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Transcriptional analyses of *Trypanosoma brucei gambiense* from infected mice and *in vitro* culture

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ABSTRACT

With the hypothesis that African trypanosomes could have *in vivo* specific genes for adaptation to host's environment, the present study was conducted by using suppressive subtractive hybridization (SSH) technique to seek the highly expressed genes especially in host. A total of 328 clones from the *in vivo* SSH library and that of 160 clones from the *in vitro* SSH library were analyzed in order to determine their expression levels, but none of the above-mentioned genes showed differential expression. This indicates that no trypanosome genes could be differentially expressed either the *in vivo* or *in vitro* propagated trypanosomes. Alternatively, there might be limitation for detecting specifically expressed genes in African trypanosomes using this method, because of their polycistronic gene expression.

Key Words: differential expression; infection; *Trypanosoma brucei*

The development of axenic culture methods for both bloodstream form (BSF) and procyclic form (PCF) of African trypanosomes (Hirumi and Hirumi 1989; Hirumi and Hirumi 1991) has contributed to further investigation of African trypanosomes. However, this invention has given rise to a question of whether biological characteristics of *in vitro* propagated parasites are the same as those in host. Indeed, prolonged *in vitro* cultivation occasionally results in the loss of infectivity and/or virulence for the host. For example, prolonged *in vitro* growth (14 months) of *Trypanosoma evansi* (*T. evansi*), evolutionarily related parasite to *T. brucei*, led to reduction or loss of the kinetoplast (dyskinetoplasty), and a loss of infectivity for mice (Zweygarth *et al*. 1990). In general, trypanosomes (hemoflagellates) have to be maintained without extensive *in vitro* passage from their original isolate, since their phenotypic change can occur in parasite populations derived from prolonged *in vitro* cultivation (Schuster and Sullivan 2002). Although it was reported that PCF of *T. brucei* cultivated extended periods was phenotypically stable (Pearson and Jenni 1989), it is unknown whether the same results will be obtained from BSF of African trypanosomes or not. It has been reported that certain genes of some pathogens were specifically expressed only after infection to their mammalian host. Several pathogenic genes in *Salmonella typhimurium* (Heithoff *et al*. 1997; Hensel *et al*. 1995; Mahan *et al*. 1993; Valdivia and Falkow 1997) *Yersinia enterocolitica* (Darwin and Miller 1999), and *Escherichia coli* (Dozois *et al*. 2003), such as putative adhesins, lipopolysaccharide core synthesis, iron-responsive, plasmid- and phage-encoded genes, are specifically expressed in the mammalian host, but not *in vitro*. In case of protozoan parasites, such pathogenic genes have also been reported from *Trypanosoma cruzi* (*T. cruzi*) (Contreras *et al*. 1998), *Entamoeba histolytica* (Bruchhaus *et al*. 2002) and *Trichomonas vaginalis* (Garcia *et al*. 2003). These findings imply that African trypanosomes also have *in vivo* specific genes for adaptation and/or pathogenesis to their mammalian hosts. Therefore, the present study

was conducted in order to identify the genes specifically expressed and/or up-regulated by *Trypanosoma brucei gambiense* (*T. b. gambiense*) in host (*in vivo*), but not *in vitro*.

The BSF of *T. b. gambiense* IL2343 was previously obtained from the International Livestock Research Institute, Nairobi, Kenya (Inoue 2000). The parasites were cultured *in vitro* using an HMI-9 growth medium (Hirumi and Hirumi 1989; Hirumi and Hirumi 1991) composed of Iscove's modified Dulbecco's medium (IMDM; Sigma, St. Louis, MI) supplemented with 20% fetal bovine serum (FBS, Biological Industries, Ashrat, Israel), penicillin-streptomycin (100 U/ml-100 μg/ml) (Invitrogen, Carlsbad, CA), 2 mM L-glutamine (Sigma), 0.1 mM bathocuproine (Sigma), 1 mM pyruvic acid (Sigma), 10 ml/l HT supplement (0.1 mM sodium hypoxanthine and 0.016 mM thymidine) (Invitrogen), 0.4 mg/ml bovine serum albumin (BSA; Sigma), 1 μg/ml bovine holo-transferrin (Sigma), 0.2 mM 2βmercaptoethanol (Wako, Osaka, Japan), 2 mM L-cysteine (Sigma), and 60 mM HEPES (Sigma) at pH 7.2. The parasites were harvested from *in vitro* culture, and centrifuged at 1,000 xg for 10 min, and this preparation was designated as the in vitro parasite. For preparation of the *in vivo* propagated parasites (*in vivo* parasite), female BALB/c mice (6 weeks old) were injected intraperitoneally (i.p.) with 5 x 10^3 cell/head of the *in vitro* propagated parasites. At the peak parasitemia (more than 1.0 x 10^8 cells/ml), whole blood was collected by cardiac puncture, and *in vivo* parasites were purified by using DE52 anion exchange column chromatography (Whatman, Brentford, UK)(Lanham and Godfrey 1970). The purified parasites were collected by centrifugation at 1,000 xg for 10 min. According to the manufacturer's instructions, total RNA was isolated from both *in vitro* and *in vivo* parasites by using TRIZOL (Invitrogen).

For construction of subtractive library derived from *in vivo* and the *in vitro* parasites, poly $(A)^+$ RNAs were purified from the parasites using oligo (dT) spin columns (Invitrogen). Using 2 μg poly $(A)^+$ RNA from each preparation, a subtracted library was constructed by using PCR-Selected™ (Clonetech, Mountain view, CA) according to the manufacturer's instructions. In order to perform exhaustive comparison of gene expression levels between *in vivo* and *in vitro* derived parasites, we used a method based on suppressive subtractive hybridization (SSH) (Diatchenko *et al.* 1996). Differential complimentary DNA (cDNA) sequences obtained by this method were inserted into pGEM®-T easy vector (Promega, Madison, WI) to prepare the subtracted cDNA libraries. The nucleic acid sequence of each cDNA clone was determined by using the Big Dye terminator cycle sequencing kit (Applied Biosystems Japan Ltd., Japan). Sequence homology searches were performed using the BLASTn program of the DNA Data Bank of Japan (National Institute of Genetics, JAPAN, URL: http://www.ddbj.nig.ac.jp/Welcome-j.html). cDNA inserts were randomly picked up from *in vivo* and *in vitro* SSH libraries, and amplified by polymerase chain reaction (PCR) with primers that bind to T7 promoter region (5'-TAA TAC GAC TCA CTA TA-3') and SP6 promoter region (5'-ATT TAG GTG ACA CTA TAG AAT-3') under the following conditions: 94°C for 10 min as an initial denaturation step and then subjected to 30 cycles consisting of 45 sec at 94°C, 1 min at 55°C, and 1 min at 72°C, and finally a terminal elongation for 7 min at 72°C. These amplified products were dotted on the Hybond-N^+ (GE Healthcare, Little Chalfont, UK) nylon with Bio-Dot® microfiltration apparatus (BIO-RAD, Hercules, CA). On the other hand, total RNAs were prepared from the *in vivo* and *in vitro* trypanosomes with TRIZOL (Invitrogen). From these RNAs, ^{32}P-deoxycytidine triphosphate (dCTP)-labeled cDNAs were synthesized with oligo (dT) primer and avian myeloblastosis virus (AMV) reverse transcriptase (Invitrogen), and used as probes for dot blot analysis of each cDNA clone. Hybridization was performed at 42°C for 12 hrs in hybridization solution containing 50% formaldehyde, 0.6 M NaCl, 0.2 M Tris-HCl (pH

8.0), 0.02 M ethylenediaminetetraacetic acid (EDTA), 0.5 % sodium dodecyl sulfate (SDS) and 100 μg/ml heat-denatured salmon sperm DNA. Filters were washed with 2x SSC and 0.1% SDS three times for 10 min each, and subsequently washed 0.2x SSC and 0.1% SDS 4 times for 15 min. Detection was done by autoradiography. The expression levels of selected cDNA clones were also examined by Northern blot analysis. Briefly, the *in vivo* and *in vitro* total RNAs were electrophoresed in 1.0% gel and transferred to the Hybond-N^+ nylon membrane with 20x SSC. From the cDNA clones of the *in vivo* subtractive library, inserts were amplified by PCR and followed by synthesizing ^{32}P-dCTP-labeled inserts with AMV reverse transcriptase. These inserts were used as the probe for the Northern blot analysis. Hybridization was

Table 1. The clones in the *in vivo* subtracted library.

Gene name (Accession number)	Gene name (Accession number)
Ribosomal L, S and P protein	*T. brucei* RNA binding protein **(AF020695)**
Trypanosoma brucei (*T. brucei*) ribosomal protein L1 protein **(Z54340)**	*T. brucei* exosome-associated protein 4 protein **(AJ516002)**
T. brucei ribosomal protein L30 protein **(Z54339)**	*T. cruzi* putative eukaryotic translation factor 6 protein **(AF117890)**
T. brucei ribosomal protein S12 protein **(AF031925)**	
T. brucei 40S ribosomal protein S13, putative protein **(AE017170)**	**Other function**
Trypanosoma cruzi (*T. cruzii*) ribosomal L27 protein **(AY197727)**	*T. b. rhodesiense* activated kinase C protein receptor homolog (TRACK) protein **(AF049901)**
T. cruzi ribosomal protein P0 protein **(L15558)**	Bloodstream-specific protein 1,3-4-*T. brucei* **(B37416)**
T. cruzi ribosomal protein P1 protein **(X65025)**	*T. b. rhodesiense* prohibitin protein **(AF049901)**
Leishmania major (*L. major*) probable 40S ribosomal protein S6 protein **(AL163505)**	*T. b. rhodesiense* aldolase locus for fructose 1,6 bisphosphate aldolase **(X52586)**
L. major ribosomal protein S2 protein **(AF467951)**	*T. b. rhodesiense* alpha (complete) and beta tubulin genes in 3.7 kb tandem repeat **(K02836)**
L. major homologous to rat ribosomal protein S8 protein **(X62942)**	*T. brucei* HSP83 gene **(X14176)**
L. major 40S ribosomal protein S4, copy 2 protein **(AL365154)**	*T. brucei* malate hydrogenase protein **(AF027739)**
Argopecten irradians ribosomal protein L12 protein **(AF526229)**	*Urechis caupo* 34/67 kD laminin binding protein protein **(U02370)**
Myxine glutinosa ribosomal protein L17 protein **(AY130353)**	*Kluyveromyces marxianus* S10 protein **(S53430)**
Podocoryne carnea ribosomal protein L18a protein **(AJ009692)**	*Bradyrhizobium japonicum* USDA 110 heat shock protein protein **(AP005937)**
	Schizosaccharomyces pombe SPAC19B12.04,rps30-1,rps3001 protein **(AL390814)**
Protease	Probable ubiquitin-protein ligase (EC 6.3.2.19) fission yeast (*Schizosaccharomyces pombe*) **(T37499)**
T. b. rhodesiense cysteine protease protein **(AJ297265)**	
T. brucei ubiquitin EP52/2 **(X56511)**	**Conserved protein**
Trypanosoma rangeli (*T. rangeli*) cathepsin B-like protease protean **(AF400046)**	*T. brucei* conserved hypothetical protein **(AL929604)**
	T. cruzi TC3_70K14.2 protein **(AC116971)**
RNA binding protein / transcription	*L. major* hypothetical predicted protein P265.14, unknown function protein **(AL359716)**
T. brucei RNA editing complex protein MP24 protein **(AY228168)**	*L. major* hypothetical protein L8342.05 protein **(AL122012)**

performed under the same condition described as dot blot analysis. As summarized in Fig. 1, these clones were arbitrarily picked up from the *in vivo* library (328 clones) and the *in vitro* library (160 clones), and analyzed following BLASTn search and their transcriptions. Firstly, from 328 clones of the *in vivo* library, 134 clones were arbitrarily sequenced and analyzed in BLASTn search (Table 1). The result showed that the library possesses 37 differential unique genes that share sequence homology to genes in the DNA database. The genes consisted of ribosomal protein genes (26.9%), RNA binding / transcription protein genes (4.5%), protease genes (4.5%), other functional genes (10.4%), conserved protein genes (3.7%) and unknown genes (50.0%). On the other hand, the BLASTn search of arbitrary selected 52 clones of the *in vitro* library showed total 6 known genes, such as ribosomal protein genes (7.7%) containing *L. donovani* 60S ribosomal protein L26 protein (Accession number, AF499606), protease genes (7.7%) including *T.*

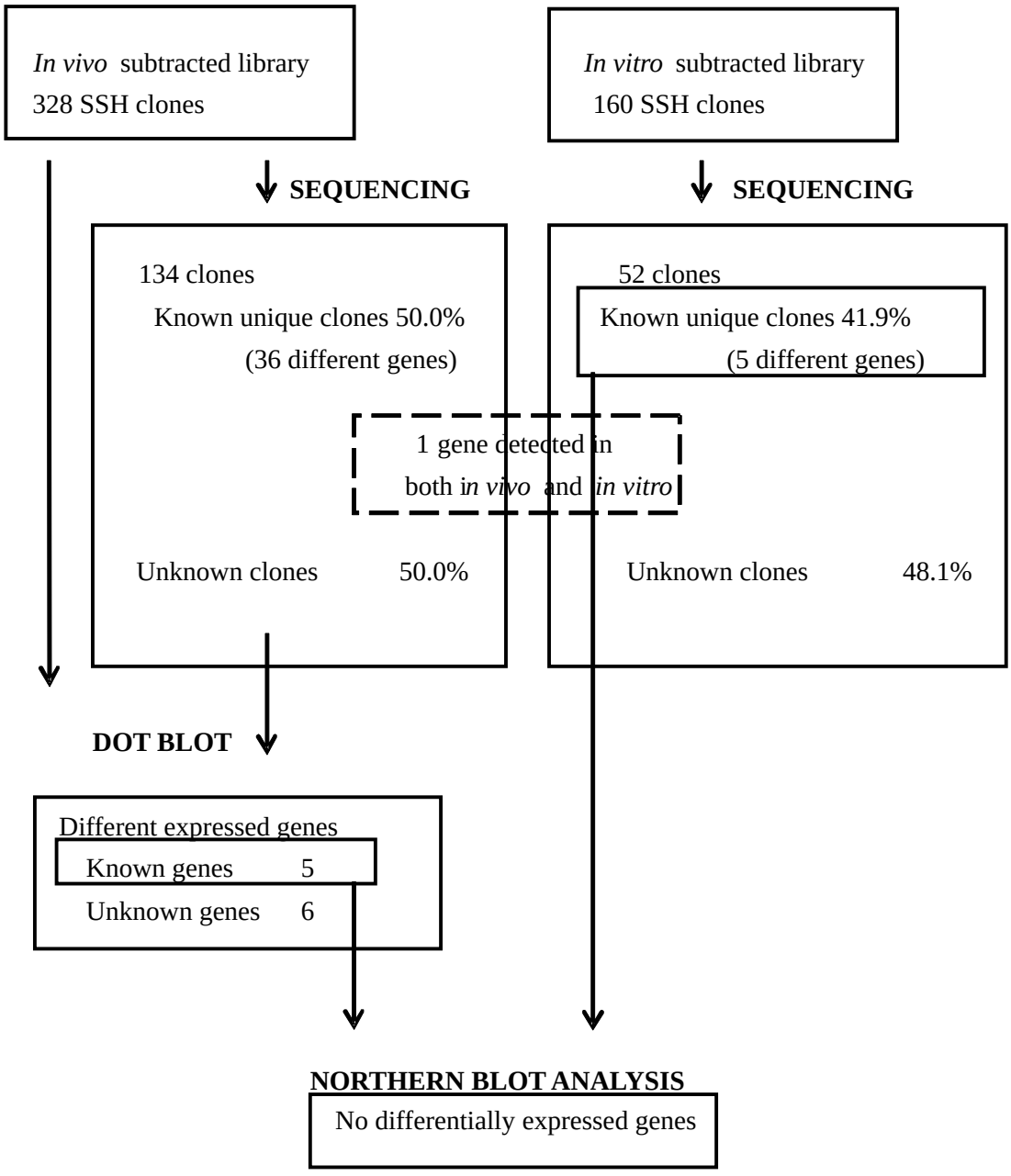

Fig. 1. Summary of the SSH strategy.

brucei proteasome activator protein PA26 protein gene (AF085608), other functional genes (32.7%) such as *T. b. rhodesiense* activated kinase C protein receptor homolog (TRACK) protein gene (AF049901), *T. brucei* hypoxanthine-guanine phosphoribosytransferase protein gene (L10721), *T. cruzi* laminin receptor precursor-like protein/p40 ribosome associated-like protein gene (AF133210), and conserved protein genes (3.8%) including *T. brucei* hypothetical protein conserved protein gene (AE017168), as well as unknown genes (48.1%). According to the results, RNA binding/transcription protein genes were only found in the *in vivo* library. In addition, 2 SSH libraries shared the same gene, such as TRACK protein coded gene.

Expression levels of the cDNA clones listed above were examined by using cDNA probes synthesized from total mRNA obtained from *in vivo* and *in vitro* parasites (Fig. 1). As a result, 11 clones showed lower intensity in hybridization with *in vivo* cDNA probes as compared to that in hybridization with *in vitro* cDNA probes. In detail, lower intensities were observed in 6 unknown genes (data not shown) and 5 known genes, containing *T. brucei* ribosomal protein L1 protein gene, *Leishmania major* (*L. major*) probable 40S ribosomal protein S6 protein gene, *T. brucei* exosome-associated protein 4 protein gene, *Trypanosoma rangeli* (*T. rangeli*) cathepsin B-like protease protein gene, and *L. major* hypothetical predicted protein gene P265.14, unknown function protein gene.

To confirm *in vivo* or *in vitro* specific expression of the genes selected by dot blot analysis described

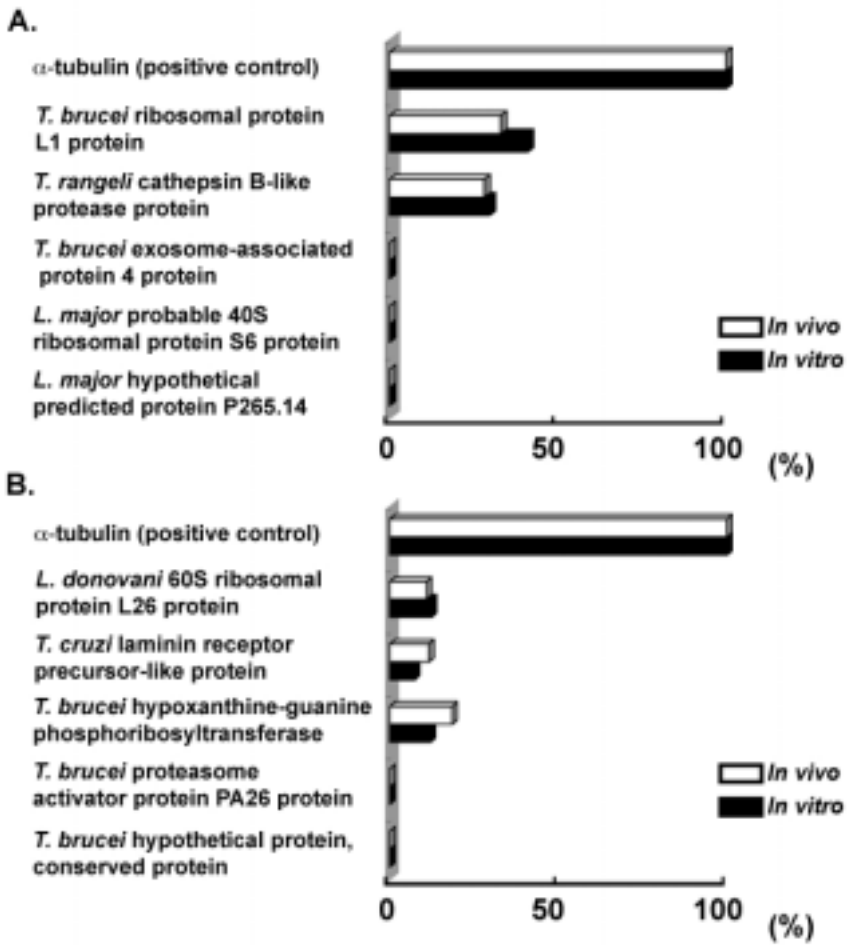

Fig. 2. Northern blot analyses of the mRNA transcription levels of cDNA clones from the subtracted libraries. Total trypanosome RNAs from *in vivo* and *in vitro* derived parasites were electrophoresed, and transferred on a membrane. The membrane was probed with either cDNA clones from *in vivo* (A) or *in vitro* (B) subtracted library. Expression levels of all clones were normalized based on the level of alpha-tubulin mRNA.

above, the Northern blot analysis was conducted by hybridizing the total RNA of the trypanosome with the probes that were derived from the cDNA clones selected by dot blot analysis. As an internal standard, α-tubulin gene of *T. brucei* (Accession number: Tb927.1.2360) was used. As a result, 2 genes (*T. brucei* ribosomal protein L1 protein gene, and *T. rangeli* cathepsin B-like protease protein gene) were detected by corresponding probes, but 3 genes were not detectable in Northern blot analysis (Fig. 2A). However, these 2 genes showed almost the same expression levels between *in vivo* and *in vitro*. In addition, the expression level of the 5 genes from *in vitro* SSH library was also examined (Fig. 2B). Of the 5 cDNA clones, 3 genes (*L. donovani* 60S ribosomal protein L26 protein gene, *T. cruzi* laminin receptor precursor-like protein gene, and *T. brucei* hypoxanthine-guanine phosphoribosyltransferase gene) were detectable in Northern blot analysis, but expressed at almost the same levels (Fig. 2B). The rest of 2 genes were not detected.

In this study, in order to identify genes specifically expressed either *in vivo* or *in vitro*, we have applied SSH library method. The SSH gave rise to the group of candidate genes, and these genes appeared to be differentially expressed by dot plot analysis. However, the Northern blot analysis showed that these genes are neither specifically expressed *in vivo* nor *in vitro*. This result indicates that there might be no differentially expressed genes between the *in vivo* and the *in vitro* propagated *T. brucei.* However, it is also considerable that the expression levels of the candidate genes might not be able to be differentiated by Northern blot analysis due to its low sensitivity to detect gene expression. It is possible that more sensitive methods, such as nuclear run-on assay, real-time PCR or micro array, might reveal differentiation of gene expression. In case of *T. cruzi*, which also belongs to order Kinetoplastida, gene and protein expression levels in the *in vivo* and *in vitro* parasites have been compared in previous studies (Contreras *et al*. 1998; Weston *et al*. 1999). However, these previous reports did not give the consistent results. This emphasized the importance of protein expression analysis for identification of *in vivo* specific factors, such as a two-dimensional gel electrophoresis and subsequent proteome analysis. In addition, SSH might not be a suitable method for detecting stage specific genes in the order Kinetoplastida, because their genes are expressed polycistronically, and regulated at post-transcriptional level (D'Orso *et al*. 2003). In conclusion, this is the first step to examine gene expression level between *in vivo* and *in vitro* derived African trypanosomes. However, any genes were not identified as *in vivo* or *in vitro* specific gene.

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