-Cl, pH 8.0, 20 mM imidazole, 300 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture). The protein was eluted with increasing concentrations of imidazole. Imidazole was

removed, and the purified protein was found to be >95% pure as judged by SDS-PAGE. The purified protein was aliquoted and stored at −80 °C in 50 mM Tris-Cl, pH 8.0, 300 mM NaCl buffer.

Deoxyhypusine Synthase Assay—Deoxyhypusine synthase activity was assayed by measuring incorporation of radioactivity from [1,8-^{3}H]spermidine into *L. donovani* eIF5A (accession number ABO34138.1) precursor protein (38, 39). A typical reaction mixture contained, in a total volume of 50 μl, 0.2 M glycine-NaOH buffer, pH 9.2, 1 mM dithiothreitol, 1 mM NAD, 200 μg of bovine serum albumin, ~5 μM (4 μCi) [1,8-^{3}H]spermidine, 5 μM eIF5A, and the indicated amount of recombinant DHS. The reaction was incubated at 37 °C for 60 min. The reaction mixture was terminated by the addition of 10% trichloroacetic acid containing putrescine, spermidine, and spermine (1 mM each). The samples were then centrifuged at $10,000 \times g$ for 5 min at 4 °C. The precipitate obtained was washed repeatedly with 10% trichloroacetic acid containing polyamines to remove the [^{3}H]spermidine bound to the pellet. The [^{3}H]deoxyhypusine formed was measured by dissolving the pellet in 100 μl of 0.1 N NaOH, and the counts were taken. One unit of enzyme activity is defined as the amount of enzyme catalyzing the formation of one pmol/h of deoxyhypusine. Deoxyhypusine formation was also confirmed by detection of radiolabeled eIF5A by fluorography after SDS-PAGE.

In Vivo Labeling of eIF5A—Wild type and spermidine synthase-deficient ($\Delta spdsyn$) parasites (40) were incubated in growth medium containing radiolabeled [1,4-^{14}C]putrescine and 100 μM unlabeled spermidine for 4 days. Subsequently, parasites were harvested and sonicated, and lysates were fractionated by SDS-PAGE. The gel was stained with Coomassie Blue and subsequently dried and exposed for autoradiography.

Molecular Constructs for the Replacement of the DHS34 Alleles—To construct drug resistance cassettes for targeted *DHS34* gene replacement, the 5′- and 3′-flanking regions of *DHS34* were amplified by PCR. The 5′-flank was amplified using genomic DNA as a template and the following primers: AAGCTTCCAGAGTACACTGGATAGGC (HindIII site underlined) and GTCGACTCTCTTGTCTCACCGCC (SalI site underlined). The 423-bp 5′-flanking region was then cloned into the HindIII/SalI sites of pX63HYG to generate pX63HYG-5′*DHS34*. The 3′-flank was amplified using the following primer pair: CCCGGGGGAGTGGGCTGGTGGCG (SmaI site underlined) and AGATCTCGCGCCGCAACCTCGTAAAAG (BglII site underlined). The 584-bp 3′-flanking region was cloned into the SmaI/BglII site of pX63HYG-5′*DHS34* to generate the pX63HYG$\Delta dhs34$ gene replacement construct. The same 5′-flank was cloned into the HindIII/SalI sites of the pX63PHLEO vector to generate pX63PHLEO-5′*DHS34*. Because of the presence of a SmaI site within the coding region of *PHLEO*, a different strategy was employed to insert the *DHS34* 3′-flanking region into the pX63PHLEO-5′*DHS34*. For the generation of the 3′-flanking fragment, the following primers were used: GGATCCGGAGTGGGCTGGTGGCG (BamHI underlined) and AGATCTCGCGCCGCAACCTCGTAAAAG (BglII underlined) to generate a 580-bp PCR fragment. The fragment was cloned into pX63PHLEO-5′*DHS34* to generate the gene replacement construct pX63PHLEO$\Delta dhs34$. PCRs were performed using the Advantage^{TM} PCR kit (BD) at the following conditions: 94 °C for 10 min, 30 cycles of 94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 10 min. Restriction mapping and limited nucleotide sequence analysis was used to confirm the correct orientation of the 5′ and 3′ *DHS34* flanking region within the pX63HYG$\Delta dhs34$ and pX63PHLEO$\Delta dhs34$ vectors.

Cloning of the pX63PAC-DHS34 Episome—To generate the pX63PAC-*DHS34* complementation construct, the following primers were used to amplify the *DHS34* coding region by PCR: GGATCCATGGCGAATATTGCGGAATCTG (BamHI site underlined) and AGATCTTCATTCTCCAAGACACGGGTGG (BglII site underlined). PCRs were performed using the high fidelity HFAdvantage^{TM} PCR kit (BD Biosciences) under the following conditions: 94 °C for 10 min, 30 cycles of 94 °C for 1 min, 60 °C for 2 min, 72 °C for 1 min, and a final extension at 72 °C for 10 min. The 1.8-kb BamHI-BglII fragment was subcloned into the BamHI/BglII sites of pX63PAC. The correct orientation and sequence fidelity of the insert was verified by nucleotide sequence analysis.

Generation of Genetically Manipulated Parasites—The pX63HYG$\Delta dhs34$ and pX63PHLEO$\Delta dhs34$ plasmids were digested with HindIII and BglII to liberate the deletion fragments containing the drug resistance cassette and *DHS34* flanking regions (designated HYG$\Delta dhs34$ and PHLEO$\Delta dhs34$). These fragments were gel-purified and transfected into wild type *LdBob* parasites according to standard protocols (26, 41). The HYG$\Delta dhs34$ and PHLEO$\Delta dhs34$ gene replacement cassettes were individually transfected into wild type parasites, and colonies were selected from semisolid agar plates in the presence of 50 μg/ml hygromycin or 50 μg/ml phleomycin, respectively. The genotype of the putative heterozygous parasites, *DHS34/HYG::dhs34* and *DHS34/PHLEO::dhs34*, was confirmed by Southern blot analysis using the 5′- and 3′-flanking regions as probes. The growth phenotype of heterozygous parasites was established by seeding 2×10^5 cells/ml, and parasites were counted after 4 days on a hemocytometer. The heterozygous parasites, *DHS34/HYG::dhs34* and *DHS34/PHLEO::dhs34*, were subsequently transfected with the PHLEO$\Delta dhs34$ and HYG$\Delta dhs34$ replacement cassettes, respectively, in an attempt to generate homozygous gene deletion mutants. Colonies were selected on semisolid agar plates containing 50 μg/ml hygromycin and 50 μg/ml phleomycin, and parasite genotypes were established by Southern blotting. The pX63PAC-*DHS34* episome was transfected into *DHS34/HYG::dhs34* heterozygous parasites, and parasites containing the plasmid were selected in 20 μM puromycin. These *DHS34/HYG::dhs34*[pX63PAC-*DHS34*] parasites were subsequently transfected with the purified PHLEO$\Delta dhs34$ gene replacement construct, and colonies were selected on semisolid agar plates containing 50 μg/ml hygromycin, 20 μM puromycin, and 50 μg/ml phleomycin to isolate triple drug-resistant parasites. The genotypes of several clones were established by Southern blotting.

Molecular Characterization of Genetically Manipulated Parasites—Genomic DNA from wild type and genetically manipulated parasites was prepared for Southern blot and PCR analysis using the DNAeasy kit (Qiagen Inc., Valencia, CA).

```
L.donovani    MLASVPAPRPAKKDSAASRRKSASKSTGAAVKDGSSARVSASGAAESPKQSCAQVHGVDF  60
T.cruzi       ------------------------------------------------METVDALDY      9
T.brucei      -MSGVPFP--------------------------------------------SRVIGDLDY 16
H.sapiens     ------------------------MEGSLEREAPAGALAAVLKHSSTLPPESTQVRGYDF  36
S.cerevisiae  -----------------------MSDINEKLPELLQDAVLKASVPIPDDFVKVQGIDY    35
                                                                         :   *:

L.donovani    QSLVHATQE----ETLRAVVSSLPTTGLQATQIGRARQLVQQILHHR-------------  103
T.cruzi       SEIVALNQE----EALRRVLASYPRIGLQATEIGRARIRVQRALYHK-------------  52
T.brucei      SNLLNIGQE----EAIRCVLNAYPNIGLEATNLGRARIRVQRALNDNG------------  60
H.sapiens     NRG---------VVNYRALLEAFGTTGFQATNFGRAVQQVNAMIEKKLEPLSQDEDQHAD  86
S.cerevisiae  SKPEATN------MRATDLIEAMKTMGFQASSVGTACEIIDSMRSWRGKHIDELDDHEKK  89
                 .             ::  :     *::*:..  *    ::         

L.donovani    -------SPEDRVFLAYTSNMISCGLRDTFTYLARERLVDCFISSAGCIEEDVIKCGGST 156
T.cruzi       -------RAGDAVFLAYTSNLISSGLRDTFACLARDRLIDGFISTAGCIEEDAIKCLGKT 105
T.brucei      -------MDGNKVMLAYTSNLISSGLRDTFACLARENRIGAVVTTAGGVEEDVIKCLGDT 113
H.sapiens     LTQSRRPLTSCTIFLGYTSNLISSGIRETIRYLVQHNMVDVLVTTAGGVEEDLIKCLAPT 146
S.cerevisiae  GCFDEEGYQKTTIFMGYTSNLISSGVRETLRYLVQHKMVDAVVTSAGCVEEDLIKCLAPT 149
                       :::.****:**.*:*:*:.  *.:. :. .:::***:*** **  . *

L.donovani    ILGQFGQDGRALRRRGINRIGNLLVPNDNYCWFEDFFTPVLESVQEAQRASR-------- 208
T.cruzi       LVGQFSLDCRELRRCGVNRTGNLLVPNDNYCHFENFFMPVLKHLHELQRESR-------- 157
T.brucei      LVGDFALNDHALRNNCLNRVGNLLVPNDNYRNFEDFFVPLLRRLHEQQRDSR-------- 165
H.sapiens     YLGEFSLRGKELRENGINRIGNLLVPNENYCKFEDWLMPILDQMVMEQN----TEG---- 198
S.cerevisiae  YLGEFALKGKSLRDQGNRIGNLLVPNENYCKFEEWIVPILDKMLEEQDEYVKKHGADCL 209
                :*:*     **   *:  ***:***:**  **::  *.*        *

L.donovani    ------WKLHTAPSEIIEAMGAAIAKNHPDTCTSSLVYWCYRNGISVFSPAFTDGSMGDM 262
T.cruzi       ------WELMTAPSEMIAAIGAALGCKHPETCSDSLLYWCYRNNIPVFSPALTDGSIGDM 211
T.brucei      ------WTTKTTPSQIIAEIGAALESVRPNDCGSSLIYWCYRNDIPVFSPAFTDGSMGDM 219
H.sapiens     -------VKWTPSKMIARLGKEINNP------ESVYWAQKNHIPVFSPALTDGSLGDM 244
S.cerevisiae  EANQDVDSPIWTPSKMIDRFGKEINDE------SSVLYWAHKNKIPIFCPSLTDGSIGDM 263
                       :**.:  :*  :              .*. **. :* *.::.:::****:***

L.donovani    IYFYNFSHK--GLVVDPLEDVVRLRKLAAKER---------------GRNLAIVLGGGLP 305
T.cruzi       IYFYNYSKK--GLVLDEIVDVVRLRELGCRNRRCDDSQGGNRSQNNNGRTTCIVLGGGLP 269
T.brucei      IYFYNYSRK--GLVVDPVPDVVRLRLQLGCKS-------------TNVGRITCIVLGAGLP 264
H.sapiens     IFHSYKNP--GLVLDIVEDLRLINTQAIFAK---------------CTGMIILGGGVV 286
S.cerevisiae  LEFHTEKASPKQLRVDIVGDIRKINSMSMAAY---------------RAGMIILGGGLI 307
                ::*:::.    * :*  *:  :  .      .        *:**.*:

L.donovani    KFHLLRN----VPMDAVVMVTTGLEADGCVSSGVLADDVACGLLREETETVRVQGDALVV 361
T.cruzi       KFHLLQN----VRADTVVYVSTGLEVDASPESCNVAEDRANGVILDNCEVVRVHGDASFV 325
T.brucei      KFHLLRN----VQADAVVYVTTGSDADGCESSCNVMADRANGLLSPNCDVVRVHGDATII 320
H.sapiens     KFHIANANLMRNGADYAVYINTAQEFDGSDSGARPDEAVSWGKIRVDAQPVKVYADASLV 346
S.cerevisiae  KFHIANACLMRNGADYAVYINTGQEYDGSDAGARPDEAVSWGKIKAEAKSVKLFADVTIV 367
                ***:          :  *  .:*  *.:.: * .. :.    :*  :   : *::.:

L.donovani    FPLMLIAETAATREGAAA----- 379
T.cruzi       FPILLCKAETSADTHKDVAA--- 345
T.brucei      SPILLLRSSDGKEKVGVREDGN- 342
H.sapiens     FPLLVAETFAQKMDAFMHEKNED 369
S.cerevisiae  LPLIVAATFASGKPIKKVKN--- 387
                **::: 
```
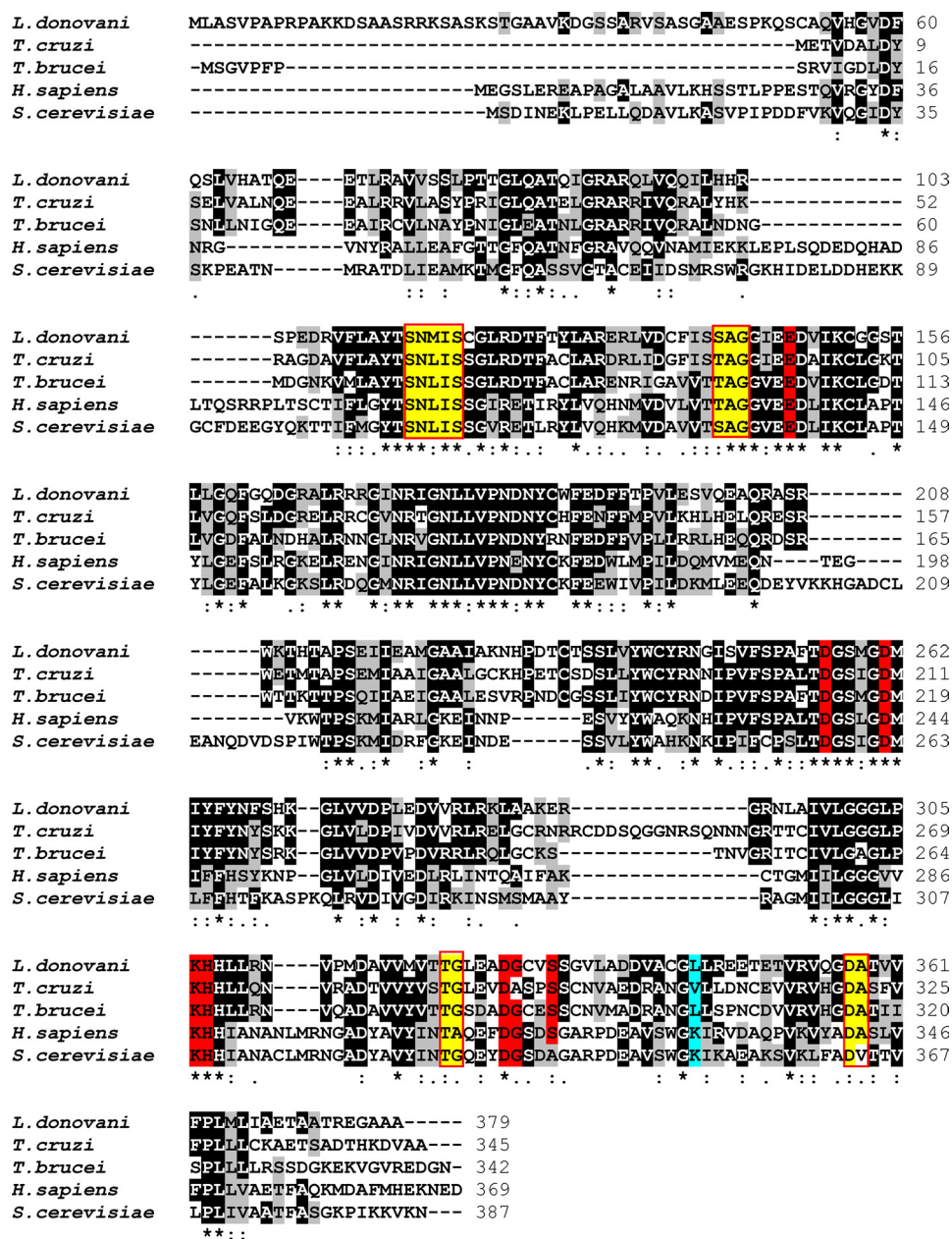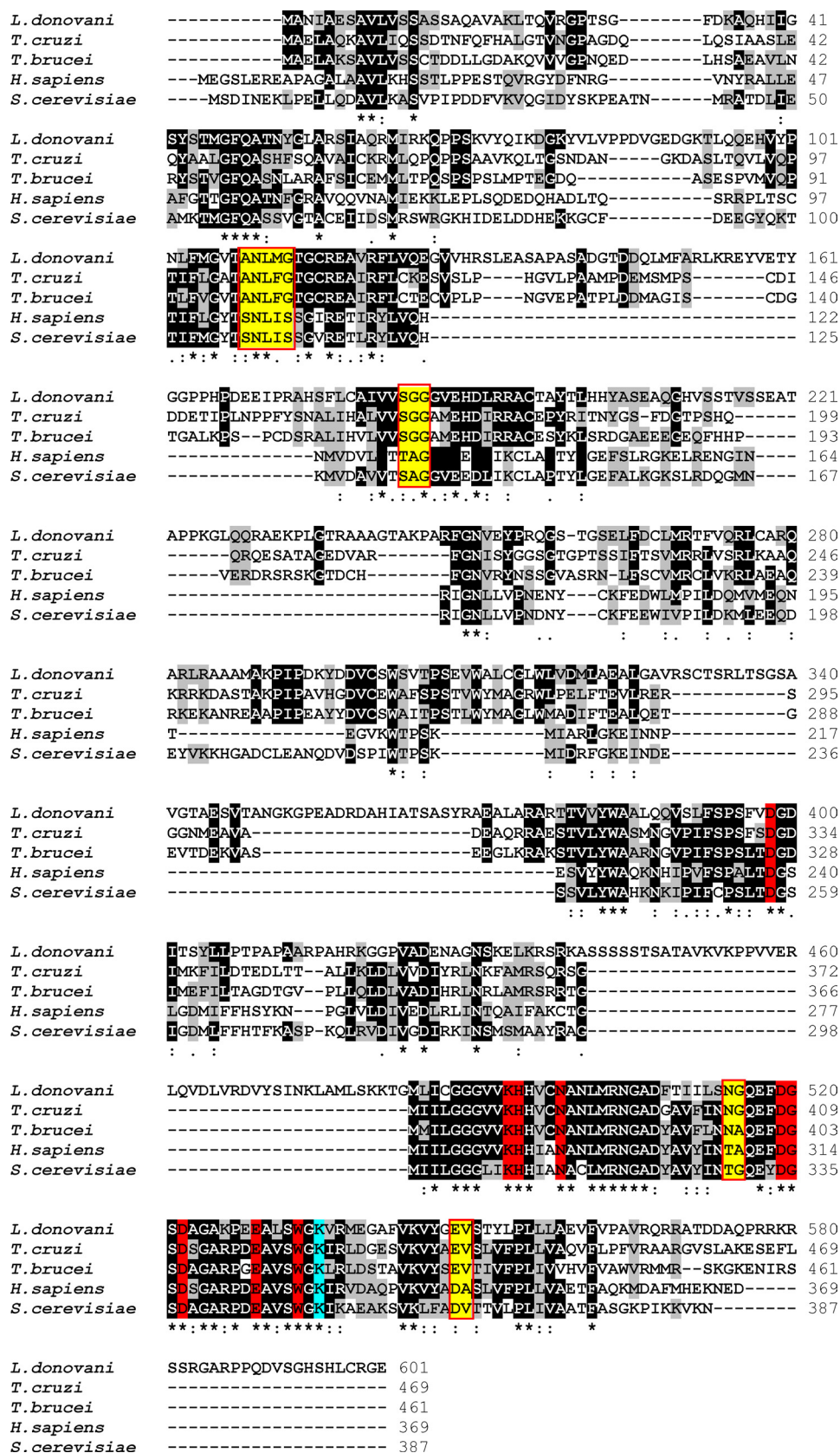
FIGURE 1. **Multiple sequence alignment of DHS-like protein (DHSL20) sequences from *L. donovani* (ABP65295), *T. cruzi* (Tc00.1047053511421.60), *T. brucei* (Tb927.1.870), *S. cerevisiae* (P38791), and human (P49366).** Conserved residues are *shaded* in *black,* showing complete identity, and residues *shaded* in *gray* are similar. NAD$^+$ binding regions are enclosed in *yellow rectangular boxes*. The catalytic lysine residue is shown on a *cyan background*. Other residues important for the overall functioning of enzyme are highlighted on a *red background*.

DNA was digested with the appropriate restriction enzymes and probed with either 5′- or 3′-flanking regions or the *DHS34* coding region probe using highly stringent hybridization conditions. Furthermore, a PCR strategy was devised to confirm the results obtained by Southern blotting. The following primers were designed to amplify DNA fragments within the *DHS34* coding region: the top primer 1 (ATGGCGAATATTGCGGA-GTCT) and the bottom primers 7 (CAACACCCTCCTGCAC-AAGAA), 8 (GGCGTAGTAGTGCAGTGTG), and 10 (CAC-AGCCCTCAGCGCCTGCGC). Another set of primers was designed to amplify a fragment spanning from the 5′-flanking region into the coding region, the top primers 2 (cctcagtctgct-

atgctccc) and 4 (agaaagagcgatgtcgt-tgcctc) to hybridize to the 5′-flanking region, and the bottom primers 5 (cagattccgcaatattcgcCAT) and 6 (caccctcctgcacaagaaagcg) to hybridize to the *DHS34* coding region. PCRs were performed using the Advantage™ PCR kit (BD Biosciences) at the following conditions: 94 °C for 10 min, 30 cycles of 94 °C for 1 min, 58 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 10 min.

RESULTS

Sequence Analysis and Genomic Organization—Two *DHS* sequences have been identified in each of the *Leishmania infantum* and *Leishmania major* genomes (see the EMBL-EBI Web site). Two DHS-like sequences have also been identified in the *Trypanosoma brucei* (Tb927.1.870 and Tb10.70.4900) and *Trypanosoma cruzi* genomes (Tc00.1047053511421.60 and Tc00. 1047053504119.29), whereas most other organisms have only one *DHS* gene copy. BLAST analysis of *L. major* and *L. infantum* genomes revealed that the deoxyhypusine synthase-encoding genes are present on chromosomes 20 (*DHSL20*) and 34 (*DHS34*). Southern blot analysis of the *DHSL20* and *DHS34* showed that these were single copy genes (data not shown). Sequence analysis, data base search, and alignment of the *L. donovani* DHSL20 and DHS34 amino acid sequences were performed. The DHSL20 amino acid sequence had a single open reading frame consisting of 1140 bp (*L. donovani DHS*, EF512031). The ORF encoded a putative polypeptide of 379 amino acids, with a predicted molecular mass of ~42 kDa. Amino acid sequence alignment of DHSL20 protein with homologous sequences from other species revealed that it shares 63.4% identity with the *T. cruzi* (Tc00.1047053511421.60), 56.1% identity with *T. brucei* (Tb927.1.870), and 30.7% with *S. cerevisiae* (P38791) proteins (Fig. 1).

Furthermore, we proceeded with the characterization of DHS34, which is located on chromosome 34. The sequence identity between DHSL20 protein and DHS34 is only 21.7%. A single open reading frame consisting of 1806 bp was identified (*L. donovani DHS*, EU864544). The ORF encoded a putative polypeptide of 601 amino acids, with a predicted

```
L.donovani    ----------MANIAESAVLVSSASSAQAVAKLTQVRGETSG--------FDKAQHITG  41
T.cruzi       ---------MAELAQKAVLIQSSDTNFQFHALGTVNGEAGDQ------LQSIAASLE     42
T.brucei      ---------MAELAKSAVLVSSCTDDLLGDAKQVVVGPNQED-------LHSAEAVLN    42
H.sapiens     ---MEGSLEREAPAGALAAVLKHSSTLPPESTQVRGYDFNRG----------VNYRALLE 47
S.cerevisiae  ----MSDINEKLPELLQDAVLKASVPIPDDFKVQGIDYSKPEATN-----MRATDLIE   50
                         **:     *       *                 :

L.donovani    SYSTMGFQATNYGLARSIAQRMIRKQPPSKVYQIKDGKYVLVPPDVGEDGKTLQQEHVYP 101
T.cruzi       QYMAALGFQASHFSQAVAICKRMLQPQPPSAAVKQLTGSNDAN-----GKDASLTQVLVQP 97
T.brucei      RYSTVGFQASNLARAFSICEMMLTPQSPSPSLMPTEGDQ----------ASESPVMVQP  91
H.sapiens     AFGTTGFQATNFGRAVQQVNAMIEKKLEPLSQDEDQHADLTQ----------SRRPLTSC 97
S.cerevisiae  AMKTMGFQASSVGTACEIIDSMRSWRGKHIDELDDHEKKGCF------DEEGYQKT    100
                 ****    *   *     *             *

L.donovani    NLFMGVIANLMGTGCREAVRFLVQEGVVHRSLEASAPASADGTDDQLMFARLKREYVETY 161
T.cruzi       TIFLGAIANLFGTGCREAIRFLCKESVSLP----HGVLPAAMPDEMSMPS-------CDI 146
T.brucei      TLFVGVIANLFGTGCREAIRFLCTECVPLP----NGVEPATPLDDMAGIS-------CDG 140
H.sapiens     TIFLGYISNLISSGIRETIRYLVQH----------------------------------- 122
S.cerevisiae  TIFMGYISNLISSGVRETLRYLVQH----------------------------------- 125
               .:*:*  ::**.  :*  *:..:*:

L.donovani    GGPPHPDEEIPRAHSFLCAIVVSGGGVEHDLRRACTAYTLHHYASEAQGHVSSTVSSEAT 221
T.cruzi       DDETIPLNPPFYSNALIHALVVSGGAMEHDTRRACEPYRITNYGS-FDGTPSHQ------ 199
T.brucei      TGALKPS--PCDSRALIHVLVVSGGAMEHDTRRACSYKLSRDGAEEEGEQFHHP----- 193
H.sapiens     --------------NMVDVLTTAGE-IKCLATYGEFSLRGKELRENGIN---- 164
S.cerevisiae  --------------KMVDAVVTSAGGVEHDIIKCLAPTYLGEFALKGKSLRDQGMN---- 167
                           :   *:..:*.:.:  .   :

L.donovani    APPKGLQQRAEKPLGTRAAAGTAKPARFGNVEYPRQGS-TGSELFDCLMRTFVQRLCARQ 280
T.cruzi       ------QRQESATAGEDVAR------FGNISYGGSGTGPTSSIFTSVMRRLVSRLKAAQ  246
T.brucei      --VERDRSRSKGTDCH--------FGNVRYNSSGVASRN-LESCVMRCLVKRLAEAQ    239
H.sapiens     ------------------RIGNLLVPNENY---CKFEDWLMPILDQMVMEQN        195
S.cerevisiae  ------------------RIGNLLVPNDNY---CKFEEWIVPILDKMIEEQD        198
                                 **:       ..       :   :  :  :   :

L.donovani    ARLRAAAMAKPIPDKYDDVCSWSVTPSEVVWALCGLWLVDMIAEALGAVRSCTSRLTSGSA 340
T.cruzi       KRRKDASTAKPIPAVHGDVCEWAFSPSTVWYMAGRWLPELFTEVLRER----------S  295
T.brucei      RKEKANREAAPIPEAYYDVCSWAITPSTLWYMAGLWMADIFTEALQET----------G  288
H.sapiens     T--------------EGVKWTPSK--------MIARLGKEINNP------------     217
S.cerevisiae  EYVKKHGADCLEANQDVDSPIWTPSK--------MIDRFGKEINDE-----------    236
                            *:               :       :  :  :

L.donovani    VGTAESVTANGKGPEADRDAHIATSASYRAEALARARTVVYWAALQQVSLFSPSFVDGD  400
T.cruzi       GGNMEAVA--------------------DEAQRRAESTVLYWASMNGVPIFSPSFSDGD  334
T.brucei      EVTDEKVAS--------------------EEGLKRAKSTVLYWAARNGVPIFSPSLTDGD 328
H.sapiens     ----------------------------ESVYYWAQKNHIPVFSPALTDGS         240
S.cerevisiae  -----------------------SSVLYWAHKNKIPIFCPSLTDGS             259
                                       ::  ***   :  :.::.*::  **.

L.donovani    ITSYLLPTPAPAARPAHRKGGPVADENAGNSKELKRSRKASSSSSTSATAVKVKPPVVER 460
T.cruzi       IMKFILDTEDLTT--ALLKLDIVVDIYRLNKFAMRSQRSG-------------------- 372
T.brucei      IMEFILTAGDTGV--PLIQLDVAIDIHRLNRLAMRSRRTG-------------------- 366
H.sapiens     LGDMIFFHSYKN---PGIVLDIVEDLRLINTQAIFAKCTG-------------------- 277
S.cerevisiae  IGDMIFFHTFKASP-KQIRVDIVGDIRKINSMSMAAYRAC-------------------- 298
                :  :           . * *    *      :

L.donovani    LQVDLVRDVYSINKLAMLSKKTGMIICGGGVVKHHVCNANLMRNGADFTIILSNGQEFDG 520
T.cruzi       -------------------MIILGGGVVKHHVCNANLMRNGADGAVFINNGQEFDG     409
T.brucei      -------------------MMILGGGVVKHHVCNANLMRNGADYAVFLNNAQEFDG     403
H.sapiens     -------------------MIILGGGVVKHHIANANLMRNGADYAVYINTAQEFDG     314
S.cerevisiae  -------------------MIILGGGLIKHHIANACLMRNGADYAVYINTGQEYDG     335
                                :* ***  *** ** ******:  :::   *:**

L.donovani    SRPAGAKPEBALSWGKVRMEGAFVKVYGEVSTYLPLLIAEVFVPAVRQRRATDDAQPRRKR 580
T.cruzi       SRPSGARPDEAVSWGKIRLDGESVKVYAEVSLVFPLLVAQVFLPFVRAARGVSLAKESEFL 469
T.brucei      SRPAGARPGEAVSWGKIRLDSTAVKVYSEVTIVFPLIVHVFEVAAWVRMMR--SKGKENIRS 461
H.sapiens     SRPSGARPDEAVSWGKIRVDAQPVKVYADASLVFPLLVAETFAQKMDAFMHEKNED----- 369
S.cerevisiae  SRPAGARPDEAVSWGKIKAEAKSVKLFADVTTVLPLIVAATFASGKPIKKVKN-------- 387
                **:**:* **:****::      **::  : :     **::  *

L.donovani    SSRGARPPQDVSGHSHLCRGE 601
T.cruzi       -------------------- 469
T.brucei      -------------------- 461
H.sapiens     -------------------- 369
S.cerevisiae  -------------------- 387
```

FIGURE 2. Multiple sequence alignment of DHS34 protein sequences from *L. donovani* (ACF75531), *T. cruzi* (Tc00.1047053504119.29), *T. brucei* (Tb10.70.4900), *S. cerevisiae* (P38791), and human (P49366). Conserved residues are *shaded* in *black*, showing complete identity, and residues *shaded* in *gray* are similar. NAD⁺ binding regions are enclosed in *yellow rectangular boxes*. The catalytic lysine residue is shown on a *cyan background*. Other residues important for the overall functioning of enzyme are highlighted on a *red background*.

molecular mass of ~66 kDa. Amino acid sequence alignment of DHS34 with homologous sequences from other species revealed that it is 46.5% identical to *T. cruzi* (Tc00.1047053504119.29), 45% with *T. brucei* (Tb10.70.4900), and 33.3% with *S. cerevisiae* (P38791). Interestingly, DHSL20 protein has closer identity with the *T. brucei* (56.1%) and *T. cruzi* genes (63.4%) compared with DHS34 (45% with *T. brucei* and 46.5% with *T. cruzi*).

Alignment of DHSL20 and DHS34 protein with the human DHS (P49366) shows conservation of important residues, which are essential for the overall functioning of the enzyme (Figs. 1 and 2). The leishmanial DHS34 and DHSL20 protein share 32.8% (Fig. 2) and 34.7% (Fig. 1) sequence identity with the human DHS sequence, respectively.

Interestingly, although the DHSL20 protein with 379 amino acid residues is of similar length as the human enzyme, the DHS34 is much longer with 601 amino acids. Alignment of DHS34 with the human DHS shows that there is a big insertion in DHS34 from residue 130 to 354 (Fig. 3). No homologs were found for this region using standard homology detection methods like PSI-BLAST. A reverse transcriptase PCR on *DHS34* (parasite RNA as template) confirmed that the entire gene (with all insertions) was transcribed (data not shown).

The residues that have been identified as important for NAD⁺ binding in the human DHS, Asn[106], Asp[238], His[288], and Asp[313], are conserved in DHS34 (42). Residues equivalent to human DHS, His[288], Trp[327], Lys[329], Asp[316], and Glu[323], which participate in spermidine binding and reaction mechanism, are also conserved (23, 42–44). Amino acids corresponding to Lys[287] (important for covalent intermediate formation) (45) and the catalytic center (Lys[329]) (43) of the human DHS and the *L. donovani* DHS34 (Lys[535]) show the conservation.

In the case of the DHSL20 protein, some additional residues corresponding to Glu[137], Asp[243] (im-
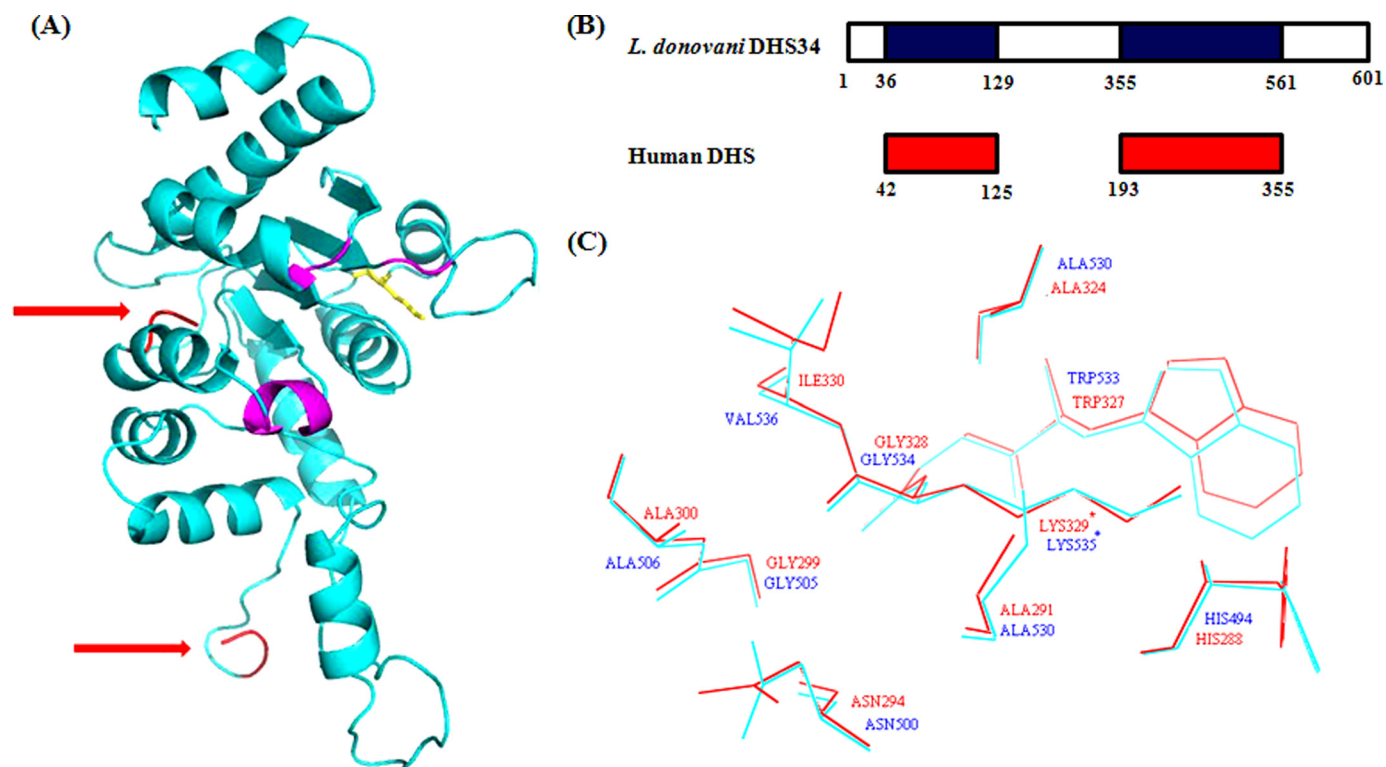
(A)

(B)

(C)

FIGURE 3. **Structural modeling of DHS34.** *A*, the DHS34 model was generated using MODELLER (version 8.0). The catalytic lysine residue is shown in *yellow sticks*. The regions of insertion are depicted by *red arrows*. The probable NAD$^+$ binding region is shown in *magenta*. *B*, alignment showing homologous residues from *L. donovani* DHS34 (*blue*) and human DHS (*red*). *C*, residues around active site lysine (indicated by *asterisks*) of both human DHS (shown in *red*) and modeled DHS34 (shown in *cyan*).

portant for spermidine binding), and Ser^{317} (mutation of which leads to reduced NAD$^+$ binding) are also conserved (42). Most importantly, however, the catalytic lysine (Lys^{329} in humans), which is indispensable for the enzymatic reaction and is present in the active center for catalysis (enzyme-substrate intermediate formation), is substituted by a non-polar amino acid, leucine (Leu^{344} in *Leishmania*). This leucine residue, Leu^{344} (Lys^{329} in humans), in DHSL20 is highlighted in *cyan* (Fig. 1). Such a mutation is likely to render the enzyme inactive (43, 46).

The computer model that we generated for DHS34 shows that the probable NAD$^+$ binding regions are spatially closer in the tertiary structure. Alignment of DHS34 with human DHS shows that there is a big insertion in DHS34 from residues 130–354 (total 225 residues) (Fig. 3*B*). The large 225-residue insertion is marked in Fig. 3*A*. Fig. 3*B* shows the homologous regions between DHS34 of *L. donovani* and the human DHS. Region 36–129 (94 residues) of *L. donovani* DHS34 is homologous to region 42–125 of the human DHS (84 residues), and region 355–561 (total 207 residues) is homologous to region 193–355 (163 residues) of the human DHS. However, residues 1–36 and 562–601 (40 residues) of DHS34 have no homology to the human DHS. There are four NAD$^+$ binding regions in the human DHS: 1) SNLIS (residues 105–109), 2) TAG (residues 131–133), 3) TA (residues 308 and 309), and 4) DA (residues 342 and 343). The alignment of the human DHS and DHS34 shows the presence of SGG of DHS34 aligning with TAG of human DHS, which is the nucleotide binding motif. This region matches with a very small fragment of human DHS and lies

within a big insertion. The remaining three regions come together to form the NAD$^+$ binding region in DHS34; however, the NAD$^+$ binding regions are interrupted by the large insertion of 225 residues.

Analysis of residues around the catalytic center (Lys^{329}) (within a 4-Å radius) of the human DHS and the modeled *L. donovani* DHS34 (Lys^{535}) shows the conservation of all of the residues except Ile^{330} of human DHS, which is substituted by Val^{536} in the case of *Leishmania* (Fig. 3*C*).

A phylogenetic tree has been constructed for both putative DHS sequences (Fig. 4) using representative DHS sequences. The tree for DHSL20 sequence indicates a close evolutionary relationship of *L. donovani* with trypanosome species among the kinetoplastid protozoa (Fig. 4*A*). Another phylogenetic tree has been constructed (Fig. 4*B*) using the *L. donovani* DHS34 sequence and other representative DHS sequences. This tree also indicates a close evolutionary relationship of *L. donovani* and *T. cruzi* among the kinetoplastid protozoa. In both cases, the kinetoplastid DHS sequences are conserved among each other and are distantly related with human DHS.

Overexpression and Purification of the Deoxyhypusine Synthase Enzymes in E. coli—Both *DHSL20* and *DHS34* genes were overexpressed in *E. coli* cells. A protein with an estimated molecular mass of ~48 kDa was induced; the size correlated well with the amino acid composition of DHSL20 protein (~42 kDa) with a His$_6$ tag (~6 kDa) at the C terminus (Fig. 5*A*). Purification of DHSL20 by metal affinity chromatography yielded ~1 mg of purified protein/liter of bacterial culture. The recombinant DHSL20 from *L. donovani* did not show any activ-
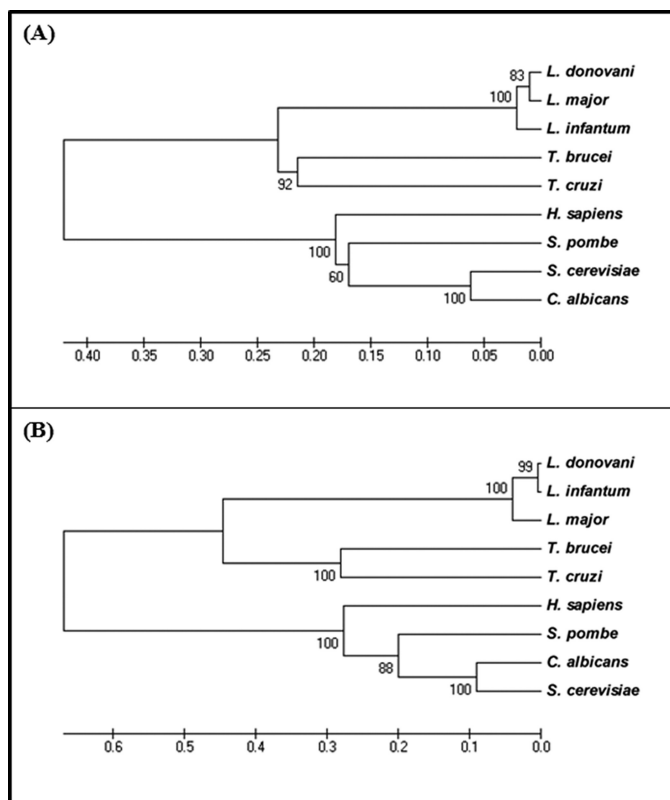
FIGURE 4. *A*, phylogenetic analysis by Unweighted Pair Group Method with Arithmetic Mean of DHS-like gene, *DHSL20*. The *phylogram* presented was a consensus of 500 bootstrap replicates constructed using the MEGA program (version 4.0). The *numbers* at the node present the percentage of trees with the same node among all of the bootstraps. The accession numbers of the gene sequences used are as follows: *L. donovani* (ABP65295), *L. major* (LmjF20.0250), *L. infantum* (LinJ20.0270), *T. brucei* (Tb927.1.870), *T. cruzi* (Tc00.1047053511421.60), *Homo sapiens* (P49366), *Schizosaccharomyces pombe* (CAA22194), *S. cerevisiae* (P38791), *Candida albicans* (XM_716612.1). *B*, phylogenetic analysis by Unweighted Pair Group Method with Arithmetic Mean of the *DHS34* gene. The phylogram presented was a consensus of 500 bootstrap replicates constructed using the MEGA program (version 4.0). The *numbers* at the node present the percentage of trees with the same node among all the bootstraps. The accession numbers of the gene sequences used are as follows: *L. donovani* (ACF75531), *L. major* (LmjF34.0330), *L. infantum* (LinJ34.0350), *T. brucei* (Tb10.70.4900), *T. cruzi* (Tc00.1047053504119.29), *H. sapiens* (P49366), *S. pombe* (NP_595146.1), *C. albicans* (XP_721705.1), *S. cerevisiae* (P38791).

ity under varying pH, eIF5A, and spermidine concentrations (data not shown). This is to be expected because DHSL20 lacks the catalytic lysine residue at residue 344 and contains leucine instead. In an effort to determine whether the lack of the catalytic lysine is solely responsible for the lack of its activity, we have converted this leucine residue (Leu-344) to lysine by site-directed mutagenesis. This mutant enzyme of DHSL20 (L344K) was still devoid of any DHS activity (data not shown), suggesting that other differences in amino acid sequences also contribute to its inactivity.

Upon induction of *E. coli* transformed with the *DHS34* expression vector, a protein that matched the estimated ~73 kDa of DHS34 with a His tag was induced (Fig. 5*B*). Purification yielded ~3 mg of purified protein per liter of bacterial culture.

The kinetic parameters of the enzyme DHS34 were determined using [1,8-^{3}H]spermidine and *L. donovani* eIF5A as the substrates. The enzyme showed a specific activity of 569 units/mg of protein, which is much lower than the specific

activity of the human recombinant DHS. Low specific activity of DHS34 could be due to the fact that one of the NAD$^+$ binding regions in the DHS34 is present in the big long insertion when compared with human DHS, and how this region positions itself to carry out this reaction is not clear. The K_m value of DHS34 for spermidine was estimated to be 23.2 μM, which was much higher than that of human (7.2 μM) (21) and *S. cerevisiae* (12.5 μM) (22), whereas the K_m value of DHS34 for eIF5A was 1.77 μM, higher than human (0.6 μM) (21) but comparable with that of *S. cerevisiae* (1.01 μM) (22). Deoxyhypusine formation was also confirmed by running the reaction mixture on an SDS-polyacrylamide gel, and the labeled eIF5A was detected by fluorography. Fig. 5*C* shows ~24-kDa radiolabeled protein in *lane 2*, confirming the deoxyhypusine formation on *L. donovani* eIF5A by DHS34. A similar ~24-kDa labeled protein was confirmed in the reaction mixture containing human DHS (Fig. 5*C*, *lane 4*). Fig. 5*D* shows a comparison of trichloroacetic acid-precipitable radioactivity obtained in the DHS reaction mixture with different concentrations of the recombinant human DHS, DHS34, and DHSL20. Interestingly, N^1-guanyl-1,7-diaminoheptane, a potent and competitive inhibitor of spermidine binding for human DHS, had little inhibitory effect on the leishmanial recombinant DHS34 (data not shown).

Human DHS native enzyme is a homotetramer consisting of two dimers of 40-kDa subunits. We questioned if *Leishmania* DHS34 native enzyme also exists as a tetramer. Gel exclusion chromatography of DHS34 on a Sephacryl S-300 column resulted in a peak corresponding to a molecular mass of 137 ± 7.6 kDa (supplemental Fig. 1), suggesting that DHS34 exists as a dimer in its native form.

In Vivo Activity of Deoxyhypusine Synthase—To assess if the hypusination of eIF5A occurs in intact *Leishmania* parasites, a labeling experiment was performed in both wild type and spermidine synthase-deficient parasites (Δ*spdsyn*) using radiolabeled putrescine (40). The Δ*spdsyn* parasites are not able to convert putrescine to spermidine, which donates the aminobutyl group for the hypusination of eIF5A, and depend on exogenous supply of spermidine for growth. Fig. 5*E* shows that 18-kDa eIF5A was radiolabeled upon culture with ^{14}C-labeled putrescine in the wild type parasite but not in the Δ*spdsyn* mutant.

Gene Deletion Studies for Deoxyhypusine Synthase 34—We attempted to delete the *DHS34* gene by targeted gene replacement techniques to determine if this enzyme is vital for the parasite. Heterozygous parasites were generated, in which one *DHS34* gene was replaced with either the phleomycin drug resistance gene or the hygromycin drug resistance gene: *PHLEO::dhs34/DHS34* (*PHLEO/DHS34*) and *HYG::dhs34/DHS34* (*HYG/DHS34*) (Fig. 6*B*). Interestingly, the heterozygous parasites consistently showed a slight growth delay compared with wild type parasites (Fig. 6*C*). It is reasonable to assume that a gene dosage effect resulted in the production of less DHS protein, and such a conjecture would suggest that *DHS34* is involved in optimal cell proliferation.

Several attempts to replace both *DHS34* gene copies to generate homozygous gene deletion mutants (Δ*dhs34*) failed. Although several clones resistant to both drugs were obtained, Southern blot analyses demonstrated that the *DHS34* gene was
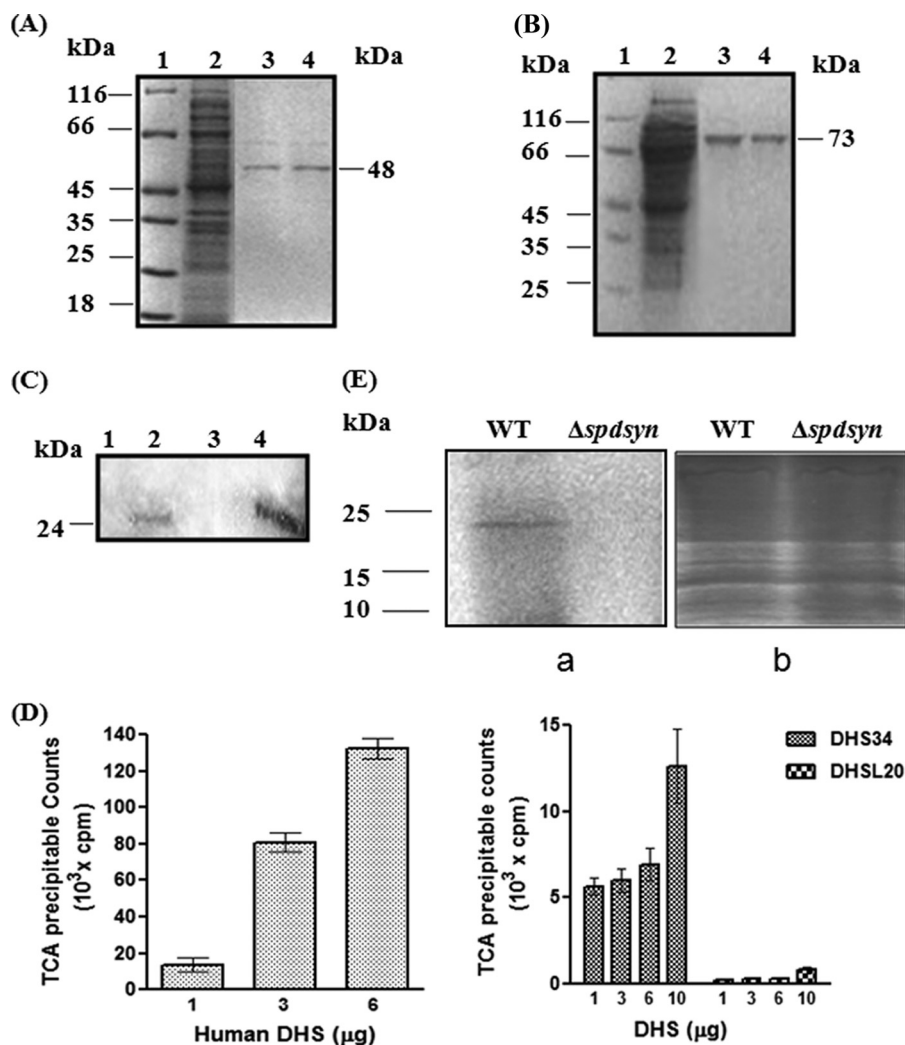
FIGURE 5. **Characterization of DHSL20 and DHS34 proteins.** *A*, purification of DHSL20 protein on Ni^{2+}-nitrilotriacetic acid affinity resin. *Lane 1*, molecular weight protein marker (MBI Fermentas); *lane 2*, flow-through; *lanes 3* and *4*, eluted fractions showing purified protein with buffer containing 300 mM imidazole. *B*, purification of DHS34 protein on Ni^{2+}-NTA affinity resin. *Lane 1*, molecular weight marker; *lane 2*, flow-through; *lanes 3* and *4*, eluted fractions showing purified DHS34 protein with buffer containing 300 mM imidazole from the affinity column. *C*, identification of ^{3}H-labeled deoxyhypusine-containing eIF5A product from DHS reaction mixture by SDS-PAGE and fluorography. *Lane 1*, DHS34 minus eIF5A; *lane 2*, DHS34 plus eIF5A; *lane 3*, human recombinant DHS minus eIF5A; *lane 4*, human recombinant DHS plus eIF5A. *D*, comparison of trichloroacetic acid-precipitable counts obtained in the DHS enzyme assay using different concentrations of recombinant enzymes, human DHS (*hDHS*), DHS34, or DHSL20. A DHS enzyme assay was performed as reported under "Experimental Procedures." Results are mean ± S.D. of triplicate samples. *E*, *in vivo* metabolic labeling of 18-kDa protein in the wild type cells (*WT*) but not in the (Δ*spdsyn*) mutant after culture with [1, 4-^{14}C]putrescine. The gel was stained with Coomassie Blue (*b*) and subsequently dried and exposed to autoradiography (*a*).

from wild type and heterozygous parasites showed a ~11 kb band hybridizing with *DHS34*, corresponding to the chromosomal *DHS34* gene. This ~11-kb *DHS34* fragment was replaced with a higher molecular weight band, suggesting that some clones had indeed lost the chromosomal *DHS34* gene (clones 2, 4, and 7). The hybridizing intensity of this band was similar to that of wild type DNA, suggesting that the *DHS34* gene was present as a single copy, either on a single episome or integrated into the genome. Since the size of pX63PAC-*DHS34* was predicted to be ~8 kb and contained a single BamHI restriction site, the ~12 kb band hybridizing to *DHS34* was indicative of a random integration into the chromosome. Other clones (3, 8, 11) showed an intense band at ~8 kb, suggesting the presence of several copies of the pX63PAC-*DHS34* episome in these parasites, in addition to the ~11-kb chromosomal *DHS34* fragment. A control cell line, the heterozygous *HYG/DHS34* parasites transfected with pX63PAC-*DHS34*, exhibited the ~11-kb *DHS34* chromosomal fragment and a ~9 kb and an intense ~8 kb episomal band hybridizing to the *DHS34* coding region probe. To further bolster the premise that a chromosomal *DHS34* knock-out had occurred in complemented clones 2, 4, and 7, a PCR strategy was applied (Fig. 7*B*). Primer pairs designed to the *DHS34* coding region (primers 1 and 7, 1 and 8, and 1 and 10) generated fragments when genomic template DNA was used derived from *DHS34/HYG* heterozygous para-

still present in the genome of these parasite lines (data not shown), indicating that *DHS34* may be an essential gene.

To further test this conjecture, a chromosomal *DHS34* gene deletion was attempted in the presence of a rescuing episome that contained the leishmanial *DHS34* gene (pX63PAC-*DHS34*). *HYG/DHS34* heterozygous parasites, containing pX63PAC-*DHS34*, were then transfected with the *PHLEOΔdhs34* gene deletion constructs. Southern blot and PCR analysis was performed to investigate the genotype of these triple drug-resistant parasites. Genomic DNA derived from wild type, *HYG/DHS34* heterozygotes, *HYG/DHS34* heterozygotes transfected with pX63PAC-*DHS34*, and several triple drug-resistant clones was probed with the *DHS34* coding region probe (Fig. 7*A*). DNA

sites and clones 2 and 4, as expected. Additional primer pairs were designed to generate fragments only if the *DHS34* gene was present in its chromosomal context because the upstream primers (primers 2 and 4) were complementary to a region upstream of the gene, and the downstream primers were complementary to the coding region (primers 5 and 6). The primer combinations 2 and 5, 2 and 6, and 4 and 5 generated fragments only from DNA derived from the heterozygous cell line but not from clones 2 and 4. The PCR analysis demonstrated that *DHS34* in the chromosomal location could only be found in the heterozygous cell line. Thus, although heterozygous parasites could be created for both the *HYGΔdhs34* and *PHLEOΔdhs34* gene deletion constructs, a chromosomal knock-out of *DHS34*
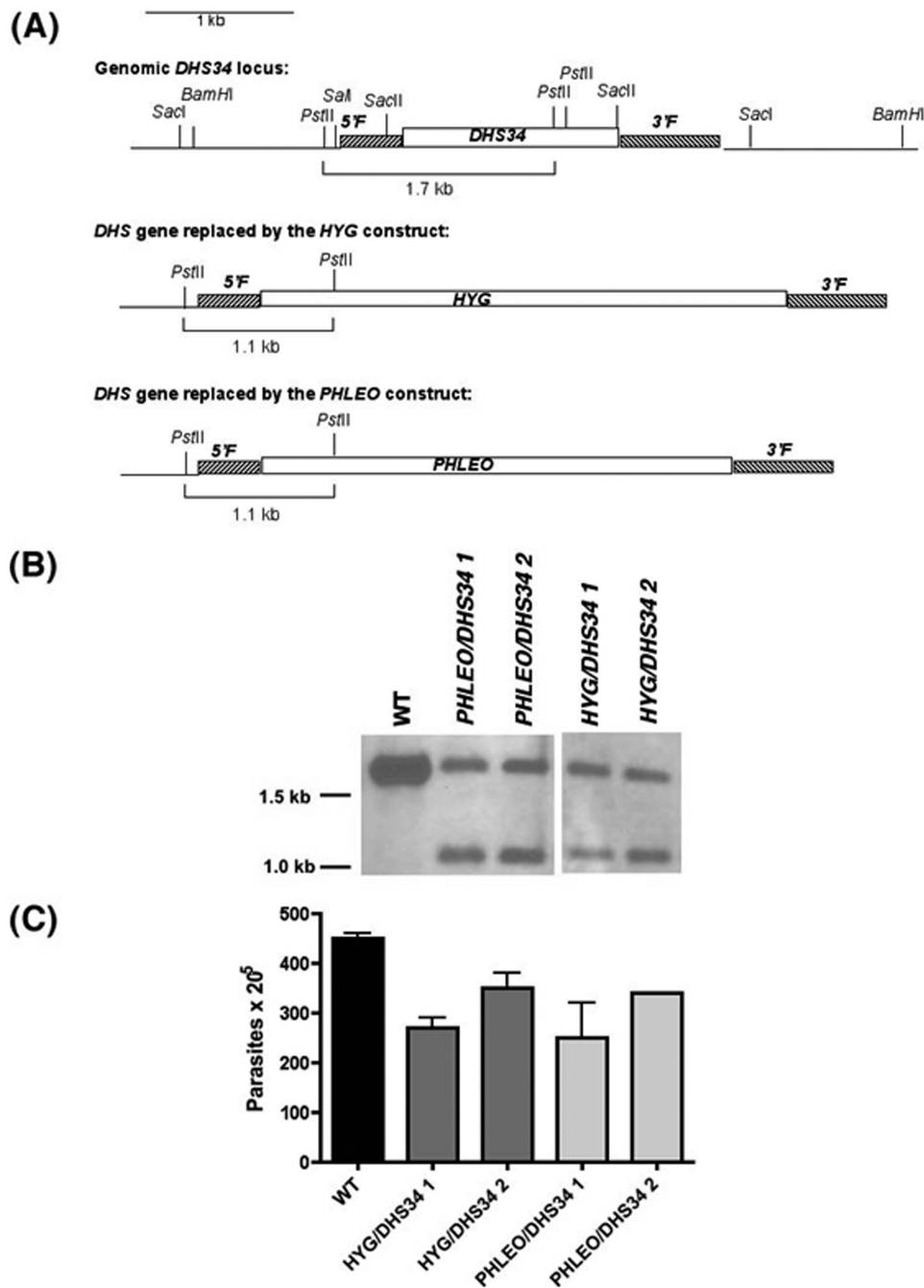
(A)

FIGURE 6. *A*, restriction map of the *DHS34* locus and the predicted rearrangements occurring during *HYG* and PHLEO replacement of *DHS34* in the chromosomal locus. *B*, Southern blot analysis of wild type (*WT*) and heterozygous parasites. Genomic DNA from wild type parasites, two *PHLEO/DHS34* (*1* and *2*), and two *HYG/DHS34* (*1* and *2*) heterozygous cell lines were digested with PstI and probed with a PCR fragment corresponding to the 5'-flanking sequence. Molecular weight markers are indicated to the *left* of the blot. *C*, proliferation of heterozygous parasites. Wild type parasites, two *HYG/DHS34* (*1* and *2*), and two *PHLEO/DHS34* (*1* and *2*) heterozygous cell lines were incubated at 2×10^5 parasites/ml and counted after 4 days on a hemocytometer. The experiment was set up in duplicates and repeated several times with similar outcomes.

was only achieved when the gene was provided on a complementing plasmid. Taken together, these data provide strong evidence that *DHS34* is indeed an essential gene.

DISCUSSION

In the present study, we, for the first time, report the identification, cloning, and characterization of DHS from *L. donovani*. Two DHS-like genes have been identified in *L. donovani*,

but only one, *DHS34*, exhibits deoxyhypusine synthase activity. The ORF of this leishmanial *DHS* encodes a putative polypeptide of 601 amino acids and contains long intervening sequences compared with the human DHS, which is only 369 amino acids long (Fig. 2). DHS is an essential enzyme in mammalian and yeast cells and also appears to be vital for the parasite. Attempts to delete the *DHS34* gene from the parasite genome failed, and chromosomal null mutants of *DHS34* could only be obtained in the presence of an episome that contained the *DHS34* gene (Figs. 6 and 7). Thus, the *L. donovani* DHS34 appears to be an essential protein, whose structure is expected be significantly different from its human counterpart.

Leishmania has two distantly related putative DHS genes. One, *DHS34*, is a true DHS because its recombinant protein showed DHS activity (Fig. 5), whereas the other, DHSL20 protein, does not function as such but may carry out a different unknown function. The phylogenetic tree analysis showed a close evolutionary relationship of both DHSL20 and DHS34 protein with trypanosome species among kinetoplastid protozoa. It also illustrates their common evolutionary origin and that they are distantly related to the higher eukaryotic DHS sequences. In most eukaryotes, a single *DHS* gene has been found. Only kinetoplastid genomes contain two DHS-like genes, and the *DHS34* sequence with its extensive insertions appears to be unique to these organisms. The question that needs to be addressed is why would an organism have two of the DHS-like genes when only one of them shows DHS activity? It is possible that the DHSL20 protein performs a

different, as of yet unknown function. The sequence identity between DHSL20 and DHS34 is only 21.7%. These two distantly related copies might have originated by a gene duplication event followed possibly by functional divergence of one of the copies. Alternatively, it is also plausible that one of the genes is a DHS cognate gene like that of cyanobacteria, myxobacteria, etc. and has been laterally transferred from the archaea (47). In certain plants, it has been proposed that homospermidine syn-
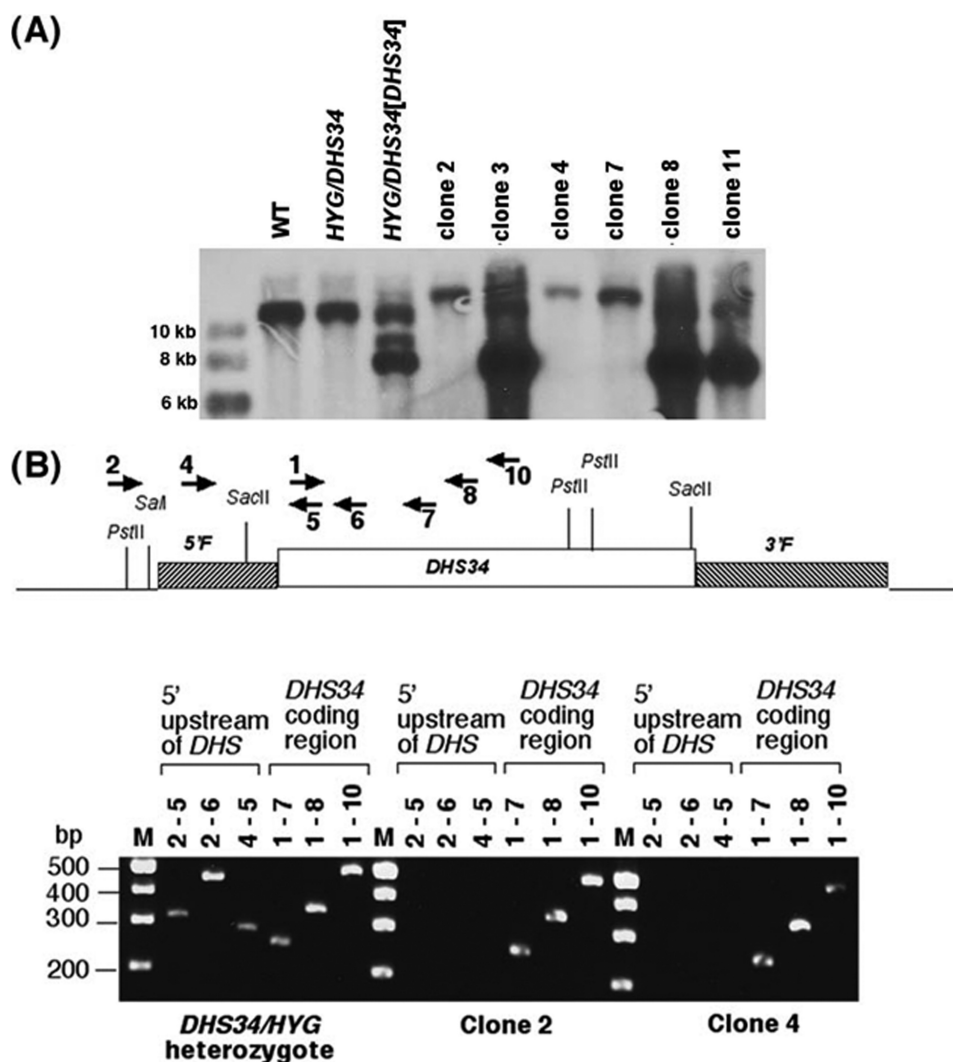
(A)

(B)

FIGURE 7. *A*, Southern blot of genetically manipulated parasites. Genomic DNA was extracted from the following cell lines: wild type parasites (*WT*), heterozygous parasites (*HYG/DHS34*), heterozygous parasites transfected with pX63PAC-*DHS34* (*HYG/DHS34*[*DHS34*]), and six clonal cell lines (clones 1, 3, 4, 7, 8, and 11) derived from the *HYG/DHS34*[*DHS34*] strain transfected with the *PHLEOΔdhs34* gene replacement construct. *B*, the *top panel* shows a map of the *DHS34* genomic locus and the locations of the primers used for PCR analysis. Genomic DNA from *HYG/DHS34* heterozygous parasites (*first panel*) and from clones 2 and 4 was used as a template for PCR analysis. Primers 2 and 4 were designed to match the upstream region of the *DHS34* gene, with primer 4 corresponding to a region (5′*F*) that was present in the gene deletion construct used for the chromosomal *DHS34* replacements. Primer 1 was designed as a forward primer corresponding to the early coding region. Primers 5–10 were reverse primers corresponding to various regions of the *DHS34* coding region. Molecular size markers (in kb) are indicated on the *left*.

are conserved in both DHSL20 and DHS34 proteins. Furthermore, the residues that take part in spermidine binding and reaction mechanism are also conserved in the leishmanial proteins. However, a critical lysine residue (Lys[329]), which is the site of the enzyme-intermediate formation in human DHS (43), is replaced by a non-polar amino acid leucine (Leu[344]) in the case of the DHSL20 protein. It has been shown earlier in the human DHS that a Lys[329] → Arg or Ala substitution abolishes the covalent enzyme-substrate intermediate formation and DHS activity (45). Site-directed mutagenesis of leucine residue to lysine (L344K) of DHSL20 showed no activity, thereby suggesting that other differences in amino acid sequences also contribute to its inactivity.

The DHS34 sequence codes for 601 amino acids and is much longer than the human DHS (369 amino acids) and DHSL20 (379 amino acids). The deoxyhypusine synthase domain is interrupted by a long intervening sequence that spans from amino acid 130 to 354, which does not show any significant homology with any other sequence. It is unknown whether this intervening sequence of DHS34 adds a special function unique to the parasite DHS. The computer-generated model of DHS34 shows the conservation of residues around the catalytic center. The amino acid sequence alignment of DHS34 with human DHS shows that one of the NAD$^+$ binding regions is present in

thase evolved from *DHS* by gene duplication (48). However, amino acid sequence alignment of DHSL20 and DHS34 protein showed only 35.5 and 22.6% identity, respectively, with the homospermidine synthase from *Senecio vernalis*. Because of the recent discovery that the kinetoplastid AdoMetDC requires an inactive AdoMetDC-like protein for dimerization and activation (49), we tested the possibility of DHSL20 having a regulatory role in the activity of DHS34. DHS enzyme assays were performed in the presence of both DHSL20 and DHS34 at varying ratios; however, no significant increase in DHS34 enzyme activity was observed (data not shown).

DHS34 shares only 34.7% sequence identity with the human DHS. DHS34 exists as a dimer in its native form in contrast to human DHS, which is a tetramer. The residues that have been found to be important for NAD$^+$ binding in the human DHS

the 225-amino acid-long insertion of DHS34 that might interrupt the NAD$^+$ binding regions as they come together spatially to form the perfect NAD$^+$ binding site.

Disruption of eIF5A genes (19, 50) and DHS genes (20, 51) in *S. cerevisiae* leads to a lethal phenotype, demonstrating the importance of deoxyhypusine modification in eukaryotic cell survival. Deoxyhypusine hydroxylase is an essential gene in the fruit fly and *C. elegans* but not in yeast, suggesting an evolutionary progression of the requirement of hypusine modification in eukaryotes. In *Leishmania*, chromosomal null mutants of *DHS34* could only be obtained in the presence of a rescuing episome that contained the *DHS34* gene, suggesting that *DHS34* is essential for *L. donovani* and that the hypusination pathway also plays a vital role in *L. donovani* survival and proliferation.

Despite conservation of some of the active site amino acid residues between the human and leishmanial DHS (Fig. 3), a potent inhibitor of human DHS, N^1-guanyl-1,7-diaminoheptane, had little inhibitory effect on either *L. donovani* proliferation or recombinant DHS34. This finding suggests a topological difference in the spermidine binding sites between the human and the leishmanial enzymes and opens the possibility that the differences between the two enzymes could be exploited for drug development for visceral leishmaniasis. Inhibitors of hypusine biosynthetic enzymes like spermidine analogs (for DHS) and metal ion chelators (for deoxyhypusine hydroxylase) have been shown to exert antiproliferative effects (9, 15), making the pathway a potential target. Further studies are required to elucidate the leishmanial enzyme structure, its interaction with its substrates, and its reaction mechanism. This will pave the way to conclusively establish the hypusine pathway as a chemotherapeutic target for the treatment of leishmaniasis and perhaps even African sleeping sickness and Chagas disease.

REFERENCES

1. Sundar, S., More, D. K., Singh, M. K., Singh, V. P., Sharma, S., Makharia, A., Kumar, P. C., and Murray, H. W. (2000) *Clin. Infect. Dis.* **31,** 1104–1107
2. Murray, H. W. (2001) *Antimicrob. Agents Chemother.* **45,** 2185–2197
3. Sundar, S. (2001) *Trop. Med. Int. Health* **6,** 849–854
4. Müller, S., Coombs, G. H., and Walter, R. D. (2001) *Trends Parasitol.* **17,** 242–249
5. Fairlamb, A. H., and Cerami, A. (1992) *Annu. Rev. Microbiol.* **46,** 695–729
6. Park, M. H., Cooper, H. L., and Folk, J. E. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78,** 2869–2873
7. Shiba, T., Mizote, H., Kaneko, T., Nakajima, T., and Kakimoto, Y. (1971) *Biochim. Biophys. Acta* **244,** 523–531
8. Cooper, H. L., Park, M. H., Folk, J. E., Safer, B., and Braverman, R. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80,** 1854–1857
9. Park, M. H., Cooper, H. L., and Folk, J. E. (1982) *J. Biol. Chem.* **257,** 7217–7222
10. Chen, K. Y., and Dou, Q. P. (1988) *FEBS Lett.* **229,** 325–328
11. Murphey, R. J., and Gerner, E. W. (1987) *J. Biol. Chem.* **262,** 15033–15036
12. Abbruzzese, A., Park, M. H., and Folk, J. E. (1986) *J. Biol. Chem.* **261,** 3085–3089
13. Byers, T. L., Lakanen, J. R., Coward, J. K., and Pegg, A. E. (1994) *Biochem. J.* **303,** 363–368
14. Jakus, J., Wolff, E. C., Park, M. H., and Folk, J. E. (1993) *J. Biol. Chem.* **268,** 13151–13159
15. Park, M. H., Wolff, E. C., Lee, Y. B., and Folk, J. E. (1994) *J. Biol. Chem.* **269,** 27827–27832
16. Nishimura, K., Murozumi, K., Shirahata, A., Park, M. H., Kashiwagi, K., and Igarashi, K. (2005) *Biochem. J.* **385,** 779–785
17. Hanauske-Abel, H. M., Park, M. H., Hanauske, A. R., Popowicz, A. M., Lalande, M., and Folk, J. E. (1994) *Biochim. Biophys. Acta* **1221,** 115–124
18. Hauber, I., Bevec, D., Heukeshoven, J., Krätzer, F., Horn, F., Choidas, A., Harrer, T., and Hauber, J. (2005) *J. Clin. Invest.* **115,** 76–85
19. Schnier, J., Schwelberger, H. G., Smit-McBride, Z., Kang, H. A., and Hershey, J. W. (1991) *Mol. Cell. Biol.* **11,** 3105–3114
20. Park, M. H., Joe, Y. A., and Kang, K. R. (1998) *J. Biol. Chem.* **273,** 1677–1683
21. Joe, Y. A., Wolff, E. C., and Park, M. H. (1995) *J. Biol. Chem.* **270,** 22386–22392
22. Kang, K. R., Wolff, E. C., Park, M. H., Folk, J. E., and Chung, S. I. (1995) *J. Biol. Chem.* **270,** 18408–18412
23. Liao, D. I., Wolff, E. C., Park, M. H., and Davies, D. R. (1998) *Structure* **6,** 23–32
24. Umland, T. C., Wolff, E. C., Park, M. H., and Davies, D. R. (2004) *J. Biol. Chem.* **279,** 28697–28705
25. Goyard, S., Segawa, H., Gordon, J., Showalter, M., Duncan, R., Turco, S. J., and Beverley, S. M. (2003) *Mol. Biochem. Parasitol.* **130,** 31–42
26. Boitz, J. M., Yates, P. A., Kline, C., Gaur, U., Wilson, M. E., Ullman, B., and Roberts, S. C. (2009) *Infect. Immun.* **77,** 756–763
27. Tamura, K., Dudley, J., Nei, M., and Kumar, S. (2007) *Mol. Biol. Evol.* **24,** 1596–1599
28. Gowri, V. S., Krishnadev, O., Swamy, C. S., and Srinivasan, N. (2006) *Nucleic Acids Res.* **34,** D243–D246
29. Finn, R. D., Tate, J., Mistry, J., Coggill, P. C., Sammut, S. J., Hotz, H. R., Ceric, G., Forslund, K., Eddy, S. R., Sonnhammer, E. L., and Bateman, A. (2008) *Nucleic Acids Res.* **36,** D281–D288
30. Marchler-Bauer, A., Anderson, J. B., DeWeese-Scott, C., Fedorova, N. D., Geer, L. Y., He, S., Hurwitz, D. I., Jackson, J. D., Jacobs, A. R., Lanczycki, C. J., Liebert, C. A., Liu, C., Madej, T., Marchler, G. H., Mazumder, R., Nikolskaya, A. N., Panchenko, A. R., Rao, B. S., Shoemaker, B. A., Simonyan, V., Song, J. S., Thiessen, P. A., Vasudevan, S., Wang, Y., Yamashita, R. A., Yin, J. J., and Bryant, S. H. (2003) *Nucleic Acids Res.* **31,** 383–387
31. Berman, H. M., Westbrook, J., Feng, Z., Gilliland, G., Bhat, T. N., Weissig, H., Shindyalov, I. N., and Bourne, P. E. (2000) *Nucleic Acids Res.* **28,** 235–242
32. Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997) *Nucleic Acids Res.* **25,** 3389–3402
33. Sali, A. (1995) *Mol. Med. Today* **1,** 270–277
34. Wiederstein, M., and Sippl, M. J. (2007) *Nucleic Acids Res.* **35,** W407–W410
35. Sippl, M. J. (1993) *Proteins* **17,** 355–362
36. Morris, A. L., MacArthur, M. W., Hutchinson, E. G., and Thornton, J. M. (1992) *Proteins* **12,** 345–364
37. Laskowski, R. A., MacArthur, M. W., Moss, D. S., and Thornton, J. M. (1993) *J. Appl. Crystallogr.* **26,** 283–291
38. Park, M. H., and Wolff, E. C. (1988) *J. Biol. Chem.* **263,** 15264–15269
39. Wolff, E. C., Park, M. H., and Folk, J. E. (1990) *J. Biol. Chem.* **265,** 4793–4799
40. Roberts, S. C., Jiang, Y., Jardim, A., Carter, N. S., Heby, O., and Ullman, B. (2001) *Mol. Biochem. Parasitol.* **115,** 217–226
41. Robinson, K. A., and Beverley, S. M. (2003) *Mol. Biochem. Parasitol.* **128,** 217–228
42. Lee, C. H., Um, P. Y., and Park, M. H. (2001) *Biochem. J.* **355,** 841–849
43. Wolff, E. C., Folk, J. E., and Park, M. H. (1997) *J. Biol. Chem.* **272,** 15865–15871
44. Wolff, E. C., Wolff, J., and Park, M. H. (2000) *J. Biol. Chem.* **275,** 9170–9177
45. Joe, Y. A., Wolff, E. C., Lee, Y. B., and Park, M. H. (1997) *J. Biol. Chem.* **272,** 32679–32685
46. Wolff, E. C., and Park, M. H. (1999) *Yeast* **15,** 43–50
47. Brochier, C., López-García, P., and Moreira, D. (2004) *Gene* **330,** 169–176
48. Ober, D., Harms, R., Witte, L., and Hartmann, T. (2003) *J. Biol. Chem.* **278,** 12805–12812
49. Willert, E. K., Fitzpatrick, R., and Phillips, M. A. (2007) *Proc. Natl. Acad. Sci. U.S.A.* **104,** 8275–8280
50. Wöhl, T., Klier, H., Ammer, H., Lottspeich, F., and Magdolen, V. (1993) *Mol. Gen. Genet.* **241,** 305–311
51. Sasaki, K., Abid, M. R., and Miyazaki, M. (1996) *FEBS Lett.* **384,** 151–154