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Isolation and Characterization of a Species-Specific DNA Fragment for Detection of *Candida albicans* by Polymerase Chain Reaction

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A 2-kbp DNA fragment, EO3, that was present in multiple copies in the *Candida albicans* genome was isolated for use in developing a detection method for *C. albicans* by polymerase chain reaction (PCR). Dot blot hybridization revealed that EO3 was specific for the 40 isolates of *C. albicans* serotypes A and B used. Using a set of primers (20-mer each) derived from the nucleotide sequence of EO3, we performed specific amplification of a 1.8-kbp DNA fragment within EO3 by PCR. All 40 isolates belonging to *C. albicans* serotypes A and B contained amplifiable 1.8-kbp fragments, although the DNA of the amplified products exhibited small variations in size, yielding three different fragment groups. Southern blot hybridization probed with EO3 showed that these 1.8-kbp fragments were derived from the EO3 region. Conversely, the 1.8-kbp fragment was not amplified from 38 isolates belonging to seven other medically important *Candida* species or from isolates of *Cryptococcus neoformans*, *Saccharomyces cerevisiae*, various bacteria, and a human cell line. The detection limit of the PCR assay for *C. albicans* with the EO3 fragment was shown to be approximately 2 to 10 cells and 100 cells in saline and human urine, respectively, by ethidium bromide staining and 2 and 10 cells, respectively, by Southern blot analysis. In addition, EO3 was assumed to originate from mitochondrial DNA on the basis of the results of its characterizations. These results indicate that the PCR system using the 1.8-kbp fragment as a target is a reliable method for identifying *C. albicans* isolates, thereby suggesting its potentials for specific and sensitive detection of *C. albicans* in samples from patients with candidiasis.

Candida albicans continues to be recognized as an important pathogen for the immunocompromised host. Although isolation and identification of the causative yeasts are thought to be the definitive method for diagnosis of candidiasis and sensitive blood culture systems are available (1, 20, 31), the detection of yeasts in clinical samples is not always successful; blood cultures from patients with proven disseminated candidiasis are often negative (6). One reason for this phenomenon may be intermittent shedding of the organisms into blood (6). In addition, chemotherapy may result in negative blood cultures. Therefore, detection of the organisms without culture is sometimes necessary for diagnosis. Although methods for antibody detection have been extensively investigated (11, 12), such methods have the following shortcomings. Firstly, the ability of antibody production is markedly reduced in some immunocompromised patients (11). Secondly, a high titer of antibodies against *C. albicans* is frequently found in healthy individuals (12). On the other hand, detection of specific antigens such as cell wall mannans by enzyme immunoassay has been recommended as a useful method for laboratory diagnosis of candidiasis by the Centers for Disease Control (Atlanta, Ga.) (12). However, its sensitivity is often not sufficient for routine diagnosis (2, 12, 21).

As a new approach for detecting infectious agents, DNA fragments specific for *C. albicans* have been isolated (3, 5, 17, 24, 27, 29). However, hybridization techniques with these fragments as probes still suffer from poor sensitivity, because approximately 10^5 cells are required for reliable identification (3). The recently developed polymerase chain reaction (PCR) (25) has brought a new vista for the diagnosis of microbial infections. Theoretically, it is possible to detect a single microorganism in clinical samples by using PCR (14). This technique has been applied to a number of microorganisms (8, 34), including pathogenic fungi such as the *Cryptococcus* species, for which a specific fragment derived from ribosomal DNA has been amplified (33). However, the use of PCR for the detection of *Candida* species has not yet been reported. In the present study, we isolated a *C. albicans*-specific DNA fragment, which we have shown to be derived from mitochondrial DNA (mtDNA), and showed that the PCR system with a part of this fragment as a target sequence may be useful for specific identification and sensitive detection of *C. albicans* in clinical samples.

MATERIALS AND METHODS

Isolates and a plasmid. *Candida* isolates used as references and for comparative experiments came from stock cultures maintained in our laboratory, Meiji College of Pharmacy (Tokyo, Japan), the Iatron Laboratories (Tokyo, Japan), and the Japan Collection of Microorganisms (Saitama, Japan), as described previously (9, 18). All of the *Candida* isolates were identified by conventional biological methods (13) and serological methods by using polyclonal factor sera (Candida Check; Iatron Laboratories), as described previously (18). Yeast cells were grown in modified Sabouraud glucose broth (2% glucose, 1% polypeptone, 0.5% yeast extract [Difco Laboratories, Detroit, Mich.]) at 27°C (9), and bacteria were grown in a nutrient broth (Eiken Chemical Co. Ltd., Tokyo, Japan) at 37°C. *Escherichia coli* JM109 was used as a host, and a plasmid, pUC118, was used as a vector. *Saccharomyces cerevisiae* LL20 (30), *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, *Cryptococcus neoformans* NIH-68 (10), and a human mouth squamous carcinoma HSC-2 cell line (Japanese Cancer Resources

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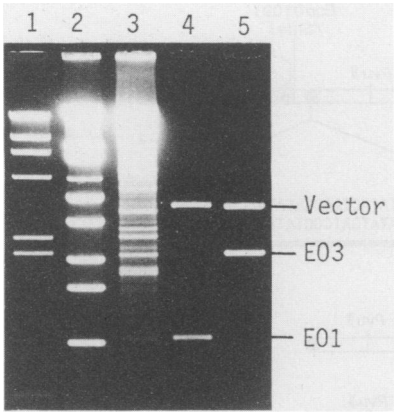

FIG. 1. Agarose gel electrophoresis of cloned fragments with multiple copies derived from *C. albicans* DNA. Lanes: 1 and 2, DNA size standards of *Hin*dIII- and *Eco*T14I-digested λ DNA, respectively; 3, *Eco*O109I-digested whole DNA of *C. albicans* M1012; 4 and 5, *Eco*RI-*Pst*I- and *Eco*RI-*Hin*dIII-digested plasmids with the inserted fragments EO1 and EO3, respectively, derived from discrete bands with the corresponding size.

Bank, Tokyo, Japan) (19) were used as reference strains for testing the species specificities of the probes.

Preparation of DNA. Whole DNA of *C. albicans* M1012 serotype A was prepared from spheroplasts (32), as described by Cryer et al. (4). Briefly, spheroplasts were lysed with 1% sodium dodecyl sulfate (wt/vol) in the presence of proteinase K (Merck & Co. Inc., Rahway, N.J.). The lysate was deproteinized with chloroform-isoamyl alcohol (24:1) and then treated with pancreatic RNase (50 μg/ml; Sigma Chemical Co., St. Louis, Mo.), RNase T1 (100 U/ml; Boehringer Mannheim, Germany), and α-amylase (0.03 U/ml; Boehringer Mannheim). Chloroform-isoamyl alcohol extraction and RNase treatment were repeated three times. The purified DNA was collected by ethanol precipitation, dissolved in TE buffer (10 mM Tris-HCl–1 mM Na_2 EDTA, pH 8.0), and used for library construction. In other *Candida* isolates, DNAs were prepared from the lysate (see above) by chloroform-phenol (1:1) extraction, treated with pancreatic RNase, ethanol precipitated, and then used as crude DNAs for hybridization and PCR analysis. Crude DNAs of *C. neoformans*, *S. cerevisiae*, the human cell line, and various bacteria were prepared according to the method described by Restrepo and Barbour (23) and Maniatis et al. (16). The crude DNAs were used in dot blot analyses and PCR assay to determine their species specificities.

Cloning of DNA fragments with multiple copies. Whole DNA of *C. albicans* M1012 was digested with *Eco*O109I and subjected to electrophoresis through a 1% agarose gel. Among discrete DNA fragments generated on agarose, the fragments of 0.9 and 2 kbp in length (indicated as EO1 and EO3, respectively, in Fig. 1) were extracted and filled in with Klenow fragment. Each of the DNA fragments was ligated into the *Sma*I site of pUC118, and the ligation mixture was introduced into *E. coli* JM109.

Labeling of DNA probes. The 0.9- and 2-kbp DNA fragments, EO1 and EO3, respectively, derived from *C. albicans* were labeled with [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham International plc, Amersham, United Kingdom) by using a random primer DNA labeling kit (Takara Shuzo Co., Ltd., Kyoto, Japan) and purified on a nick column (Pharmacia LKB, Uppsala, Sweden).

Hybridization. **(i) Dot blotting.** Each 5 μl (0.1 and 1.0 μg) of heat-denatured crude DNA was spotted onto a Hybond-N membrane (Amersham) pretreated with alkali and then baked at 80°C for 20 min.

(ii) Southern blotting. The PCR products with or without restriction digestion were separated on an agarose gel, denatured, blotted onto the membrane, and baked.

(iii) Hybridization. The membranes were subjected to hybridization with the labeled probes at 68°C as described previously (16). Specific activity for each probe was 10^9 cpm/μg of DNA. After hybridization and washing, the membranes were exposed to Fuji RX X-ray film (Fuji Photo Film Co., Ltd., Tokyo, Japan) at −80°C for various lengths of time.

Synthetic oligonucleotides. The partial nucleotide sequence of EO3 was determined by the dideoxy sequencing method (26). On the basis of the partial nucleotide sequence of EO3, a pair of primers (20-mer each) (Fig. 2A) was synthesized by a DNA synthesizer (Applied Biosystems, Inc., Foster City, Calif.).

PCR. *Taq* polymerase (0.625 U; Perkin-Elmer Corp., Norwalk, Conn.) was added to 25 μl of solution consisting of 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 0.001% (wt/vol) gelatin, 0.20 mM each of the deoxynucleotides dGTP, dATP, dTTP, and dCTP (Perkin-Elmer Corp.), and unless otherwise stated, 250 pg of target DNA and 1 μM of each primer. DNA was amplified in a PCR processor Bio Oven (Bio Therm Corp., Arlington, Va.) by using 35 cycles as follows: 45 s of denaturation at 92°C, 30 s of annealing at 50°C, and 90 s of primer extension at 72°C. At the final cycle, an additional 7 min of incubation at 72°C was done to ensure complete polymerization of any remaining PCR products.

Rapid procedure for preparation of PCR samples from *C. albicans* cells in saline or urine. *C. albicans* (10^5 to 2 cells) in 1 ml of saline or human urine was washed twice with distilled water. Spheroplasts were prepared by treating the cells with 0.5 mg of Zymolyase 60000 per ml (Seikagakukougyo Co., Ltd., Tokyo, Japan) and 2% 2-mercaptoethanol in 100 μl of 1 M sorbitol–0.1 M EDTA (pH 7.5) at 37°C for 15 min. After centrifugation at 12,500 × *g* for 2 min, the spheroplasts were washed twice with 1 M sorbitol–0.1 M EDTA (pH 7.5), suspended in PCR buffer containing 0.1% Nonidet P-40 and 0.1 mg of proteinase K per ml, and incubated at 55°C for 30 min. To inactivate the proteinase K, the samples were heated at 95°C for 10 min and then used for the PCR assay.

RESULTS

Isolation of DNA fragments with multiple copies from *C. albicans*. In an attempt to isolate the fragments that were present in multiple copies in the *C. albicans* genome, we constructed plasmid libraries containing 0.9- and 2-kbp insert fragments derived from discrete bands on agarose, which were generated with *Eco*O109I digestion of *C. albicans* whole DNA (Fig. 1). Then, plasmids were extracted from several transformants, including each plasmid library, and were analyzed with *Hin*cII digestion. From the library containing the 0.9-kbp inserts, we obtained four plasmids with identical digestion patterns and designated the insert of a representative plasmid EO1. In a similar procedure, we obtained three identical plasmids from the library containing the 2.0-kbp inserts and designated the insert of a representative plasmid EO3.

Specificity of EO3. Dot blot analysis for DNAs (each pair with 0.1 and 1.0 μg of DNA) from a number of isolates was performed to determine the species specificity of EO3. Representative data are shown in Fig. 3A. The probe EO3

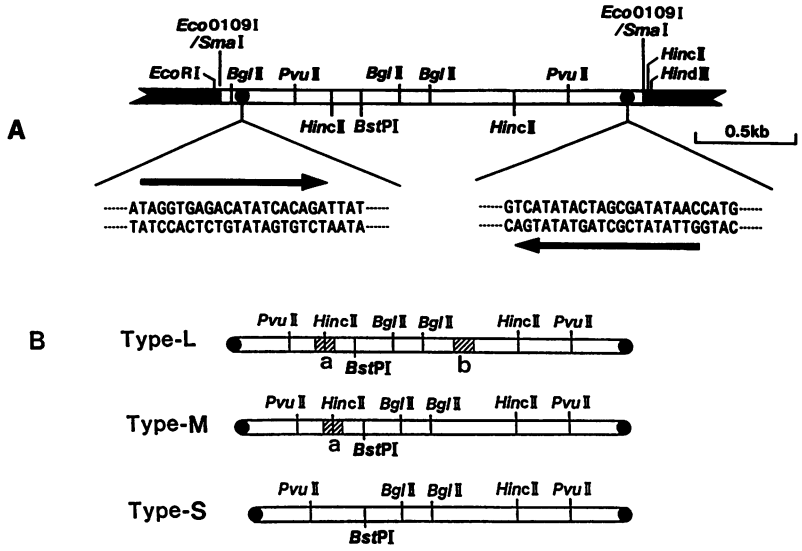

FIG. 2. (A) Physical map of *C. albicans*-specific fragment EO3 and its partial nucleotide sequence. Closed bar, pUC118; open bar, cloned fragment EO3; closed circles, position of a pair of primers for PCR. Nucleotide sequences with arrows (20-mer each), PCR primers. (B) Comparison of three types of PCR products with small size variations, based on the restriction analysis for the PCR products shown in Fig. 6. Striped bars, small regions absent in PCR products of type S (a) or in type S and M products (b).

specifically hybridized with DNAs from reference isolates belonging to *C. albicans* serotypes A and B (Fig. 3A, lane a, blots 1 and 2) but did not hybridize with DNA from any other reference isolates of six medically important *Candida* species (lane a, blots 3 and 4, and lane b), *S. cerevisiae* (lane c, blot 1), *S. aureus* (lane c, blot 2), *E. coli* (lane c, blot 3), *P. aeruginosa* (lane c, blot 4), *C. neoformans* (lane f, blot 3), and the human cell line (lane f, blot 4). EO3 also hybridized with DNA from 10 stock strains tested belonging to *C. albicans* serotypes A (Fig. 3A, lane d, blots 1 to 4, and lane e, blot 1) and B (lane e, blots 2 to 4, and lane f, blots 1 and 2). Furthermore, comparative study of the dot blot analysis with EO3 indicated that EO3 hybridized with the DNAs from all the *C. albicans* isolates tested but did not hybridize at all with DNAs from isolates of the other species (Table 1), indicating that EO3 is specific for both serotypes of *C. albicans* tested.

Similar results were demonstrated when EO1 was used as a probe (Fig. 3B). Although EO1 appeared to be specific for

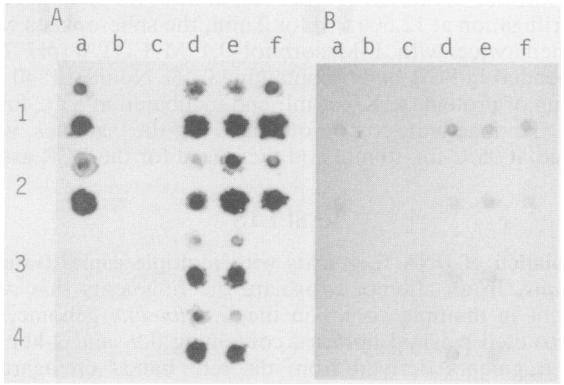

FIG. 3. Species specificities of probes EO3 (A) and EO1 (B) analyzed by dot blot hybridization. Specific radioactivity was 10^9 cpm/μg of DNA for both probes. Each pair of DNA, 0.1 μg (upper blot) and 1.0 μg (lower blot), from various isolates of the reference strains was dot blotted onto membranes. Lanes a: blot 1, *C. albicans* serotype A M1012; blot 2, *C. albicans* serotype B M1445; blot 3, *C. tropicalis* M1017; blot 4, *C. guilliermondii* M1023. Lanes b: blot 1, *C. krusei* M1005; blot 2, *C. parapsilosis* M1015; blot 3, *C. kefyr* M1004; blot 4, *C. glablata* M4002. Lanes c: blot 1, *S. cerevisiae* LL20; blot 2, *S. aureus* ATCC 25923; blot 3, *E. coli* JM109; blot 4, *P. aeruginosa* ATCC 27853. Lanes f: blot 3, *C. neoformans* NIH-68; blot 4, human cell line HSC-2. DNAs from *C. albicans* isolates, five isolates each of serotype A (lanes d, blots 1 to 4, and lanes e, blot 1) and serotype B (lanes e, blots 2 to 4, and lanes f, blots 1 and 2), were used for comparative experiments.

TABLE 1. Species specificities of the fragment EO3 and amplification of the 1.8-kbp fragment by PCR

Species or cell line	No. of isolates used	No. of isolates whose DNA hybridized with EO3[a]		No. of isolates in which the 1.8-kbp fragment was detected by:			
				EB stain[b]		Southern[c]	
		+	−	+	−	+	−
C. albicans, type A	23	23	0	23	0	23	0
C. albicans, type B	17	17	0	17	0	17	0
C. tropicalis	8	0	8	0	8	0	8
C. guilliermondii	5	0	5	0	5	0	5
C. krusei	5	0	5	0	5	0	5
C. parapsilosis	5	0	5	0	5	0	5
C. kefyr	5	0	5	0	5	0	5
C. glabrata	5	0	5	0	5	0	5
C. lusitaniae	5	0	5	0	5	0	5
C. neoformans	1	0	1	0	1	0	1
S. cerevisiae	1	0	1	0	1	0	1
S. aureus	1	0	1	0	1	0	1
E. coli	1	0	1	0	1	0	1
P. aeruginosa	1	0	1	0	1	0	1
Human cell line	1	0	1	0	1	0	1

[a] Determined by dot blot hybridization probed with EO3.
[b] EB stain, Ethidium bromide staining.
[c] Southern, Southern blot hybridization probed with EO3.

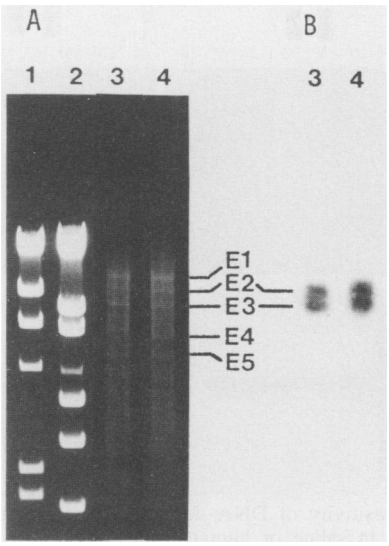

FIG. 4. Hybridization of EO3 with *Eco*RI-digested *C. albicans* DNA. *Eco*RI-digested *C. albicans* whole DNAs were subjected to electrophoresis (A) and analyzed by Southern blot hybridization probed with EO3 (B). Data for the representative isolates are shown. Lanes: 1 and 2, *Hin*dIII- and *Eco*T14I-digested λ DNA, respectively, as size standards; 3 and 4, *C. albicans* serotype A and B isolates, respectively. Discrete fragments E1 through E5 originated from mtDNA are indicated in the center, according to the sizes reported by Wills et al. (36).

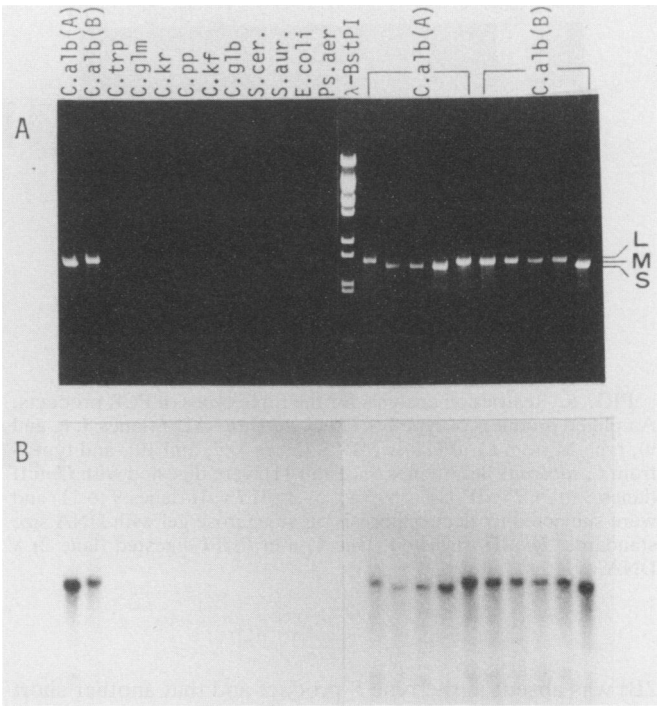

FIG. 5. Specific amplification of 1.8-kb fragments from crude DNAs of *C. albicans* serotypes A and B by PCR. (A) Ethidium bromide-stained DNA fragments produced by PCR. (B) Southern blot analysis of the products probed with EO3. λ-BstPI, λ DNA digested with *Bst*PI (DNA size standard). C. alb(A), *C. albicans* serotype A; C. alb(B), *C. albicans* serotype B; C. trp, *C. tropicalis*; C. glm, *C. guilliermondii*; C. kr, *C. krusei*; C. pp, *C. parapsilosis*; C. kf, *C. kefyr*; C. glb, *C. glabrata*; S. cer., *S. cerevisiae*; S. aur., *S. aureus*; Ps. aer, *P. aeruginosa*. Lanes to the right of the size standard, a set of five stock strains for *C. albicans* serotypes A and B, as indicated. L, M, and S indicate types of PCR products, classified according to their DNA sizes (see text).

C. albicans as well, the hybridization signal of EO1 with 1.0 μg of *C. albicans* DNA (lower blot of each pair) was weaker than that of EO3 with 0.1 μg of the corresponding *C. albicans* DNA (upper blot of each pair), irrespective of use of the same specific radioactivity of EO1 with EO3. These results suggest that the copy number of EO3 is much higher than that of EO1. Therefore, we decided to use EO3 as a primary target for PCR for the detection and identification of *C. albicans*.

Origin of EO3. To determine whether EO3 originated from nuclear DNA or mtDNA, we performed Southern blot analysis of EO3 for *Eco*RI-digested products of whole DNA from several isolates of *C. albicans* serotypes A and B. By agarose gel electrophoresis of the *Eco*RI digestion products, five discrete fragments corresponding in DNA sizes to the five *Eco*RI fragments of *C. albicans* mtDNA (designated E1 to E5) reported by Wills et al. (35, 36) were detected by ethidium bromide staining (Fig. 4A). By Southern blot analysis, EO3 hybridized with two of five such fragments in these isolates: the sizes of the two fragments were identical to those of E2 (10.0 kbp) and E3 (8.1 kbp), respectively (Fig. 4B). Moreover, we constructed a physical map of EO3 (Fig. 2A). EO3 contained two *Pvu*II sites, being 1.3 kbp apart, and one *Bst*PI site; i.e., *Bst*PI cleaved this 1.3-kbp *Pvu*II-fragment into two fragments of 1.0 and 0.3 kbp in length. Such a physical map is found to correspond to the map of E2 and E3 (Fig. 7 and 4A in references 36 and 28, respectively). It can be assumed from these results that EO3 originated from *C. albicans* mtDNA. The result of the dot blot hybridization experiment showing the presence of EO3 as multiple copies in the *C. albicans* cell (Fig. 3) appears to support this possibility.

Application of EO3 for PCR. Nucleotide sequences close to both ends of EO3 were determined and a pair of oligonucleotides (20-mer each), which are 1.8 kbp apart from each other, were synthesized (Fig. 2A). By using these oligonucleotides as primers, 1.8-kbp fragments within EO3 were amplified from whole DNA by PCR. Crude DNAs were extracted from various isolates (the same as those listed in Table 1) and subjected to PCR. After 35 cycles of PCR, the products were analyzed by agarose gel electrophoresis. As shown in Fig. 5A and Table 1, the 1.8-kbp fragments were amplified in all 40 isolates tested belonging to *C. albicans* serotypes A and B. Conversely, no amplified fragment was detected in the isolates not belonging to *C. albicans*, i.e., several isolates in each of the other seven *Candida* species and one isolate in each of *C. neoformans*, *S. cerevisiae*, *S. aureus*, *E. coli*, *P. aeruginosa*, and the human cell line (Table 1; Fig. 5A).

Identification of the 1.8-kbp products with small size variations. The PCR products generated from *C. albicans* exhibited small size variations among the isolates tested and were classified into three types, L, M, and S, according to their DNA sizes (indicated on the right of Fig. 5A). To determine size variations in detail, we constructed a physical map of EO3 (Fig. 2A). *C. albicans* M1012, from which EO3 was derived (Fig. 1), generated a type M product by PCR (Fig. 5). Then, we analyzed each PCR product of type L, M, or S by *Hin*cII, *Pvu*II, and *Bgl*II-*Pvu*II digestion (Fig. 6). Together with the physical map of EO3 (Fig. 2A), it was revealed that a short fragment with one *Hin*cII site (striped bar a in Fig.

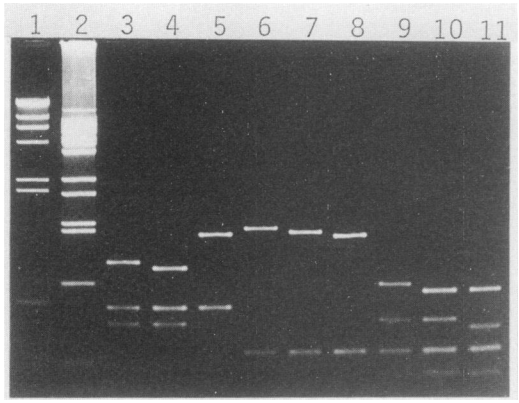

FIG. 6. Restriction analysis for the three types of PCR products. Amplified products of type L from *C. albicans* A207 (lanes 3, 6, and 9), type M from *C. albicans* M1012 (lanes 4, 7, and 10), and type S from *C. albicans* 8624 (lanes 5, 8, and 11) were digested with *Hin*cII (lanes 3 to 5), *Pvu*II (lanes 6 to 8), or *Bgl*II-*Pvu*II (lanes 9 to 11) and were subjected to electrophoresis on an agarose gel with DNA size standards, *Hin*dIII-digested (lane 1) and *Bst*PI-digested (lane 2) λ DNA.

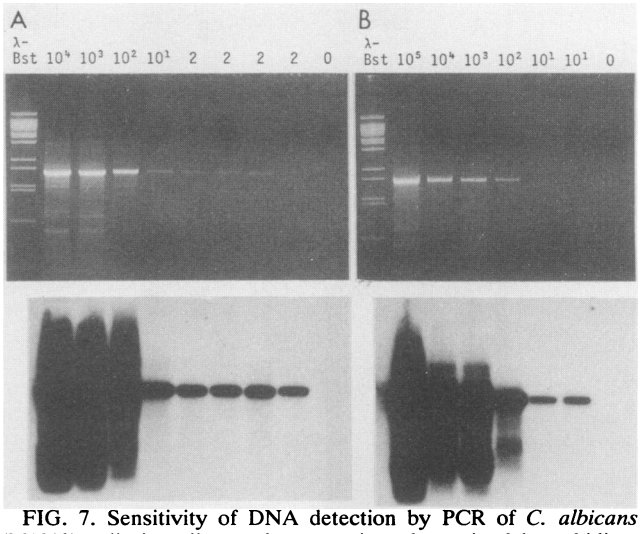

FIG. 7. Sensitivity of DNA detection by PCR of *C. albicans* (M1012) cells in saline or human urine, determined by ethidium bromide staining and Southern blot analysis. Template DNAs were prepared from 1 ml of saline containing 10^4 to 2 cells (A) or from 1 ml of urine containing 10^5 to 10 cells (B) by a rapid procedure as described in Materials and Methods. Samples were amplified by PCR and applied to agarose gel with DNA size standard *Bst*PI-digested λ DNA (λ-Bst) and analyzed by ethidium bromide staining (upper panels) or by Southern blot hybridization probed with EO3 (lower panels). The numbers above the lanes indicate the number of yeast cells in each test tube from which template DNAs were prepared.

2B) was absent in the type S product and that another short fragment within the *Bgl*II-*Hin*cII fragment (striped bar b in Fig. 2B) was absent in type M and type S products. Restriction analysis of each of the amplified products from the remaining 37 PCR-positive isolates resulted in patterns identical to that of one of the three types shown in Fig. 6 (data not shown). These results suggest that the loci responsible for such size variations are present on the restricted region within the *Bgl*II-*Hin*cII fragment and/or the region around the *Hin*cII site on the map of EO3 (Fig. 2B).

To confirm that these amplified fragments were derived from the EO3 region, products from all 40 PCR-positive isolates tested were subjected to Southern blot hybridization probed with EO3. As shown in Table 1 and Fig. 5B, EO3 hybridized with all of the amplified 1.8-kbp fragments, although small size variations were observed in the hybridized fragments (Fig. 5B), which corresponded to the fragments stained with ethidium bromide (Fig. 5A). Thus, we conclude that the amplifiable 1.8-kbp fragments are derived from the same region of EO3 and suggest that the fragments are contained in all of the *C. albicans* isolates tested.

Sensitivity of detection for *C. albicans* DNA from cells in saline or in human urine by PCR assay. To examine the utility of the *C. albicans*-specific PCR test for clinical samples, we attempted to establish a rapid procedure for preparation of PCR samples from *C. albicans* cells in saline or urine. The treatment of the spheroplasts prepared from *C. albicans* cells in saline with Nonidet P-40 in the presence of proteinase K, followed by the PCR assay, resulted in extensive amplification of the target sequence of the 1.8-kbp DNA fragment (Fig. 7A). To determine the detection limit, 10-fold dilution of *C. albicans* cells was made either in saline or in human urine. As shown in Fig. 7, the detection limit of *C. albicans* cells in saline was 2 to 10 cells per test tube and that of *C. albicans* cells in 1 ml of urine was 100 cells, when the PCR products were stained with ethidium bromide. Moreover, Southern blot hybridization probed with EO3 showed that the amplified products of 1.8 kbp were derived from the EO3 region and that only 2 and 10 cells were able to be detected from saline and urine, respectively.

DISCUSSION

We demonstrated that a 2-kbp fragment, EO3, isolated from *C. albicans* mtDNA was specific for the isolates of *C. albicans* serotypes A and B tested. Using a set of primers derived from the nucleotide sequence of EO3, we performed amplification of a 1.8-kbp fragment within EO3 by PCR. The amplifiable 1.8-kbp fragment was involved specifically in *C. albicans* serotypes A and B.

Wills et al. (35, 36) have shown that *Eco*RI digestion of *C. albicans* whole DNA generated nine discrete bands on agarose gel, among which six fragments (E1 to E6) originated from mtDNA. Our Southern blot hybridization showed that EO3 hybridized with both DNA fragments corresponding to E2 (10.0 kbp) and E3 (8.1 kbp) in terms of sizes of DNA fragments (Fig. 4). Shaw et al. and Wills et al. have demonstrated that these two fragments (E2 and E3) contained identical restriction sites for several enzymes over 5 kbp in length, indicating that *C. albicans* mtDNA contains large duplicated sequences (28, 36). In comparing the physical map of EO3 (Fig. 2A) with those of E2 and E3 reported by Wills et al. (36), we noticed that EO3 corresponds to the 2-kbp region within the duplication of E2-E3 (36) containing two *Pvu*II sites. These results suggest that EO3 is a part of E2-E3 and is contained within a duplicated region of *C. albicans* mtDNA.

Several DNA probes of *C. albicans* have been isolated (3, 5, 17, 24, 27, 29). The 2.9-kbp *Msp*I fragment isolated by Cutler et al. (5) has been shown to be one of the repeated elements of *C. albicans*. However, the EO3 fragment appears to be different from the 2.9-kbp *Msp*I fragment of Cutler et al., because of a distinct difference in the physical maps of EO3 (Fig. 2A) and the 2.9-kbp fragment (5) and in the size of fragment hybridized with *Msp*I-digested *C. albi-*

cans DNA; EO3 hybridized with a 1.4-kbp fragment but not with the 2.9-kbp fragment (data not shown).

In the present PCR assay, the sizes of the PCR products exhibited small variations among the isolates used. This variation is interpreted as that of size polymorphism, frequently observed in the repeated elements and mtDNA of *C. albicans* (7, 15, 22). In the 1.8-kbp fragment, such size polymorphism occurred within the specific regions, as shown in Fig. 2B. The products from all the PCR-positive isolates tested could be classified into three types according to their sizes: L, M, or S. However, we wish to emphasize that the 1.8-kbp fragment within EO3 could be successfully amplified by PCR assay for all the *C. albicans* isolates tested, irrespective of the small size variations of the PCR products. Although the biological significance and the mechanism for the small size variation within EO3 are thought to be interesting issues, we have not yet obtained any information to elucidate such problems, and we are now attempting to compare the sequences among three types of PCR products. In addition, these size variations may be applicable to epidemiological studies of candidiasis and remain to be investigated further.

The detection limit of the PCR assay with the EO3 fragment was shown to be 2 to 10 cells and 100 cells in saline and human urine, respectively, by ethidium bromide staining and 2 and 10 cells, respectively, by Southern blot analysis. Since fewer than 10^3 yeast cells per ml of urine are suspected to colonize in an asymptomatic patient with renal candidiasis (6), our PCR assay is thought to be useful for detecting *C. albicans* cells in these cases. Moreover, the fact that the amplifiable 1.8-kbp fragment is not contained in human cells (Fig. 3 and Table 1) suggests a possibility for the clinical application of our PCR system.

Although the detection of *C. albicans* in clinical samples other than urine has not yet been performed, it is expected that the PCR assay with the EO3 fragment may be a useful and valuable tool for the detection of *C. albicans* in clinical samples, especially when *C. albicans* cannot be detected by culture because of administration of antifungal chemotherapeutics (6). However, a target sequence of 1.8 kbp may be too long for detecting the small amount of DNA in clinical samples, such as blood. Very recently, we have found *C. albicans*-specific 0.4- to 0.5-kbp fragments within EO3. These shorter fragments may amplify more efficiently than the 1.8-kbp fragment does. Such studies are now underway in our laboratory.

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