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Cloning and Nucleotide Sequence of a Specific DNA Fragment from *Paracoccidioides brasiliensis*†

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We cloned and sequenced a species-specific 110-bp DNA fragment from *Paracoccidioides brasiliensis*. The DNA fragment was generated by PCR with primers complementary to the rat β-actin gene under a low annealing temperature. Comparison of the nucleotide sequence, after excluding the primers, with those in the GenBank database identified approximately 60% homology with an exon of a major surface glycoprotein gene from *Pneumocystis carinii* and a fragment of unknown function in *Saccharomyces cerevisiae* chromosome VIII. By Southern hybridization analysis, the ^{32}P-labelled fragment detected 1.0- and 1.9-kb restriction fragments within whole-cell genomic DNA of *P. brasiliensis* digested with *Hin*dIII and *Pst*I, respectively, but failed to hybridize to genomic DNAs from *Candida albicans*, *Blastomyces dermatitidis*, *Cryptococcus neoformans*, *Aspergillus fumigatus*, *Saccharomyces cerevisiae*, *Pneumocystis carinii*, rat tissue, or humans under low-stringency hybridization conditions. Additionally, the specific DNA fragment from three different *P. brasiliensis* isolates (Pb18, RP18, RP17) was amplified by PCR with primers mostly complementary to nonactin sequences of the 110-bp DNA fragment. In contrast, there were no amplified products from other fungus genomic DNAs previously tested, including *Histoplasma capsulatum*. To date, this is the first species-specific DNA fragment cloned from *P. brasiliensis* which might be useful as a diagnostic marker for the identification and classification of different *P. brasiliensis* isolates.

Paracoccidioides brasiliensis, a thermally dimorphic pathogenic fungus, is the etiologic agent of paracoccidioidomycosis, the most prevalent systemic mycosis in South America (7–10). Paracoccidioidomycosis is a disorder that primarily involves the lungs and then disseminates to other organs. Secondary lesions appear frequently in the mucous membranes, lymph nodes, skin, and adrenals (1). Both the clinical presentation and the course of the disease vary from patient to patient. *P. brasiliensis* grows as a yeast form in host tissues and in the laboratory when it is incubated at 37°C (4). Growth of the yeast form may first be recognized after 10 to 15 days and, as a mold, after 20 to 30 days when cultures are incubated at room temperature (4). Culture and histological diagnoses rely upon the most characteristic feature of the yeast form, which is the pilot's wheel appearance: a mother cell surrounded by multiple peripheral daughter cells. Serological diagnosis has shown deficiencies with regard to sensitivity and specificity, especially with cross-reactivity with other fungi such as *Histoplasma capsulatum* (3–6). Rapid and efficient molecular biological methods for identifying and distinguishing different isolates of *P. brasiliensis* are needed for diagnosis, epidemiologic investigations, and determination of mechanisms of infection. To date, there have been no reports of a *P. brasiliensis* DNA sequence that might function as a useful tool that can be applied to these problems. We report on the cloning and sequencing of a specific DNA fragment from *P. brasiliensis.*

P. brasiliensis (Pb18) was grown as yeasts in 500 ml of Sabouraud broth at 37°C for 5 days. The yeast cells were pelleted by centrifugation (1,800 × *g*), washed with distilled deionized water, resuspended in 3.0 ml of 0.9 M sorbitol–0.1 M EDTA–50 mM dithiothreitol (pH 7.5)–15 mg of Zymolase 20T (Seikagaku Kogyo, Ltd., Tokyo, Japan), and incubated at 37°C for 120 min. Protoplasts were pelleted and resuspended in 3.0 ml of 50 mM Tris-HCl–50 mM EDTA (pH 8.0)–1% sodium

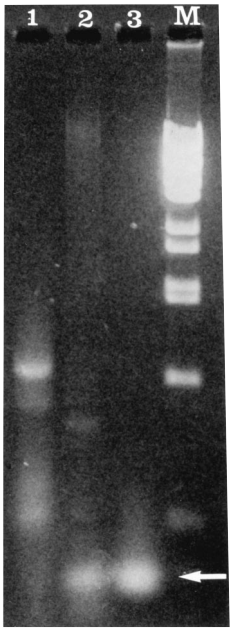

FIG. 1. Ethidium bromide-stained gel of the PCR control β-actin fragment from rat cDNA (lane 1), the 110-bp fragment generated by PCR with rat β-actin primers from *P. brasiliensis* genomic DNA (lane 2), and the purified 110-bp DNA fragment (lane 3); lane M, bacteriophage lambda DNA *Bst*EII digest marker (New England BioLabs). The arrow indicates 110 bp.

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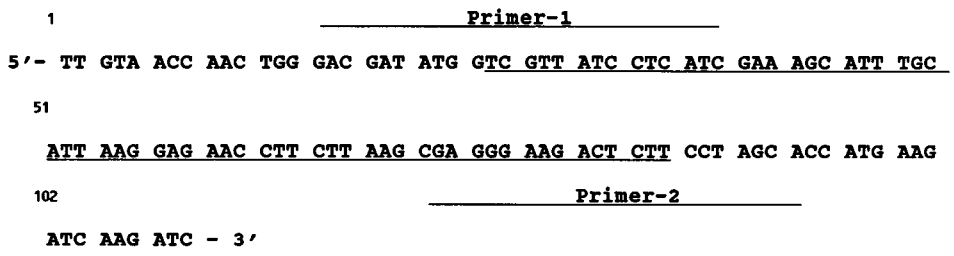

FIG. 2. Nucleotide sequence of the DNA fragment cloned from *P. brasiliensis*. The nucleotide sequence that does not include the rat β-actin primer region is underlined. Note the primers mostly complementary to the nonactin sequences of the DNA fragment.

dodecyl sulfate (SDS). The suspension was incubated at 65°C for 30 min. Genomic DNA was extracted with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) and was precipitated from the aqueous phase with a 1/10 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of ethanol. The DNA pellet was resuspended in TE buffer (0.01 M Tris, 0.001 M EDTA [pH 7.5]). The *P. brasiliensis* 110-bp DNA fragment was generated from genomic DNA by PCR in a total volume of 50 μl, which contained 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.4 mM (each) nucleotide, 1.25 U of Amplitac (Perkin-Elmer Cetus, Norwalk, Conn.), 1 μM (each) primer encoding a rat β-actin gene exon (5′-TTGTAACCAACTGGGACGATATGG-3′ and 5′-GATCT TGATCTTCATGGTGCTAGG-3′, which correspond to upstream bases 1552 to 1575 and the downstream bases 2992 to 2844 in rat genomic DNA [Clontech, Palo Alto, Calif.], respectively), and 300 ng of *P. brasiliensis* genomic DNA. The primers were expected to amplify a 764-bp β-actin gene fragment from rat cDNA (5). The DNA amplification was initiated with a 5-min denaturation step at 96°C; this was followed by 35 cycles at 96°C for 60 s, 37°C for 60 s, 72°C for 2 min, and a final 10-min extension step at 72°C. The 110-bp amplified DNA fragment (10 μl) was analyzed by electrophoresis in 1% agarose in 1× TBE buffer at 120 V for 30 min (Fig. 1).

Cloning of the DNA fragment was carried out by ligating the DNA fragment into pGEM-T vector (Promega, Madison, Wis.) and transforming *Escherichia coli* XL1-blue. The identity of the cloned insert was confirmed by Southern blot hybridization. The DNA sequence was determined from single-stranded sequencing templates by using Sequenase T7 polymerase (U.S. Biochemicals) (8). DNA sequencing revealed a fragment of 110-bp including the rat β-actin primers (Fig. 2). Comparison of the nucleotide sequence, after excluding the primers from the DNA fragment, against sequences in the GenBank database identified an approximately 60% identity with an exon of a major surface glycoprotein gene from *Pneumocystis carinii* and a fragment with an unknown function in *Saccharomyces cerevisiae* chromosome VIII (2). Initially, the objective of the present study was to amplify actin gene-related sequences from *P. brasiliensis* genomic DNA by using rat β-actin primers. Interestingly, only one DNA fragment was amplified, and this fragment has no identity with any actin gene from heterologous species. Besides the 110-bp DNA fragment, other amplified fragments with different sizes were occasionally obtained with this PCR. These might be actin gene-related sequences. However, we have not yet cloned and sequenced these DNA fragments.

To determine the specificity of the 110-bp DNA fragment for *P. brasiliensis*, *Hin*dIII-digested genomic DNA from rat (Wistar), humans, clinical isolates of *Candida albicans*, *P. carinii* and *Aspergillus fumigatus*, *H. capsulatum* (G184AS), *Cryptococcus neoformans* (B-3501), *Blastomyces dermatitidis* (ATCC 26199), *S. cerevisiae* (ATCC 64665), and *P. brasiliensis* (Pb18) were transferred to a nylon membrane by the method of Southern (9) and were hybridized to ^{32}P-labeled 110-bp DNA fragment from *P. brasiliensis* at 42°C under aqueous hybridization conditions. Washings were performed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) plus 0.1% SDS twice for 20 min at 37°C and twice in 1× SSC plus 0.1% SDS at 65°C. Filters were exposed to Kodak XAR-5 film at −70°C in the presence of an intensify ing screen for 48 h. Southern blot analysis revealed that the 110-bp fragment detected restriction fragments of 1.0 and 1.9 kb in the *Hin*dIII- and *Pst*I-digested genomic DNAs from *P. brasiliensis*, respectively, but that it failed to hybridize to the other genomic DNAs, including *P. carinii* and *S. cerevisiae* (Fig. 3). Additionally, the specific DNA fragment was amplified by PCR from three different *P. brasilien sis* isolates (Pb18, RP18, RP17) (Fig. 4) by using primers mostly complementary to nonactin sequences of the 110-bp DNA fragment, 5′-ACGATATGGT CGTTATCCTCATC-3′ (primer 1) and 5′-GTGCTAGGAAGA GTCTTCCCT-3′ (primer 2), which correspond to upstream bases 16 to 28 and the downstream bases 74 to 94, respectively (Fig. 3). In contrast, there were no amplified products from other fungal genomic DNAs previously tested, including *H. capsulatum* (Fig. 4).

Identification of a specific DNA fragment for *P. brasiliensis* may provide an attractive tool for the detection and classification of *P. brasiliensis* strains in different clinical specimens. Further investigations with this specific DNA fragment are under way to develop a PCR method that can be used to diagnose paracoccidioidomycosis.

Nucleotide sequence accession number. The DNA sequence of the 110-bp DNA fragment from *P. brasiliensis* has been submitted to GenBank (accession number U23426).

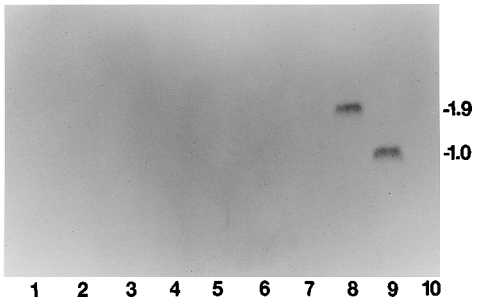

FIG. 3. Hybridization of 110-bp DNA fragment under aqueous conditions at 42°C to *Hin*dIII-digested whole-cell DNAs from *C. albicans* (lane 1), *C. neoformans* (lane 2), *B. dermatitidis* (lane 3), *A. fumigatus* (lane 4), *S. cerevisiae* (lane 5), *P. carinii* (lane 6), rat tissue (lane 7), and humans (lane 10). Note the 1.9-kb *Pst*I (lane 8) and 1.0-kb *Hin*dIII (lane 9) fragments from *P. brasiliensis* Pb18.

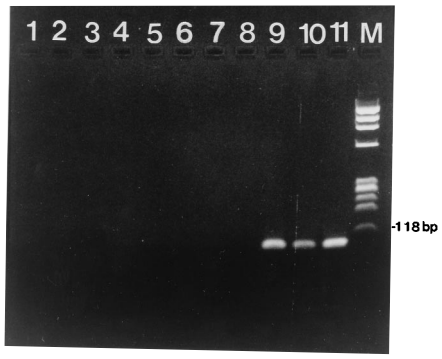

FIG. 4. Ethidium bromide-stained gel of the PCR-specific DNA fragment from genomic DNAs of *P. brasiliensis* Pb18 (lane 9), RP18 (lane 10), and RP17 (lane 11) by using primers mostly complementary to nonactin sequences of the 110-bp DNA fragment. Note that there is no detectable amplified DNA fragments from *C. albicans* (lane 1), *C. neoformans* (lane 2), *B. dermatitidis* (lane 3), *A. fumigatus* (lane 4), *S. cerevisiae* (lane 5), *P. carinii* (lane 6), *H. capsulatum* (lane 7), or rat (lane 8) genomic DNA. Lane M, *Hae*III ϕ174 digest marker (VWR Scientific).

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