FULL PAPER *Parasitology*

Expression of a Gene Encoding *Trypanosoma congolense* Putative Abc1 Family Protein is Developmentally Regulated

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ABSTRACT. During the attempt to seek *T. congolense* species-specific diagnostic antigens, we discovered one cDNA clone (*P74*) encoding 74 kDa putative abc1 protein (p74) from *T. congolense* PCF cDNA library. It has been suggested that members of the abc1 family are novel chaperonins and essential for both the mitochondrial electron transfer in the bc 1 complex and the coenzyme Q biosynthesis. Although abc1 protein in yeast has a nuclear or mitochondrial subcellular location, neither nuclear localization signal nor mitochondrial targeting signal was found within p74. Northern blot analysis revealed that the transcription level of *P74* mRNA in bloodstream form (BSF) cells were 4 times higher than that in procyclic form cells. Western blot analysis also indicated that p74 was only expressed in *T. congolense* BSF cells, and revealed that molecular mass of native p74 was not 74 kDa but 56 kDa. This indicates extensive post-translational modification in p74. Although further characterization of p74 will be required, our findings provide implications for CoQ biosynthesis pathway in *T. congolense*.

KEY WORDS: *Trypanosoma congolense*, abc1 family protein, Coenzyme Q.

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Trypanosoma congolense is one of the major causative agents of nagana in Africa. Animal trypanosomoses caused by either *T. brucei* or *T. congolense* are generally regarded as nagana. However, *T. congolense* has been less investigated than *T. brucei*, and only limited number of its gene and protein sequences appeared in public databases, such as GenBank [3]. Considering the equal importance of *T. congolense* and *T. brucei*, more intense investigations of *T. congolense* must be done in order to develop effective diagnostic and treatment strategies to protect livestock against trypanosomosis. Although *T. congolense* infection is indistinguishable from *T. brucei* infection by clinical symptoms, previous studies have indicated that *T. congolense* and *T. brucei* substantially differ from each other at the molecular and genetic levels, their tissue tropism, and pathogenicity in their mammalian hosts. For example, it is well known that *T. brucei* crosses blood-brain barrier and invades tissues [5] while, *T. congolense* does not cross blood-brain barrier and less tissue tropic, but adheres to endothelial cell layer of peripheral blood vessel [7, 18]. These two African trypanosome species are biologically transmitted by tsetse fly (*Glossina* spp). When bloodstream forms (BSFs) of the parasite are ingested by a tsetse fly, they rapidly undergo striking morphological and biological changes in order to survive in an insect vector.

During the last two decades, differences in the energy metabolism between BSFs and procyclic forms (PCFs) of the parasite have been investigated [20]. PCF, which is the first differential stage in tsetse fly, synthesizes ATP by oxidative phosphorylation, a common process in eukaryotic cells. Thus their mitochondria possess developed cristae containing the respiratory chain and ATP synthetase systems. In contrast, mitochondria of BSFs, especially of the long slender BSFs of *T. brucei*, do not synthesize ATP by oxidative phosphorylation, and lack both classical respiratory chain and cytochrome systems. Alternatively, BSFs largely depend on glycolysis for their ATP synthesis in a unique organelle called glycosome in which glycolytic pathway actively takes place. Moreover, BSFs utilize a plant-like alternative oxidase, called trypanosome alternative oxidase, as a key enzyme for an alternative respiratory chain [10]. Coenzyme Q (CoQ) functions as a member of the mitochondrial respiratory chain [21].

All genes encoding enzymes responsible for the biosynthesis of CoQ has been isolated from yeast (*COQ1 – 8*) [21]. *ABC1* gene, which is equivalent to the *COQ8*, was first described in the yeast *Saccharomyces cerevisiae* as a novel nuclear gene [1, 4]. The exact molecular functions of abc1 is not clear, however yeast abc1 suppresses a cytochrome b mRNA translation defect and is essential for the electron transfer in the bc 1 complex [1]. It was also reported that yeast abc1 mutants have a defect in CoQ biosynthesis [4]. In addition, yigQ and yigR, which are abc1 homologue in *Escherichia coli*, are required for CoQ production [14]. However, enzymes responsible for CoQ biosynthesis have not been isolated from trypanosomes.

In this study, we have cloned a cDNA encoding yeast *ABC1* homologue, named *P74*, from *T. congolense* PCF cDNA library. We present here, that the expression of *P74* is developmentally regulated. Since only a few *T. congolense* genes have been characterized as differentially

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expressed among its life-cycle stages, further investigation of *P74* will provide implications not only for CoQ biosynthesis pathway in *T. congolense* but also for regulatory mechanisms of stage-specific gene expression.

MATERIALS AND METHODS

Experimental animals: Female 8 week old BALB/c, ICR or SCID mice were obtained from CLEA Japan, Inc. (Tokyo). The animal room was maintained at 22 ± 3°C with a 12:12 hr light-dark cycle. All experiments were conducted according to the guidelines for the care and use of laboratory animals, Obihiro University of Agriculture and Veterinary Medicine.

Trypanosomes: Isolates of trypanosome used in this study are listed in Table 1. All the trypanosomes were grown in mice up to the first peak of parasitemia. The mice were bled by cardiac puncture and blood collected in EDTA. Trypanosomes were purified from whole blood on a DE-52 (Whatman plc., Middlesex, U.K.) anion exchange column chromatography [13] and preserved in Tris-EDTA (TE) buffer (10 mM Tris-HCl pH 8.0, 5 mM EDTA) at –30°C. Insect form (PCF and epimastigote form) cultures were maintained following the methods of Hirumi and Hirumi [8] with slight modifications. Briefly, TVM-1 culture medium was prepared with the following composition: Eagle's minimum essential medium (Sigma, St. Louis, MO, U.S.A.), 60 mM HEPES (Sigma) pH 7.2, 2 mM L-glutamine (Invitrogen Co., Carlsbad, CA, U.S.A.), 10 mM L-proline (Wako Pure Chemical Industries, Ltd., Osaka, Japan), 20% v/v heat inactivated fetal bovine serum (Biosource International Inc., Camarillo, CA, U.S.A.), 100 IU/m*l* penicillin (Meiji Seika Kaisha, Ltd., Tokyo, Japan) and 100 μg/m*l* streptomycin (Meiji Seika Kaisha, Ltd., Tokyo, Japan). About 0.5 m*l* of infected blood was aseptically collected from a *T. congolense*-infected mouse at peak parasitemia (>100 parasites/microscope field with a × 400 magnification on a wet blood smear) and blood suspended in 30 m*l* TVM-1 medium in a 25 cm^2 flask. The flask was maintained in an up-right position in an incubator at 27°C overnight to allow blood cells to sediment. In order to differentiate BSF to PCF, the supernatant (containing trypanosomes) was transferred to new flasks at a volume of 25 m*l* and incubated further at 27°C for 5–7 days without changing the medium. The cultures were maintained by replacing the culture fluid with fresh medium every other day.

Preparation of total trypanosome DNA: More than 1 × 10^7 trypanosomes were suspended with DNA extraction buffer (0.2 M NaCl, 10 mM Tris-HCl pH 8.0, 10 mM EDTA, 1% sodium dodecyl sulfate (SDS)), and then proteinase K (final concentration: 100 μg/m*l*) (Invitrogen) were added and incubated at 55°C for 6 hr with gentle agitation. Proteinase K (final concentration: 200 μg/m*l*) was added again and the samples incubated at 55°C overnight. The mixture was extracted twice with phenol-chloroform-isoamylalcohol (Sigma) and total DNA was precipitated by addition of 0.1 volume of 3 M sodium acetate (pH 5.5) and 1 volume of isopropanol (Wako Pure Chemical Industries). After centrifugation at 15,000 × g for 15 min at 4°C, the pellet was rinsed with 70% (v/v) ethanol, air-dried and re-suspended in 50 μ*l* of autoclaved distilled water (D.W.). DNA concentration was determined by spectrophotometry.

Table 1. Trypanosome species and strains used in this study

Species	Strain	Place of isolation	Year of isolation
T. b. brucei	GUTat3.1	Uganda	1966
T. b. gambiense	IL1922	Ivory Coast	1952
	IL3248	Nigeria	1969
	IL3250	Nigeria	1969
	IL3254	South Sudan	1982
	IL3301	Nigeria	1969
	IL3707	Nigeria	1968
	Welcome	ND	ND
T. b. rhodesiense	IL1501	Kenya	1980
	IL2343	Ivory Coast	1978
T. evansi	IL1695	Kenya	1978
	IL1934	South America	1971
	IL3354	Mali	1988
	IL3382	Mali	1988
	IL3960	Kenya	1980
	IL3962	Sudan	1976
	Tansui	Taiwan	ND
T. congolense	IL1180	Tanzania	1971
	IL3000	Kenya-Tanzania border	1966
	IL3338	ND	ND

Except for record of Tansui and Welcome strains, all information was obtained from International Livestock Research Institute Biological Service Unit. ND: No data.

cDNA cloning: In a process of immunoscreening procedure of *T. congolense* IL3000 PCF cDNA expression library using several monoclonal antibodies previously reported (9), one false positive cDNA clone includes 1,935 bp of ORF was identified as a putative *T. congolense* species-specific gene by PCR analysis.

PCR amplification: DNA samples extracted from various trypanosome species BSFs (listed in Table 1) as well as DNA samples extracted from *in vitro* cultures of *T. congolense* strains IL3000, IL3338 and IL1180 PCFs were used as templates. A 499 bp fragment of the *P74* gene (from 1,062 to 1,560 bp of the *P74* ORF) was chosen for PCR amplification of *P74* from various trypanosomes using primers P74F: 5'-GGC AAA CAT TCT CGT TCG-3' and P74R: 5'-AGC ACT ACG AGC AAA CAT AC-3'. To express deletion versions of recombinant p74 proteins, the following three primer pairs were used: 1) p74 deletion clone 1 primer pair (from 1 to 630 bp of the *P74* ORF), Δp74-1F: 5'-CTC GAG ATG AAA AAG AGC TTG ATC CGG-3' and Δp74-1R: 5'-CTG CAG CTA ATC TGC TGG CTG GAG AAG AGC-3'; 2) p74 deletion clone 2 (Δp74–2) primer pair (from 631 to 1,260 bp of the *P74* ORF), Δp74–2F: 5'-CTC GAG TCC CAG CAA GAT CCT GTC GAG-3' and Δp74–2R: 5'-CTG CAG CTA GTC CTG CAA CAT CAT AGC TTT-3'; 3) p74 deletion clone 3 primer pair (from 1,261 to 1,935 bp of the *P74* ORF), Δp74–3F: 5'-CTC GAG ATG AAA

ACT GTA AAG AAA ATT-3' and Δp74–3R: 5'-CTG CAG TTA CCT TCT CAT CAG AGG GGC-3'. PCR amplifications were performed in a 100 μl reaction mixture containing 1 μl of template DNA (10 *n*g), 10 μl of 2 mM dNTPs mixture, 10 μl of primer pair (10 pmol/μl each primer), 10 μl of ×10 PCR buffer, 67 μl of D.W. and 1 μl of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, U.S.A.). The amplification was performed in a thermal cycler (GeneAmp PCR system 9700, Applied Biosystems) programmed for 30 cycles of denaturation at 94°C for 30 sec, annealing 53°C for 30 sec and extension at 72°C for 30 sec, preceded by an initial denaturation of 10 min at 94°C. Final extension was for 7 min at 72°C. Products were fractionated by electrophoresis in 1.5% agarose gel in Tris-borate-EDTA (45 mM Tris, 45 mM borate, 1 mM EDTA; TBE) buffer and stained in TBE buffer containing 1 μg/m*l* ethidium bromide (Wako Pure Chemical Industries). The gel was examined and photographed under UV light.

DNA sequencing and analysis: DNA sequencing was performed with the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) and the automated DNA sequencer (ABI PRISM 310 Genetic Analyzer, Applied Biosystems). Obtained DNA sequences were assembled and edited using MacVector (Accelrys Co., Cambridge, U.K.) and AssemblyLIGN (Accelrys Co.). Homology search of both cDNA and predicted amino acid sequence to known sequences in the public database was done by using NCBI BLAST (http://www.ncbi.nlm.nih.gov/BLAST). Conserved domains in the predicted amino acid sequence were examined by using NCBI Structure Conserved Domain Database and Search Service v2.00 (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml).

Southern blot analysis: *T. congolense* IL3000 PCF genomic DNA were digested by the following restriction enzymes (Roche Diagnostics Co., Mannheim, GmbH): *Eco* RI and *Dra* I (*P74* single cutters); *Bam* HI and *Hin*d III (*P74* non-cutters). The Δp74–2 (648 bp) was used as probe. Five micrograms of DNA from each enzyme digest were loaded and separated in 1% Tris-acetic acid-EDTA (40 mM Tris, 40 mM acetic acid, 1 mM EDTA; TAE) agarose gel. DNA sizes were estimated according to the migration of 1 kbp DNA ladder (Takara Bio Inc., Shiga, Japan). The gel was processed and transferred onto nylon membrane (Hybond-N+, Amersham Biosciences, Buckinghamshire, U.K.) as previously described [17]. Hybridization and labeling of the probe were performed using AlkPhos Direct Labeling and Detection Systems (Amersham Biosciences). Imaging and analysis of the results were carried out using VersaDoc 5000 Gel Documentation System (BIO-RAD Laboratories Inc., Hercules, CA, U.S.A.) and Quantity One software (Bio-Rad Laboratories Inc.).

Northern blot analysis: Parasite cells of 1–5 × 10^8 were collected and processed for total RNA extraction. Total RNA was extracted using TRizol reagent (Invitrogen) following the manufacturers instructions. In every 10 μg of total RNA, 200 units of deoxyribonuclease I (Takara Bio Inc.) was added. Total RNA of 10 μg from BSF or PCF was fractionated electrophoretically on 1% TAE agarose-formaldehyde gel with 0.20–10 kb RNA marker (Novagen Inc., San Diego, CA, U.S.A.). The RNA samples were blotted on a nitrocellulose membrane (Protran, Schleicher and Schuell BioScience Inc., Keene, NH, U.S.A.), and were hybridized with ^{32}P-labelled Δp74–2 fragment (630 bp). Then, the same membrane was reprobed with ^{32}P-labelled *T. congolense* ribosomal P0 gene fragment (1,035 bp, GenBank accession number AB056702) as normalization probe. Labeling of the probes was performed using Random Primed DNA Labeling kit (Roche Diagnostics Co.). The procedure including hybridization, film imaging and analysis of the sample were done as previously described [17].

Expression and purification of recombinant p74: The pRSET A bacterial expression vector (Invitrogen) was utilized to express His-tagged Δp74 deletion clones. The recombinant protein was purified by ProBond purification system (Invitrogen). All procedures for protein expression and purification were performed following the manufacturers instructions.

Production of chicken antisera against P74 deletion clones: Chicken anti-Δp74 antibody was produced by Sigma-Aldrich Japan K.K. Genosys Division.

SDS polyacrylamide gel electrophoresis (SDS-PAGE) and Immunoblotting: BSFs and insect forms (PCF and EMF) of trypanosomes were obtained as described above. Parasites were washed three times with PBS, and then suspended in sample buffer (0.0625 M Tris-HCl (pH 6.8), 10% glycerol, 5% 2β-mercaptoethanol, 2% (w/v) SDS, 0.05% (w/v) bromophenol blue) at the concentration of 5 × 10^8/m*l*. Parasite lysates were heated at 95°C for 5 min. Ten μl of parasite lysate was applied to each sample well. SDS-PAGE was performed under reducing conditions on 12% separating gel following Laemmli's standard protocol [12] and stained with Coomassie brilliant blue R-250 solution. For immunoblot analyses, chicken anti-Δp74 serum was used as the 1st antibody. The chicken sera used in this study were pre-absorbed with *E. coli* lysate in order to eliminate non-specific reactions. After the addition of horseradish peroxidase-conjugated sheep anti-chicken IgY secondary antibody (ICN Biochemicals Inc., Aurora, OH, U.S.A.), results were developed and visualized by using 3,3'-diamino-benzidine (Wako Pure Chemical Industries). Apparent molecular masses were quantified by comparison with molecular mass marker proteins (Amersham Biosciences).

RESULTS

Cloning and sequence analyses of P74: In the search for novel antigens for *T. congolense* specific diagnosis, *T. congolense* PCF cDNA expression library has been screened using anti-*T. congolense* monoclonal antibodies [9]. During the process, one cDNA clone, named *P74*, encoding 1,935 bp ORF was identified from false positive clones (Fig. 1) (GenBank accession number AB183873). *P74* did not display significant homology to known nucleic acid sequences

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ACGAGGTTTC TGTACTATAT TGCAGGGAGA CGTAACTACA CCTGTACGGT GCGATCAGGA GA            -1
ATGAAAAAGA GCTTGATCCG GGGTGGGCTC CTCCGTGGGC GTCTGGCTAC TTACCTCACC AACCACCACC    70
      Δp74-1F
ACCACCACGT GGCGATGAAA TACGCGTTCA GAACGCAGTT TCCTTCTATT GGCAGCGCCA ATGCACAGGT   140
TGCAGCGAAG TCACGTTCGC GAAAGTGGGG CCGCCGTATT CTTTTTTGCA CGTGTGCTGG TGTCTCCCTT   210
TATATTTTTG TGGACTGTAT GACAGCTCAT TCGTTGACGC GTTCTTTACG GACGGTGCAG ACGATGATAT   280
ACATCATATA CCTTTATAAG GTCATGTCAC CAGAAACTAT GGAGGAATAT TCAGAGCTTC ACCAAACTGT   350
TGCGGCCTCC CTACTAAACC TTTTTCTAAA GAACGAGGGG TTGTACATCA AATTGGGACA GATGTTCACT   420
TCAATGAACC ACTTCTTGCC GGGGGAGTAC ATCGACACAA TGAAAGCGCT GCTGGACAGC GCTCCCTCGG   490
TGCCGCTTGA TGACATCCAG CAGGTTATTC TGGAGGAAAC AGGAAAGACG TGCGAGGAGT TGTTTGTACA   560
CTTCGATCCC GAGCCTGTTG CATCCGCGTC CATCGCGCAG GTGCACCGAG CTCTTCTCCA GCCAGCAGAT   630
                                                    Δp74-1R
TCCCAGCAAG ATCCTGTCGA GGTTTGTGTC AAAATCCAGA AGCCATACAT TCGCCGCCAA GTGTTTTGGG   700
      Δp74-2F
ACCTTCAGAC GTACCGGTTT GTAATGTTTG TTCTTGGCGC TGCATTCAAT ATGCCTGTGA CGTGGGCGAA   770
GGAAACAATT ATTGAAGGCA TCAATCGTGA GGTGAATTTC TCTATGGAGG CAGCTAATGC TGTGCGGATT   840
AAAAATGATT TCGCTGATCG GGAAGATTTT TACGTTCCTT ACGTGTACGA ACATCTTGTG ACTCCTCGGC   910
TACTCGTGAT GGAGTGGGTC AACGGTGTAA AGTTAATTGA TGTTGACAGG GTGAGGTCGC GCTACAGTGA   980
TGTAGAAATT CTTCGAATTC TGTTTGATGT TTTTGGCAGC ATGATATTTA AAAAAGGGTT CGTTCATTGC  1050
GATCCACACG GGGCAAACAT TCTCGTTCGT GACTTTGCCA GAGGTGATGT GAAGGACCCA GCTGCACATA  1120
                P74F
ATCAAGAACA TGGAAGGTGC TCCGGTAAGA CCCATCACAA GCCGCAACTG GTTCTGCTGG ACTTCGGCCT  1190
CTGCTGTCCC GAATCAGAAT GCTTCCGTGT GGAATATGCC ATTTTACTAA AAGCTATGAT GTTGCAGGAC  1260
                                                    Δp74-2R
ATGAAAACTG TAAAGAAAAT TGTCGGTTCA TGGGGCGTTG ATGACGCAGT GGCTTTCTCA AGCCTTCAAC  1330
      Δp74-3F
TGCGCAAGTC ATACGACTTG GTTCGCAGAG GAAACTACGG TGAGACTACA AGAGAGGAGG CAATTAATGA  1400
GCGCATGAAG CAGCGTGATA GCATTAGGAA CCTACTAAGC AACGAGGAAC GTTTACCTTA TGAACTGTCA  1470
CTGATAGGAA GAAGCATTGA TATTCTCCAC GGGGTTAACC GCTTGTACGG CGCGCCAGTG AACCGGGTTG  1540
GTATGTTTGC TCGTAGTGCT GTTGCTGCTC TGGGCCCTCT TAGCACTTAC GAGGATGTGC AGCAATACCT  1610
   P74R
TAGGGAGATC AATGATTTGT CTGGTGTCAC CAATGAAATC CAGTCGCTAT CAACTTCACT GAGGAGAAAA  1680
TCAATGTCCC TATTCGACAC GACGCTAGAG CAGCAACGTC GGCAGGAGGA GGCGGCCGTG GCGTGTCACC  1750
GGCACACGCG GGGGACGTCT CTTTCTCACC GTGTCTGGGA GGGTATTACT TCGATGTACT GTCGAATCCA  1820
TTTGGAAGTG TTCCTATTAA TACTCGACGT TTGTCACAGG TTAAATCAGT GGTTTGGCAG GGGTGTTCAA  1890
CCGTCCGTGG GAAGCCGTGG AGAGGCCCCT CTGATGAGAA GGTAATAGGA CTCCAGCCCC TGTTACGTGT  1960
                                  Δp74-3R
TGCCAATTTT TTAATTTCCC GGAGAACGCG AATATATGTT TGAGTGCTCT TATGGGATGA GGGAAGGACC  2030
CACTGTTTGT GAGTGTAACG TTTACTACGG CCAAGACTTA CCAGGAAAAG AATCCTTTAT TAAGCACATT  2100
AGCTTGTTCT TCACTGATTG AGCGCTTTAC GTTTAACGAA AAAGTTCGTG ACTACAATTT TGTGACACAA  2170
GAAAGAAGGA AAATTTCTCG ATGTCCATGT GTATAGTGAT GAATGTTTTA TTCGGCAACA ACTTTTTTGT  2240
AATACCATAA TTTACTATAA TTAATATTTG TTACTTTAAT TTTTCTACGC CTTTGTGTTT TATATTAATA  2310
CACATAATAT ATACATGGCC CCTCACGGCT TGCATGAATG GATTGCGTAC TGGAGCCTTC GTGGTAATAT  2380
TTCTGCCTTG TGTGCTAGCG GCTGAGCAAT TAGGAGCTTG AGGCGGAGCG CTCGGGCGGG ACCCGGTTGG  2450
TGTTCCTTTT TCCTTCCGCC GTCCCCATAA CACCTCTCAA AATAAAGCCC CTCATGCGGG GAGTGTGATG  2520
GTAAAGAGGC AGTGGAGCCT GTGATTTGGT AGGCGCTGCC GGGTAAATGT CACGGTGGTG CGAAGTTATA  2590
AATGTTAACT ATTTGCCAAC AACTTATTGA TGCTCTTCCT CGTTGCAATG TGTTCCATAT TTTTGCCACA  2660
TATCTGTTGT GTGTCGCATT TTTATTTGTT TCTTTATATA TATTGATAAG TCACATCAAG GGACGTCCTT  2730
TTACGATGAA AAAAAAAAAA AAAAAAA                                                 2757
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Fig. 1. Full-length sequence of the *P74* cDNA. Conserved spliced-leader sequence is enclosed by open box. Bold letter indicates coding region of the *P74*. PCR primer binding sites are indicated by underlines. Name of each primer is described under the underlines. GenBank accession number of the *P74* cDNA sequence is AB183873.

in GenBank, but a predicted amino acid sequence had significant homology to abc1 family proteins and ubiquinone biosynthesis proteins, such as Accession Nos. NP065154 (*Homo sapiens*), NP766548 (*Mus musculus*), NP176770 (*Arabidopsis thaliana*), and NP981191 (*Bacillus cereus*). The NCBI conserved domain database search revealed that p74 possesses one characteristic domain architecture and an AarF domain. The domain architecture is consist of ABC1 domain and serine/threonine protein kinase catalytic domain (Fig. 2). Analysis of the p74 primary structure found neither N- and C-terminal signal peptide nor translocation signal peptide.

PCR analysis of different pathogenic trypanosomes using P74-specific primers: Out of the 20 total DNAs from different trypanosome species and isolates, only 3 isolates (IL1180, IL3000 and IL3338) of *T. congolense* showed pos-

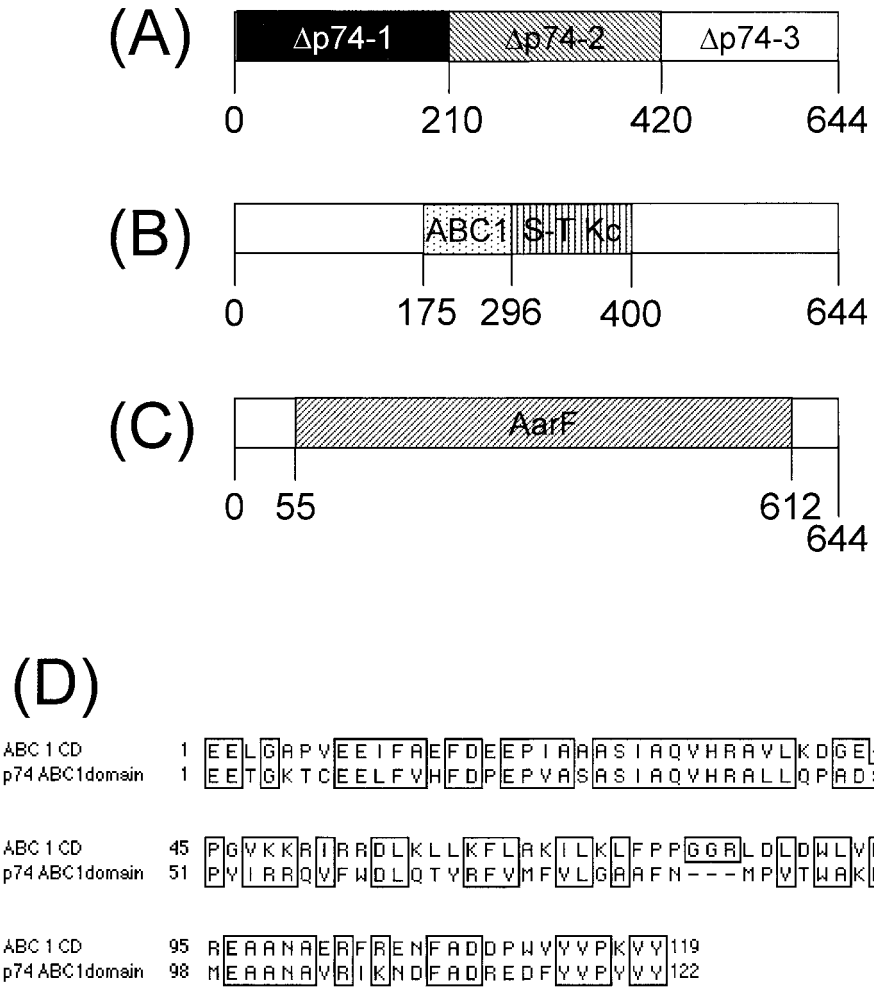


Fig. 2. Schematic presentation of domain architecture of p74. Locations of three deletion clones of p74 expressed for antiserum production (A). Unique domain architecture of p74 consists of ABC1 (dotted box) and serine/threonine protein kinase catalytic domain (S-T Kc) (striped box) (B). Location of AarF domain found in p74 is indicated by shaded box (C). Numbers shown under the schemata indicate position of amino acid. Amino acid sequence alignment of conserved ABC1 domain (ABC 1 CD) and putative ABC1 domain in p74 (p74 ABC 1 domain) (D).

itive reaction in *P74*-specific PCR (Fig. 3). This result does not indicate total absence of *P74* homologue(s) in the other trypanosome species but the gene seems to be well conserved among *T. congolense* isolates.

Southern and Northern hybridization analysis of P74: Southern blot analysis indicated that *P74* is a single copy gene. There is probably an allelic polymorphism at the *Eco* RI restriction sites (Fig. 4). The *P74* mRNA was detected as a ~2.5 kb transcript in both BSF and PCF (Fig. 5). The ratio of the *P74* mRNA signals in BSF versus PCF was determined by densitometric analysis to be at about 4:1. However, the signal intensity of the *P0* mRNA was identical in the two life cycle stages, suggesting nearly equal level of the P0 gene expression in both BSF and PCF.

Expression of Δp74 and immunoblot analysis: We had tried to express full-length p74 by using several bacterial expression vectors. However our attempt was not successful due to unknown reasons. Thus we decided to produce 3 deletion clones as described in Materials and Method. Among the clones, only Δp74–2 amplified from 631–1,260 nucleotide sequence position (630 bp) was successfully expressed as 27 kDa protein. In order to determine intracellular localization and molecular mass of the native p74 by confocal laser scanning microscopy and immunoblotting respectively, a purified Δp74–2 was utilized to produce specific chicken anti-sera. Although, anti-Δp74–2 chicken serum specifically recognized several antigens of trypanosome species and life cycle stages tested, most prominent reaction was found at molecular size of 56 kDa (Fig. 6). As shown also in the Northern blot analysis, Immunoblot analysis revealed that p74 was only expressed in *T. congolense* BSF. Immunofluorescent staining followed by confocal

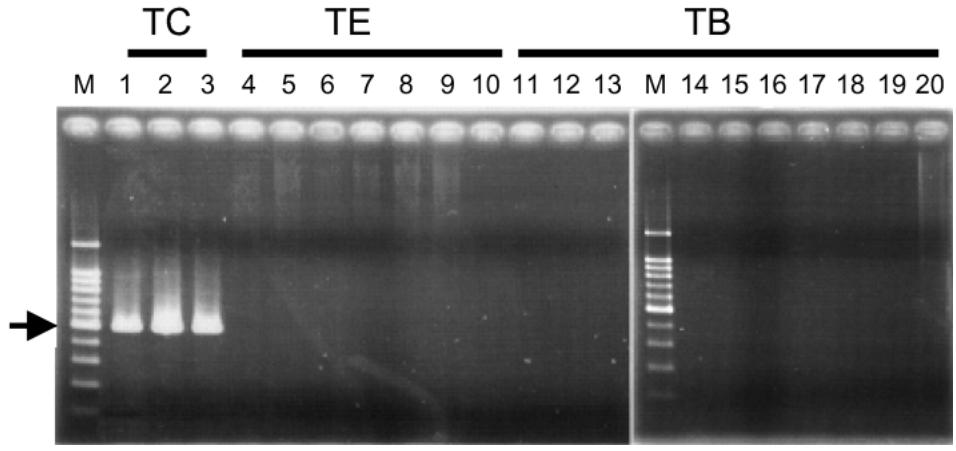
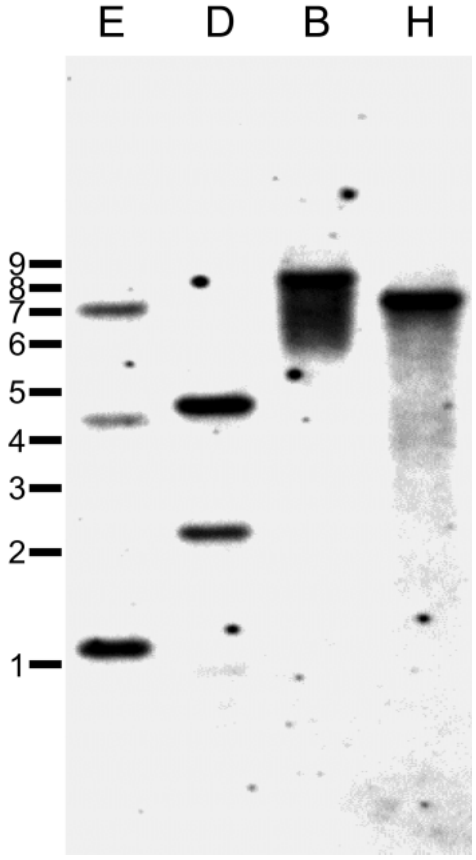


Fig. 3. Species specific detection of *P74* by PCR. P74F and P74R primers were used for PCR. Expected 499 bp PCR product was only obtained from *T. congolense* strains (lanes 1–3). TC, TE, TB, and M indicate *T. congolense*, *T. evansi, T. brucei*, 100 bp ladder DNA size marker, respectively. Lanes 1–20 indicate *T. congolense* (TC) IL1180 (1), TC IL3000 (2), TC IL3338 (3), *T. evansi* (TE) IL1695 (4), TE IL1934 (5), TE IL3354 (6), TE IL3382 (7), TE IL3960 (8), TE IL3962 (9), TE Tansui (10), *T. brucei* (TB) IL1922 (11), TB IL3250 (12), TB IL3248 (13), TB IL3254 (14), TB IL3707 (15), TB IL2343 (16), TB IL3301 (17), TB IL1501 (18), TB Welcome (19), and TB GUTat3.1 (20), respectively.



laser scanning microscopy was not successful due to very strong nonspecific reaction of normal chicken serum to flagellum (data not shown).

DISCUSSION

This study reports cloning and characterization of a gene, named *P74*, encoding putative abc1 family protein (or putative CoQ synthetase) in *T. congolense*. The gene has an open reading frame of 1,935 bp and thus the putative abc1 protein consists of 644 amino acid residues. Molecular mass (Mr) of native p74 was 56 kDa, which is18 kDa less than the Mr of predicted amino acid sequence (74 kDa). Although, computer-based analysis of the p74 primary structure predicted no N-terminal signal peptide, this result appears to indicate extensive post-transcriptional modification, such as proteolytic cleavage.

The p74 was only expressed in *T. congolense* BSF (Fig. 6). In addition, its mRNA level in *T. congolense* BSF was four times higher than that in the *T. congolense* PCF as shown by Northern hybridization (Fig. 5). These results suggest that p74 is a novel *T. congolense* developmental stage- and species-specific protein. According to the result of the NCBI conserved domain database search (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml), characteristic domain architecture and an AarF domain was found

Fig. 4. Southern blot analysis for determination of the copy number of the *P74* gene. Five micrograms of *T. congolense* total DNA digested with *Eco* RI (E), *Dra* I (D), *Bam* HI (B), or *Hin*d III (H) was electrophoresed. B and H are non-cutters of *P74*. While E and D are single cutters of *P74*. The numbers indicated at the left are 1 kbp ladder DNA size marker.

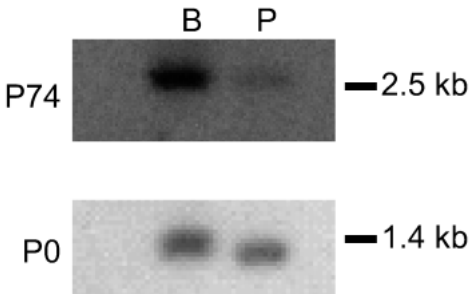


Fig. 5. Northern blot analysis of *P74* mRNA. Ten micrograms of total RNA from BSFs (B) and PCFs (P) was electrophoresed and transferred to a nylon membrane. The membrane was probed with *P74* specific probe (panel P74) and then reprobed with ribosomal *P0* specific probe as a signal standardized control (panel P0). Numbers presented at the right side indicate sizes of the transcripts.

within p74 [1, 14] (Fig. 2). The domain architecture consists of ABC1 domain and serine/threonine protein kinase catalytic domain found within Δp74–2. Interestingly, the proposed function of this domain structure and AarF domain are essential in order to synthesize CoQ [1, 14]. Thus the result implies that p74 is a putative CoQ synthetase in *T. congolense*.

CoQ is present in all cells and membranes and plays an important role in the mitochondrial respiratory chain, regulation of cellular redox balance, controlling expression level of β2-integrin on the blood monocytes, and so on [21, 22]. The mitochondrial respiratory chain is essential for the survival of all living organisms, including parasites. During life cycle development, function and morphology of the mitochondrion of African trypanosome change markedly [10, 20]. *T. brucei* BSF has no oxidative phosphorylation, no cytochrome-mediated electron transport system, and no tricarboxylic acid cycle [2, 6]. The parasite is totally dependent on glycolysis for energy production [20]. However, mitochondrion of the BSF of trypanosome has a unique electron transport chain mediated by CoQ, glycerol-3 phosphate oxidase and plant-like alternative oxidase, called trypanosome alternative oxidase [2, 10]. Since this unique electron transport chain is not present in the host, this has been considered as a possible chemotherapeutic target [2, 10, 15]. However, except for our study, there are no reports about CoQ synthesis pathway and related information in African trypanosome. It was reported that CoQ content of *T. brucei* BSF is ~200 ng/mg total BSF proteins but there is no information in PCF [2]. Nonetheless, our results of Northern blot and immunoblot analyses imply the importance of CoQ synthesis in BSF.

Apart from molecular and biochemical interests, p74 can be utilized for identification of *T. congolense* from other trypanosome species. The diagnostic techniques have successively evolved through molecular biological techniques, such as DNA hybridization, PCR and loop-mediated isothermal amplification of DNA [11, 16, 19]. The diagnosis using PCR aims to identify the parasites at the species level using various target genes. In this study, *P74* was able to be specifically amplified by PCR using the primer set, 5'-GGC AAA CAT TCT CGT TCG-3' and 5'-AGC ACT ACG AGC AAA CAT AC-3'. Since the same antigenic protein was not identified in *T. brucei*, p74 might be a candidate for *T. congolense* species-specific serodiagnostic antigen.

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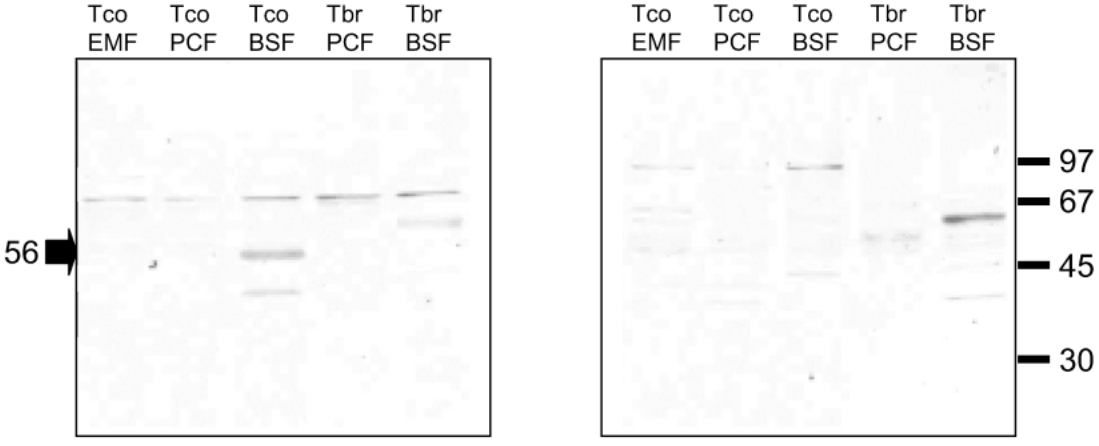

Fig. 6. Western blot analysis of the p74. Tbr, Tco, BSF, PCF, and EMF indicate *Trypanosoma brucei rhodesiense*, *T. congolense*, bloodstream form, procyclic form and epimastigote form, respectively. Left panel is probed with chicken anti-Δp74 serum raised against Δp74–2 as a 1st antibody. While, right panel is probed with normal chicken serum as a control reaction. The number and arrow indicated at the left show the size (kDa) and location of specific reaction. The numbers indicated at the right are molecular size (kDa) marker.

Century COE program from JSPS.

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