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Paracoccidioides brasiliensis translation and protein fate machineries revealed by functional genome analysis

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ABSTRACT. The translational and post-translational modification machineries of *Paracoccidioides brasiliensis* were assessed by means of comparative analyses of PbAESTs (*P. brasiliensis* assembled expressed sequence tags) with sequences deposited on different databases. Of the 79 sequences corresponding to cytosolic ribosomal proteins, we were able to find 78 in the *P. brasiliensis* transcriptome. Nineteen of the 27 *Saccharomyces cerevisiae* genes related to translation initiation were also found. All eukaryotic elongation factors were detected in *P. brasiliensis* transcriptome, with eEF1A as one of the most expressed genes. Translation termination is performed, in eukaryotes, by factors 1 and 3 (eRF1, eRF3). In *P. brasiliensis* transcriptome it was possible to identify eRF3, but not eRF1. Sixteen PbAESTs showing aminoacyl-tRNA synthetase-predicted activities were found in our analyses, but no cysteinyl-, leucyl-, asparagyl- and arginyl-tRNA synthetases were detected.

Among the mitochondrial ribosomal proteins, we have found 20 and 18 orthologs to *S. cerevisiae* large and small ribosomal subunit proteins, respectively. We have also found three PbAESTs similar to *Neurospora crassa* mitochondrial ribosomal genes, with no similarity with *S. cerevisiae* genes. Although orthologs to *S. cerevisiae* mitochondrial EF-Tu, EF-G and RF1 have been found in *P. brasiliensis* transcriptome, no sequences corresponding to functional EF-Ts were detected. In addition, 64 and 28 PbAESTs associated to protein modification and degradation, respectively, were found. These results suggest that these machineries are well conserved in *P. brasiliensis*, when compared to other organisms.

Key words: *Paracoccidioides brasiliensis*, Transcriptome analysis, Translation, Protein modification, Protein degradation, 26S proteasome

INTRODUCTION

Paracoccidioides brasiliensis, the etiological agent of paracoccidioidomycosis, is a dimorphic fungus, which is found as mycelia at 22-26°C and as yeasts at 37°C, or upon host tissue invasion. A remarkable feature common to several pathogenic fungi is their ability to differentiate from mycelium to yeast morphologies or vice-versa (Gow et al., 2002). Dimorphism seems to be of major importance for the establishment of infection and disease, since the blockage of this process leads to a decrease or loss of virulence (Salazar et al., 1988; Rooney and Klein, 2002). *Paracoccidioidomycosis* is the most prevalent mycosis in Latin America, with Brazil, Venezuela and Colombia corresponding to the areas of highest disease incidence (Franco, 1987; Restrepo et al., 2001).

In order to obtain information concerning the *P. brasiliensis* mycelium and yeast transcriptomes, a laboratory network from the central region of Brazil has carried out an expressed sequence tag (EST) genome project (Felipe et al., 2003). Single-pass 5' sequencing from non-normalized cDNA libraries of mycelium and yeast cells generated a total of 19,718 high-quality ESTs. Upon CAP3 assembly, 2,655 contigs and 3,367 singlets, which constitute the so-called 6,022 *P. brasiliensis* assembled EST (PbAEST) database, was generated (Felipe et al., 2005). We analyzed the translational and post-translational machineries of *P. brasiliensis*, comparing our transcriptome findings with *Saccharomyces cerevisiae* MIPS functional database. We were able to describe almost all of the components involved in the major steps of these processes, showing that our approach in the sequencing and identification of *P. brasiliensis* genes involved in these processes was successful.

RIBOSOMAL PROTEINS

In translation, the codons in the mRNA direct protein synthesis, generating a polypeptide chain. This process takes place in ribosomes, a large complex composed of rRNA and proteins. In all species, ribosomes are organized into two structural components, the small and

large subunits. Eukaryotic ribosomes have a sedimentation coefficient of 80S, reversibly split in the two, 60S and 40S, subunits (Lafontaine and Tollervey, 2001).

Ribosomes possess three regions where the charged tRNA can be positioned, the A, P and E sites. The aminoacylated tRNA carrying the amino acid to be incorporated into the nascent polypeptide chain binds to the A site. The P site is where the peptidyl-tRNA carrying the nascent polypeptide chain is positioned. The peptide bond formation transfers the peptide from the P site to the aminoacylated tRNA positioned at the A site. Finally, the exit site E, is the location from where the deacylated tRNA is released (Wilson and Nierhaus, 2003).

Recently, it was demonstrated that the large rRNA itself possess a peptidyl transferase activity, being responsible for peptide bonds formation (Nissen et al., 2000). In this sense, proteins may increase the efficiency of the process, maintaining the rRNA in an optimal conformation (Gallie, 2002). The eukaryotic subunits have not been obtained in high resolution structures until now, but the high degree of similarity observed among the various components suggests that ribosomes function in a similar way in these kinds of cells (Doudna and Rath, 2002).

Saccharomyces cerevisiae ribosomes are composed of 78 proteins, 32 in the small subunit and 46 in the large one. These proteins are conserved in all genomes studied so far (Lecompte et al., 2002). However, there is no equivalent to the L28e protein in *S. cerevisiae*, though it is found in almost all other organisms (Planta and Mager, 1998).

We were able to identify orthologs corresponding to all *S. cerevisiae* ribosomal proteins in *P. brasiliensis* transcriptome, except that encoding L23 of the large subunit. Table 1 shows all PbAESTs related to ribosomal proteins and associated to translation. Additionally, an ortholog to the mammal L28e protein was also found (PbAEST 981).

There are 80 different ribosomal proteins in eukaryotes but, since one of them (P3) is found only in plants (Lecompte et al., 2002), we expected to find 79 ribosomal protein orthologs in *P. brasiliensis* transcriptome. We were able to find 78 of the 79 predicted cytosolic ribosomal proteins, corresponding to the entire small subunit and almost all of the large subunit set of proteins. Consequently, we believe that our approach in the sequencing and identification of new genes was successful, allowing the characterization of a significant number of genes in this microorganism.

In *S. cerevisiae*, ribosomal protein genes correspond to almost 50% of total RNA polymerase II transcripts (Warner, 1999). Similarly, several ribosomal proteins are among the highly expressed genes in *P. brasiliensis* transcriptome. PbAESTs encoding the L24, P1 and P2 proteins of the large subunit, and S2, S3, S10, and S14 of the small subunit are among the 24 groups in the transcriptome that are represented by a high number of ESTs (Table 2).

Differential ribosomal protein gene expression was demonstrated in various fungi as related to cell differentiation (Kusuda et al., 2000), sexual development (Jeong et al., 2001) and stress responses (Yale and Bohnert, 2001). The L19 cytosolic ribosomal protein of *P. brasiliensis* (PbAEST 1704) was found to be differentially expressed, since we have detected 14 reads in the yeast phase of this fungus, and none in the mycelial phase (Table 3).

TRANSLATION INITIATION

The initiation of eukaryotic translation is a complex process, involving ribosomal subunits assembly along with an initiator methionyl-tRNA (Met-tRNAi), and initiation factors (eIFs), resulting in a functional protein synthesis apparatus (Pestova et al., 2001).

Table 1. Genes related to protein synthesis in the *Paracoccidioides brasiliensis* transcriptome.

Function	*Saccharomyces cerevisiae* gene	Corresponding PbAEST ortholog
40S Ribosomal proteins		
S0-S10	Rps0, Rps1, Rps2, Rps3, Rps4, Rps5, Rps6, Rps7, Rps8, Rps9, Rps10	1196, 733, 754, 1378, 2539, 1394, 1083, 2122, 530, 2394, 808
S11-S20	Rps11, Rps12, Rps13, Rps14, Rps15, Rps16, Rps17, Rps18, Rps19, Rps20	747, 292, 3826, 2543, 1006, 777, 2140, 690, 2443, 1324
S21-S31/S27a	Rps21, Rps22, Rps23, Rps24, Rps25, Rps26, Rps27, Rps28, Rps29, Rps30, Rps31	1998, 1679, 4969, 654, 467, 489, 857, 897, 615, 664, 1484
60S Ribosomal proteins		
L1-L11	Rpl1, Rpl2, Rpl3, Rpl4, Rpl5, Rpl6, Rpl7, Rpl8, Rpl9, Rpl10, Rpl11	436, 1325, 1940, 1206, 1052, 4, 127, 2599, 662, 2315, 1571
L12-L22	Rpl12, Rpl13, Rpl14, Rpl15, Rpl16, Rpl17, Rpl18, Rpl19, Rpl20, Rpl21, Rpl22	195, 1708, 2627, 2333, 1556, 1298, 571, 1704, 1290, 477, 2050
L24-L34	Rpl24, Rpl25, Rpl26, Rpl27, Rpl28, Rpl29, Rpl30 Rpl31, Rpl32, Rpl33, Rpl34	1932, 458, 1433, 206, 1318, 1892, 993, 2444, 650, 2128, 1933
L35-L43	Rpl35, Rpl36, Rpl37, Rpl38, Rpl39, Rpl40, Rpl41, Rpl42, Rpl43	551, 1412, 1538, 3084, 1563, 1483, 750, 81, 45
L28e/L44	Rpl44[1]	981[1]
P0, P1-α, P2-β	Rpp0, Rpp1, Rpp2	443, 1023, 1255
Translation initiation factors (cytoplasm)		
eIF1 and eIF1A	Sui1, Tif11	158, 52
eIF2 (γ subunit)	Gcd11	3228
eIF2B, (α, β, γ, and ε subunits)	Gcn3, Gcd7, Gcd1, Gcd6	575, 5257, 3998, 2147
eIF3 (core subunits: eIF3a; eIF3b; eIF3c; eIF3g; eIF3h)	Rpg1, Prt1, Nip1, Tif35, Tif3h[2]	884, 2183, 2890, 688, 5211[2]
eIF3 auxiliary subunits	Clu1, Gcd10	5951, 5210
eIF4A and E	Tif1, Cdc33	2336, 17
eIF5 and 5A	Tif5, Hyp2	2119, 2500
eIF6	Tif6	1837
mRNA poly(A) binding protein (PABP)	Pab1	634
Translation elongation and termination factors (mitochondrion)		
EF-TU, mitochondrial	Tuf1	346
EF-G, and EF-G-like, mitochondrial	Mef1, Mef2	1949, 4904
Strong similarity to *E. coli* EF-type GTP-binding protein lepa	Guf1	4980
RF1, mitochondrial	Mrf1	2163
Translation elongation and termination factors (cytoplasm)		
eEF1A	Tef2	1841
eEF1B, chains: β, γ	Efb1, Cam1	2007, 1644

Continued on next page

Table 1. Continued.

Function	*Saccharomyces cerevisiae* gene	Corresponding PbAEST ortholog
eEF2	Eft2	647
eEF4 (eEF2-like)	Ria1	4741
eEF3 and eEF3-like	Yef3, New1	845, 4525
eRF3	Sup35	975
tRNA Aminoacylation		
Phenylalanyl-tRNA synthetase α and β chain, cytosolic	Frs1, Frs2	3970, 973
tRNA synthetase, cytosolic	Krs1, Ses1, Ths1, Gln4, Wrs1, Ils1, Tys1, Mes1, Dps1, Gus1, Yhr020w	192, 4297, 2720, 5860, 4242, 5302, 3820, 5457, 426, 5886, 4978
tRNA synthetase, cytosolic and mitochondrial	Hts1, Grs1, Vas1, Ala1	510, 4512, 218, 249
tRNA synthetase, mitochondrial	Ism1, Mse1, Msf1, Nam2, Ycr024c	3226, 2137, 1012, 5152, 3561
Glutamyl-tRNA amidotransferase, mitochondrial	Pet112	756
Mitochondrial large ribosomal subunit proteins		
Mitochondrial ribosomal protein (L2, L4-L6)	Rml2, Mrpl4, Yml6, Mrpl5[3], Mrpl6	820, 5831, 4836, 789[3], 5018
Mitochondrial ribosomal protein Mrp7 (YmL2)	Mrp7	1427
Mitochondrial ribosomal protein (L8-L12, L14)	Mrpl8, Mrpl9, Mrpl10, Mrpl19, Mnp1, Mrpl38	2726, 554, 2250, 3124, 979, 1076
Mitochondrial ribosomal protein (L16, L17, L19)	Mrpl16, Mrpl17, Img1	4542, 1996, 2379
Mitochondrial ribosomal protein (L23-L25, L31, L36) family	Mrp20, Mrpl23, Mrpl24, Mrpl25, Mrpl31, Mrpl36	129, 2519, 1575, 788, 5015, 430
Related to ribosomal protein MRP49	Mrp49[4]	3760[4]
Mitochondrial small ribosomal subunit proteins		
Mitochondrial ribosomal protein, Mrp family	Mrp4, Mrps8, Mrps9, Mrp10, Mrps15[5], Mrps16, Mrp17, Mrps17, Mrps35	790, 1655, 2758, 5111, 3066[5], 4792, 1464, 2344, 285
Mitochondrial ribosomal protein, Nam9	Nam9	1981
Mitochondrial ribosomal protein, Rsm family	Rsm10, Rsm19, Rsm22, Rsm24, Rsm25, Rsm26, Rsm27	681, 232, 2029, 1999, 1914, 1557, 2156
Similarity to ribosomal protein (S12 and S13)	Ynr036c, Ynl081c	2571, 5540
Other protein-synthesis activities	Dom34, Gcn20, Gis2, Map2, Msg5, Mss1, Mss51, Mto1, Nam7, Nop14, Nsa2, Pan2, Pth2, Shp1, Spb1, Ssl1, Ykl056c, Rrs1, Nmd3, Brx1, Nop1, Ubi4, Asc1, Rlp24	772, 2906, 1019, 414, 1106, 3612, 1950, 1350, 885, 1809, 1488, 5058, 5293, 223, 2239, 1201, 1583, 1222, 3288, 1069, 1013, 388, 2208, 959

[1]NP_593124 (*S. pombe*). [2]NP_563880 (*A. thaliana*). [3]CAD11403 (*N. crassa*). [4]CAB99177 (*N. crassa*). [5]CAD21143 (*N. crassa*).

Table 2. Over expressed genes related to translation in *Paracoccidioides brasiliensis* transcriptome and the corresponding PbAEST.

Annotation	PbAEST	Number of ESTs
60S acidic ribosomal protein P2	1255	141
40S ribosomal protein S10	0808	104
60S ribosomal protein L24	1932	78
40s ribosomal protein S2	0754	60
eEF1A	1841	58
60S acidic ribosomal protein P1-a	1023	57
40S ribosomal protein S3	1378	57
40S ribosomal protein S14	2543	52

Table 3. Differentially expressed genes involved in translation and protein modification in yeast cells of *Paracoccidioides brasiliensis*, detected by electronic subtraction and cDNA microarray analysis.

Function	PbAEST	Number of reads[1]		P value[2]
		Y	M	
60S ribosomal protein L19	1704[3]	14	0	0.000032
Strong similarity to *Cricetus* mitochondrial ribosomal L12 protein	979[4]	8	1	0.008358
Glutamyl-tRNA amidotransferase	756[3]	4	0	0.031496
Glycosyltransferase	1422[4]	11	4	0.019803
Protein disulfide isomerase	2398[4]	8	3	0.038949

The PbAESTs were analyzed for differential expression by two methods: a statistical analysis of the number of mycelium and yeast ESTs clustered in each PbAESTs (Audic and Claverie, 1997) and a cDNA microarray analysis.
[1]Number of mycelium (M)- and yeast (Y)-derived ESTs in the PbAEST. [2]P value for the Audic and Claverie (1997) test. [3]Electronic subtraction differential pattern and not assayed in cDNA microarray analysis. [4]Electronic subtraction and cDNA microarray analysis - differential pattern in both analyses.

The Met-tRNAi is the only aminoacylated tRNA that is directly charged in the P site of the ribosome, as a ternary complex with eIF2-GTP. During this step, eIF3 and eIF1A bind to the 40S subunit, while eIF6 binds to the 60S subunit, preventing the early assembly of the complete 80S ribosome (Ganoza et al., 2002).

In *S. cerevisiae*, eIF2 is composed of three subunits (α, β, and γ), while eIF3 comprehends a core constituted of five subunits associated to auxiliary factors (Browning et al., 2001; Preiss and Hentze, 2003). The eIF3, eIF1, eIF1A, and eIF5, together with the ternary complex, bind to the 40S subunit, giving rise to the 43S complex (Preiss and Hentze, 2003). Orthologs of all these factors were found in *P. brasiliensis* transcriptome, with the exception of Tif34 eIF3 subunit and eIF2 α and β subunits. We also found a PbAEST similar to the *Arabidopsis thaliana* eIF3H subunit, which is absent in *S. cerevisiae* (Browning et al., 2001).

The association of eIF4E, which directly binds to the mRNA 5' cap, with eIF4G and eIF4A, forms a stable ternary complex, named eIF4F. The eIF4A protein is an RNA helicase, which is stimulated by eIF4B and eIF4H factors (Preiss and Hentze, 2003) and facilitates the ribosome scanning in the search of an initiation codon. The eIF4G is an adaptor protein, which

interacts with the other subunits of eIF4F and with eIF3, promoting the recruitment of the 43S complex to the 5' cap. In addition, eIF4G also interacts with PABP, altering the mRNA to a circular structure, where the 5' and 3' ends are positioned very closely (Sonenberg and Dever, 2003). We were able to find orthologs to *S. cerevisiae* eIF4A and E, as well as a PABP, in the *P. brasiliensis* transcriptome.

When the Met-tRNAi is positioned on the AUG codon, eIF5 induces the GTPase activity of eIF2, generating eIF2-GDP, which releases most of the eIFs that are interacting with the 40S subunit. The hydrolysis of a second GTP molecule attached to eIF5B is activated by the 60S subunit, which induces the release of the remaining initiation factors, allowing the assembly of an 80S functional ribosome. We have detected four (α, β, γ, and ε) of the five eIF2B subunit orthologs in *P. brasiliensis* transcriptome. Orthologs to the eIF5B factor have not been found in our transcriptome analysis.

The putative initiation factor eIF5A, which is not essential for general protein synthesis, seems to be required for the translation of specific sets of mRNAs (Watkins and Norbury, 2002). This factor is also essential to yeast viability, being highly conserved in *Eukarya* and *Archaea*. It is the only known protein that is post-translationally modified by the incorporation of a hypusine amino acid (Thompson et al., 2003). An ortholog to eIF5A was found in *P. brasiliensis* transcriptome.

Based on the analysis of 27 genes related to translation initiation in *S. cerevisiae*, we have found 19 orthologs in *P. brasiliensis* transcriptome, as is shown in Table 1.

TRANSLATION ELONGATION AND TERMINATION

The translation elongation requires several non-ribosomal proteins, named eukaryotic elongation factors (eEFs). The elongation process involves eEF1A, responsible for the recruitment of the aminoacylated tRNA to the ribosomal A site, and eEF2, which mediates ribosomal translocation (Browne and Proud, 2002). Both proteins show GTPase activity, while eEF1B promotes a GDP-GTP exchange in eEF1A (Jeppesen et al., 2003). All eukaryotic elongation factors were detected in the *P. brasiliensis* transcriptome, with eEF1A as one of the most expressed genes (PbAEST 1841), as shown in Table 2.

In fungi, ribosomal translocation requires a third factor, eEF3. Besides its role in each elongation cycle, it was demonstrated that this protein is also essential to cell viability in *S. cerevisiae*, being also required in cell-free translation systems (Anand et al., 2003). The PbAEST 845 is the ortholog of *S. cerevisiae* eEF3 gene. Probably, eEF3 functions either facilitating the aminoacyl-tRNA attachment to the A site, or helping in the release of the deacylated tRNA from the E site (Ganoza et al., 2002). Since this protein is not found in animal cells, the development of drugs with anti-eEF3 potential is promising for the treatment of mycoses (Sturtevant, 2002).

Translation termination in eukaryotes is performed by release factors 1 and 3 (eRF1, eRF3). The termination signals correspond to the three possible stop codons (UAA, UAG, UGA) in the mRNA molecule. All these codons are recognized by eRF1, which triggers a reaction catalyzed by the ribosome, resulting in the release of the nascent peptide (Kisselev et al., 2003). The eRF3 corresponds to a GTPase which increases the termination efficiency, stimulating eRF1 activity in a GTP-dependent manner (Kong et al., 2004). In *P. brasiliensis* transcriptome it was possible to identify eRF3 (PbAEST 975), but not eRF1, as shown in Table 1.

AMINOACYL-tRNA SYNTHETASES

The enzymes aminoacyl-tRNA synthetases (aaRS) connect amino acids to the 3' end of tRNAs, establishing the specificity of these molecules, and strongly contributing to protein synthesis fidelity. All aaRS proceed through essentially the same two-step reaction. The condensation of an amino acid to an ATP molecule, generating an activated aminoacyl-adenylate intermediate, is the first step. Second, the transfer of the aminoacyl group to the 3' terminal ribose of tRNA generates an aatRNA (Ibba and Söll, 2000). Most organisms possess 20 aaRS activities, each responsible for a specific esterification of an amino acid with its cognate tRNA. These enzymes are subdivided into two classes, corresponding to different evolutionary solutions to the aminoacylation reaction (Francklyn et al., 2002). The aaRS play different roles in the cell, including RNA splicing, RNA transport, transcription and translation regulation, as well as cell signaling (Ewalt and Schimmel, 2002). Sixteen PbAESTs, showing aaRS predicted activities were found in our transcriptome analysis (Table 1). No cysteinyl-, leucyl-, asparagyl-, and arginyl-tRNA synthetases were detected in *P. brasiliensis* transcriptome.

MITOCHONDRIAL TRANSLATION APPARATUS

Mitochondria are organelles originated from a remote ancestor, probably a member of the α-proteobacteria subdivision, related to rickettsiae (Emelyanov, 2003). This organelle contains its own translational machinery and employs an alternative genetic code. In spite of the different characteristics of mitochondrial translation, it is considered a process similar to the bacterial one (Chacinska and Boguta, 2000).

Saccharomyces cerevisiae mitochondrial ribosomes are 74S, being composed of a 54S large subunit and a 37S small subunit (Chacinska and Boguta, 2000). There are approximately 75 mitochondrial ribosomal protein-encoding genes in yeast (Gan et al., 2002), almost all located in the organism nuclear genome. The only known translational factor encoded by the mitochondrial genome corresponds to the ribosomal protein Var1p (Fiori et al., 2003). In this sense, most mitochondria-associated genes are transcribed in the nucleus as polyadenylated mRNAs, which are translated in the cytoplasm and later transported to the organelle (Zhang et al., 2002).

Since the *P. brasiliensis* transcriptome was generated from cDNA molecules synthesized using polyadenylated mRNAs, it contains various PbAESTs related to mitochondrial translation. We have found 20 and 18 orthologs to the *S. cerevisiae* large and small mitochondrial ribosomal subunit proteins, respectively, in *P. brasiliensis* transcriptome (Table 1). We have also found three PbAESTs similar to *Neurospora crassa* mitochondrial ribosomal genes, which have not shown any similarity with *S. cerevisiae* genes.

PbAEST 979 was identified as the putative mitochondrial ribosomal protein L7/L12, and revealed a pattern of differential expression. This behavior was confirmed by microarray experiments (Felipe et al., 2005), revealing that PbAEST 979 synthesis was higher during the yeast phase of *P. brasiliensis*. The *S. cerevisiae* ortholog (protein YGL068W) was not directly identified as a mitochondrion ribosomal protein. It was suggested that this protein is a precursor of L7/L12 due to its high homology with the bacterial ribosomal protein L7/L12 (Sato and Miyakawa, 2004). Although the disruption of *S. cerevisiae YGL068W* gene was found to be lethal by Giaever et al. (2002), other authors described that the elimination of mitochondrial ribosomal proteins in yeasts generally results in a non-lethal pet phenotype. A *pet* gene is de-

fined as a mutated nuclear gene, at least in one allele, conferring a respiratory deficiency (Tzagoloff and Dieckmann, 1990). In such cases, the defect in the translational machinery prevents the electron transport chain protein synthesis. In this sense, we might expect a second physiological role for *YGL068W* of *S. cerevisiae*, and by consequence, of *P. brasiliensis* PbAEST 979. Stevenson et al. (2001) suggested that *YGL068W* is associated to *S. cerevisiae* cell cycle progression.

Three translation initiation factors are known in bacteria, IF1, IF2 and IF3, which are functionally equivalents to the eIF1A, eIF5B and eIF1 cytosolic eukaryotic factors, respectively (Sonenberg and Dever, 2003). In mitochondria, no ortholog to IF1 has been found in any species studied so far (Koc and Spremulli, 2002). On the other hand, orthologs to IF2 and IF3 have been described in several organisms (Koc and Spremulli, 2002; Garofalo et al., 2003). Analyses of the *P. brasiliensis* transcriptome did not reveal any of these proteins associated to the mitochondrial initiation of translation.

The elongation step in mitochondria involves the EF-Tu, EF-Ts, and EF-G factors, which are functional equivalents to the cytoplasmic eEF1A, eEF1B and eEF2, respectively (Hammarsund et al., 2001). No EF-Ts/eEF1B homologs have been found in *S. cerevisiae* mitochondria (Cai et al., 2000). Only one factor has been described so far for the translation termination step in mitochondria, the RF1 factor (Towpik et al., 2004), which plays the same role as cytoplasmic eRF1.

Although orthologs to the *S. cerevisiae* mitochondrial EF-Tu, EF-G and RF1 have been found in *P. brasiliensis* transcriptome, no sequences corresponding to functional EF-Ts were detected. We have also found orthologs of *S. cerevisiae* GUF1 and MEF2 factors, which are probably involved in elongation, but when disrupted they did not promote any phenotypic alteration (Kiser and Weinert, 1995; Hammarsund et al., 2001).

A common feature observed in eukaryotes is the occurrence of several genes encoding cytoplasmic aaRSs. Nevertheless, it is also known that the same aaRS can be found either in the cytoplasm or in mitochondria. In *S. cerevisiae*, the cytoplasmic and mitochondrial alanyl-, glycyl-, histidyl-, and valyl-tRNA synthetase are each encoded by the same gene (Turner et al., 2000). The *S. cerevisiae* histidyl-tRNA synthetase gene contains two potential ATG start codons that could generate two different proteins. The longer mitochondrial isoform presents a mitochondrial N-terminal localization signal, which is absent in the cytoplasmic protein (Chiu et al., 1992).

The analysis of *P. brasiliensis* transcriptome revealed five mitochondrial aaRSs, and if we consider that in *P. brasiliensis* the cytoplasmic and mitochondrial aaRS activities occur in the same way as described above for *S. cerevisiae*, the number of PbAESTs corresponding to putative mitochondrial aaRS activities increases to nine (Table 1).

The putative mitochondrial aaRS were analyzed by MITOPRED (Guda et al., 2004a,b) and PSORT (Nakai and Horton, 1999), in a search for mitochondrial targeting signals. We found such signals in five *P. brasiliensis* aaRS, two of them related to cytosolic and mitochondrial aaRS, and three to mitochondrial aaRS of *S. cerevisiae* (Table 4).

We believe that this low number of mitochondrial targeting sequences detected could be explained by their N-terminal localization, and most of our PbAESTs correspond to incomplete ORFs, probably lacking this region.

Many bacteria, *Archaea*, mitochondria and chloroplasts do not have the glutaminyl-tRNA synthetase. They produce glutaminyl-tRNAglutaminyl by employing a glutamyl-tRNA

Table 4. Putative mitochondrial aminoacyl-tRNA synthetases found in the *Paracoccidioides brasiliensis* transcriptome, based on identification of the mitochondrial localization signal using PSORT and MITOPRED programs.

Function	PbAEST	% Mitochondrial	Localization (PSORT)
Histidyl-tRNA synthetase, cytosolic and mitochondrial	510	92.3%	Mitochondrial
Phenylalanyl-tRNA synthetase α chain, mitochondrial	1012	92.3%	Mitochondrial
Glutamyl-tRNA synthetase, mitochondrial	2137	84.6%	Mitochondrial
Glycyl-tRNA synthetase, cytosolic and mitochondrial	4512	84.6%	Mitochondrial
Leucyl-tRNA synthetase, mitochondrial	5152	99.0%	Mitochondrial

amidotransferase reaction (Tumbula et al., 2000; Francklyn et al., 2002). An ortholog corresponding to a putative subunit of this enzyme has been found in *P. brasiliensis* transcriptome (PbAEST 756, Table 1). This PbAEST was found only in the yeast phase of *P. brasiliensis* (Table 3).

PROTEIN MODIFICATION

Co- and post-translational modifications have long been recognized as essential steps for the translocation, activation, regulation and, ultimately, the degradation of proteins, particularly in eukaryotic cells (Krishna and Wold, 1993). Since all protein synthesis begins at the N-terminus, this region provides an early and important site of protein processing, including: residue-specific alterations, removal of the initiator methionine, modification of the α-amino group, and cleavage of the signal sequence. Two of these modifications, excision of the initiation methionine and Nα-acetylation, potentially affect most proteins (Arfin et al., 1995; reviewed by Kouzarides, 2000).

Nα-acetylation is characteristic of eukaryotic cells, and it has been estimated that as many as 70% of soluble proteins bear this modification (Mullen et al., 1989). It occurs during protein synthesis and involves the transfer of an acetyl group from acetyl-coenzyme A to the protein α-NH2 group (Bradshaw et al., 1998). Acetylation neutralizes a positive charge and, like protein phosphorylation, it may influence protein function by altering stability or interactions with other molecules (Kouzarides, 2000). PbAESTs related to *S. cerevisiae* methionine aminopeptidase and acetyltransferase were found in *P. brasiliensis* transcriptome. In Table 5, six PbAESTs associated with the *S. cerevisiae* acetyltransferase activities: ARD1, GCN5, HAT1, HAT2, ESA1, and ELP3 are shown. Among these, we emphasize ARD1, a member of a large and diverse super-family of acetyltransferases that includes histone acetyltransferases (Neuwald and Landsman, 1997). Another study on ARD1 strongly suggests that it is an essential gene in the insect to mammalian-stage of *Trypanosoma brucei.* This study proposes that ARD1 may provide a target for novel chemotherapy agents against this parasite (Ingram et al., 2000).

Glycosylation is another protein modification of fundamental importance. It is the most complex post-translational modification known to occur in eukaryotes. Mannosylation of proteins can be subdivided into *O* (serine/threonine)-linked and *N* (asparagine)-linked glycosylation. The biosynthetic events leading to these modifications are coupled to the secretory path-

Table 5. PbAEST related to protein modification.

Function	*Saccharomyces cerevisiae* gene	Corresponding PbAEST ortholog
Transferases	ARD1, MAK3, MNN9, ESA1, GCN5, HAT1, HAT2, ELP3, PMT1, PMT2, PMT4, PMT5, ALG1, ALG5, ALG7, ALG8, ALG11, RHK1, KTR3, OCH1, BET2, BET4, RAM1, STT3, OST1, RAM2, UGP1	1102, 107, 3220, 3484, 5286, 4091, 357, 491, 2980, 2980, 2980, 2980, 3157, 2557, 3461, 5016, 1422, 696, 1063, 4478, 1496, 3164, 3641, 5197, 1100, 1926, 3668
Peptidases	MAP2, APE2	414, 587
Protein glycosylation in the Golgi	ANP1	3498
GPI anchor attachment	GPI8, GAA1	3107, 3403
Proteases	IMP1, STE23, PEP4	197, 3046, 1765
Long-chain-fatty-acid-CoA ligase	FAA4	3646
Miscellaneous	PSK1, RAD18, SGF29, PDI1, BPL1, GRX3, SKP1, PPM1, PRB1, SEC53, CYC3, ARL1, KEX1, CAX4, TRX2, MAS2, SDP1, MNS1, OCT1, SDS22, TRX1, SPC3, YPS1, MAS1, SSQ1, VIP1, PTC5, ARL3	2164, 1123, 5284, 2398, 2650, 1333, 486, 1835, 1385, 5088, 2239, 949, 1494, 2077, 1812, 934, 1007, 930, 4126, 3325, 1812, 2470, 5557, 2206, 2210, 1299, 686, 2357

way. Early stages of *N*-linked glycosylation and the formation of glycosylphosphoinositol anchors have been conserved through evolution of eukaryotes (Herscovics and Orlean, 1993).

In fungi, the attachment of *O*-linked mannosyl residues to proteins of the secretory pathway is essential for cell viability, in particular it is indispensable for cell wall integrity and rigidity (Gentzsch and Tanner, 1996). In these organisms, *O*-mannosylation is initiated in the lumen of the endoplasmic reticulum, where the transfer of mannose to seryl and threonyl residues of secretory proteins is catalyzed by a family of protein mannosyltransferases (PMTs), which were first characterized in *S. cerevisiae*. Besides *S. cerevisiae,* where seven PMT family members have been identified, orthologs are known in many other fungi, such as *Candida albicans* and *Schizosaccharomyces pombe* (Timpel et al., 1998, 2000), as well as in many multicellular eukaryotes, such as *Drosophila melanogaster*, mice and humans (Martín-Blanco and García-Bellido, 1996; Jurado et al., 1999; Willer et al., 2002). The analysis of *P. brasiliensis* transcriptome revealed four *S. cerevisiae* ortholog genes related to members of the PMT family, Pmt1p, Pmt2p, Pmt4p, and Pmt5p, as shown in Table 5. In *C. albicans,* which is a human pathogenic fungus, as *P. brasiliensis*, correct *O*-mannosylation is important for morphogenesis, adherence to host cells and, consequently, for virulence (Timpel et al., 1998, 2000). Since this process is fundamentally different in fungi and mammalian cells, the PMT family would be especially attractive as a target for the development of new antifungal drugs for both models (Herscovics and Orlean, 1993; Hounsell et al., 1996).

N-linked glycosylation, which is carried out by enzymes highly conserved through evolution, involves the addition of large oligosaccharides to proteins, being of great importance to eukaryotic cells. In *S. cerevisiae*, ALG11 is a gene that is required for normal glycosylation and is essential for growth at high temperatures. A deletion of an ALG11 gene leads to poor growth and temperature-sensitive lethality (Cipollo et al., 2001). A *S. cerevisiae* ALG11 ortholog gene was detected and shown to be a differentially expressed gene in *P. brasiliensis* transcriptome. The analyses were proceeded by electronic subtraction and cDNA microarray analysis (Table 3; Felipe at al., 2005), revealing that the *S. cerevisiae* ALG11 ortholog gene was present only in the pathogenic *P. brasiliensis* yeast form. These data reinforce the idea that in *P. brasiliensis* this gene is also required for growth at higher temperatures, as in *S. cerevisiae*.

UBIQUITINATION AND PROTEIN DEGRADATION

Ubiquitin is a 76-amino acid protein with remarkable evolutionary conservation throughout the eukaryotic kingdoms (Goldstain et al., 1975; Finley and Chau, 1991). This small protein is conjugated to lysine residues on a wide assortment of substrates through a three-enzyme cascade. Some substrates are conjugated to only one ubiquitin, while others are conjugated to multiple ubiquitins, resulting in a polyubiquitin chain (Sloper-Mould et al., 2001). Protein modification by ubiquitination plays important roles in gene transcription, chromatin structure organization, stress resistance and protein degradation (Finley and Chau, 1991). PbAEST orthologs to *S. cerevisiae* ubiquitin system have been identified in *P. brasiliensis* transcriptome (Table 6).

Table 6. PbAEST related to protein degradation by 26S proteasome.

Function	*Saccharomyces cerevisiae* gene	Corresponding PbAEST ortholog
Ubiquitination system	UBC1, UBC4, UBC5, UBC6, UBC9, UBC11, UBC13, UBP8, UMP1, PRD1, DOA4, QRI8, SKP1, UBA2, SZI11	1792, 1795, 1890, 550, 3203, 2736, 1890, 2809, 2219, 5630, 2809, 821, 486, 4532, 3541
20S subunits - proteasome core	PRE1, PRE6, PRE7, PRE9, PUP1	1542, 1517, 2211, 843, 2591
19S subunits - regulator complex	RPN2, RPN3, RPN6, RPN7, RPN9, RPN10, RPT1, RPT3	1238, 725, 4376, 369, 2586, 2212, 1941, 4205

Intracellular protein degradation is frequently used to regulate key biological processes such as cell cycle control, DNA repair, the stress response, differentiation, and metabolic control (Hochstrasser, 1996; Hershko and Ciechanover, 1998). In eukaryotic cells, intracellular proteolysis occurs via two major processes: a lysosomal and a non-lysosomal ATP-dependent pathway. The latter, which is known to degrade most cell proteins, including regulatory proteins, requires first the covalent linking of multiple ubiquitin molecules to the target protein. This modification marks the protein for rapid degradation by the Ub-26S proteasome pathway (reviewed by Coux et al., 1996).

Protein degradation by the Ub-proteasome pathway comprises two phases: in the initial phase, ubiquitin is covalently attached to an acceptor protein by three classes of enzymes. The

active E1 enzyme transfers the ubiquitin to E2 (ubiquitin conjugating), and E3 selects the substrate for ubiquitin ligation. In the second phase of the cycle, the 26S proteasome recognizes the substrate via its polyUb chain, which is degraded to small peptides in a processive manner, with Ub regeneration by specific deubiquitinating enzymes (reviewed by Hochstrasser, 1996 and Coux et al., 1996). PbAEST orthologs to *S. cerevisiae* UBC1, UBC4, UBC5, UBC6, UBC9, UBC11, and UBC13 were found in *P. brasiliensis* transcriptome (Table 6).

After the conjugation of target proteins with the polyubiquitin, this system will be addressed to the 26S proteasome, where target protein degradation occurs. The 26S proteasome is constituted by the 20S and 19S complexes: the 20S core complex is a cylindrical stack of four heptameric rings, harboring the proteolytic active sites and the 19S complex, which is located at both ends of the 20S complex (Coux et al., 1996). In yeast, there are 14 genes encoding 20S proteasome subunits, with seven different α-type subunits and seven different β-type subunits (Chen and Hochstrasser, 1995). The PbAEST orthologs to the *S. cerevisiae* genes related to 20S proteasome subunits found in *P. brasiliensis* transcriptome were PRE1, PRE6, PRE7, PRE9, and PUP1, as shown in Table 6. The 19S regulator complex contains 15-20 subunits, including six ATPases that are located proximal to the 20S barrel, and it is likely to promote ATP-dependent substrate unfolding and translocation (Coux et al., 1996; Pickart, 2000). The 19S complex is divided into the lid and base subcomplexes, the lid subcomplex consists of eight non-ATPase subunits (RPN3, RPN5-9, RPN11, and RPN12), while the base subcomplex consists of six ATPases (RPT1-6) and three non-ATPases (RPN1, RPN2 and RPN10) subunits (Pickart, 2000). PbAEST orthologs corresponding to 19S regulator complex subunits genes, such as RPN2, RPN3, RPN6, RPN7, RPN9, and RPN10 of *S. cerevisiae*, were found in *P. brasiliensis* transcriptome (Table 6). Two PbAEST orthologs to the *S. cerevisiae* Rpt ATPase subunits (RPT1and RPT3) were also described (Table 6).

In all eukaryotes examined so far, proteases specific for linear ubiquitin fusions (ubiquitin carboxyl-terminal hydrolases) have been described. Many of these proteases have been characterized, including members of a large family of deubiquitinating enzymes, known as the ubiquitin-specific proteases (Gilchrist and Baker, 2000). Several ubiquitin-specific proteases from *S. cerevisiae* have been cloned and characterized by homology to the conserved Cys and His boxes (Hochstrasser, 1996). The PbAEST ortholog to *S. cerevisiae* UBP8 has been detected in *P. brasiliensis* transcriptome (Table 6).

CONCLUDING REMARKS

The transcriptome of *P. brasiliensis*, a dimorphic fungus responsible for a severe systemic mycosis in Latin America, has provided important information about this microorganism's physiology. Several aspects related to the biology of this fungus were inferred by comparative analyses of PbAESTs with sequences deposited in different databases. The translational apparatus and post-translational machineries are very similar to those of other eukaryotes, such as *S. cerevisiae*. The results presented in this report could be used as a starting point for further studies in order to better understand the molecular biology of this important human pathogen.

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