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ARTÍCULO ORIGINAL

Molecular cloning and characterization of two *hsp 70* homologous genes from the dimorphic fungus *Paracoccidioides brasiliensis*

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Paracoccidioides brasiliensis, a dimorphic fungus, is the etiologic agent of Paracoccidioidomycosis (PCM), one of the most important systemic mycosis in Latin America. Two genes (2.2 and 1DB5) were cloned, characterized and sequenced; they showed homology with members of *hsp70* gene family. By using several probe fragments derived from these genes, levels of expression for each gene were determined by Northern blot during transition to the yeast phase. The highest level of *hsp70* transcript occurred between 30 min to 6 hours after temperature shift, with significant reduction after 36-48 hours. However, after 72 hours, the level of the transcription increased until yeast phase was reached. As a response to temperature increase, *hsp 70* genes are expressed during the transition phase and possibly play a role in the differentiation process.

Key Words: Heat Shock Proteins, dimorphic fungus, *Paracoccidioides brasiliensis*, differential expression, *hsp70* genes.

Clonaje y caracterización molecular de dos genes homólogos *hsp70* del hongo dimórfico *Paracoccidioides brasilensis.*

Paracoccidiodes brasiliensis es el agente etiológico de la Paracoccidioidomicosis (PCM), una de las micosis sistémicas mas importantes en Latinoamérica. Dos genes *hsp70* (Clones 2.2 y 1DB5) fueron clonados, caracterizados y secuenciados. El análisis de secuencia mostró que los clones 2.2 y 1DB5 poseen una alta homología con la familia de genes de choque térmico *hsp70*. Mediante la utilización de fragmentos de ADN provenientes de ambos clones, se determinó la expresión por Northern blot durante la fase de transición a levadura. Nosotros encontramos los niveles mas altos de transcriptos ocurre entre los 30 min y 6 h después de cambiar la temperatura a 37°C. Entre las 36 y 48 h, se reducen los niveles de ARNm. Sin embargo, los niveles de ARNm aumentan a las 72 h hasta alcanzar la fase de levadura. Como resultado del aumento de la temperatura durante la fase de transición micelio a levadura, se incrementa la expresión de genes *hsp70* sugiriendo que las proteínas de choque térmico tienen un papel durante el proceso de diferenciación.

Palabras clave: Proteínas de Choque Térmico, hongo dimórfico, *Paracoccidioides brasiliensis*, expresión diferencial, gene *hsp70*.

Paracoccidioides brasiliensis is a dimorphic fungus responsible for paracocidioidomycosis, one of the most important systemic mycoses in Latin America (1,2). *P. brasiliensis* grows as a yeast (Y) in cultures at 37°C and in the host tissues, while at lower temperatures, the fungus grows as a mold (M) (3). Dimorphism is an adaptive mechanism, whereby the fungus developing its saprophytic existence in nature is able to adjust to the 37°C temperature of its casual homeothermic host. Dimorphic pathogenic fungi such as *P. brasiliensis*, *Blastomyces dermatitidis* and *Histoplasma capsulatum* have the unique ability to colonize host tissues in parallel with the development of M→Y morphological transition. In these fungi the temperature is an important factor because it induces phase transition and as a consequence, a set of biochemical events occur during this process. In *P. brasiliensis* these events are fundamentally similar to those reported for *B. dermatitidis* and *H. capsulatum* (4). It is known that the triggering event in the M→Y phase transition by heat insult leads to various degrees of oxidative phosphorylation uncouplings depending on the incubation temperature (4). When mycelia are shifted to 37°C, respiration is partially uncoupled during the M→Y transition and fully coupled when the fungus reaches the yeast form. In *B. dermatitidis*, respiration becomes completely uncoupled if the temperature used for transition is 43°C (4), while in *P. brasiliensis*, a temperature of 41°C is sufficient to uncouple respiration during the phase transition. In these fungi, temperature shift causes a decline in the concentration of electron transport components, due to reduced ATP levels and respiration rates (stage 1). The cells then enter stage 2 during which respiration decreases or ceases when reaching temperatures between 41° and 43°C. Finally, in stage 3 cells recover and transformation to the yeast phase begins. There are also profound similarities in the biochemical behavior of both *P. brasiliensis* and *B. dermatitidis* during phase transition (4). When respiration stops (stage 2), cysteine and/or sulfhydryl-containing compounds activate the shunt respiratory pathways, thus allowing the utilization of mitochondrial substrates that provide energy to complete transition. Additionally, in *B. dermatitidis* heat shock proteins are induced upon temperature shift (4).

In *H. capsulatum*, *hsp70* mRNA transcription is transiently induced at 37°C showing the maximal peak at 6 hours when the mycelium to yeast transition occurs (5,6). At the same time, *hsp 82* mRNA transcription is induced 3 hours after temperature shift (7,8).

These biochemical events suggest alterations in the patterns of gene expression by a heat-related insult, leading to expression of specific genes. These genes, in turn, control changes along different stages of the cell's metabolic processes, as well as, the morphology of the fungus. It is known that membrane components may be involved in the activation and repression of transcription of heat shock and other genes (9). Thus, membrane lipid composition and their ratio between saturated (SFA) and unsaturated (USF) fatty acids have received significant attention, when considering the evolution of species (10). Abrupt changes occurring during transition processes in the dimorphic fungus *H. capsulatum* can cause modifications in the membrane structure with consequent either repression or induction of specific genes (11,12). In general, the cells can compensate for stress-induced cellular disturbances through physiological and biochemical adjustments.

In dimorphic organisms the transduction of temperature changes affecting differential gene expression is a central problem in regulating heat shock genes; this problem has important consequences. In fact, temperature works not only as a signal for adaptation processes such as the induction of the heat shock phenomenon but also as triggering mechanisms of the phase transition (13,14). As a first approach, the present study reports the differential expression of two *hsp 70*-like genes during the process of differentiation in the dimorphic fungus *P. brasiliensis*.

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Materials and methods

P. brasiliensis isolate (Pb339) ATCC32069 was grown in liquid SMV medium (15), at either 22°C or 37°C. The cDNA libraries were constructed using the mycelial phase grown for two hours after the temperature was shifted to 37°C.

Cloning procedures

Two cDNA libraries from Pb339 were constructed using 5 micrograms of mRNA. Total RNA was extracted from both non-induced mycelia and mycelia at 37°C (induced). Both types of cDNA libraries were done using lZAPII synthesis kit (Stratagene, La Jolla, CA). The first cDNA library was probed with a pool of sera from patients with paracoccidioidomycosis diagnosed by direct observation and isolation of *P. brasiliensis,* as well as by serological tests. The initial screening was performed with secondary rabbit anti-human peroxidase-labeled antibody. Further screenings were carried out with the streptavidin-alkaline phosphatase system (Amersham, Aylesbury, UK). The second cDNA library was screened following the standard procedures (16) using heterologous probes of *hsp 82*, *hsp 70* from *H. caspulatum* (6,8), *hsp 26* from *Saccharomyces cerevisiae* (17) and homologous *hsp 70* isolated from the first cDNA library. The DNA fragments were labelled using Fluorescein Gene Images™ (Asmersham, Aylesbury, UK). Analysis and characterization of isolated cDNA fragments were carried out on the phagemide pBluescript SK+/-™ (Stratagene, La Jolla, CA). Clones were cut with the restriction enzymes *EcoRI* and *XhoI* (New England Biolabs Inc, Beverly, MA.USA) (16).

Sequencing and homology analysis

The cDNA fragments were sequenced using the dideoxynucleotide chain-termination method (18) with alkaline denaturation of double stranded DNA and the Sequenase kit version 2.0™ (United States Biochemicals, Inc, Cleveland, OH) in the presence of [a^{35}S]-dATP (1200 Ci/mmol, Amersham) and fractionated on standard 6% denaturing polyacrylamyde/urea gels. The entire sequence was determined in both strands. Progressive unidirectional deletions with Erase-a-Base System™ (Promega), subcloning of several fragments upon digestion with restriction enzymes and primer "walking" with synthetic oligo-nucleotides, were used to sequence positive clones. Sequence comparisons with the gene data bank were performed using SWISS-PROT, EMBL and BLAST databases (19-21). Sequence analysis was carried out with the programs DNA Strider™ (1.2.Ch. Marck and C.E.A., 1991) and DNAsis (Hitachi, Software Engineering Co. USA).

Isolation of total RNA

Total RNA was extracted from mycelium grown at 22±2°C and from mycelia incubated at 37°C, 30 min, 1, 2, 6, 12, 24, 48, and 72 hours and later on when fully transformed to the yeast phase. Approximately 3 g of cells were collected and washed in bidestilled H_2O with diethylpyrocarbonate (DEPC). All cells were then frozen in liquid nitrogen and lysed using 10 ml of lysis buffer. Lysis was obtained at 65°C by adding 0.5 vol of glass beads (Sigma Type I.W, 50/150 microns), 1 ml of 20% Lauryl sulfate, 15 ml of 2dH_2O-saturated phenol to the frozen cells with vortexing. The upper phenol phase, containing nucleic acids, was recovered by centrifugation at 4000 rpm for 10 min and then extracted with 2 ml of cloroform:isoamyl alcohol (24:1). Two volumes of cold ethanol was added to the supernatant and incubated at -20°C overnight before centrifugation at 7,500 rpm at 4°C for 20 min. The pellet was washed twice with 75% EtOH, dissolved in 200 µl of 2dH_2O-DEPC containing 40 U RNasin™ (Promega) and 5 mM DTT. DNAse treatment of RNAs was performed using the DNase I™ (Boheringer) according to the standard procedures (16). After DNAse treatment, small aliquots of each sample were loaded onto a 1% agarose gel. Total RNA was quantified using the Gel Analyzer program™ and Kodak Photo enhanced™ (Kodak).

Northern blot analysis

Ten micrograms of total RNA from extracted mycelium at 22±2°C and from mycelia incubated at 37°C at 30 min, 1, 2, 6, 12, 24, 48, and 72 hours and from the yeast phase were run on denaturing 1.2% agarose gel and blotted onto Hybond™membrane (Amersham, UK) following standard procedure (16). Filters were dried at 37°C and the RNAs was fixed to the membrane by UV crosslinking (16). The RNA

was normalized in denatured agarose gels and fluorescense was quantified (pixels^2/area) using a Scion Corporation based on NIH Image 1.61/ppc computer program (Program Macintosh, National Technical Information Service, Springfield, VA).

Labeling and hybridization

A 540 bp DNA fragment from clone 2.2 and clone 1DB5 from Pb339, *hsp 82*, *hsp 70* from *H. caspulatum* and *hsp 26* from *S. cerevisiae* were labeled using Fluorescein Gene Images System™ (Amersham). Approximately 10 ng/ml of probe was used under hybridization conditions at 65°C overnight. Filters were washed two times for 30 min at 63°C in each wash solution as follow: 2XSSC/0.2% SDS; 1XSSC/0.2% SDS; 0.5XSSC/ 0.2% SDS and 0.1XSSC/0.2% SDS. The northern blots were detected as described the detection System to Fluorescein Gene Images System™ (Amersham) and exposed to X-ray film using intensifying screens (16).

Protein extraction

The protein extracts were obtained from mycelium at 22±2°C and from mycelia incubated at 37°C at 0 time, 1, 2, 3, and 24 hours as described (22). In brief, after harvesting from the SMV medium, the Pb339 cells were washed in Tris-HCl buffer pH 8.8 and frozen in liquid nitrogen containing a protease inhibitor 4 mM phenyl-methyl-sulfonyl-fluoride (PMSF). After mechanical maceration the cellular debris was removed by centrifugation at 10,000 rpm at 4°C and the total protein concentration was determined by Bradford's method (23).

Western blot analysis

Ten micrograms of total proteins were electrophoresed and blotted to nitrocellulose membranes. After staining with Ponceau S (Sigma), the nitrocellulose were incubated in 25 mM Tris-HCl pH 7.5 containing 5% powdered milk for 1 hour at room temperature. They were incubated overnight in 1:2000 dilution with polyclonal antibody MAb 69F against 70 kDa protein family (24). Then, the membranes were washed three times with washing solution containing Tween 20 in TBS 1x and incubated for 1 hour with an antimouse alkaline phosphatase-labeled secondary antibody, diluted 1:1000. After washing three times with Tween 20 in TBS1x, the membranes were developed with 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetratzolium (BCIP/NBT (Sigma).

Nucleotide sequence accession numbers

The cDNA sequence data from homologous *hsp70* clones 2.2 and 1DB5 reported in this paper have been submitted to GeneBank (21) and assigned the accession numbers: AF004553 and AF111194, respectively (figures 1 and 2).

Results

Isolation of recombinant clones containing a sequence homology to genes of the hsp 70 gene family

Positive clones were taken from the non-induced mycelial phase DNA library of *P. brasiliensis* using sera from patients with PCM. We identified two positive recombinants (clones 2.2 and 3.3). Clone 3.3 has been extensively characterized (25,26). Clone 2.2 (*hsp70* a), containing a 1.9 kb insert, was analyzed by using *Eco* RI-*Xho* I restriction enzymes which released 2 DNA fragments of approximately 1.7 kb and 0.2 kb. Sequence analysis of clone 2.2 revealed the presence of an open reading frame translated 480 aminoacids (aa) (ORF480). Comparison analysis revealed that ORF480 has a high degree of homology to other HSP70 proteins from *H. capsulatum* (85%), *Neurospora crassa* (85%), *Penicillium citrinum* (83%), *Cladosporium herbarum* (82%), *S. cerevisiae* (81%), *Cryptococcus curvatum* (78%), *Candida albicans* (72%), and also to a previously identified *P. brasiliensis* 70 kDa heat shock protein (90%) (27).

The induced cDNA library (2 hours after shifting temperature at 37°C) was screened using heterologous *hsp82*, *hsp70*, *hsp26* genes and the homologous clone 2.2, as probes. Positive phages were obtained using only clone 2.2 (540 bp) as a probe. These were processed and excised using Gigapack system kit™ (Stratagene). Restriction analysis of positive clones was performed using various enzyme digestions. Clone 1DB5 (*hsp70* b) released two *Eco* RI-*Xho* I fragments of 1.8 and 0.4 Kb that were used for further investigation. cDNA sequence analysis showed that clone 1DB5

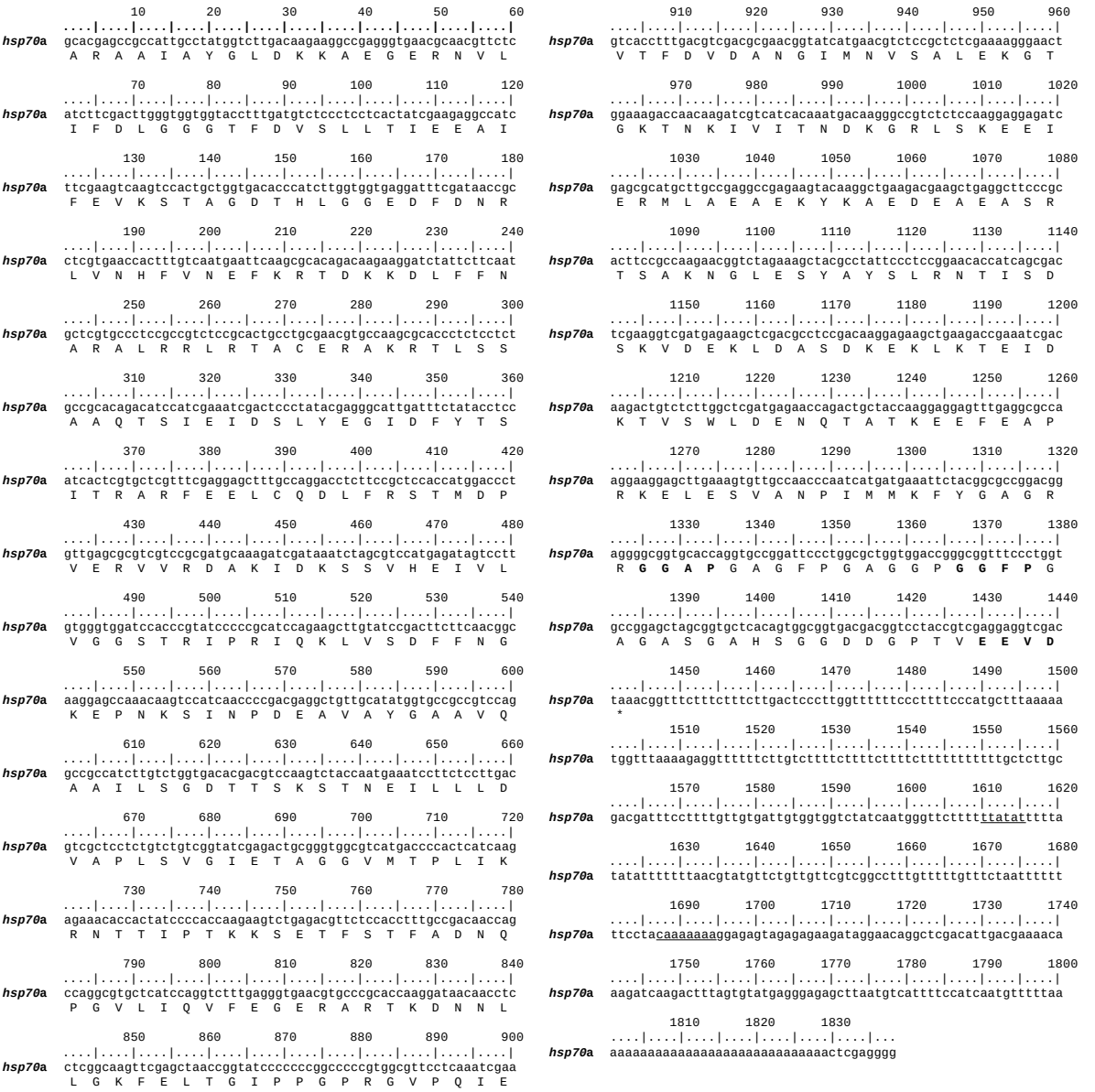

Figure 1. Nucleotide and deduced aminoacid sequence (in single letter code) of *hsp70*a (clone 2.2). Coding regions are referred by caps. In the 3' adjacent region possible putative polyadelination signal as described by Guo and Sherman are underlined and stop codon are referred by asterisc. In the extreme carboxy-terminus degenerated repeats of tetrapeptide GGAP/GGFP, as well as a EEVD motif, are referred by bold single letters.

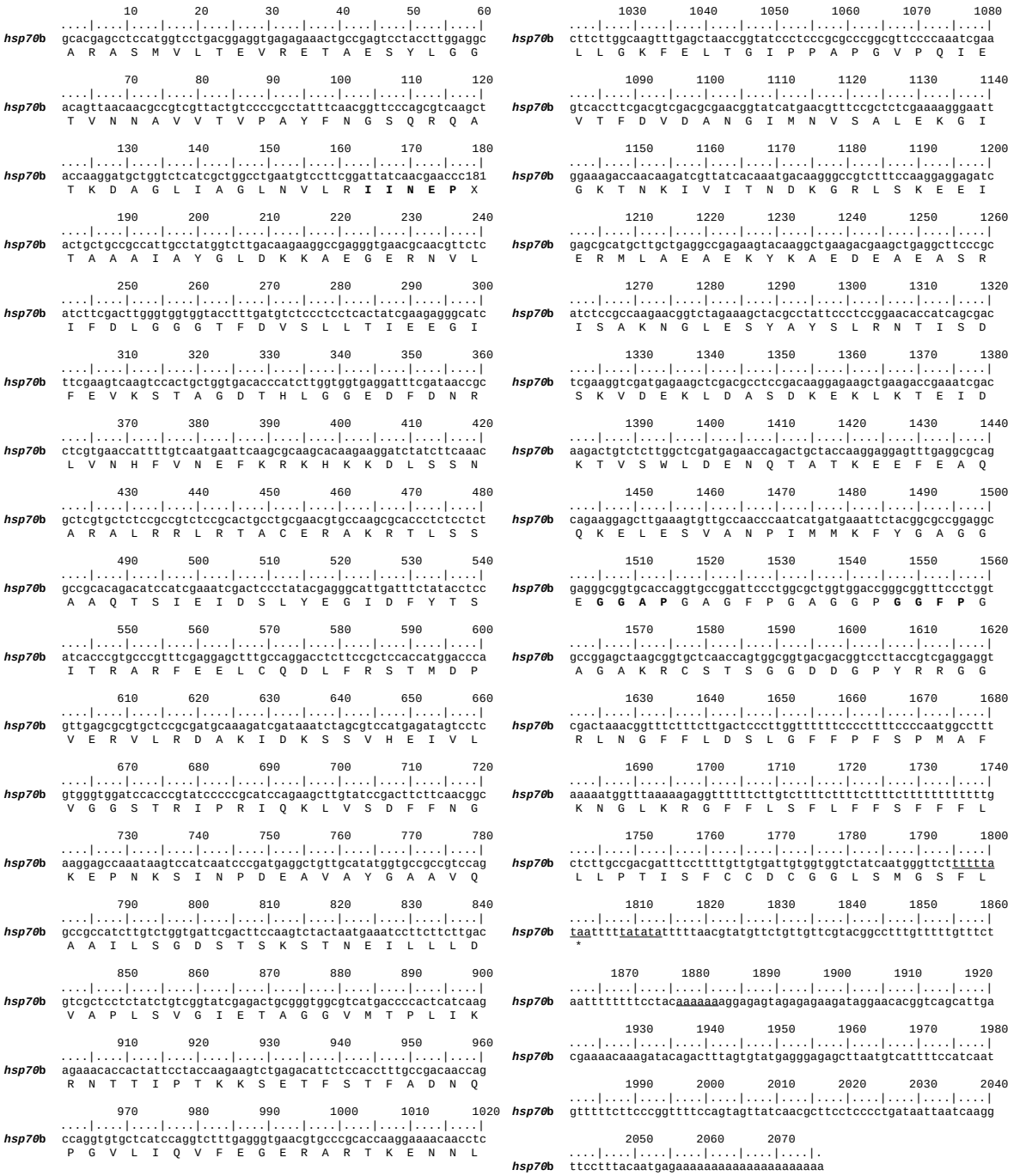

Figure 2. Nucleotide and deduced aminoacid sequence (in a single letter code) of *hsp70*b (clone 1DB5). Coding region are referred by caps letters. In the 3' adjacent region possible putative polyadelination signals as described by Guo and Sherman are underlined and stop codon are referred by asterisc. In the extreme carboxy-terminus degenerated repeats of tetrapeptide GGAP/GGFP as well as the putative ATP binding domain IINEP, are referred by bold single letters.

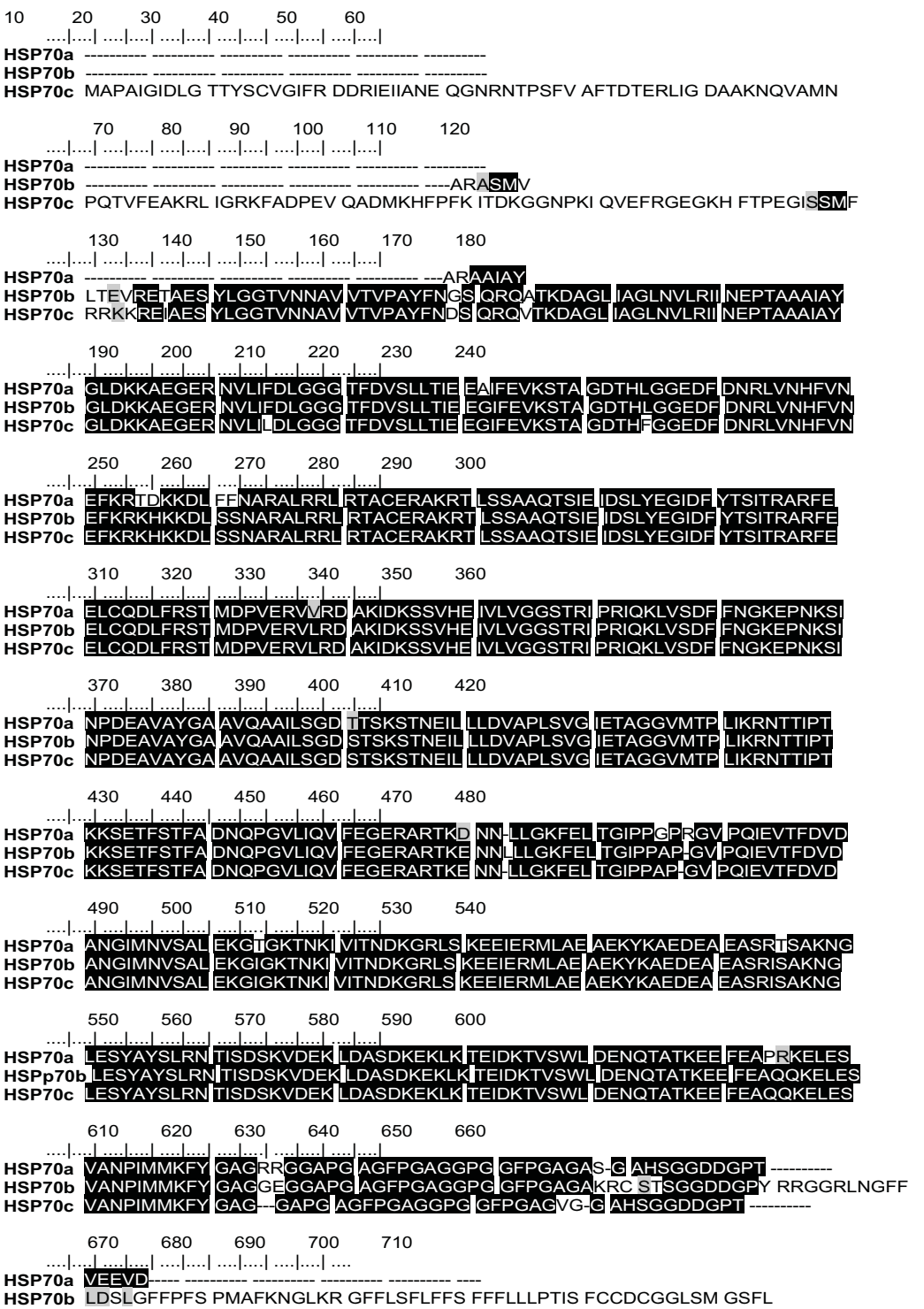

Figure 3. Multiple alignments between HSP70a and HSP70b with HSP70c deduced aminoacid sequence from *P. brasiliensis* accession number U91560. Identical aminoacids are indicated within black boxes.

contained an open reading frame which was translated into 600 aa (ORF600) with high homology with other *hsp70* from *H. capsulatum* (84%), *N. crassa* (80%), *C. herbarum* (79%), *P. citrinum* (79%), *Puccinia graminis* (74%), and *Schizosaccharomyces pombe* (74%). Moreover, proteins translated from the sequence of *hsp70*a and *hsp70*b showed a high degree of identity with the identified *P. brasiliensis* HSP70 (HSP70c) (27) (figure 3).

Identification of the hsp 70-mRNA transcripts during mycelium to yeast phase transition

Total RNA was isolated from mycelia grown at 22°C and from mycelia recovered 30 min, 1, 2, 6, 12, 24, 48, and 72 hours after 37°C temperature shift and yeast phase were hybridized using as probes a conserved 5'end region (*Sal* I fragment) from clones 2.2 and 1DB5 respectively.

The highest level of induction of the *hsp 70*-like transcripts was observed 30 min after temperature shift. The level of mRNA induction remained constant for 6 hours after temperature shift, whereas at 48 hours, transcripts were dramatically reduced. However, 72 hours after temperature shift, the normal level of induction was restored (figures 4A and B). We also found that HSP70 protein is present during the transition phase showing a correlation with mRNA levels after the temperature was shifted at 37°C (figures 5A and B).

Discussion

Previous works in dimorphic fungi have demonstrated the level of transcription of the *hsp70* genes by Western and Northern blot techniques. These works have also shown some correlation among protein and gene expression of *hsp70* during the transition phase and their relationship with the virulence of the strains that infect causal host. In this study we have identified in the dimorphic fungus *P. brasiliensis*, two *hsp*70 genes homologues, clones *hsp70*a and *hsp70*b that are differentially expressed during the mycelial and yeast phase transition. To determine the level of expression of the *hsp* during the differentiation process, DNA fragments of clones *hsp70*a and *hsp70*b were used as probes in a Northern blot analysis. It was shown that mRNA transcripts were expressed more in

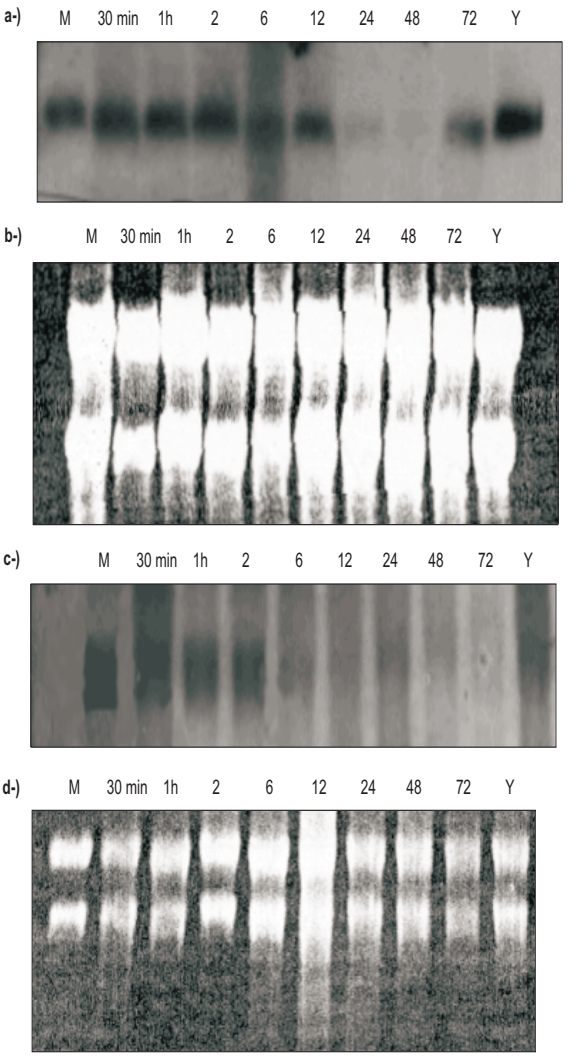

Figure 4 A. Analysis of expression *hsp70* a and *hsp70* b mRNAs. **a**) *hsp70* a mRNA levels from mycelia and yeast (96 hours) and *hsp70* a mRNA levels of the mycelial/yeast phase transition 30 min, 1, 2, 6, 12, 24, 48 and 72 hours after temperature shift at 37°C. **b**) Normalization of total RNA in desnaturalized agarose gel by etidium bromide staining. **c**) Analysis of expression *hsp70* b mRNA. *Hsp70* b mRNA levels from mycelia and yeast (96 hours) and *hsp70* b mRNA levels of the mycelial/yeast phase transition 30 min, 1, 2, 6, 12, 24, 48, and 72 hours after temperature shift at 37°C. **d**) Normalization of total RNA in desnaturalized agarose gel by etidium bromide staining.

the yeast than by the mycelium form. The early expression of these genes in *P. brasiliensis* occurs during stage I of the transition step. In fact, we found that mRNA expression is higher after 30

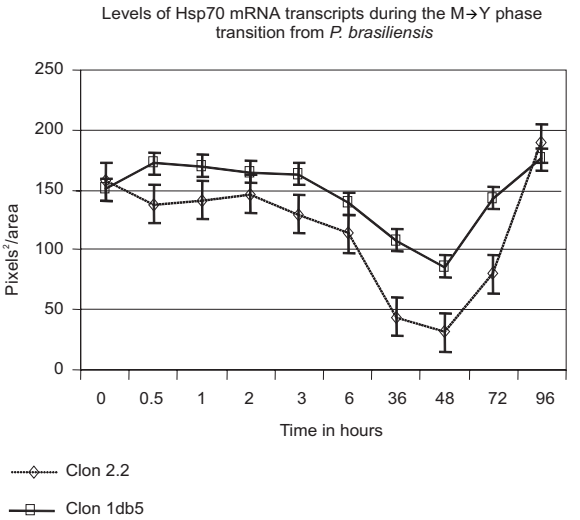

Figure 4B. Semiquantitation of mRNA expression of hsp70 genes from *P. brasiliensis* during M→Y transition. The relative semiquantitation was determined for three times measuring the intensity of bands in pixels²/area using a Scion Corporation based on NIH Image 1.61/ppc computer program (Program Macintosh, National Technical Information Service, Springfield, VA).

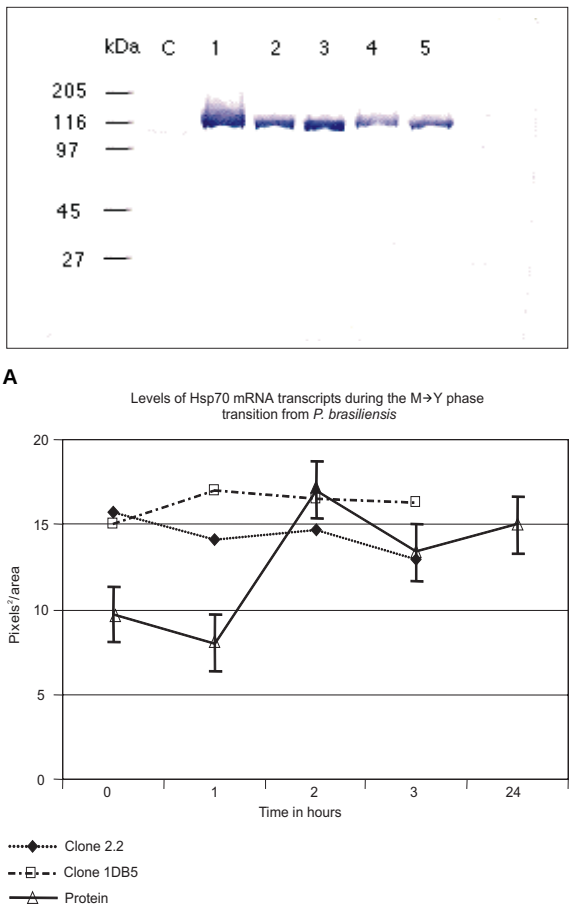

Figure 5 A. Inmunodetection of HSP70 from *P. brasiliensis* during the initial steps of phase transition using M Ab69F. Line 1: 24 hours; Line 2: 3 hours; Line 3: 2 hours, and Line 4: 1 hour after temperature shifting at 37°C; Line 5: mycelium.

B. Semiquantitation of HSP70 proteins and *hsp70* mRNAs from *P. brasiliensis* during the early steps of M→Y transition. The values correspond to Western blot analysis using M69F-antiHsp70 policlonal antibody from *H. capsulatum*. The relative semiquantitation of proteins was determined for three times measuring the intensity of bands by pixels²/area using a Scion Corporation based on NIH Image 1.61/ppc computer program (Program Macintosh, National Technical Information Service, Springfield, VA).

min of heat shock and remained high until 6 hours after shifting the temperature to 37°C. These data correlate with several biochemical events and heat shock responses that have been described in other dimorphic fungi during the transition phase (4,6). However, in contrast to higher levels in the yeast phase, studies done by Northern blot analysis in the fungus *P. brasiliensis* strain pb01, have demonstrated the absence of *hsp70*-mRNA in the mycelium phase and lower levels at 24 and 48 hours after shift to 37°C (27). In this study, they found that there was initial accumulation of the unspliced *hsp70* mRNA transcripts that decreased after 24 days. They suggested that the yeast forms seem to be more proficient in mRNA processing. Moreover, at 42°C, heat shock induced *hsp70* overproduction with a dramatic shutdown of the remain cellular proteins (27).

The mechanism that depends on the spliceosome function has been correlated with the thermotolerance conditions in most fungi (28). This mechanisms may be different to Pb01 that probably depends on the nature of the strain and its nutritional factors. It has been suggested that isolates of *P. brasiliensis* consist of several genetically distinct groups according to their geographical origin. Most of the Brazilian and Colombian strains belong to the cluster V and IV respectively (29). We found by Clustal W analysis that HSP70s are similar but not identical. The HSP70b are more phylogenetically related with HSP70c from Pb01 than other HSP70 even

produced by the same Pb01 (AF386787) and *H. capsulatum* (figure 6).It is possible that genetic variation provide differences between them, such as pathogenicity, virulence, morphogenesis and DNA variation. The induction at 37°C in SMV culture medium, induce the *hsp70* mRNA expression during the initial steps of cellular differentiation in Pb339 strain, contrary to the findings in Pb01. In fact, *hsp70* mRNA transcripts of Pb01 are visualized in yeast form only. It has been also reported that there are two protein isoforms and different *hsp70* genes in the Pb01 suggesting that either they are similar mechanisms of isoforms production as it has been described to chitin synthases genes (30) or the presence of a possible mixture of transcripts from paralogous genes (31).

The presence of *hsp70* mRNA transcripts suggests that mycelium and transitional cells are sufficient to protect the spliceosome avoiding the *hsp70* accumulation. These findings are in agreement with *H. capsulatum* where *hsp70* is properly spliced and induced during the first hours of the heat shock (6). In this fungus, a correlation between temperature sensitivity, degree of virulence and the amount of mRNA of specific genes have been demonstrated in different strains. In the avirulent strains the peak levels of *hsp70* mRNA occurs at 34°C whereas in virulent strains it occurs at 37°C (11). These strains also show differences between unsaturated (UFA) and saturated fatty acids (SFA) ratio at membrane level (12). In an avirulent strain the saturated fatty acids are dramatically reduced after 1 hour at 37°C with a defect on the heat shock response. However, the heat shock response is restored when the cells are incubated with SFA. It has been found that D^9- desaturase gene; a lipohilic microsomal enzyme that converts saturated into saturated fatty acids, is transcriptionally inactive in mycelia of virulent strain while it is actively transcribed in the avirulent strain (12). It is possible that a major difference in the regulation of the activation of the heat shock response does exist among strains with different virulence (low thermotolerance of less pathogenic strains), possibly due to a reduced level of induction of the "factor(s)" that regulate(s) the transcription of heat shock genes. It has been suggested that the membrane cell acts as a sensor during heat shock response altering the fatty acids composition (9).

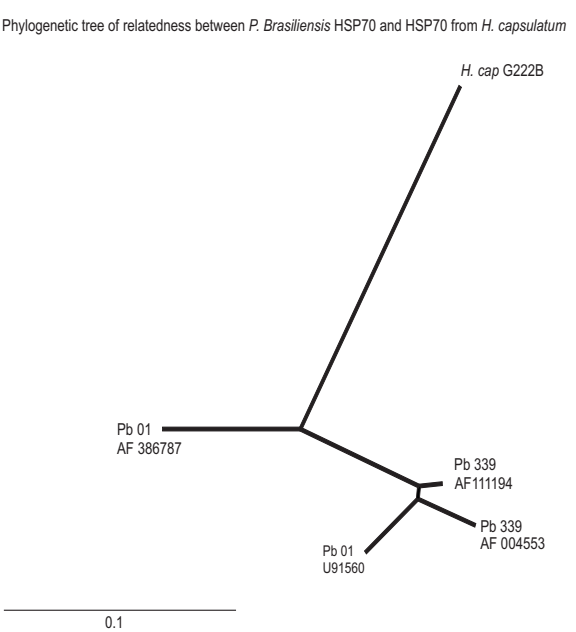

Figure 6. Phylogenetic tree constructed by neighbour-joining method in the Clustal W program that showed the relatedness between *P. brasiliensis* (*Pb)* HSP70 proteins and HSP70 from *H.capsulatum* (*H. cap.*).

Recently, homologues to *Candida* virulence and pathogenicity genes have been identified in *P. brasiliensis* by expressed sequence Tag analysis (32). The authors found that several genes either are induced or repressed during mycelium, transitional and yeast phases. They have found that in Pb18 strain, *hsp70*, *hsp82* and *hsp104* genes are differentially expressed with lower levels in mycelium and high levels during the transition phase. In fact, the maximum peaks levels were at 5 hours with a decrease of levels at 48 hours after the temperature shift to 37°C. The same behavior was also found to *Ole1* (desaturase), *Ubi* (ubiquitin), *Oxi* (oxidase), *Hydro* (Hydrophobin) and *Rbt* genes (32). The expression of *hsp70* mRNA in *P. brasiliensis* Pb339 is in agreement with these results suggesting that they are differentially expressed and that they may play an important role in the dimorphic phase transition as an adaptive response to a higher temperature.

We found functional protein domains based on the deduced aminoacid sequence from *hsp70*'s. All 3 proteins presented a high degree of homology in

the ATPase domain. In fact, we found a putative ATP binding domain localized between aa 121 and aa 256, a highly conserved sequence IING^{176}-PT, that was identified as an ATP acceptor region (33). We also found a consensus sequence: GID$_{11}$LG, that it has been described as essential for hydrolysis (34).

In the amino-terminus of the HSP70 proteins, we detected two conserved aminoacids known to be involved in the formation of a salt bridge and a loop between aminoacids QG$_{65}$DR$_{67}$. It has been also suggested that the internal part of the protein, between aa 335 and aa 401, is important for structural integrity (35). We also observed high homology in the peptide binding domain, suggesting that these proteins may have similar properties. This peptide binding domain is localized in the carboxy-terminal of the HSP70's, between aa 457 and 535 that seems to be responsible for nucleolar localization (35). However, the extreme 10 kDa carboxy-terminal appears to control binding to cofactors (36,37). It has been found that HSP70's have several helical subdomains with multiple degenerate repeats of tetrapeptide GGAP of unknown function and EEVD motifs at the extreme of carboxy-terminus that determine the functional specificity of individual HSP70 (37). In fact, we found degenerated repeats GGFP/GGAP at the extreme of carboxy-terminus in all deduced sequences. However, EEVD motifs at the extreme carboxy-terminus were observed only in two sequences, HSP70a and HSP70c. It has been suggested that only HSP70 proteins that have COOH- terminal sequence EEVD are from eukaryotic cytosol (38). These data suggest that encoded proteins from clone *hsp70*a and *hsp70*c share the same specificity to binding cofactors and probably are located in the cytosol. In the yeast *S. cerevisiae*, this notion has been supported by studies on cytosolic HSPs 70 which interact with other HSPs such as HSP40 and HSP90 (39) and with tetratricopeptide repeat (TRP) cofactors (38). The existence of distinct binding sites for different HSP70 cofactors appears to provide the structural basis for the functional cooperation of HSP70 with multiple chaperone cofactors. Further works in order to establish the mechanisms of induction of heat shock proteins from different isolates of *P. brasiliensis* from different geographical origins may provide important information about pathogenicity, virulence and mechanisms to adapt to different environment conditions. The behavior of the morphological appearance of yeast even when grown at room temperature, will give new insights into the phenomenon of dimorphism.

Based on these results we suggest that the *hsp70* genes are differentially expressed during the temperature dependent mycelia/yeast phase transition of *P. brasiliensis* that may be play an important role during the adaptation to the casual host.

Acknowledgments

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