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Identification of transcription elements in the 5′ intergenic region shared by *LON* and *MDJ1* heat shock genes from the human pathogen *Paracoccidioides brasiliensis*. Evaluation of gene expression

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Abstract

The *MDJ1*/*LON* locus is conserved among pathogenic dimorphic fungi. We have mapped using DNase I footprinting and mobility shift assays three putative heat shock elements and one AP-1 binding domain (ARE) in the 5′ intergenic region shared by Pb*MDJ1*and Pb*LON* (ML) from *Paracoccidioides brasiliensis*. The region bearing an ARE-like towards Pb*LON* also has an opposite skn-1-like element. We studied genetically and pathogenically distinct isolates Pb18 and Pb3, where ML is polymorphic and the number of elements detected was higher. The functionality of the elements was suggested by the stimulatory response of both genes to heat shock and oxidative stress. Co-regulation occurred upon heat shock from 36 to 42 °C and, only in Pb3, also during mycelium to yeast transformation (26–36 °C). In Pb18, Pb*MDJ1* seemed to be preferentially expressed in yeast. Our study might help understand regulation of genes involved in fungal adaptation to the host.

Keywords: *Paracoccidioides brasiliensis*; *MDJ1*; *LON*; Transcription elements; Gene expression; Heat shock; Oxidative stress

1. Introduction

Paracoccidioides brasiliensis is the fungal species responsible for paracoccidioidomycosis (PCM), which is prevalent in endemic areas of Latin America. Recently, three distinct *P. brasiliensis* lineages S1, PS2 and PS3 have been recognized using a combined data set of polymorphism in five nuclear loci (Matute et al., 2006). PS2 corresponds to a cryptic phylogenetic species that assembles six isolates so far. The genetic variation in *P. brasiliensis* is largely reflected at the Pb*GP43* gene, especially in exon 2 and promoter region (Morais et al., 2000; Matute et al., 2006). This gene encodes gp43, a specific diagnostic antigen involved in both immune protection against experimental PCM and adhesion (Travassos et al., 2004). PS2 isolates (e.g. Pb3) contain highly substituted Pb*GP43* sequences and provoked regressive, Th1-driven infection in B10.A mice, in contrast with isolates from S1 group (e.g. Pb18), which evoked a progressive, lethal and Th2-driven type of disease (Carvalho et al., 2005 and unpublished data).

Paracoccidioides brasiliensis grows as a multi-budding yeast when in parasitism or cultivated at 37 °C and as mycelium when incubated below 28 °C. Environmental fungal conidia reach the pulmonary alveoli upon inhalation and

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transform to the yeast pathogenic phase before infection can be established. A number of studies have recently been undertaken to unravel genes that are differentially expressed during temperature-induced mycelium-to-yeast transition and that might participate in maintenance of the yeast phase (Felipe et al., 2005; Goldman et al., 2003; Marques et al., 2004; Nunes et al., 2005). These studies have been possible due to the establishment of EST databases from the yeast and mycelium fungal phases of isolates Pb18 and Pb01 (Felipe et al., 2005; Goldman et al., 2003). So far, further genetic analyses of candidate genes have been hampered by the difficulties to optimize transformation systems for *P. brasiliensis*, which are still inefficient and tend to produce unstable clones (Leal et al., 2004; Soares et al., 2005).

Stress responses of microorganisms are particularly important in their adaptation to the host. For *P. brasiliensis*, survival at higher temperatures is an obvious stress that the fungus has to cope with in the host, leading to the expression of heat shock proteins. Indeed, genes like *HSP10*, *HSP30*, *HSP60*, *HSP70*, *HSP82* and *HSP104* are up regulated during in vitro temperature-induced mycelium-to-yeast formation (Felipe et al., 2005; Goldman et al., 2003; Marques et al., 2004; Nunes et al., 2005). Our group has characterized the *LON* and *MDJ1* gene homologues from *P. brasiliensis* (Barros and Puccia, 2001; Batista et al., 2006). Lon proteins are conserved ATP-binding, heat-inducible serine proteinases that control proteolysis in the mitochondrial matrix of *Saccharomyces cerevisiae* by mediating cleavage of misfolded or unassembled proteins (van Dyck and Langer, 1999). Mdj1p is a type I DnaJ protein that co-localizes with Lon and is essential for substrate degradation (alone or as an Mdj1/Ssc1 complex) by this and other stress inducible ATP-dependent proteinases (revised by Voos and Rottgers, 2002). DnaJ (or J-domain) members of the Hsp40 chaperone family have a primary function of regulating the activity of their cognate Hsp70 members in various cellular compartments by stimulating ATPase activity.

We have recently shown that PbLon and PbMdj1 are heat shock proteins that are sorted to the mitochondria of *P. brasiliensis*, however PbMdj1 was also detected in large amounts along the cell wall and the budding region (Batista et al., 2006). Anti-recombinant PbMdj1 antibodies specifically recognized a single 55-kDa mitochondrial and cell wall component, compatible with the predicted size of the protein devoid of its matrix peptide-targeting signal. We observed an extremely conserved gene organization of *MDJ1* and *LON* among dimorphic pathogenic fungi (*P. brasiliensis*, *Histoplasma capsulatum*, *Blastomyces dermatitidis*, *Coccidioides immitis*) and *Aspergillus*, where the number and position of the introns are conserved (Batista et al., 2006). Interestingly, the whole *MDJ1*/*LON* locus is conserved in these species, where the genes are adjacent, inversely orientated and separated by a common 5′ intergenic (ML) region ranging between 400 and 485 nucleotides. In *P. brasiliensis*, the ML region between each start codon is 485-nt long. It should contain most of the regulatory elements that control transcription of *LON* and *MDJ1*, considering that computer analysis shows the presence of many putative transcription factor-binding proteins in both strands, many of them related to stress response (Barros and Puccia, 2001). Therefore, transcription of *LON* and *MDJ1* might be either coordinately triggered by the same elements for both genes, or individually regulated, as determined by the stimulus.

Individually, both Lon and Mdj1 are essential for the biogenesis of functional mitochondria in *S. cerevisiae*. Lon-deficient yeasts are unable to utilize non-fermentable carbon sources and to keep functional mitochondrial DNA (van Dyck and Langer, 1999). In human lung fibroblasts, early stages of *LON* down regulation causes massive apoptosis, probably due to the lack of both proteinase and chaperone functions (Bota et al., 2005). Therefore, the gene should be under tight control. On the other hand, inactivation of yeast *MDJ1* also results in deficient respiration due to loss of mitocondrial DNA and generates non-viable growth at 37 °C (Voos and Rottgers, 2002). In *P. brasiliensis*, Mdj1 is additionally found in the cell wall, where its role is merely speculative.

In the present work, we have identified transcription elements in the Pb*MDJ1*/Pb*LON* 5′ intergenic region and correlated them with gene expression in a way to understand their transcriptional control and how it might influence their functions. We compared the results in two genetically distinct isolates that show diverse patterns of virulence in mice.

2. Materials and methods

2.1. Fungal isolates and growth condition

Clinical isolates of *P. brasiliensis* Pb18 and Pb3 (original name Pb608) were analyzed in this work, but we also used DNA from Pb339 (B-339), Pb5 (AP) and Pb12 (Argentina) for polymorphism studies. Description of the isolates and their genetic group according with the Pb*GP43* phylogeny can be found in Morais et al. (2000). The isolates were maintained in the yeast phase in slants of modified YPD medium (0.5% yeast extract, 0.5% casein peptone and 1.5% glucose, pH 6.3) either at 36 °C, with sub-culturing every 20 days, or for a couple of months at 4 °C. For experimental purposes, fungal cells were incubated in liquid media with shaking (120–150 rpm).

2.2. RNA extraction of stressed P. brasiliensis

Fully formed yeast cells were mechanically disrupted by vortexing with glass beads for 10 min in the presence of TRizol reagent (Gibco-BRL). For fully formed mycelia or mycelia undergoing phase transition, cell pellets of *P. brasiliensis* were packed in aluminum foil, frozen in liquid N_2, ground and the resulting fine powder was defrosted in TRizol. Ground cells in TRizol were processed as suggested by the manufacturer for purification of RNA, which was then

quantified in a GeneQuant apparatus (Amersham–Pharmacia), analyzed for integrity in formaldehyde agarose gels and stored at −70 °C in H_2O.

For evaluation of gene expression in *P. brasiliensis* undergoing heat shock at 42 °C, logarithmic yeast cells growing in modified YPD at 36 °C under shaking were transferred to 42 °C and aliquots were collected after 0, 30 and 60 min for RNA extraction. For expression studies during mycelium-to-yeast phase transition after heat shock from 26 to 36 °C, *P. brasiliensis* cultures in modified YPD were processed as described previously (Nunes et al., 2005). For oxidative stress, yeast cells growing in logarithmic phase in F12 Nutrient Mixture (Gibco) supplemented with 1.5% glucose were subdivided in aliquots and incubated for another 20 min at 36 °C after addition of 0, 1, 2.5 or 5 mM of H_2O_2. A separate set of aliquots was incubated in the presence of H_2O_2 only after replacement of growth medium with fresh medium. Another set of cells was used to analyze gene response to 10 μg/mL of itraconazole for 0, 30, 120 and 360 min. The concentrations tested are within the range of minimal inhibitory concentrations reported for *P. brasiliensis* (Silva et al., 2006).

2.3. Preparation of protein extracts for EMSA and DNAseI footprinting assays

Total protein extracts used in assays meant to identify transcription elements were obtained from *P. brasiliensis* using the protocols described by Srikantha et al. (1995) and Tosco et al. (1997), with modifications. Briefly, yeast cells growing in modified YDP medium at 36 °C for 5–7 days under shaking (log to late-log phase) were pelleted by centrifugation and washed three times in phosphate buffered saline (PBS). The cells (1 mL of wet cell pellet) were mechanically broken with glass beads in a solution (2 mL) containing 200 mM Tris–HCl, pH 8.0, 400 mM $[NH_4]_2SO_4$, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM *ortho*-phenanthroline and 7 mM β-mercaptoethanol. Cell lysis was achieved under vigorous agitation in vortex (10 cycles of 30 s of agitation alternating with 30 s of incubation in ice). Cell debris was discarded following centrifugation (3000*g*, 15 min) and the supernatant was ultracentrifuged at 100,000*g* for 3 h. The supernatant was dialyzed in 20 mM Hepes, pH 8.0, 5 mM EDTA, 10% glycerol, 1 mM PMSF, 1 mM *ortho*-phenanthroline, 7 mM β-mercaptoethanol and stored at −20 °C, or long term at −70 °C. Protein contents were estimated by Bradford (1976) method, using Coomassie brilliant blue G-250 (Sigma) and BSA as standard.

2.4. DNAseI protection footprinting assays

Dnase I protection assays were carried out using the "Core Footprinting System" kit (Promega). A fragment of 434 bp from the 5′ intergenic region shared by Pb*MDJ1* and Pb*LON* was elongated by PCR using as template total DNA from Pb18 or Pb3, and the specific primers sense 5′-CGTGAGCAAAGACCAGT-3′ (D4) and anti-sense 5′-CTAGAGGCAGATGGTAAG-3′ (2A9). The amplicons were cloned in pGEM-T easy (Promega), which was then amplified and used to obtain the insert by *Eco*RI restriction. Restricted DNA fragments were purified from agarose gels with the Perfect Gel Cleanup Kit (Eppendorf). The Dnase I protection assays were previously optimized following the manufacturer's guidance and our best experimental conditions are briefly described below. Approximately 1 μg of purified DNA fragment was 5′-dephosphorylated with CIAP (Promega), extracted with phenol/chloroform, ethanol-precipitated and the dry pellet suspended in 20 μL of H_2O. The dephosphorylated DNA fragment was radiolabeled with 1 μL of [γ-^{32}P]dATP (10 mCi/ml, Amersham) in the presence of T4 polynucleotide kinase (Promega). The labeled fragment was 3′-digested with *Spe*I (40 U) and extracted one time with phenol:chloroform:isoamylic alcohol (25:24:1). The labeled DNA probe was precipitated from the aqueous phase with ethanol, dried, diluted in 100 μL of Tris–EDTA buffer (TE) and kept at 4 °C. For the DNase I protection experiments, the probe was incubated 10–40 min in the presence or absence of *P. brasiliensis* total protein extracts in ligation buffer (50 mM Tris–HCl, pH 8.0, 100 mM KCl, 12.5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT and 20% de glycerol). The samples were then digested with Dnase I (0.01 U/μL) for 1 min and phenol/chloroform purified. The ethanol-precipitated DNA was diluted in 0.1 M NaOH:formamide (1:2, v:v), 0.1% xyleno cyanol and 0.1% bromophenol blue. The reactions were resolved in 6% polyacrylamide–urea gels run in Tris-borate/EDTA (TBE) buffer. The gels were dried and exposed to an X-Omat film (Kadak) at −70 °C. Manual sequencing reactions of the corresponding DNA fragment were carried out using the T7 Sequencing kit (Amersham) as previously described (Morais et al., 2000) and run in parallel with the DNAse I protection products.

2.5. Electrophoretic mobility shift assays (EMSA)

Sense and anti-sense oligonucleotides were initially annealed to form double stranded DNA (Table 1). For that purpose, equimolar amounts (10 or 20 μM) of each strand were incubated for 10 min at 95 °C and then cooled at room

Table 1
Oligonuleotides used in EMSA reactions

	Double-stranded sequences (5′ → 3′)
HSE1	CTTTGTTCCACGAAAAATGGCATCTT
	GAAACAAGGTGCTTTTTACCGTAGAA
HSE2	TACTTGATCAACTCGGAGAACCTCGTCGAATAG
	ATGAACTAGTTGAGCCACTTGGAGCAGCTTATC
HSE3	AGAAGAAGGGAATTTGCCGAGTGA
	TCTTCTTCCCTTAAA CGGCTCACT
AP1	ATCTTAGTCATCCAACGCCC
	TAGAATCAGTAGGTTGCGGG
BS10	TGTATAAATATCTGCTGTCAACATATTTATAGA
(inespecific)	CGACAGT

temperature. Pairing was confirmed by electrophoresis in non-denaturing 6% polyacrylamide gels (37.5:1 acrylamide/bis-acrylamide) impregnated with ethidium bromide. Double-stranded oligonucleotides (2 μL of a 1.75 pmol/μL solution) were radiolabeled with 1 μL of [γ-^{32}P]dATP (10 mCi/ml, Amersham) in the presence of T4 polynucleotide kinase (Promega). Free nucleotides were separated from the oligonucleotides in a Sephadex G-50 (Amersham) column. Labeled oligonucleotides were used in EMSA reactions carried out as previously described (Tosco et al., 1997). In a microcentrifuge tube, we added 2–3 μg of total protein extract from *P. brasiliensis* (Pb18: 3.7 μg/μL; Pb3: 5.5 μg/μL), 1 μL of poly(dI-dC) (25 U/mL), 1 μL of BSA (10 mg/mL) and 2 μL of a solution containing 125 mM Hepes, pH 7.5, 5 mM EDTA, and 50% glycerol. The mixture was kept in ice for 15 min, mixed with radio labeled oligonucleotide (double-stranded), with or without cold competitor at 250–500 molar excess, and incubated for another 15 min in ice. The mixture was resolved in 6% non-denaturing polyacrylamide gels (37.5:1 acrylamide/bis-acrylamide) in TBE buffer for 45 min at 100 V in a mini Protean II apparatus (Bio-Rad). The gels were dried and exposed to an X-Omat (Kodak) film at −70 °C.

2.6. Quantitative real time RT-PCR

Experiments of quantitative real time RT-PCR were performed using the Syber Green detection system, according to the manufacturer's instructions. To a final volume of 20 μL, we added 10 μg of total DNA-free RNA, 100 μM of oligo(dT) and 200 U of reverse transcriptase (SuperScript, Invitrogen). The volume was diluted to 50 μL when the reaction was complete. For quantification, we added 2 μM of each specific oligonucleotide (Table 2), 5 μL of the kit's master mix (Applied Biosystems) and 1 μL of cDNA. Cycling was carried out in an ABI Prism 7000 Sequence Detection System (Perkin-Elmer Applied Biosystem, USA) starting with one cycle of 50 °C (2 min) and 95 °C (1 min), followed by 40 cycles at 95 °C (15 s) and 60 °C (1 min). The dissociation curve was determined with an additional cycle of 95 °C (15 s), 60 °C (20 s) and 95 °C (15 s). DNA contamination was evaluated by PCR amplification of the Pb*GP43* gene (Accession No. U26160). Negative controls did not contain DNA or RNA. Reactions were run in duplicates in parallel with an endogenous control of α-tubulin used as calibrator. Basic procedures and calculations were performed as described previously (Semighini et al., 2002). The data are represented as means ± SD. Statistical significance was determined by the Student's *t* test. A $p < 0.05$ indicated statistical significance.

Table 2
Primers used in quantitative real-time RT-PCRs

Pb genes	Primer sequence
Pb*MDJ1* (AF334811)[a]	5′ ACATAAAGATCACCAACGCG 3′
	5′ CGAGTGCCTGGAGAGGGAGAT 3′
Pb*LON* (AF239178)[a]	5′ CTATCACATCTCTATCCGC 3′
	5′ GCAGCCATACAGGAAATGATG 3′
HSP70 (PbCC006–G05)[a]	5′ GACCACACCCTCGTTCGTTG 3′
	5′ ACTTGATTTTTGGCGGCATC 3′
HSP82 (PbCC005–F04)[a]	5′ACCGCTGGTGCCGATATTT 3′
	5′GGCCACGAGGTAGGCAGAG 3′
HSP104 (PbCC012–B06)[a]	5′ CGAAATCCATGCTCTGGCTC 3′
	5′TGTTTGGCAGCCTCGAGTC 3′
α-TUB	5′ TAATGGAAAATACATGGC 3′
	5′ GTCTTGGCCTTGAGAGATGCA 3′

[a] GeneBank accession numbers or contigs from the *P. brasiliensis* EST GeneBank http://143.107.203.68/pbver2/default.html.

2.7. Analysis of sequence polymorphism

DNA polymorphism in the ML region was investigated as described (Morais et al., 2000) by sequencing the correspondent PCR-elongated fragments from *P. brasiliensis* isolates Pb18, Pb339, Pb3, Pb12 and Pb5, using total DNA as template and the primers sense 5′- CGTGAGCAAA GACCAGT-3′ (D4) and anti-sense 5′-CTAGAGGCA GATGGTAAG-3′(2A9). Nucleotide sequencing was carried out in the facilities of the Center of Human Genome at the São Paulo University (USP). Sequences were analyzed using the EditSeq, SeqMan and MegAlign programs of the Lasergene System (DNAstar Inc.).

3. Results

Previously, we showed by Northern blot that Pb*MDJ1* and Pb*LON* expression was increased upon heat shock at 42 °C of *P. brasiliensis* yeast cells growing at 36 °C (Batista et al., 2006), which seemed to indicate the presence of functional heat shock element(s) in their promoter region working in both directions. Therefore, a DNAse I footprinting assay was optimized to map these elements in the Pb*MDJ1*/Pb*LON* 5′ intergenic (ML) region.

We analyzed ML amplicons from two isolates, Pb18 and Pb3, against their respective yeast cell protein extracts. We tried to work with nuclear protein extracts as well, however the yields obtained were insufficient to be used in the assays. Our best results are presented in Fig. 1, where two protection regions can be seen for Pb18 (later called AP1 and HSE2) and five for Pb3 (later called AP1, HSE1, HSE2, HSE3 and GT). The sequences correspondent to these regions can be seen in Fig. 2.

Analysis of the footprinting and manual sequencing reactions suggested the existence of polymorphic sites in the ML region of Pb3, which prompted us to broaden the investigation. We originally characterized the Pb*MDJ1*/Pb*LON* locus from isolate Pb339 (Barros and Puccia, 2001; GenBank Accession No. AF239178, later updated by Batista et al., 2006, GenBank Accession No. AF334811). Presently, we have PCR-amplified, cloned and sequenced the ML region from Pb18, Pb3, Pb5 and Pb12 and compared them with that of Pb339. As seen in Fig. 2, the sequences were identical among Pb339, Pb5, Pb12 and Pb18, whereas in Pb3 we found threee nucleotide substitutions, one insertion of two nucleotides and three neighboring gaps. These differences were confirmed by sequencing

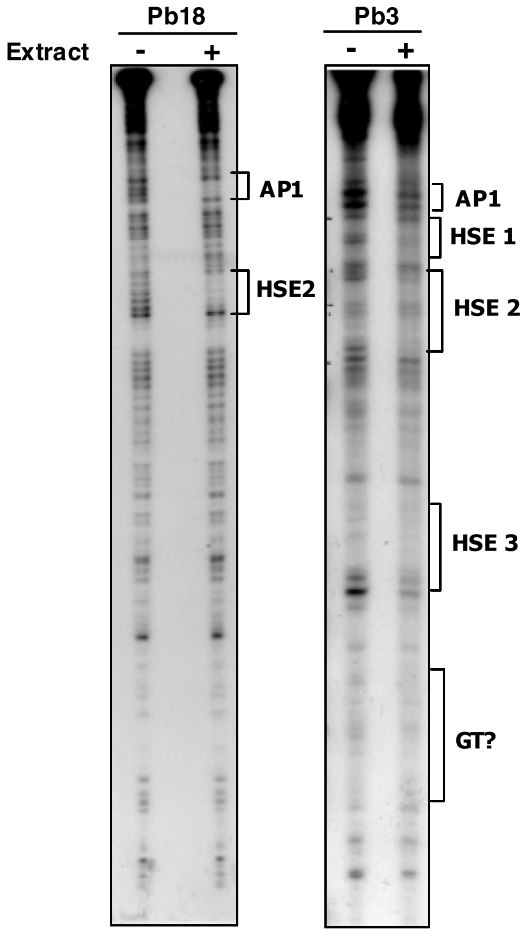

Fig. 1. DNAse I footprinting assays of the Pb*LON*/Pb*MDJ1* 5′ intergenic region (ML). Urea–polyacrylamide gel profiles of the experiments carried out without (−, reference) or with (+) total protein extracts from Pb18 (100 μg) or Pb3 (120 μg) and the correspondent radiolabeled ML region. Protected regions are indicated. The corresponding nucleotide sequences were predicted by analyzing a sequencing reaction run in parallel (not shown). The regions not resolved in the gel have not been further analyzed.

the insert of four distinct clones with three different primers. However, none of these differences was within the motifs mapped by DNase I footprinting.

We next compared the motifs mapped by DNase I footprinting with putative transcription elements localized in the ML region from Pb18 and Pb3 using the TFSEARCH computer program (http://molsun1.cbrc.aist.go.jp/research/db/TFSEARCH.html). We verified that AP1 contains an AP-1-like binding domain (GATGACTAAGA), in Pb*LON* direction at −265 pb from its start codon, which shows high score of identity with mammalian AP-1 recognition elements, or ARE (G/AC/GTGACTNC/ANA/T). In the opposite strand, i.e., to the Pb*MDJ1* direction, there is a putative binding domain (CTTAAGTCATCCA) to skn-1 (C/TAA/TTGTCATNA/CN). Fragments HSE1, HSE2 and HSE3 contain motifs with high scores of homology with HSF-binding motifs (Fig. 2). The HSE-like sequences are palindromic and might therefore work in both directions. The fragment called GT was apparently protected in Pb3 and contains GATA-1 and GATA-2-like elements (score of 85) to the Pb*MDJ1* direction. As seen below, however, this region has not been validated by EMSA.

In order to validate the putative transcription elements mapped by DNase I footprinting assays, we carried out EMSA experiments with the oligonucleotides listed in Table 2 of Section 2. Each double-stranded oligonucleotide was tested for mobility shift with protein extracts from Pb3, Pb18 (Fig. 3) and also from Pb339 (not shown). Shifted bands could be observed with all tested probes except for GT. It is of note that the probes have different sizes and so the complexes would migrate differently even if the same proteins were involved. The indicated bands in Fig. 3 have been totally inhibited with cold homologous oligonucleotides in a dose-response manner (not shown), but not with an unrelated sequence (BS10). The tested oligonucleotides bound to protein(s) from both Pb18 and Pb3 extracts, except for HSE3, which was positive only with Pb3. On the other hand, although we have not seen any DNAse I protection of HSE1 with Pb18 protein extracts, there was a shifted EMSA band. It is noticeable that the gel migration of the bands shifted by Pb18 proteins were slower than those shifted by Pb3 proteins, suggesting the presence, in different isolates, of heat shock isoforms or different number of proteins in the transcription complexes. All these EMSA assays have been further repeated once with dephosphorylated Pb18 and Pb3 extracts. We observed inhibition of the HSE3 and HSE2 complexes, but in the latter case that was more evident with the Pb18 extract. HSE1 and AP1 complexes have not been affected by protein dephosphorylation (results not shown).

In order to indirectly evaluate the functionality of the elements mapped previously, we used real time RT-PCR to quantify Pb*LON* and Pb*MDJ1* responses to heat shock and oxidative stress. Both Pb18 and Pb3 were tested in a way to correlate the differences observed before with the actual transcription expression of each gene in response to stress. The results can be seen in Fig. 4. The first useful information contained in panel A is that the *HSP70*, *HSP82* and *HSP104* responses to heat shock are much stronger than those from Pb*LON* and Pb*MDJ1*. In addition, for the genes encoding mitochondrial Lon, Mdj1 and Hsp104 the response was clearly slower in Pb3, for which a more significant higher level of transcription was only achieved after 60 min. These results are in accordance with the presence of functional heat shock element(s) in the Pb*MDJ1*/Pb*LON* promoter region working in both directions, although they seem to be either weaker than those found in classical *HSPs* or partially repressed. They also suggest that some differences exist in transcription regulation or RNA stability in Pb3. On the other hand, Pb*LON* and Pb*MDJ1* are apparently co-regulated in both Pb3 and Pb18 when stimulated at 42 °C.

We then compared Pb*LON* and Pb*MDJ1* gene expression in mycelia (M) undergoing phase transition to yeast (Y) upon heat shock from 26 to 36 °C (Fig. 4B). In our experimental conditions, at 120 h the cultures had about

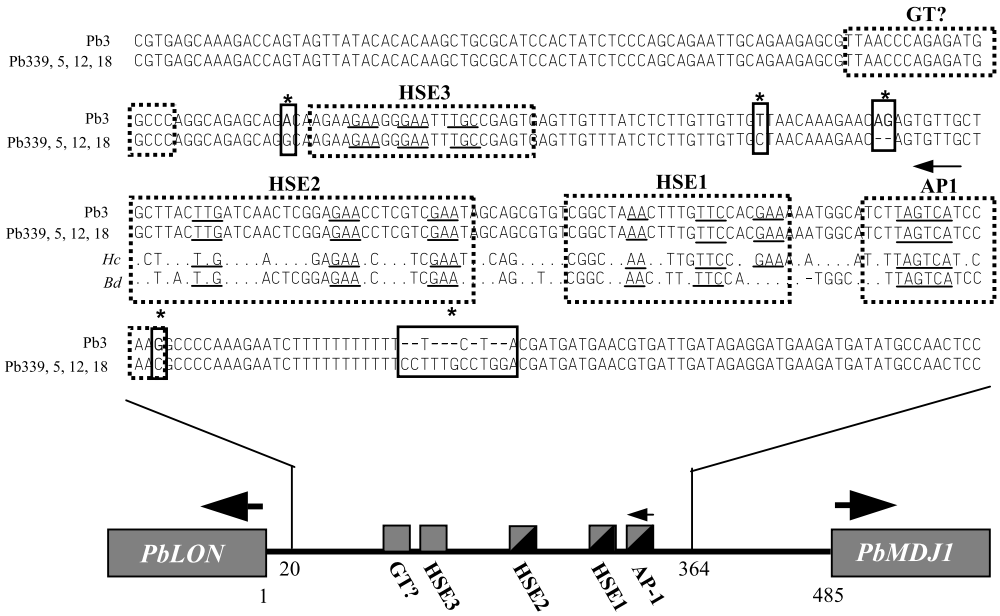

Fig. 2. Schematic representation of the Pb*MDJ1*/Pb*LON* 5′ intergenic region (ML) and localization of the mapped transcription elements. Bicolor boxes denote elements confirmed for both Pb18 and Pb3 with at least one detection method. Numbers denote nucleotide positions. The alignment of the region indicated shows the sequence polymorphism found in Pb3 when compared with Pb5, Pb18, Pb12 and Pb339. Gaps (−) and substitutions are boxed and marked with an asterisk. The regions that have been protected against Dnase I are indicated with dotted boxes. ARE- (AP-1 binding), HSE1- and HSE2-like sequences were also found in the ML region from *H. capsulatum*, *Hc*, (http://genome.wustl.edu/blast/histo_client.cgi) and *B. dermatitidis*, *Bd*, (http://genome.wustl.edu/blast/blasto_client.cgi), as seen in the alignment, where dots indicate lack of identity. The underlined ARE core is the complementary strand, as indicated by the arrow. Number denote nt positions in the ML region.

30% of transtioning forms among fully formed yeasts. It was interesting to notice that in Pb3, but not in Pb18, the expression profile of Pb*LON* and Pb*MDJ1* was similar during M to Y (and also during Y to M transitions, not shown), when the transcription level of both genes reached a peak of expression at 24 h and then went down to near the initial levels. In Pb18, M to Y transition, Pb*MDJ1* was progressively up regulated and reached a peak of 2.5-fold increase at 120 h, while Pb*LON* had only a modestly higher mRNA accumulation at 5 h. Our results suggest that Pb*MDJ1* is preferentially expressed in yeast, specially considering that it was significantly down regulated during the whole Y to M transition (not shown). It is worth mentioning that the profiles shown here differ from those previously reported for *HSP70*, *HSP82* and *HSP104* in Pb18 (Goldman et al., 2003), where the genes responded with rapid and high mRNA accumulation by 5 h of temperature increase, remained high until 24 h and then decreased.

Both Pb*LON* and Pb*MDJ1* responded to oxidative stress (addition of 5 mM H_2O_2) with fast increase in transcription expression, however the Pb*LON* response was stronger, as expected for the presence of an ARE motif in its direction (Fig. 4C). The stimulus was only possible, however, when hydrogen peroxide was added to the cells growing in fresh medium, in agreement with the secretion of functional catalase in *P. brasiliensis* (reviewed in Campos et al., 2005). Considering that AP-1 has been implicated in drug resistance (Puri et al., 1999; Tosco et al., 1997), we evaluated Pb*LON* and Pb*MDJ1* expression after incubation with itraconazole. The only noticeable effect was a fast downregulation of Pb*LON* and a late upregulation of Pb*MDJ1* in Pb3 (Fig. 4, panel D). At 400 μg/mL, sulfamethoxazole evoked a significant decrease of both Pb*LON* and Pb*MDJ1* accumulation by, respectively, 2 and 6 h of incubation (not shown).

Fig. 2 summarizes the transcription elements mapped so far in the ML region of *P. brasiliensis*. We included in the alignment a fragment of the ML region from *H. capsulatum* and *B. dermatitidis* where similar ARE, HSE1 and HSE2 were found, but not HSE3 or GT. Therefore, transcriptional regulation of *LON* and *MDJ1* might be similar in these dimorphic fungi.

4. Discussion

We have mapped by DNase I footprinting and validated by EMSA three putative heat shock factor-binding elements (HSEs) and one AP-1 binding domain (ARE) in the 5′ intergenic region shared by the Pb*MDJ1* and Pb*LON* (ML) genes from *P. brasiliensis*. The region bearing an ARE-like to the Pb*LON* direction also has an opposite skn-1-like element. We studied isolates Pb18 and Pb3, which are genetically distinct and differ in their virulence capacity (Carvalho et al., 2005). The functionality of the elements was indirectly suggested by the stimulatory response of both genes to heat shock and oxidative stress, as determined with real time RT-PCR experiments. We detected sequence differences in the ML from Pb3 and although mutations have not been found in the transcription

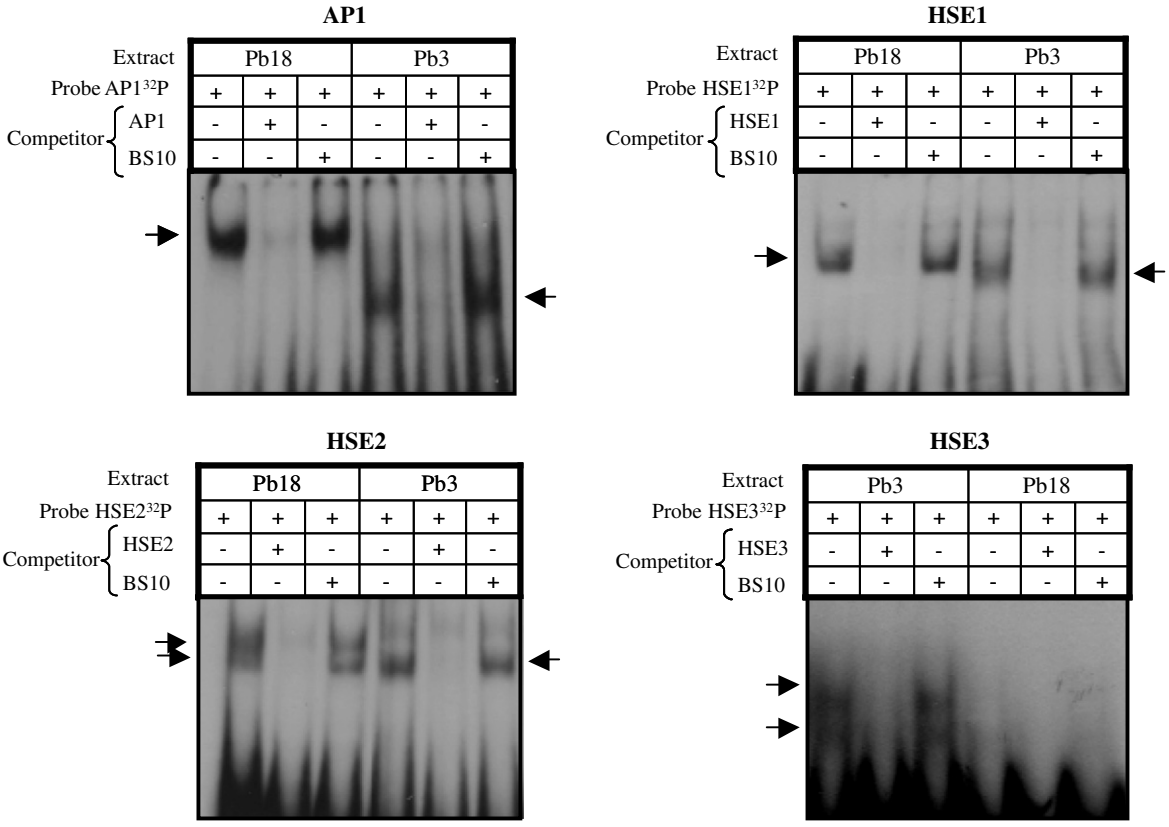

Fig. 3. Validation by EMSA of the transcription elements mapped by DNAse I footprinting. Urea–polyacrylamide gels showing the interaction between double-stranded oligonucleotides with protein extracts from both Pb18 and Pb3, in the absence (−) or in the presence (+) of an excess amount (250 to 500×) of cold test (AP1, HSE1, HSE2 and HSE3) or unspecific (BS10) oligonucleotide. For each radiolabeled oligonucleotide, a dose–response competition curve with excess of cold sample was performed (not shown). The arrows point at the bands specifically inhibited.

elements mapped so far, their contribution to the observed expression differences cannot be discarded.

HSE motifs are typically composed of at least three continuous and/or inverted pentameric units of nGAAn. Classical consensus sequences found in *S. cerevisiae HSP70*, *HSP60*, *HSP104* and *HSP10* promoters are of the nGAAnnTTCnnGAAn type, where the number of pentameric units varies from 3 to 6 (Amin et al., 1994). Other yeast heat shock genes are also regulated by non-canonical heat-shock elements (ncHSE), where the gaps between repetitive units can vary (Yamamoto et al., 2005). The HSE2 mapped in the present work (C**TTG**ATC AACTCGGA**GAA**CCTCGTC**GAA**TAGCAG) is similar to an ncHSE found by Tachibana et al. (2002) in the *MDJ1* yeast promoter (A**TTC**TTTACATCCTGTG**GAA**CTCTA TTG**GAA**A), where the first gap is two-nucleotide longer. HSE1 (CGGC**TAA**ACTTTG**TTC**CAC**GAA**AAA) would also be considered non-canonical, as the gaps between pentameric units are longer than 2 nt, while HSE3 is similar to classical heat shock elements (A**GAAGGGAATTTGC**).

Variations in the alignment of pentameric units in *P. brasiliensis* HSE1, HSE2 and HS3, the distance between them and the gap from the transcription start sites might account for the differences in response to heat shock when compared to *HSP70*, *HSP82* and *HSP104*. Ultimately, these characteristics should determine the degree of affinity for the corresponding factor (s), explaining why there was protection of HSE1 only with Pb3 extracts, while EMSA reactions with HSE1 were positive for both Pb18 and Pb3, or why HSE3 does not seem to be functional in Pb18. In our assays, Pb*MDJ1* and Pb*LON* seemed to be coordinately regulated in Pb3 during heat shock or temperature-driven phase transition, but that was only true for Pb18 in response to heat shock at 42 °C. When transcriptional response is similar, the same transcription elements are likely to be activated for both genes. Therefore, regulation of the *MDJ1*/*LON* locus is probably more sophisticated in Pb18 than in Pb3. We could speculate that the functional HSE3 element in Pb3 is responsible for the coordinated Pb*MDJ1* and Pb*LON* responses during phase transition, but the existence of other functional elements not detected here cannot be discarded, including a not validated GATA-like.

On the other hand, the differences between Pb18 and Pb3 in transcriptional response of Pb*LON* and Pb*MDJ1* could be related to polymorphism in the transcription factors. In *S. cerevisiae*, there is only one heat shock factor, but it can induce transcription of a wide variety of genes involved in cell division, half of which do not contain typical heat shock elements (Hahn et al., 2004). This plasticity probably relies in the fact that two regions—NTA (N-terminal) and CTA (C-terminal)—are responsible for

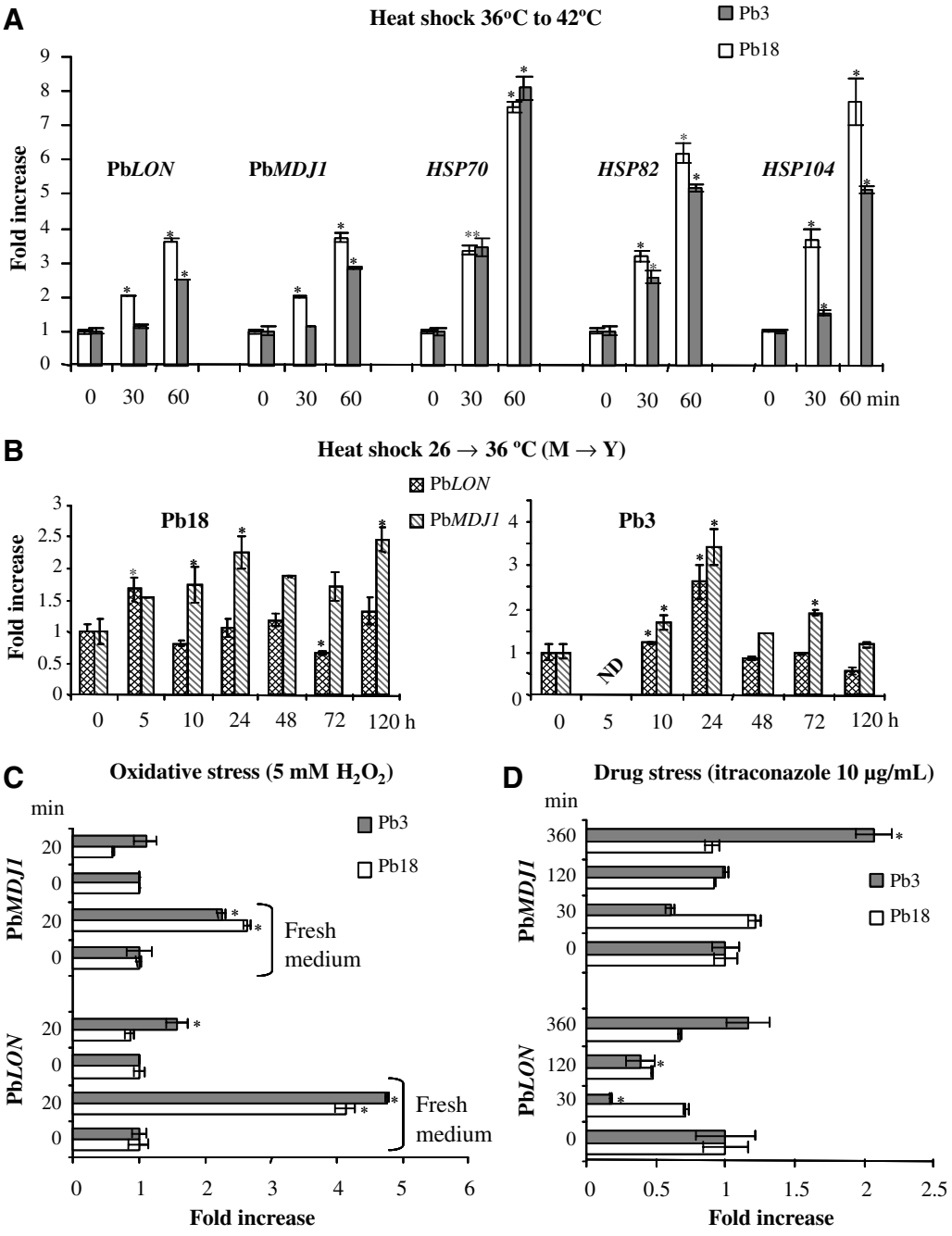

Fig. 4. Quantitative real time RT-PCR of Pb*MDJ1* and Pb*LON* from Pb18 and Pb3 yeast cells after heat shock at 42 °C (A), oxidative stress (C) and drug stress (D), as indicated. Comparison with *HSP70*, *HSP82* and *HSP104* can be seen in (A). In (B), heat shock of mycelia (M) growing at 26 °C shifted to 36 °C for transition to the yeast phase (Y). The figure shows the fold increases in mRNA accumulation when compared to that from cells at time 0 (value 1). The absolute mRNA amounts were calculated as values relative to reference α-tubulin (mean values of duplicates). Statistically significant values ($p < 0.05$) relative to time 0 are indicated by an asterisk.

transcription activation, where CTA is apparently responsible for activation of genes containing gapped HSE (ncHSE) and for sustained response (reviewed by Estruch, 2000; Tachibana et al., 2002). In *P. brasiliensis*, ESTs of heat shock factors have not been detected so far, but at least 3 *HSF* genes seem to exist in the related fungi *Coccidiodides immitis* http://www.broad.mit.edu/annotation/genome/coccidioides_immitis/Home.html) and *Aspergillus nidulans* (http://www.broad.mit.edu/annotation/genome/aspergillus_nidulans/Home.html). Consequently, regulation of heat shock response in these fungi probably relies on the interaction of different factors with the promoter region. The interaction between the trimeric Hsf and HSE seems to depend on phosphorylation (Kiang and Tsokos, 1998). We observed that dephosphorylation of protein extracts has not prevented the formation of HSE1 complexes in EMSA assays. Further analyses will be necessary to figure out if HSE1 really bears a heat shock or another type of element.

AP-1 refers to a family of transcription factors that bind to an ARE (G/AC/GTGACTNC/ANA/T) domain (Gutman and Wasylyk, 1990). In fungi, AP-1 has been directly related to the induction of genes that encode proteins expressed during oxidative stresses, for e.g., *C. albicans* thioredoxin reductase (*TRR1*) and thiol-specific anti-oxidant (*TSA1*) (Urban et al., 2005). Consequently, in *S. cerevisiae*, *yap1* and *yap2* mutants are more sensitive to oxidation by hydrogen peroxide (Stephen et al., 1995). We observed that transcription of both Pb*LON* and Pb*MDJ1* was stimulated by H_2O_2, however Pb*LON* response was over two-fold more intense than that of Pb*MDJ1*. In fact, Pb*LON* response to oxidative stress reached near 5-fold and was the most intense we have obtained in the course of our work, suggesting that ARE is highly active in regulation of that gene. PbLon is localized solely in the mitochondria (Batista et al., 2006), where proteins are constantly attacked by free radicals that result from normal aerobic metabolism, response to drugs or to a toxic environment in the host. Oxidized and damaged proteins are selectively degraded by mitochondrial proteinases as part of a protection mechanism, where Lon might play an important role as suggested previously (Bota and Davies, 2002). The presence of itraconazole, however, caused a rapid decrease of Pb*LON* transcripts, which might be related to a negative regulation yet to be determined. In a fluconazole resistant *C. albicans* strain, for e.g., the CAP1 factor acted as a negative regulator of *MDR1* (Alarco and Raymond, 1999) and not as an inducer, as seen with *CDR1* (Puri et al., 1999).

The Pb*MDJ1* response to oxidative stress could be due to the presence of an active skn-1-like binding domain, which lies in the opposite strand of the ARE-containing oligonucleotide, however the participation of another element or even of HSE elements (Liu and Thiele, 1996) cannot be discarded. Transcription factor skn-1 has recently been reported to play a role in resistance to oxidative stress in *Caenorhabditis elegans*, where it initiates mesodermal development (An and Blackwell, 2003). It has not been found in other organisms so far. In Pb18, Mdj1 protein has been found in the yeast cell wall and budding neck (Batista et al., 2006), where it supposedly acts as a chaperone. However, a possible enzymatic function should be considered, based on the fact that bacterial DnaJ has been shown to possess a thiol-reductase activity in the zinc-finger repeats (Crouy-Chanel et al., 1995). Therefore, Pb*MDJ1* might also be under oxidative stress regulation.

This is the first study of transcription elements in *P. brasiliensis*. Our results open the doors to a better understanding of the regulation of stress genes that could be implicated in fungal responses to the host environment. The differences in transcriptional regulation between Pb18 and Pb3 in Pb*LON* and Pb*MDJ*, Pb*GP43* (Carvalho et al., 2005) and probably others, could also help explain the differential disease outcome they evoke in mice (Carvalho et al., 2005). Considering the similarities in transcription elements found in the ML region from *H. capsulatum* and *B. dermatitidis*, future functional studies might be carried out in these genetically related fungi, such as those aiming at evaluating the *BAD-1* promoter (Rooney and Klein, 2004).

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