Fungal Genetics and Biology **20,** 125–131 (1996)
Article No. 0027

Molecular Cloning, Nucleotide Sequencing, and Characterization of a 27-kDa Antigenic Protein from *Paracoccidioides brasiliensis*

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Accepted for publication March 29, 1996

McEwen, J. G., Ortiz, B. L., García, A. M., Florez, A. M., Botero, S., and Restrepo, A. 1996. Molecular cloning, nucleotide sequencing, and characterization of a 27-kDa antigenic protein from *Paracoccidioides brasiliensis. Fungal Genetics and Biology* 20, 125–131. A gene encoding a 27-kDa antigenic protein from *Paracoccidioides brasiliensis* was cloned, sequenced, and characterized. A cDNA library of the mycelial phase was produced and packed in Uni-Zap-XR vector, λ Zap II synthesis kit (Stratagene, La Jolla, CA). The screening of the library was carried out using a pool of sera from paracoccidioidomycosis patients that had proven reactive in serological testing. Among 44,000 immunoscreened clones from the library, 2 were positive (clones 2 and 3). The former was not characterized further. The latter has a 1-kb DNA insert with an open reading frame encoding a protein of 259 amino acids with a predicted molecular mass of 28.6 kDa (27 kDa by SDS–PAGE). This protein corresponds to a 25-kDa protein in antigenic preparations of *P. brasiliensis* as determined by Western blot analysis. Comparison of the transcribed sequence with different gene banks failed to reveal a high degree of homology with other proteins. The cloned DNA fragment was easily expressed in *Escherichia coli* without the need of induction by isopropyl-β-D-thiogalactopyranoside. These findings suggest that the gene encodes a *P. brasiliensis*-specific protein.

Paracoccidioides brasiliensis is the dimorphic fungus responsible for paracoccidioidomycosis (PCM),[2] one of the most important systemic mycosis in Latin America. Both the disease and its etiologic agent have been the subject of many studies (Brummer *et al.*, 1993; Restrepo, 1994). There are still many unsolved problems in the field of paracoccidioidomycosis. One of them is the lack of information on the natural habitat of the etiologic agent, *P. brasiliensis* (Restrepo, 1994). Others are the difficulties encountered in the production of reliable and reproducible antigens for diagnosis and immunological evaluations (Travassos, 1994). Molecular biology is consequently an interesting tool to explore these and other, unsettled questions. However, there have been only a few studies on the molecular biology aspects of this fungus. Taba *et al.* (1989) generated a genomic library of *P. brasiliensis* in its yeast phase in λgt11 and obtained a clone that expressed the antigenic protein gp43. Further work with this clone showed that it encoded a polypeptide of 416 amino acids and possessed a 78-bp intron (Travassos *et al.*, 1995). Using primers complementary to the rat β-actin gene, Goldani *et*

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[2] Abbreviations used: PCM, paracoccidioidomycosis; CIB, Corporación para Investigaciones Biológicas; IPTG, isopropyl-β-D-thiogalactopyranoside.

1087-1845/96 $18.00

al. (1995) amplified and cloned a 110-bp fragment from *P. brasiliensis* that did not hybridize with other fungi. The present report describes the construction of a *P. brasiliensis* mycelial cDNA expression library and the cloning and expression of genes coding for *P. brasiliensis* antigenic proteins.

MATERIALS AND METHODS

Culture Conditions

P. brasiliensis (ATCC 32069) from the collection of the Mycology Section of the Corporación para Investigaciones Biológicas (CIB) was grown in the mycelial phase, in the modified liquid synthetic medium of McVeigh and Morton (Restrepo and Jimenez, 1980). The fungus was subcultured weekly. For antigen preparation, cultures were grown for 10–12 days at room temperature (22 ± 4°C) in a gyratory shaker set at 120 rpm.

Isolation of mRNA and Library Construction

Total RNA was obtained as indicated in the technique described by Promega (Promega Corp., Madison, WI). Briefly, cells from a 10-day-old *P. brasiliensis* mycelial culture incubated as indicated above were harvested, frozen in liquid nitrogen, and broken with mortar and pestle. The mRNA was isolated using an oligo(dT) cellulose column. Five micrograms of mRNA were used for the construction of the library. The cDNA library was constructed using a λ Zap II synthesis kit and packed in Uni-Zap-XR vector. Plating, titering, and amplification of the library was performed in *Echerichia coli* SURE strain using 90-mm NZY dishes (Stratagene, La Jolla, CA).

Screening of the cDNA Library and Selection of Clones

Nitrocellulose membranes were applied to plates of recombinant plaques and left overnight at 39°C. Membranes thus prepared were probed with a pool of sera from 16 PCM patients, diagnosticated by serological test, and treated at the CIB. The initial screening was performed with rabbit anti-human peroxidase-labeled secondary antibody. Further screenings were performed with the streptavidin–alkaline phosphatase system (Amersham, Aylesbury, UK).

Analysis of Positive Clones

Additional analyses and characterization were carried out in the phagemid pBluescript II (SK) (Stratagene). Restriction enzyme digestion of the phagemids was performed with *Eco*RI and *Xho*I and other restriction enzymes (6-base cutters).

Sequence Analysis

Nucleotide sequencing of the cDNA clone was done by the chain-termination method (Sanger *et al.,* 1977) using alkaline denaturation of double stranded DNA and the Sequenase kit Version 2.0 (United States Biochemicals, Inc., Cleveland, OH). T3 (forward) and T7 (reverse) oligonucleotide sequencing primers were used initially. The entire sequence was determined in both strands. The forward orientation was sequenced by progressive unidirectional deletions with the Erase-a-Base System (Promega). The reverse orientation was sequenced by subcloning several fragments upon digestion with restriction enzymes and by priming with synthetic oligonucleotides to fill in data gaps.

An additional run was done using an automatic sequencer at the DNA core unit of the University of Minnesotta (Applied Biosystems). The search for homologies was done through several of the E-mail servers using the NCBI, SWISS-PROT, EMBL, and PIR databases (Altschul *et al.,* 1990; Harper, 1994). The sequence analysis was carried out with the following programs: DNA Strider 1.1 by Christian Marck and DNAid+.1.8 by Frédéric Dardel.

Antigen Preparation and Antigenic Characterization of the Recombinant Protein

A cytosolic antigen preparation of *P. brasiliensis* was obtained as previously described (Restrepo *et al.,* 1984). The cytosolic antigen and the recombinant proteins synthesized by *E. coli* were analyzed by SDS–PAGE gels (12%) and by Western blot (Ausbel *et al.,* 1987) with a 1:1000 dilution of the pool of sera from paracoccidioidomycosis patients. The specific antibody reaction was developed using the blotting detection kit for human antibodies (Amersham International, UK). The antigen concentrations loaded by track varied between 0.5 and 0.2 μg. The

Bradford technique was used to quantitate the amount of protein present in the samples (Bradford, 1976).

Purification of the Recombinant Protein by Preparative Electrophoresis and Production of Hyperimmune Mouse Serum

Preparative electrophoresis was performed in a Prep Cell apparatus (Bio-Rad, Prep Cell Model 491) following the manufacturer's directions. The cylindrical electrophoresis cell was filled with a 12% polyacrylamide solution up to 5 cm, and then the solution was covered with water and allowed to polymerize overnight and overlayed with a 1-cm high stacking gel. Once polymerized the stacking gel was loaded with the protein mixture from the pelleted cells from a 20-ml *E. coli* culture in midlog phase and resuspended in sample buffer. Electrophoresis was performed at 10–12 W for 12 h. Five-milliliter fractions were collected and analyzed by SDS–PAGE gels.

Hyperimmune serum was prepared in groups of 5- to 6-week-old BALB/c mice, injected intraperitoneally with 150 μg of purified 27-kDa recombinant protein. They were first challenged with complete Freund's adjuvant; subsequent boostings were given at 3-week intervals with incomplete Freund's adjuvant. Bleeding though the lateral ocular veins was carried out 1 week after the last boosting.

Nucleotide Sequence Accession Numbers

The cDNA sequence data from the 27-kDa antigenic protein of *P. brasiliensis* reported in this paper have been submitted to GenBank and assigned the Accession No. U41503.

RESULTS

Molecular Cloning

The integrity of the total RNA and mRNA was evaluated by agarose gel electrophoresis. Upon titering and amplification, the library yielded 90–95% of recombinant plaques as determined by color selection using IPTG/X-gal. Two positive recombinants were detected upon initial screening of 44,000 plaques with a pool of sera from paracoccidioidomycosis patients, followed by screening with rabbit anti-human peroxidase-labeled antibodies. Restriction enzyme digestion of the phagemids with *Eco*RI and *Xho*I showed that clone 2 released two DNA fragments of approximately 1.8 and 0.15 kb. Clone 3 showed a 1-kb insert (data not shown).

Expression and Antigenic Characterization of the Recombinant Proteins

Characterization of the recombinant proteins was performed by Western blot analysis using total protein extracts from the transformants (Ausbel *et al.*, 1987). Upon IPTG induction, clone 2 showed an immunoreactive band corresponding to a protein of approximately 58 kDa, and clone 3, an immunoreactive band of about 27 kDa with and without IPTG induction (Fig. 1). Using 40 individual sera from patients with paracoccidioidomycosis, the antigen produced by clone 2 was recognized by only 1 of the sample sera and, consequently, it was not further characterized. On the other hand the antigen produced by clone 3 was recognized by 40 of 44 (91%) of the sera from these patients (Ortiz *et al.*, 1995).

Antigenicity of the 27-kDa Protein and Immunoblot Characterization

The hyperimmune polyclonal sera obtained from the mice immunized with the cloned purified protein reacted with a single band of 25 kDa in mycelial extracts of *P. brasiliensis* (data not shown).

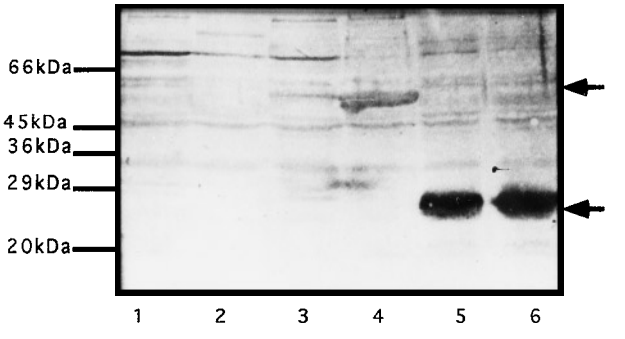

FIG. 1. Western blot analyses of *E. coli* containing the phagemid obtained from clones 2 and 3. Western blot was performed using a pool of sera from PCM patients. Secondary antibodies were labeled with streptavidin–alkaline phosphatase and developed with the appropriate substrate and color reagent. Lane 1 corresponds to *E. coli* strain XL1-blue. Lane 2 corresponds to the same *E. coli* strain containing the plasmid Bluescript II (SK). Lanes 3 and 4 correspond to *E. coli* containing phagemid from clone 2 without and with induction by IPTG. Lanes 5 and 6 correspond to *E. coli* containing phagemid from clone 3 without and with induction by IPTG.

Nucleotide and Amino Acid Sequences of the 27-kDa Protein

Double-stranded sequencing showed that the complete clone encoding the 27-kDa protein had 1151 bp. No promoter sequence was found upstream, possibly because of incomplete 5′ cloning during the construction of the cDNA library. The cloned sequenced construct is showed in Fig. 2a. It has an ORF for a 259-amino-acid polypeptide with a molecular mass of 28,646 Da. The amino acid composition predicts a p*K* of 9. There are two possible N-glycosylation sites at NXT and NXS. The recombinant protein has two main areas of hydrophilicity between the amino acids 81–86 and 193–200. The first 36 amino acids are coded by the vector and account for 3.6 kDa of the total 28.6 kDa transcribed by the clone. The final sequence contains 370 bp, from which there are 43 residues that belong to the poly(A) tail. The whole sequence and its translation to amino acids are shown in Fig. 2b.

The deduced amino acid sequence and the nucleotide sequence were used to search several of the E-mail servers using the NCBI, SWISS-PROT, EMBL, and PIR databases for homologous sequences. The search did not reveal the presence of any sequence having a significant homology.

DISCUSSION

This paper describes the cloning and sequencing of a gene encoding a 27-kDa antigenic protein from *P. brasiliensis* and the expression and characterization of this protein. When we started this study, the knowledge of the molecular biology of the fungus was limited and several difficulties were encountered when applying standard techniques to isolate the mRNA. One of the main problems for this isolation was breaking the strong cell wall of the fungus without causing degradation of the RNA. This problem was overcome by mechanical disruption using liquid nitrogen and grinding with mortar and pestle. We constructed a cDNA library from the mycelial phase cultures of the fungus and found that it exhibited a high percentage of recombinant plaques. We cloned two genes coding for antigenic proteins. One of them (clone 3) had a MW of 27 kDa in SDS–PAGE gels and seemed promising as its reactivity with 44 sera from paracoccidioidomycosis patients was 91% (Ortiz *et al.*, 1995).

Previous work (Taba *et al.*, 1989) resulted in the cloning of the antigenic protein gp43 using a genomic library of the yeast phase of this fungus, in the λgt11 vector. Unfortunately, the unstable clone was subsequently lost. A completely new approach was necessary to clone the gp43 gene again, including peptide sequencing of the protein, PCR amplification with degenerate primers, and screening of a genomic library with the amplified fragment (Travassos *et al.*, 1995). The coding sequence of this gene contains 416 amino acids, which correspond to 42.2 kDa, correlating with the MW of gp43 antigen in SDS–PAGE gels, the small difference being attributed to glycosylation at the single glycosylation site found in the sequence. The homology analysis of this corresponding sequence revealed a relatedness with *Candida albicans* and *Saccharomyces cerevisiae* exoglucanases; however, no glucanase activity was observed in this antigen preparation (Travassos *et al.*, 1995). Further information regarding such a sequence is now available at the GenBank Accession No. U26160 (Cisalpino *et al.* 1996). Gp43 is one of the most important antigenic proteins identified in culture filtrates of *P. brasiliensis* (Puccia *et al.*, 1986; Puccia and Travassos, 1991) and it is recognized by a large proportion (91%) of sera from paracoccidioidomycosis patients (Camargo *et al.*, 1989; Casotto *et al.*, 1991; Mendes-Giannini *et al.*, 1989; Stambuk *et al.*, 1988). In one of these studies it was possible to detect the presence of gp43 antigen directly in sera from paracoccidioidomycosis patients, even after 2 years of therapy. This could be due to the continuous liberation of detectable quantities of this fungal antigen, which becomes a persistent stimulus for the production of antibodies (Mendes-Giannini *et al.*, 1989).

A number of other antigenic proteins ranging from 13 to 148 kDa have been studied (Camargo *et al.*, 1989), but only some of them (23-, 43-, 57-, and 58-kDa proteins) appear to exhibit high antigenic activity, reactivity, and specificity. The main antigenic proteins of *P. brasiliensis* as identified by immunoblot are depicted in Table 1. As indicated, the main exocellular antigen, gp43, is reactive with 100% of the patient sera in some studies. Furthermore the antibody patterns differ between the acute juvenile and the chronic forms of paracoccidioidomycosis, and only gp43 was detected in the acute juvenile cases (Camargo *et al.*, 1991). Proteins with MW in the ranges of 70–72 and 23–27 are also of importance with regard to their reactivity with sera from paracoccidioidomycosis patients (Casotto *et al.*, 1991; McEwen *et al.*, 1995; Travassos, 1994).

The 23-kDa protein (Casotto *et al.*, 1991) is of particular relevance to our study because in the initial screening of our library, one of the antigenic proteins cloned had a similar molecular weight. If our clone 3 corresponds to the

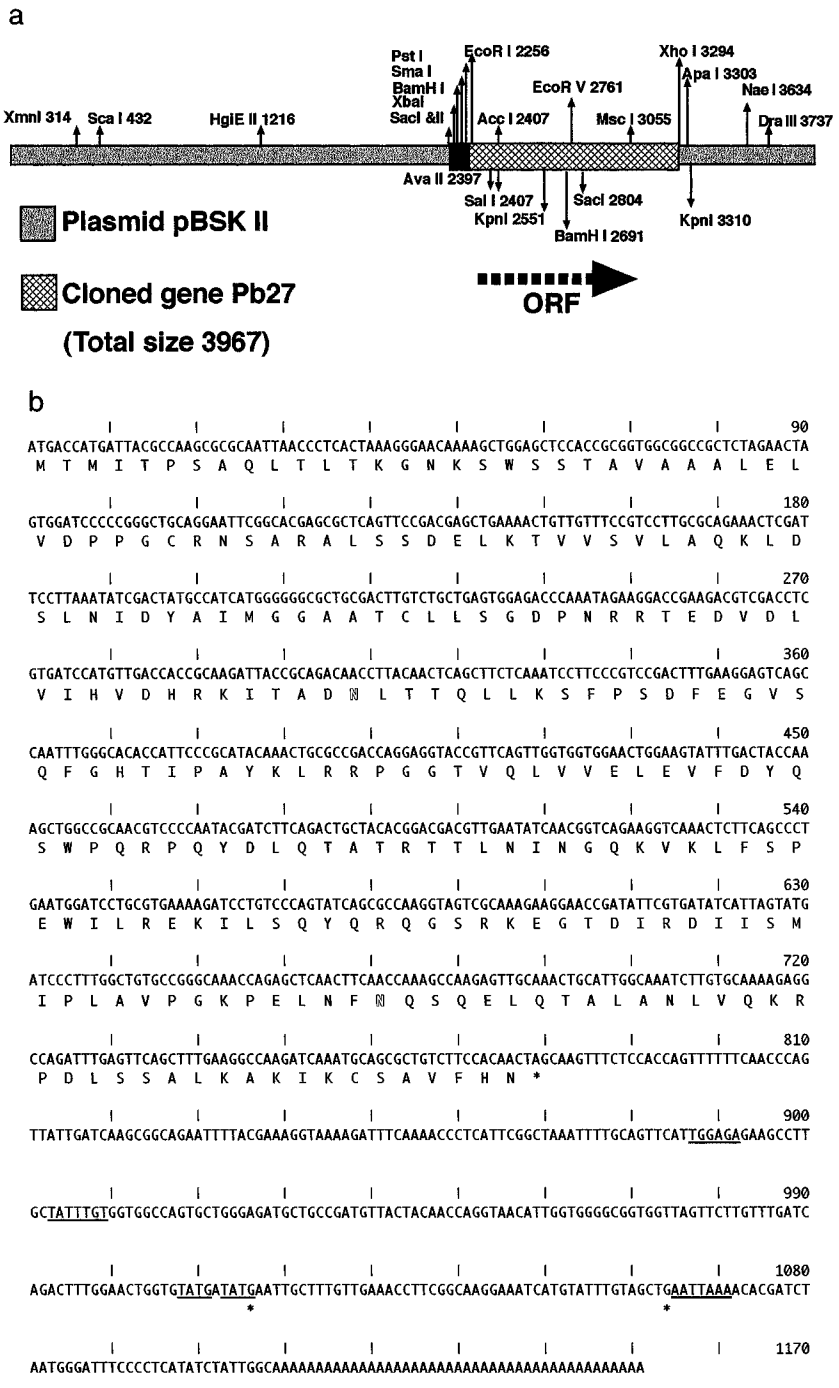

FIG. 2. Restriction map and nucleotide sequence of gene Pb 27. (a) Map of plasmid pBSKII carrying the Pb 27 gene (total 3967). Cloned between the restriction *Eco*RI (2256) and *Xho*I (3294) sites of the vector. The polylinker region is indicated by the black box. The restriction map and the position of the 6-cutter enzymes are indicated by the small arrows. The dashed arrow denotes the direction and length of the open reading frame. (b) Nucleotides and deduced amino acid sequence (in single-letter code) of the gene Pb 27 and its encoded polypeptide. Base numbers are shown on the right. Potential N-glycosylated sites are depicted as outlined characters. The first 36 amino acids are coded by the vector. In the 3′ adjacent region possible putative polyadenylation signals, according to Guo and Sherman (1995), are underlined and the putative stop codons are designated by asterisks.

TABLE 1
Immunodominant Antigens as Detected by Immunoblot

Protein	Percentage of reactivity in the presence of PCM sera	Reference
70–72 kDa	96%	Travassos, 1994
57–58 kDa	55%	Casotto *et al.*, 1991
43–45 kDa	90.6–100%	Camargo *et al.*, 1989; Casotto *et al.*, 1991; Ferreira-da-Cruz *et al.*, 1992
23–27 kDa	81–91%	Casotto *et al.*, 1991; McEwen *et al.*, 1995

23-kDa protein, it may be valuable in the study and diagnosis of chronic cases of PCM, because it is a specific antigen recognized by 81–91% of the patients with paracoccidioidomycosis (Casotto *et al.*, 1991).

The cloning and sequencing of the gene coding for a 27-kDa antigenic protein of this dimorphic pathogen are a significant contribution in a nascent field of study. The sequence and amino acid translation inferred from it indicated that the MW of the proteins is 28.6 kDa, which is in agreement with the inferred MW of the recombinant protein by SDS–PAGE gels. As indicated previously, the first 36 amino acids belong to the vector pBSK II, which accounts for the smaller size of the native protein (25 kDa) when evaluated by immunoblot using the cytosolic antigen of *P. brasiliensis.* The search for homologous sequences did not reveal any significant association with other sequences reported in the gene banks, supporting the contention that this is a specific protein for *P. brasiliensis.*

Cloning and expression of recombinant antigens from other dimorphic fungi have been described (Deepe and Durose, 1995). The H antigen of *Histoplasma capsulatum* has been already cloned, sequenced, and expressed; although it does not confer protective immunity in animal models, it may still be used for the diagnosis of histoplasmosis. Two previous studies by the same group identified an 80-kDa antigen that is homologous to HSP 70 kDa, as well as an antigen with MW of 62 kDa, which induces protective responses in mice (Gomez *et al.*, 1992).

The search and production of well-characterized antigens is a field of importance. In the future, they will probably replace most of the currently used diagnostic preparations which exhibit major drawbacks such as cross-reactivity, nonspecificity, and lack of reproducibility. By the same token, highly specific recombinant antigens are expected to play an important role in studies aimed at defining ecological problems such as spotting fungal microniches, when combined with other molecular biology techniques like PCR and probe hybridization. Furthermore, cloning of these antigenic proteins may open the way toward production of fungal vaccines.

We are currently working on the characterization of the clones reported here and some others obtained by further screenings of our library. We believe that the generation of this library could have great importance in the development of future studies on the molecular and general biology of *P. brasiliensis.*

ACKNOWLEDGMENTS

This work was supported by the "Fondo Colombiano de Investigaciones Cientificas *Antonio Jose de Caldas*" (COLCIENCIAS), Grant 2213-05-002-91. Additional financial help was provided by "Universidad Pontificia Bolivariana." We are indebted to Dr. María Mercedes Patiño for her continuous advice and encouragement, to the personnel of the section of Mycology for their advice and guidance in the handling of *P. brasiliensis*, and to Dr. Julian Molina for help with the sequencing.

REFERENCES

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. A. 1990. Basic local alignment search tools. *J. Mol. Biol.* **215:** 403–410.

Ausbel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. 1987. Analysis of Proteins. In *Current Protocols in Molecular Biology*, pp. 10.01–10.85. Wiley, New York.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248–252.

Brummer, E., Castañeda, E., and Restrepo, A. 1993. Paracoccidioidomycosis: An update. *Microbiol. Rev.* **6:** 89–117.

Camargo, Z. P., Taborda, C. P., Rodrigues, E. G., and Travassos, L. R. 1991. The use of cell-free antigens of *Paracoccidioides brasiliensis* in serological tests. *J. Med. Vet. Mycol.* **29:** 31–38.

Camargo, Z. P., Unterkircher, C., and Travassos, L. R. 1989. Identification of antigenic polypeptides of *Paracoccidioides brasiliensis* by immunoblotting. *J. Med. Vet. Mycol.* **27:** 407–412.

Casotto, M., Paris, S., and Camargo, Z. P. 1991. Antigens of diagnostic value in three isolates of *Paracoccidioides brasiliensis. J. Med. Vet. Mycol.* **29:** 243–253.

Cisalpino, P. S., Puccia, R., Yamauchi, L. M., Cano, M. I. N., Franco da Silveira, J., and Travassos, L. R. 1996. Cloning, characterization, and epitope expression of the major diagnostic antigen of *Paracoccidioides brasiliensis. J. Biol. Chem.* **271:** 4553–4560.

Deepe, G. S., and Durose, G. G. 1995. Immunological activity of recombinant H antigen from *Histoplasma capsulatum. Infect. Immun.* **63:** 3151–3157.

Ferreira-da-Cruz, M. F., Galvão-Castro, B., and Daniel-Ribeiro, C. 1992. Isolation and antigenicity of a 45-kilodalton *Paracoccidioides brasiliensis* immunodominant antigen. *Infect. Immun.* **60:** 2667–2671.

Goldani, L. Z., Maia, A. L., and Sugar, A. M. 1995. Cloning and nucleotide sequence of a specific DNA fragment from *Paracoccidioides brasiliensis. J. Clin. Microbiol.* **33:** 1652–1654.

Gomez, F. J., Gomez, A. M., and Deepe, G. S. 1992. An 80-kilodalton antigen from *Histoplasma capsulatum* that has homology to heat shock protein 70 induces cell-mediated immune responses and protection in mice. *Infect. Immun.* **60:** 2565–2571.

Guo, Z., and Sherman, F. 1995. 3′-End-forming signals of yeast mRNA. *Mol. Cell. Biol.* **15:** 5993–5990.

Harper, R. 1994. Access to DNA and protein databases on the Internet. *Curr. Opinion Biotechnol.* **5:** 4–18.

McEwen, J. G., Garcia, A. M., Ortiz, B. L., Botero, S., and Restrepo, A. 1995. In search of the natural habitat of *Paracoccidioides brasiliensis. Arch. Med. Res.* **26:** 305–306.

Mendes-Giannini, M. J., Bueno, J. P., Shikanai-Yasuda, M. A., Ferreira, A. W., and Masuda, A. 1989. Detection of the 43,000-molecular-weight glycoprotein in sera of patients with paracoccidioidomycosis. *J. Clin. Microbiol.* **27**(12): 2842–2845.

Ortiz, B. L., McEwen, J. G., Garcia, A. M., and Restrepo, A. 1995. Immunological characterization of a 27 kDa antigenic protein from *Paracoccidoides brasiliensis.* 95th General Meeting American Society for Microbiology, Washington Convention Center, Session 14 p. F-16.

Puccia, R., and Travassos, L. R. 1991. 43 Kilodalton protein from *Paracoccidioides brasiliensis:* Immunochemical reactions with sera from patients with paracoccidioidomycois, histoplasmosis or Jorge Lobo's disease. *J. Clin. Microbiol.* **29:** 1610–1615.

Puccia, R., Schenkman, S., Gorin, P. A., and Travassos, L. R. 1986. Exocellular components of *Paracoccidioides brasiliensis:* Identification of a specific antigen. *Infect. Immun.* **53:** 199–206.

Restrepo, A. 1994. Ecology of *Paracoccidioides brasiliensis.* In *Paracoccidioidomycosis* (M. Franco, C. S. Lacaz, A. Restrepo, and G. Del Negro, Eds.), pp. 121–130. CRC Press, Boca Raton, FL.

Restrepo, A., and Jimenez, B. 1980. Growth of *Paracoccidioides brasiliensis* yeast phase in a chemically defined medium. *J. Clin. Microbiol.* **12:** 279–281.

Restrepo, A., Cano, L. E., and Ochoa, M. T. 1984. A yeast-derived antigen from *Paracoccidioides brasiliensis* useful for serologic testing. *Sabouraudia* **22:** 23–29.

Sanger, F., Nicklen, S., and Coulson, A. R. 1977. 1977 DNA sequencing with chain-termination inhibitors. *Proc. Natl. Acad. Sci. USA* **74:** 5463–5467.

Stambuk, B. U., Puccia, R., de Almeida, M. L., Travassos, L. R., and Schenkman, S. 1988. Secretion of the 43 kDa glycoprotein antigen by *Paracoccidioides brasiliensis. J. Med. Vet. Mycol.* **26:** 367–373.

Taba, M. R. M., Da Silveira, J. F., Travassos, L. R., and Schenkman, S. 1989. Expression in *Escherichia coli* of a gene coding for epitopes of a diagnostic antigen of *Paracoccidioides brasiliensis. Exp. Mycol.* **13:** 223–230.

Travassos, L. R. 1994. Immunochemistry of *Paracoccidioides brasiliensis* antigens. In *Paracoccidioidomycosis* (M. Franco, C. S. Lacaz, A. Restrepo, and G. Del Negro, Eds.), pp. 67–86. CRC Press, Boca Raton, FL.

Travassos, L. R., Puccia, R., Cisalpino, P., Taborda, C., Rodrigues, E. G., Rodrigues, M., Silveira, J. F., and Almeida, I. C. 1995. Biochemistry and molecular biology of the main antigen of *Paracoccidioides brasiliensis. Arch. Med. Res.* **26:** 297–304.