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Comparative analyses of the structure of the 1,3-β-glucan synthase gene in *Paracoccidioides brasiliensis* isolates

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ABSTRACT. The evolutionary origin and significance of spliceosomal introns have been the subject of many investigations. Two theories, "introns-early" theory and "introns-late" theory, have been proposed to explain the evolution of introns in eukaryotic genes. Intron position is generally conserved in paralogue and orthologue genes. Some introns occur at similar but not necessarily identical positions in homologous genes, which were separated by great evolutionary distances. This event can be explained by insertion, loss or movement of the intron over short distances. Intron loss and gain events are unique in evolution and can be useful as markers for phylogenetic analyses. The insertion of introns at an identical position suggests a common ancestor gene. Here we analyzed, using PCR and RT-PCR, the structure of the 1,3-β-glucan synthase gene (*FKS*) in several clinical isolates of *Paracoccidioides brasiliensis* (*Pb*): isolates *Pb* 01, *Pb* 4940, *Pb* 8515, *Pb* 8311, *Pb* 8334, *Pb* 4268, *Pb* 1668, and *Pb* E. Our results showed that seven of the isolates examined showed identical structures concerning the position of introns in *PbFKS1*. *PbFKS4940* showed the intron described at the 3' end and

had lost that one at the 5' end. The presence of the *PbFKS4940* transcript suggests that it could be a functional gene. These data suggest a divergent evolution for introns with regard to the 1,3-β-glucan synthase gene in *P. brasiliensis* isolates.

Key words: *Paracoccidioides brasiliensis*, 1,3-β-glucan synthase, Gene structure, Intron evolution

INTRODUCTION

Paracoccidioides brasiliensis (*Pb*) is a dimorphic fungus, which causes paracoccidioidomycosis, the systemic mycosis with the highest mortality rate in Brazil (Coutinho et al., 2002). The cell wall of *P. brasiliensis* is composed of chitin and glucan polymers. β-glucans are present in the mycelium while α-glucans are prevalent in the yeast (Kanetsuna et al., 1972). In *Saccharomyces cerevisiae*, the synthesis of 1,3-β-glucan occurs at the plasma membrane through the activity of two highly homologous genes *FKS1* and *FKS2*. The *FKS1* is regulated in the cell cycle and predominates during growth in the presence of glucose; *FKS2* is expressed in the absence of glucose, and is essential for sporulation. *FKS2* expression is induced by Ca^{2+} and pheromones, suggesting that this gene may play a role in the remodeling of the cell wall during the mating process (Douglas et al., 1994; Mazur et al., 1995). In *Candida albicans*, three genes, *FKS1/GSC1*, *GSL1* and *GLS2*, have been described. *FKS1/GSC1* encodes the catalytic subunit of the oligomeric protein (Mio et al., 1997). Four *FKS* genes have been described in *Schizosaccharomyces pombe* (Ishiguro et al., 1997; Martin et al., 2000), while single *FKS* homologues have been characterized in *Aspergillus nidulans* (Kelly et al., 1996), *Cryptococcus neoformans* (Thompson et al., 1999) and *Yarrowia lipolytica* (León et al., 2002).

In *P. brasiliensis*, we found only one 1,3-β-glucan synthase homologous gene. The *PbFKS1* gene has three exons, separated by two introns. The first intron of 83 bp was located close to the 5' end, while the second one, of 78 bp, was found close to the 3' end of the gene. All intervening sequences were flanked by 5' GT and 3' AG, which correspond to the consensus sequence of known splicing sites. Both introns have the splicing signal for lariat formation (Pereira et al., 2000).

Although *FKS1* genes are highly conserved, some significant differences among them have been used to classify serotypes of *C. neoformans*. Comparative analyses of *FKS1* gene structure of serotypes A and D from *C. neoformans* determined the presence of four different insertion events in the sequence of serotype A (Tanaka et al., 2003). Although *C. neoformans* has been classified on the basis of the capsular polysaccharide antigen into serotypes A, B, C, D, and AD (Ikeda et al., 1982), those insertions indicated that serotypes A and D can be differentiated on the basis of *FKS1* sequences.

The study of gene structure includes exon and intron regions. The evolutionary origin and significance of spliceosomal introns have been the subject of many investigations (Logsdon, 2004). Introns sometimes contain functional sequences, such as transcriptional enhancers, al-

ternative-splicing arrangements, and special elements required for the 3' maturation of hnRNA into mRNA (Nesic et al., 1993; Antoniou et al., 1998; Matsui et al., 2005). Introns can bind to ribosomes (Woodson, 1998), and they often have a striking resemblance to motifs permitting initiation of translation at internal start codons in some eukaryotic mRNAs (Le and Maizel, 1997). Introns can also be involved in the processing of polycistronic mRNAs into freely translatable monocistrons (Blumenthal, 1995) and can play a role in mRNA 3' end cleavage and polyadenylation (Nesic et al., 1993; Antoniou et al., 1998). Two theories have been proposed to explain the evolution of introns in eukaryotic genes. According to the "introns-early" theory, the present-day exon/intron structures originated from the aggregation of short primordial minigenes (encoding 15-20 amino acids). This process has been shown to be important for generating protein diversity through exon shuffling. The "introns-late" theory proposes that ancient genes existed as uninterrupted exons and that introns had been introduced by random insertion into previously continuous genes during the course of evolution (Rogers, 1990; Cavalier-Smith, 1991; Patthy, 1991). Bacteria have no spliceosomal introns; on the other hand, protists have few introns, while higher metazoa and plants have many introns. Spliceosomal introns tend to be larger in multicellular organisms compared to those of unicellular organisms (Deutsch and Long, 1999; Vinogradov, 1999). Intron position is generally conserved in paralogue and orthologue genes. Some introns occur at similar but not necessarily identical positions in homologous genes, which were separated by great evolutionary distances (Logsdon, 1998). This event can be explained by insertion, loss (Stoltzfus et al., 1997) or movement of the intron over short distances (1-12 nucleotides) (Cerff, 1995). Intron loss and gain events (Frugoli et al., 1998; Kato et al., 2001) are unique in evolution and can be useful as markers for phylogenetic analyses (Venkatesh et al., 1999). The insertion of introns at an identical position suggests a common ancestor gene (Abrahm et al., 2005).

By using evolutionary comparisons between nematode genes, Coghlan and Wolfe (2004) demonstrated that 122 introns had been gained recently in *Caenorhabditis* genes and that 28 of them are actually derived from "donor" introns present in the same genome. Indeed, few of these new introns apparently derive from other introns in the same gene. Dibb and Newman (1989) proposed that introns tend to be inserted at a "proto-splice site" MAG↓R, where ↓ is the insertion site. The probable mechanisms responsible for creating a new DNA sequence are transposable elements, gene conversion, tandem exon duplication, insertion by a self-splicing (group II) intron, and reverse-splicing of existing introns (Logsdon et al., 1998; Stoltzfus, 2004). The finding that several novel introns are inserted into genes coding for proteins with functions related to splicing provides support for a reverse-splicing model (Coghlan and Wolfe, 2004). Although genome studies have shown that losses and gains of introns occurred during evolution (Rogozin et al., 2003), many examples of losses but few gains have been found in studies comprising more recent evolutionary periods (Roy et al., 2003; Fedorov et al., 2003).

In the present study, we searched for the structure of the 1,3-β-glucan synthase gene in several clinical isolates of *P. brasiliensis*: isolates *Pb* 01, *Pb* 4940, *Pb* 8515, *Pb* 8311, *Pb* 8334, *Pb* 4268, *Pb* 1668, and *Pb* E. Our results showed that seven of the isolates studied had the same gene structure of *PbFKS1* with regard to the two introns. *PbFKS4940* had the intron described at the 3' end but had lost that one at the 5' end. The presence of the *PbFKS4940* transcripts suggests that it could be a functional gene. These data suggest a divergent evolution for introns with respect to the 1,3-β-glucan synthase gene in *P. brasiliensis* isolates.

MATERIAL AND METHODS

Paracoccidioides brasiliensis isolates and growth conditions

Eight *P. brasiliensis* clinical isolates, *Pb* 01 (ATCC-MYA-826), *Pb* 4940 (ATCC-MYA-3044), *Pb* 8515, *Pb* 8311, *Pb* 8334, *Pb* 4268, *Pb* 1668, and *Pb* E, were obtained from M.R.R. Silva Collection, IPTSP, UFG, Goiânia, GO, Brazil, and used in this study. Yeast cells were grown at 36°C on Fava-Netto's medium (1% (w/v) peptone; 0.5% (w/v) yeast extract; 0.3% (w/v) proteose peptone; 0.5% beef extract; 0.5% (w/v) NaCl; 4% (w/v) glucose, and 1.4% (w/v) agar, pH 7.2) (Fava-Netto, 1955) and sub-cultured every 7 days.

DNA extraction

P. brasiliensis yeast cells of all isolates were harvested after seven days of cultivation, washed and frozen in liquid nitrogen. Grinding with a mortar and pestle was used to disrupt the cells, and the genomic DNA was isolated by the cationic hexadecyl trimethylammonium bromide (CTAB) method (Del Sal et al., 1989) with minor modifications. Ten milliliters of extraction buffer (2% (w/v) polyvinylpolypyrrolidone, 1.4 M NaCl, 0.1 M Tris-HCl, pH 8.0, 0.02 M EDTA, 2% (w/v) CTAB) was added to the cell powder. The mixture was incubated at 65°C for 1 h, extracted with 50% choloroform/50% isoamyl alcohol (v/v) and precipitated with 100% ethanol. After RNAse I (10 µg/mL) treatment, the DNA was precipitated with ethanol, centrifuged and resuspended in sterile water.

Polymerase chain reaction conditions

Polymerase chain reactions (PCR) were performed using oligonucleotides designed based on the 1,3-β-glucan synthase (*PbFKS1*) gene (GenBank accession number AF148715). The sense primers were: S1 (5'-GGATATTACGACCAGAATGAC-3') and S2 (5'-CGGGATGATTGCGGTTATTGC-3'). The antisense primers were: ATS1 (5'-CTTCTTCAGAATCATGACCCCTGC-3'), ATS2 (5'-TGGAGCAGGTTCATCGGGATA-3') and ATS3 (5'-CCATAGTAGTCCATATCCGCAG-3'). The primer positions are shown in Figure 1A. PCR mixtures contained 0.5 µM of each primer, 1.5 mM $MgCl_2$, 2.5 mM of each dNTP, 2 U Taq DNA polymerase (GE Healthcare, Amersham Biosciences, Uppsala, Sweden), and 50 ng *P. brasiliensis* total DNA. PCR mixtures were subjected to an initial denaturation at 94°C for 3 min followed by 35 cycles at 94°C, 1 min and 30 s, 54°C, 1 min and 30 s, 72°C, 1 min and 30 s, and a final extension at 72°C for 10 min. The resulting 218- and 562-bp products corresponding to the reactions with S1-ATS1 and S2-ATS2 primers, respectively, were subcloned into pGEM-T-Easy (Promega, Madison, WI, USA). The sequences were determined on both strands by automated DNA sequencing, applying the method of Sanger et al. (1977). Three independent clones of each isolate were sequenced three times.

Southern blot

Southern blot analysis was performed according to standard procedures (Sambrook and Russel, 2001). The PCR or reverse transcriptase (RT)-PCR products were fractionated on

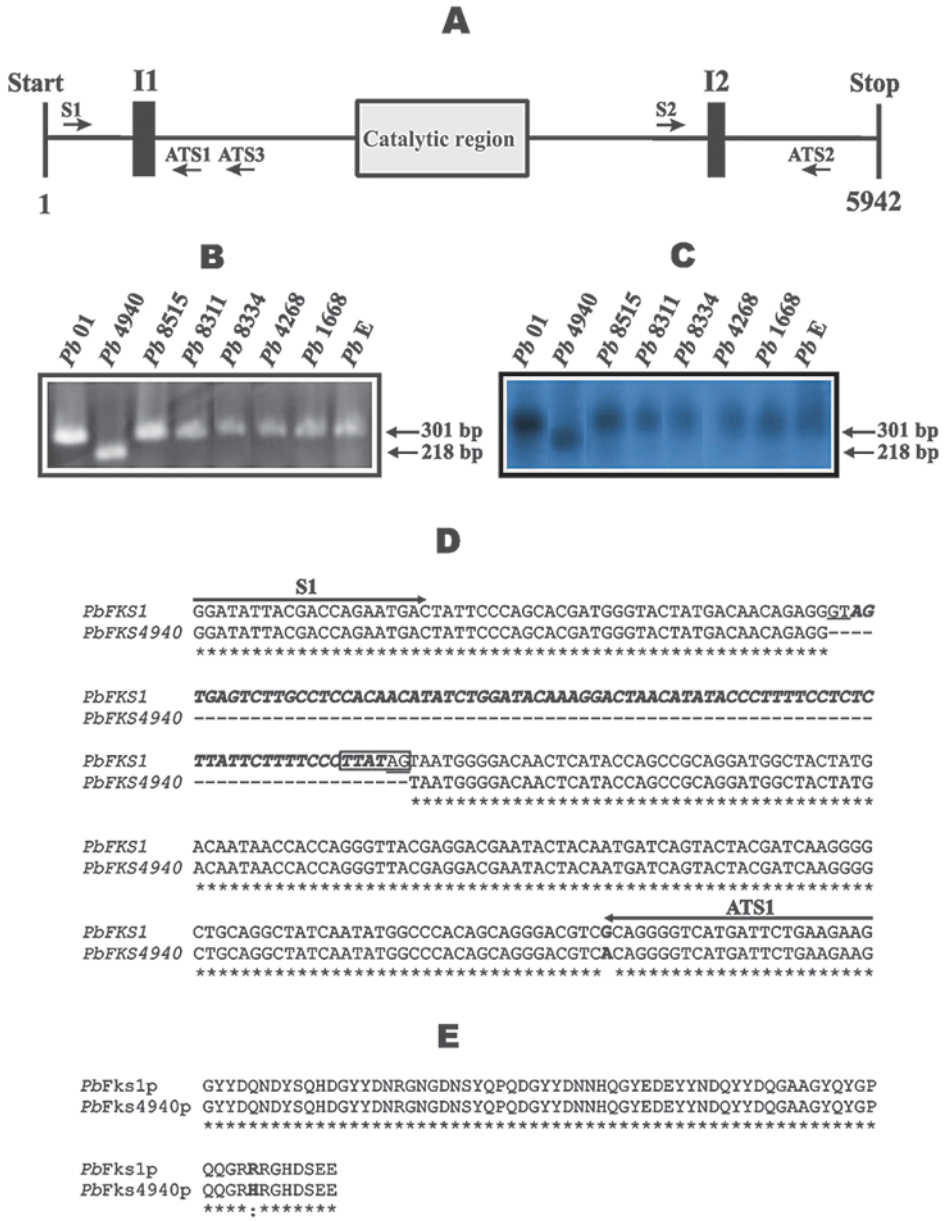

Figure 1. *PbFKS* 5' intron region analysis. **A.** Diagram representing the *PbFKS1* gene structure. The localization of the start codon, the stop codon, the introns I1 and I2 (5' and 3', respectively) and the catalytic region are shown. Numbers indicate nucleotide positions. Arrows indicate the primers sense and antisense used in PCR and RT-PCR. **B.** Agarose gel analysis of PCR of total DNA of *Paracoccidioides brasiliensis* with primers S1 and ATS1 corresponding to the 5' intron region of the *FKS* gene. Isolates: *Pb* 01, *Pb* 4940, *Pb* 8515, *Pb* 8311, *Pb* 8334, *Pb* 4268, *Pb* 1668, and *Pb* E. Arrows indicate the amplified products. **C.** Southern blot analysis. The gel in Figure 1B was transferred to a nylon membrane and hybridized to the *PbFKS1* P1 radiolabeled probe. Arrows indicate the hybridization products. **D.** Comparative analysis of the nucleotide sequences of the 5' region of the genomic fragments of *PbFKS1* and *PbFKS4940*. Primers used in the PCR are marked by arrows; 5' GT and 3' AG consensus motifs are underlined; the putative lariat sequence is boxed; intron is marked by bold italics and substituted nucleotides are in bold. The alignment was performed by using the CLUSTAL X program. **E.** Comparative analysis between the predicted amino acid sequences of the 5' region from *Pb*Fks1p and *Pb*Fks4940p. The single substituted amino acid is shown in bold. The alignment was performed by using the CLUSTAL X program.

a 0.8% (w/v) agarose gel and transferred to a nylon membrane, after denaturation for 40 min in 0.5 M NaOH. By using *PbFKS1* gene as template (Pereira et al., 2000), the P1 (218 bp) and P2 (562 bp) probes were obtained using S1-ATS1 and S2-ATS2 primers, respectively, as described above. The probes were labeled with (α-^{32}P) dCTP using a random primer DNA labeling kit RPN 1604 (Amersham Biosciences). Pre-hybridization and hybridization reactions were performed at 37° and 42°C, respectively, in blocking reagent containing 50% (v/v) formamide. The blots were washed at 42°C with 0.1X SSC (0.015 M sodium saline citrate, 0.015 M NaCl), 0.1% (w/v) SDS.

RNA extraction

Total RNA was extracted from yeast with Trizol, according to the supplier's instructions (Gibco®, Invitrogen, Carlsbard, CA, USA). RNA samples were treated with RNAse-free DNAse I at 37°C for 1 h and 30 min, followed by phenol-chloroform extraction and ethanol precipitation.

Reverse transcriptase-polymerase chain reaction

RT-PCR was performed according to standard procedures (Sambrook and Russel, 2001). Complementary DNA was synthesized from total RNA (1 µg) in the presence of the synthetic oligonucleotide antisense ATS3 derived from the *PbFKS1* sequence. A quarter of the RT reaction was amplified by PCR using the sense (S1) and antisense (ATS1) oligonucleotides derived from the *PbFKS1* sequence (see Figure 1A). The cDNA synthesis reaction was performed at 37°C for 1 h. PCR mixtures were subjected to an initial denaturation at 94°C for 3 min followed by 35 cycles at 94°C for 1 min and 30 s, 54°C for 1 min and 30 s, 72°C for 1 min and 30 s, and a final extension at 72°C for 10 min. The resulting 218- and 301-bp products corresponding to the reactions with S1-ATS1 primers, respectively, were subcloned into pGEM-T-Easy (Promega). The sequences were determined on both strands by automated DNA sequencing, applying the method of Sanger et al. (1977). Three independent clones of each isolate were sequenced three times.

Sequence analyses

The DNA sequences obtained were analyzed in the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) database using the BLAST program (Altschul et al., 1990). The similarity values of the DNA or protein sequences were based on the alignment of sequences taking into account conserved substitutions of residues, using the CLUSTAL X software (Thompson et al., 1997). The nucleotide sequences of intron 1 (5' region) and of intron 2 (3' region) of the *PbFKS4940* have been deposited at the GenBank database under accession numbers AY297814 and AY297815, respectively.

RESULTS

Analysis of the 5' region of the *PbFKS* in *Paracoccidioides brasiliensis* isolates

We analyzed the 5' region corresponding to intron 1 (5' region of *PbFKS1*) in isolates of

P. brasiliensis. Figure 1B presents the amplification of the fragment corresponding to the 5' region of the *FKS* genes of *Pb* 01, *Pb* 4940, *Pb* 8515, *Pb* 8311, *Pb* 8334, *Pb* 4268, *Pb* 1668, and *Pb* E isolates. We can observe the amplification of a 301-bp fragment, in *Pb* 01, corresponding to the expected size of *PbFKS1*. The primers derived from the *PbFKS1* gene sequence amplified identical 301-bp products in six other selected isolates of *P. brasiliensis* (Figure 1B). The *Pb* 4940 isolate provided a short fragment of 218 bp with the cited primers. Southern blotting was performed by using the P1 product corresponding to the *FKS1* gene as a probe (Figure 1C). Hybridization shows the presence of homologous fragments in all isolates. All the fragments of 301 and the one of 218 bp were gel purified, cloned and sequenced. The sequences of all isolates were very similar, except for that of the *Pb* 4940. Figure 1D presents the alignment between the 301- and the 218-bp products of *PbFKS1* and *PbFKS4940*, respectively. In contrast to all other isolates, *Pb* 4940 did not show the previously described 5' intron (Pereira et al., 2000). A 98% correlation was observed at the nucleotide level when the coding regions upstream and downstream from the 5' intron were compared between *Pb* 4940 and the other isolates. Figure 1E shows the alignment between *Pb*Fks1p and *Pb*Fks4940p predicted partial proteins. A 99% identity was observed at the amino acid level when comparing the deduced protein sequence of the amplified products. A single amino acid substitution R/H was observed when both sequences were compared.

Analysis of the 3' region of the *PbFKS* in *Paracoccidioides brasiliensis* isolates

We performed an analysis of the 3' region of the *PbFKS* in all isolates of *P. brasiliensis*. Figure 2A shows the amplification of the fragment corresponding to the 3' region of the *FKS* gene in *Pb* 4940 and *Pb* 01. The isolates exhibit the *FKS* product of 562 bp (region 3' of *PbFKS1*). Southern blotting was performed using the probe P2 (Figure 2B). The same result was obtained with all the other isolates (Figure 2C). Both fragments hybridized with the amplified 562-bp fragments corresponding to *PbFKS1* and *PbFKS4940*. Figure 2D presents the alignment between the 562-bp products of *PbFKS1* and *PbFKS4940*. An identity value of 98% was obtained by comparison of the nucleotides in both amplified PCR products. When the deduced sequences of amino acids were compared to each other, two amino acid substitutions were observed, I/T and S/N (Figure 2E).

Expression analysis of *PbFKS*

The expression of *PbFKS1* and *PbFKS4940* mRNAs was analyzed by RT-PCR. The assay provided a product of 218 bp (Figure 3A, lanes 2 and 3). Southern blot analysis showed strong hybridization of the 218-bp product to the P1 probe (Figure 3B, lanes 2 and 3). The 218-bp product was cloned into plasmid pGEM-T-easy and sequenced, showing 100% identity to the 5' region of *PbFKS4940* (Figure 3C). This result provides evidence of the presence of the *PbFKS* transcripts in the *Pb* 01 and *Pb* 4940 isolates. The same result was obtained with all the other isolates (data not shown).

DISCUSSION

In the present study, we analyzed the structure of the *FKS* gene in isolates of *P. brasi-*

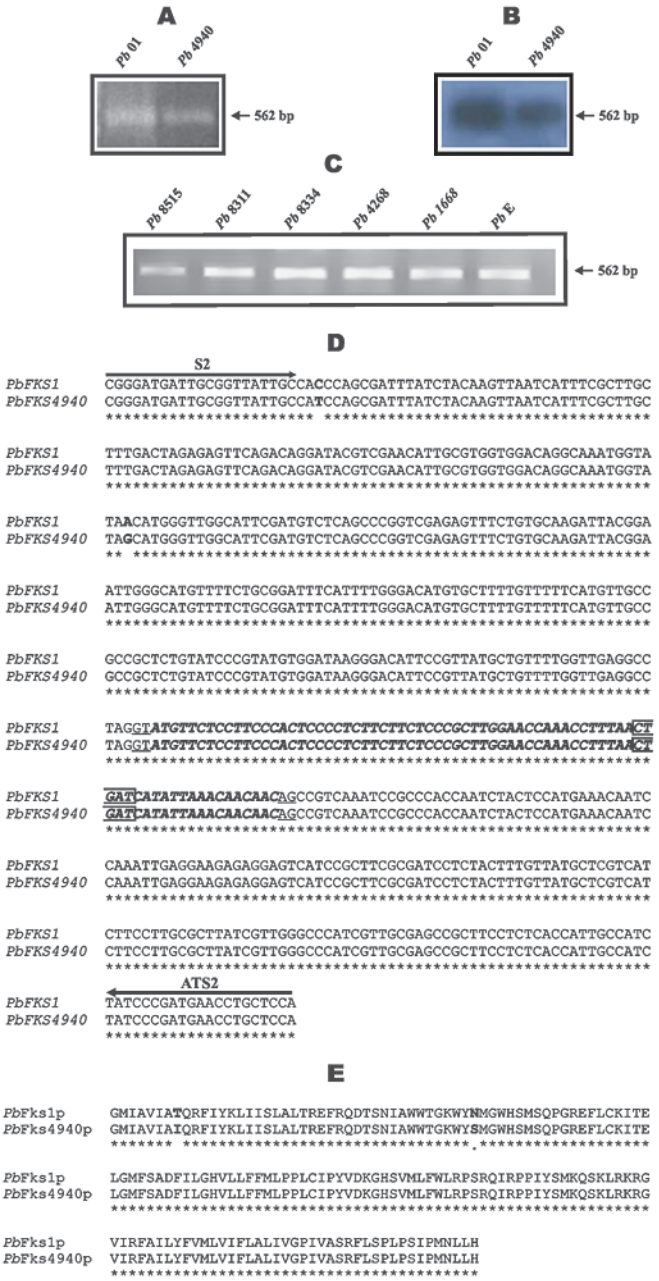

Figure 2. *PbFKS* 3' intron region analysis. **A.** Agarose gel analysis of PCR products of total DNA of *Paracoccidioides brasiliensis* isolates *Pb* 01 and *Pb* 4940 with primers S2 and ATS2 corresponding to 3' region of *FKS* gene. The arrow indicates the amplified product. **B.** Southern blot analysis. The gel was transferred to the nylon membrane and hybridized to the 3'*PbFKS1* P2 radiolabeled probe. The arrow indicates the hybridization product. **C.** Agarose gel analysis of PCR products of total DNA de *P. brasiliensis* isolates *Pb* 8515, *Pb* 8311, *Pb* 8334, *Pb* 4268, *Pb* 1668, and *Pb* E with primers S2 and ATS2 corresponding to 3' region of *FKS* gene. **D.** Comparative analysis of the nucleotide sequences of the 3' region from the genomic fragments of *PbFKS1* and *PbFKS4940*. Primers used in the PCR are marked by arrows; 5' GT and 3' AG splicing sites are underlined; the putative lariat sequence is boxed; intron is in bold italics, and substituted nucleotides are in bold. The sequence alignment was performed by using the CLUSTAL X program. **E.** Comparative analysis between the predicted amino acid sequences of the 3' region of *Pb*Fks1p and *Pb*Fks4940p. The substituted amino acids are shown in bold. The alignment was performed by using the CLUSTAL X program.

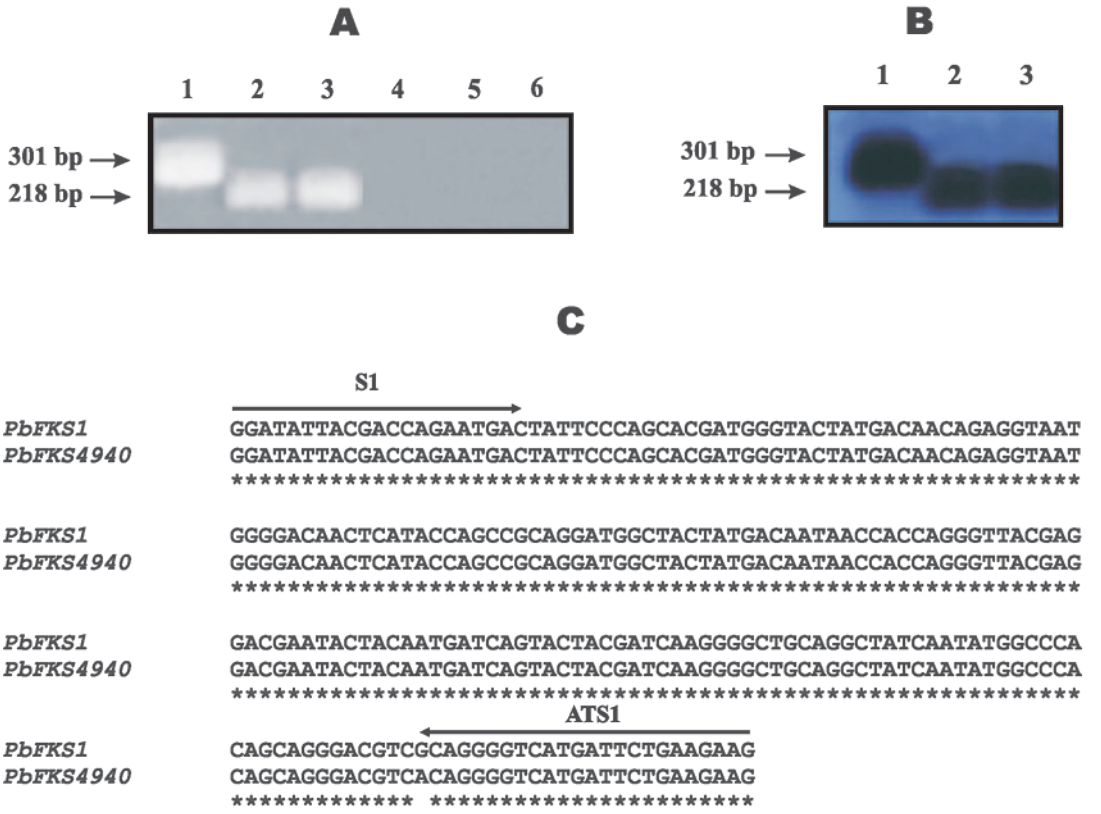

Figure 3. Expression analysis of *PbFKS* in the *Pb* 01 and *Pb* 4940 isolates. **A.** Gel analysis of RT-PCR products obtained by using primers S1, ATS1 and ATS3 corresponding to 5' region of the gene *PbFKS*. PCR control with *Pb* 01 DNA (lane 1); RT-PCR of *Pb* 01 (lane 2); RT-PCR of *Pb* 4940 (lane 3); control reaction without RT for *PbFKS1* (lane 4); control reaction without RT for *PbFKS4940* (lane 5); control reaction without RNA and RT (lane 6). The arrows indicate the amplified products. **B.** Southern blot analysis. The gel was transferred to the nylon membrane and hybridized to the *PbFKS1* P1 radiolabeled probe. The arrows indicate the hybridization product. **C.** Comparative analysis of the nucleotide sequences of the 5' region from *PbFKS* transcripts. Primers used in the PCR are marked by arrows. The sequence alignment was performed by using the CLUSTAL X program.

liensis. Since the cell wall participates in the morphogenesis and in the differentiation process in fungi (Brunner and Nurse, 2000; Goode et al., 2000), we looked at the structure of the 1,3-β-glucan synthase gene in clinical isolates of *P. brasiliensis*. This gene is involved in septation, polarized growth, mating, spore wall formation, and spore germination in fungi (Mazur et al., 1995; Cortes et al., 2002; León et al., 2002). In *P. brasiliensis*, it was observed that β-glucans are present in the mycelium while α-glucans are prevalent in the yeast cell wall (Kanetsuna et al., 1969), indicating a possible role of cell wall enzymes in fungal dimorphic transition. Six isolates examined in this study showed the same gene structure previously reported for *Pb* 01 (Pereira et al., 2000), suggesting a common *FKS* ancestor gene. The *Pb* 4940 had lost one intron at the 5' position and maintained the other one at the 3' position. In *S. cerevisiae*, 50% of the paralogous genes had lost several introns even though they were derived from recent duplication events (Bon et al., 2003).

Events of intron loss are discussed in the literature. According to Fink (1987) genes can lose their introns due to homologous recombination with reverse-transcribed cDNA by endogenous RT activity. The recombination would provide new exons and therefore, new gene and protein structures as the raw material for the evolution of new gene functions (Gilbert, 1987; Gilbert et al., 1997). According to Bon et al. (2003), the surviving introns may lead to adaptation to a specific life style (Keeling et al., 1998; The Génolevures Project, 2000) and may confer a selective advantage to the organism (Bon et al., 2003). Non-LTR retrotransposable elements (LINEs) might be the source of such endogenous RT activity (Dhellin et al., 1997). Our group developed the "*P. brasiliensis* Functional and Differential Genome Project - Brazilian Middle-West Network" (Felipe et al., 2005). By analyzing the transcriptome of the *P. brasiliensis* we detected LINE elements, which could be involved in that process.

The presence of the *PbFKS1* and *PbFKS4940* transcripts suggests that it should be a functional gene. This finding is important since the intron loss at the 5' end could not be correlated with loss of function of *PbFKS* in isolate *Pb* 4940. Intron loss can lead to gene function loss; in humans, the LINEs could be the source of the endogenous RT activity, which produces pseudogenes (Dhellin et al., 1997).

Events of intron gain and loss can be markers for phylogenetic analyses. Serotypes A and D of *C. neoformans* were classified based on *FKS1* sequence structure (Tanaka et al., 2003). Our data suggest a divergent evolution for introns with regard to the *FKS* gene in *Pb* 4940 isolate, suggesting an active mechanism for the intron loss by that isolate. In addition, the other isolates analyzed in this study could have a facilitated mechanism for intron gain or maintenance. Therefore, the present results suggest the usefulness of this intron-based PCR method for taxonomic studies. To our knowledge this is the first description of intron loss in isolates of *P. brasiliensis*. The evolutionary role of this event remains to be elucidated in *P. brasiliensis*. Future study will focus on other isolates in order to study the biological role of those findings.

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