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Molecular Cloning and Expression of a 70-Kilodalton Heat Shock Protein of *Candida albicans*

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By screening an expression library of the yeast form of *Candida albicans* with a serum directed against whole fungal cells, a cDNA (2,325 bp) encoding a stress protein of *C. albicans* was cloned and sequenced. The cloned sequence (*CaRLV130*) identified a single open reading frame with a length of 1,968 bp coding for a protein containing 656 amino acid residues (70 kDa). The deduced amino acid sequence was 84% similar to the sequence of the *Saccharomyces cerevisiae SSA1* gene, which encodes one member of the 70-kDa heat shock protein (Hsp70) family. The relevant gene (*C. albicans HSP70* gene [*CaHSP70*]) was localized on the highest-M_r (R1; approximately 3.8 Mb) chromosome of *C. albicans* as determined by pulse-field electrophoresis. *CaHSP70* was expressed after heat shock, as demonstrated by Northern (RNA) blotting and reverse transcriptase-PCR with specific pairs of oligonucleotide sequences and gene probes. A recombinant protein was obtained in *Escherichia coli* after cloning of the full coding sequence into the *Bam*HI site of the pDS56/RBSII6xhisE$^-$ plasmid and purification by nickel chelate affinity chromatography. The recombinant protein (6xhis-CaHsp70) was efficiently recognized in immunoblots by a monoclonal antibody directed against a common epitope of eukaryotic Hsp70 proteins, as well as by sera from normal human subjects. Moreover, immune mouse sera against the purified recombinant protein recognized native, heat-inducible constituents with sizes of around 70 kDa in whole-cell protein extracts of *C. albicans*. Overall, our data demonstrate that *CaHSP70* encodes one member of a family of proteins (Hsp70) which usually represent highly conserved immunodominant antigens of infectious agents.

Candida albicans is an opportunistic human pathogen of increasing medical interest. In particular, mucosal candidiasis is a frequent, early manifestation of AIDS (8, 17, 21, 30). Studies of the host-*Candida* relationship have emphasized the importance of natural as well as adaptive immunity in anti-*Candida* protection, but very few definite *Candida* immunogens have been identified and characterized (3, 27, 31, 33, 37, 39). A main antigen target of anti-*Candida* cell-mediated immunity is a 65-kDa mannoprotein (MP65), a constituent of a major mannoprotein fraction (MP-F2) of the fungus (3, 39). In human immunodeficiency virus-infected patients, failure to respond to this immunodominant antigen of *C. albicans* is associated with a high level of predisposition to oropharyngeal and esophageal candidiasis (33). The characterization of additional immunogens would be of great value for understanding the mechanisms of the anti-*Candida* immune response and would assist in the development of new diagnostic, immunoprophylactic, and/or therapeutic agents.

To this aim, a cDNA expression library with mRNA of the yeast form of *C. albicans* was constructed and screened with antibodies raised in rabbits immunized with whole heat-inactivated *C. albicans* cells. Two recombinant clones encoding a 70-kDa heat-shock protein (Hsp70) were isolated. Because Hsps are dominant, conserved antigens of infectious agents which may play an important role in the host-parasite relationship (20), we focused our attention on this new cloned gene of *C. albicans*, with the perspective of disclosing its possible relevance for host immune response to *C. albicans.*

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MATERIALS AND METHODS

Microorganisms and growth conditions. *C. albicans* ATCC 20955 was used throughout this study. Cells were grown in YPD (2% glucose; 1% yeast extract, 2% Bacto peptone; Difco, Detroit, Mich.), Winge broth (0.3% yeast extract, 0.2% glucose; Difco), or modified Lee's medium (2), as specified for single experiments.

Escherichia coli XL1 Blue (*endA1 hsdR17 supE44 thi1 recA1 gyrA96 relA1* Δ*lac* [F′ *proAB lacI9Z* ΔM15 Tn*10*]) and M15 (Nal^s Str^s Rif^s Lac$^-$ Ara$^-$ Gal$^-$ Mtl$^-$ F$^-$ RecA$^+$ Uvr$^+$ [pUHA1]) were used as host strains for recombinant plasmids, while *E. coli* Y1090 (r$^-$ m$^+$ Δ*lacU196* Δ*lon supF* [pMC9]) was the host strain for bacteriophage λ*gt11*. *E. coli* cells were usually grown in L broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl [pH 7.00]) or LB (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 1.5% agar [pH 7.00]) plates or on top agarose (1% tryptone, 0.8% NaCl, 0.6% agarose; Boehringer, Mannheim, Germany), supplemented when necessary with ampicillin (100 μg/ml), kanamycin (50 μg/ml), or tetracycline (12.5 μg/ml) (Boehringer).

Rabbit and mouse immunization. One New Zealand rabbit was immunized with heat-inactivated (70°C, 30 min) whole yeast cells harvested from a 24-h culture of *C. albicans* in Winge broth. The immunization consisted of four doses of 10^8 fungal cells injected subcutaneously, at weekly intervals, after suspension in complete (the first three doses) and incomplete (the last dose) Freund adjuvant, in a total volume of 0.5 ml. The animal was bled 3 days after the fourth inoculation of *Candida* cells. The titer of the serum was >1:64,000, as determined by an immunoenzymatic assay with a crude mannoprotein extract (GMP [3, 33]) used as a coating antigen. Hyperimmune serum against purified, recombinant CaHsp70 (see below) was raised in a CD2F1 mouse (18 to 21 g) by four intraperitoneal injections at weekly intervals of 10 μg of the recombinant protein in complete (the first two injections) and incomplete (the last two) Freund adjuvant. The titer of this serum was >24,000, as determined by an immunoenzymatic assay with CaHsp70 used as the coating antigen.

Oligonucleotides. Ca1, Ca2, Ca3, Ca5, Ca6, Ca7, and Ca9 oligonucleotides were synthesized with an Applied Biosystems 391 PCR-Mate machine (Foster City, Calif.). Their sequences and specificities are shown in Table 1.

cDNA synthesis. Poly(A)$^+$ RNA was isolated from a total nucleic acid prep-

TABLE 1. Sequence and localization of Ca oligonucleotides

Oligonucleotide	5′ to 3′ sequence	Localization[a]	Gene
Ca1	CCCGGATCCTCTAAAGCTGTTGGTATTG[b]	108–126	*CaHSP70*
Ca2	GAAATGAAAGATAAGATTGGTGCA	1770–1793	*CaHSP70*
Ca3	CCACAGTAAATTACCTATTTCTTCCTC	2104–2078	*CaHSP70*
Ca5	AATCACAACTATTTAAACAAAGGTAT	1412–1387	*TEF2*
Ca6	CCAAAGGGTTGTGACTCTTTCAAT	994–1017	*TEF2*
Ca7	GTAGTCAGTGAAAGCTTCAACACACAT	1248–1222	*TEF2*
Ca9	GGCACCACCTGGGAA	2003–1989	*CaHSP70*

[a] Numbered as in the EMBL database (accession number EMBL Z30210) and as from reference 38 for *TEF2*. The start codon of *CaHSP70* is at position 105, and its stop codon is at nucleotide position 2073.

[b] The underlined sequence, containing the *Bam*HI site, is not complementary to the *CaHSP70* gene.

aration of *C. albicans* (yeast form) (32) by an Invitrogen Micro-Fast Track mRNA isolation kit (Leek, The Netherlands) according to the manufacturer's instructions and then was retrotranscribed to double-stranded cDNA by the avian mammary virus reverse transcriptase (RT) and oligo(dT)$_{18}$-poly(dN)$_6$ random primers (Boehringer) (12, 32). The cDNA preparations were separated by electrophoresis on a 1% low-melting-point agarose gel. High-molecular-weight cDNA was bound to a DEAE filter during reverse electrophoresis and then eluted with 1.5 M NaCl–TE (10 mM Tris-HCl, 1 mM EDTA, [pH 8.00]) at 65°C. After ethanol precipitation, oligo(dT)$_{18}$ and dN$_6$-primed cDNAs were resuspended and pooled in 50 μl of 0.25× TE buffer.

***C. albicans* λ*gt11* cDNA library.** Purified *C. albicans* cDNA (described above) was ligated with dephosphorylated *Eco*RI λ*gt11* arms and incubated with in vitro packaging extracts (Boehringer) according to the manufacturer's instructions. Recombinant phage particles were amplified by preparation of plate lysates with *E. coli* Y1090, yielding a completeness of 9×10^4 plaques. The amplified library (initial density, 40,000 plaques per 13-cm-diameter plate) was immunoscreened with rabbit polyclonal anti-*Candida* serum by the Protoblot immunoscreening system (Promega Corp., Madison, Wis.).

PCR. PCRs were performed on a Gene Amp PCR system 9600 apparatus (Perkin-Elmer Cetus Corp., Norwalk, Conn.) in a volume of 100 μl containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 200 μM (each) deoxynucleotide, 50 pmol of each primer, 1 U of *Taq* polymerase (Perkin-Elmer), and 1 μl of template (antibody-positive plaque eluted into 20 μl of 0.1% sodium dodecyl sulfate (SDS). PCR with cDNA with λ*gt11* forward, λ*gt11* reverse, and Ca1 oligonucleotide primers was done in three steps of 60 s at 94°C, 60 s at 60°C, and 120 s at 72°C (25 cycles).

Cloning and sequencing. For molecular cloning of PCR fragments and λ*gt11* inserts, appropriate restriction sites of the pBluescript and pDS56/RBSII, 6xhis/E$^-$ (a derivative of the pDS56/RBSII 6xhis$^-$ plasmid family) vectors were used (15, 36) as described by Perbal (32). Double-stranded dideoxy sequencing of recombinant plasmids was performed with the Sequenase kit (U.S. Biochemical Corp., Cleveland, Ohio) by using primers flanking the polylinker regions the pBluescript and pDS56/RBSII,6xhis/E$^-$ plasmids and various internal *CaHSP70* primers (Table 1). The cDNA sequence was compared with sequences in the GenBank nucleotide and National Biomedical Research Foundation protein databases by using Mac Vector sequence analysis software (International Biotechnologies, New Haven, Conn.).

Southern blot analysis. Genomic DNA of *C. albicans* was digested with the restriction endonucleases *Eco*RI, *Bam*HI, *Hin*dIII, *Bgl*III, and *Pst*I (Boehringer), separated by agarose gel electrophoresis, and transferred onto nitrocellulose transblot membranes (Bio-Rad Laboratories, Hercules, Calif.) as described previously (12, 32). Blotted DNAs were hybridized with ^{32}P-labelled random-primed (Boehringer) *CaRLV130* full-length cDNA inserts. Hybridization and initial washing steps were carried out as described previously (32). Filters were exposed on 3M (St. Paul, Minn.) XDA plus films with 3M Trimax screens at −80°C.

Chromosome separation by TAFE. The general procedure described by Vollrath and Davis (40) was used for DNA sample preparation for pulsed-field electrophoresis. The electrophoretic karyotype was determined by using transverse alternating field electrophoresis (TAFE) in a Gene-Line apparatus from Beckman (Palo Alto, Calif.). The operating conditions were four consecutive runs on the gel (10 by 7.5 cm, 0.5 cm thick, 1% agarose; GTG Biometra, Gottingen, Germany) of DNA samples in agarose inserts and with a solution containing 25 mM Tris, 25 mM boric acid, and 0.75 mM EDTA (pH 8.2) as a running buffer. The parameters of electrophoresis were set up as follows: four consecutive runs of 24 h at 100 V and 70 mA; 10°C, 120° rotation angle; and 120-, 420-, and 600-s periods of rotation for each run. After electrophoresis, the gel was stained with ethidium bromide (0.5 mg/ml, 30 min) and then destained, photographed, and blotted to a nitrocellulose membrane (Bio-Rad) for 48 h. Hybridization was performed as described above for Southern blot analysis.

Northern (RNA) blot analysis. Total RNA from *C. albicans* cells grown in Lee's medium at 22°C with or without a temperature shift to 37°C was isolated by the protease K method as described for cDNA synthesis (32). Approximately 5 μg of RNA per lane was run on denaturing 1.5% formaldehyde-agarose gels, transblotted, and hybridized with random-primed *CaRLV130* full-length cDNA, the *Act1* gene (22), and the Ca9 oligonucleotide probe. Hybridization and initial washing steps were done as described by Perbal (32), and the final stringent washing step was carried out in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS at 70°C for 30 min. Filters were exposed overnight on 3M XDA plus films with 3M Trimax screens at −80°C.

RNA analysis by RT-PCR. Total RNA of *C. albicans* was extracted as described for cDNA synthesis and quantitated by spectrophotometric analysis. Equal amounts of RNA (approximately 1 μg) were reverse transcribed by the Moloney murine leukemia virus RT (Pharmacia Biotech, Uppsala, Sweden) with oligo(dT)$_{12–18}$ (Pharmacia) as the primer. Samples were diluted to 10, 50, and 250 ng and then amplified by 20 PCR cycles (denaturation at 94°C for 90 s, annealing at 60°C for 90 s, and extension at 72°C for 90 s) with Ca2 and Ca3 as the primers. Each sample was electrophoresed in a 2% agarose gel, transferred to a nitrocellulose filter, and hybridized with an internal ^{32}P-labeled oligomer (Ca9) as a probe. RT-RNAs were normalized against the mRNA of the protein synthesis elongation factor EF1α (*TEF2* gene) (38) by using the Ca5 and Ca6 oligonucleotide primers and Ca7 as a probe.

Expression of recombinant CaHsp70 in *E. coli*. *CaHSP70* sequences were generated by *Eco*RI restriction of *CaRLV130* and *CaRLV105* cDNA inserts or by PCR amplification of the λ*gt11*/*CaRLV130* DNA and the Ca1-λ*gt11* reverse primers. The *Eco*RI fragments were cloned into the *Eco*RI site of pDS56/RBSII6xhis/E$^-$, while the PCR product, after digestion with *Bam*HI and *Eco*RI, was cloned into the *Bam*HI-*Eco*RI polylinker sites of the expression vector pDS56/RBSII6xhis/E$^-$, which results in a fusion of six histidines at the amino termini of CaHsp70 peptides (Table 2). Expression of recombinant six-histidine-tagged (6xhis)-CaHsp70 was obtained in *E. coli* M15 carrying the *lac* repressor-producing pUHA1 plasmid (15). Induction was performed in LB medium containing kanamycin and ampicillin, by addition of isopropyl-β-D-thiogalactopyranoside (IPTG; Boehringer) at a final concentration of 1 mM to a culture with an optical density at 600 nm (1 cm) of 0.6, followed by a further 4-h incubation at 37°C.

Purification of recombinant proteins. Recombinant 6xhis-CaHsp70 proteins were purified by nickel-chelate affinity chromatography (15) as per the instructions of Diagen (Hilden, Germany) for denaturing conditions. Fractions containing the purified polypeptides were pooled and precipitated with 3 volumes of 100% ethanol, resuspended in water, and stored at −20°C.

Immunoblotting. Recombinant proteins from IPTG-induced and noninduced M15(pUHA1, pDS56/RBSII6xhis-CaHSP70) cells and their purified counterparts, as well as whole-cell extracts from *C. albicans* (described below), were resuspended in sample buffer at approximately 1 μg of protein per μl), boiled for 10 min, and subjected to SDS-polyacrylamide gel electrophoresis (PAGE [5 to 15% polyacrylamide gradient]). The electrophoresed materials were electroblotted onto nitrocellulose filters in a buffer containing 25 mM Tris, 192 mM glycine, 0.1% SDS, and 20% methanol. Filters were incubated with antibodies as detailed in single experiments. In all cases, nonspecific binding of antibodies to nitrocellulose was prevented by blocking of the filters with 1% bovine serum albumin in phosphate-buffered saline (PBS) for 2 h at room temperature. After extensive washing with PBS, bound antibodies were detected by alkaline phosphatase-conjugated second antibody.

Whole-cell extracts of *C. albicans* cells grown at 22°C in Lee's medium and after being shifted to 37°C for 3 h were obtained by breaking the cells with 0.1-mm-diameter glass beads and adsorption of mannan on a concanavalin A resin as described elsewhere (3).

Nucleotide sequence accession number. The EMBL Database accession number for the cDNA sequence used in this study is EMBL Z30210.

RESULTS

Molecular cloning of cDNA encoding a 70-kDa Hsp of *C. albicans*. The rabbit immune serum used for screening the cDNA library of *C. albicans* reacted with at least 20 different

TABLE 2. CaHsp70 polypeptides purified on Ni-nitrilotriacetic acid resin column and the corresponding codifying plasmids[a]

Recombinant plasmid designation	Cloned fragment		6-Histidine-coded protein		
	Size (bp)	Position (nt)	Designation	Size in kDa (aa)[b]	CaHsp70 polypeptide
pRLV199[c,d]	2,229	108–2325	CaHsp70	71.3 (664)	Whole protein
pRLV198[d,e]	832	1443–2325	CaHsp70/2	21.0 (202)	C terminal
pRLV197[c]	732	108–836	CaHsp70/3	28.4 (261)	N terminal
pRLV196[c]	1,027	108–1131	CaHsp70/4	39.4 (358)	N terminal

[a] Each polypeptide coding sequence was cloned in the pDS56/RBSII6xhisE$^-$ plasmid and numbered as in the EMBL database. Position 108 corresponds to the second amino acid of CaHsp70 (Table 1).
[b] Includes the six histidines plus all other amino acids (aa) coded by the recombinant plasmid construct.
[c] Corresponding clone of the library, *CaRLV130.*
[d] The stop codon of the cloned fragment is at position 2.073. This explains why pRLV197 gave a fragment with a higher molecular mass than that of the fragment encoded by pRLV198.
[e] Corresponding clone of the library, *CaRLV105.*

antigens, in the molecular mass range of 70 to 12 kDa, as well as with a polydisperse (probably mannoproteic) >100-kDa constituent in Western blots (immunoblots) of whole-cell extracts of the fungus. Sixty-eight antibody-reactive phage clones were identified and isolated by screening of the library with the serum described above. Two partially overlapping clones, as defined by restriction patterns with *Hpa*II, *Hin*fI, and *Alu*I endonucleases, were selected for further analysis. These two clones will hereafter be referred to as *CaRLV105* and *CaRLV 130.*

After subcloning of restriction fragments into pBluescript and DS56/RBSII6xhis/E$^-$ plasmids, two molecules with sizes of 832 and 2,325 bp from *CaRLV105* and *CaRLV130* respectively, were obtained. The sequence of the clone *CaRLV105* expanded the 3′ untranslated region downstream of the C-terminal coding region and was identical to that of the corresponding region of the clone *CaRLV130*. For this latter clone, sequence analysis identified a single open reading frame with a size of 1,968 bp coding for an amino acid sequence containing 656 residues. Both the coding DNA and the deduced amino acid sequence shared significant similarity with members of the eukaryotic *HSP70* family genes (23, 29). We will hereafter refer to this sequence as the *C. albicans* Hsp 70-kDa gene (*CaHSP70*). Unless otherwise indicated, the data reported in this paper refer to the *CaRLV130* clone.

The presence of initiation and stop codons at positions 105 and 2073, respectively, together with the information deduced from the alignment with similar sequences (described below) indicated that *CaRLV130* encompassed the full-length mRNA coding sequence. *CaHSP70* shared the most extensive similarity (73% identity at nucleotide level for the coding region) with the *YSCSSA1* gene, one member of the *HSP70* gene family of *Saccharomyces cerevisiae* (18). The similarity at the amino acid level between the identities of the two proteins (84%) extends over the entire sequence of the gene (Fig. 1).

Southern blot analysis and karyotype assignment. Southern blot analysis in which genomic DNA of *C. albicans* ATCC 20955 was digested with various restriction endonucleases and probed with *CaHSP70* cDNA under high-stringency conditions gave the restriction map shown in Fig. 2. Other hybridized fragments appeared under low-stringency conditions (data not shown [described below]).

With TAFE analysis, the chromosome-sized bands of the *C. albicans* ATCC 20955 genome ranged from approximately 3.8 to 1 Mb. Probing in Southern blots with *CaHSP70* cDNA gave a hybridization signal on the 3.8-Mb R1 (4, 25) chromosome-sized band. However, under low-stringency conditions, other chromosome-sized bands gave also hybridization signals (Fig. 3). Thus, Southern blots of digested DNA and of chromosome-sized bands under low-stringency conditions indicated the presence of other members of the *HSP70* gene family in *C. albicans.*

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  1 MSKAVGIDLGTTYSCVAHFANDRVEIIANDQGNRTTPSFVAFTDTERLIG 50
    |||||||||||||||||||||||||:|||||||||||||||||||||||
  1 MSKAVGIDLGTTYSCVAHFANDRVDIIANDQGNRTTPSFVAFTDTERLIG 50

 51 DAAKNQAAMNPANTVFDAKRLIGRKFDDPEVINDAKHFPFKVIDKAGKPV 100
    ||||||||||.||||||||||||.|:|||| .| |||||||| .|||
 51 DAAKNQAAMNPSNTVFDAKRLIGRNFNDPEVQADMKHFPFKLIDVDGKPQ 100

101 IQVEYKGETKTFSPEEISSMVLTKMKEIAEGYLGSTVKDAVVTVPANFND 150
    ||||:|||||.|.||:|||||.||||.||:|||..|.|||||||| |||
101 IQVEFKGETKNFTPEQISSMVLGKMKETAESYLGAKVNDAVVTVPAYFND 150

151 SQRQATKDAGTIAGLNVLRIINEPTAAAIAYGLDKKGSRGEHNVLIFDLG 200
    ||||||||||||||||||||||||||||||||||.  |.:||||||||
151 SQRQATKDAGTIAGLNVLRIINEPTAAAIAYGLDKKGK..EEHVLIFDLG 198

201 GGTFDVSLLAIDEGIFEVKASAGDTHLGGEDFDNRLVNFFIQEFKRKNKK 250
    |||||||| |::|||||||.|||||||||||||||| ||||||||||
199 GGTFDVSLLFIEDGIFEVKATAGDTHLGGEDFDNRLVNHFIQEFKRKNKK 248

251 DISTNQRALRRLRTACERAKRTLSSSAQTSIEIDSLYEGIDFYTSITRAR 300
    |:|||||||||||||||||||||||||||:|||||:|||||||||||||
249 DLSTNQRALRRLRTACERAKRTLSSSAQTSVEIDSLFEGIDFYTSITRAR 298

301 FEELCADLFRSTLDPVGKVLADAKIDKSQVEEIVLVGGSTRIPKIQKLVS 350
    |||||||||||||||||:||| |||:|||||:|||||||||||||:||||.
299 FEELCADLFRSTLDPVEKVLRDAKLDKSQVDEIVLVGGSTRIPKVQKLVT 348

351 DFFNGKELNKSINPDEAVAYGAAVQAAILTGDTSSKTQDILLLDVAPLSL 400
    |:||||| |:|||||||||||||||||||.|||||||:||||||||||
349 DYFNGKEPNRSINPDEAVAYGAAVQAAILTGDESSKTQDLLLLDVAPLSL 398

401 GIETAGGIMTKLIPRNSTIPTKKSETFSTYADNQPGVLIQVFEGERAKTK 450
    |||||||:||||||||||.||| |.|||||||||||||||||||||||||
399 GIETAGGVMTKLIPRNSTISTKKFEIFSTYADNQPGVLIQVFEGERAKTK 448

451 DNNLLGKFELSGIPPAPRGVPQIEVTFDIDANGILNVSALEKGTGKTQKI 500
    |||||||||||||||||||||||||||||:|.|||||||:||||||..||
449 DNNLLGKFELSGIPPAPRGVPQIEVTFDVDSNGILNVSAVEKGTGKSNKI 498

501 TITNDKGRLSKEEIDKMVSEAEKFKEEDEKEAARVQAKNQLESYAYSLKN 550
    ||||||||||||:|:|||.|||||||||||..|:..||||||.||||||
499 TITNDKGRLSKEDIEKMVAEAEKFKEEDEKESQRIASKNQLESIAYSLKN 548

551 TINDGEMKDKIGADDKEKLTKAIDETISWLDASQAASTEEYEDKRKELES 600
    ||.:::  ||::..||:.:||  :|||||||.. .||.||::|| |||:.
549 TISEAG..DKLEQADKDTVTKKAEETISWLDSNTTASKEEFDDKLKELQD 596

601 VANPIISGAYGAAGGAPGGAGGFPGAGGFPGGAPGAGGPGGATGGESSGP 650
    :||||:|  |.|:|::.|:|||  ::|||||||.|        .|..||
597 IANPIMSKLYQAGGAPGGAAGG..APGGFPGGAPPA.........PEAEGP 636

651 TVEEVD 656
    ||||||
637 TVEEVD 642
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FIG. 1. Alignment of the predicted amino acid sequences of CaHsp70 (upper) and YSCSSA1 (lower). Since the two proteins had different lengths, it was necessary to introduce gaps for the alignment of the sequences (dots). There were 14 insertions and 101 amino acid differences between the *C. albicans* and *S. cerevisiae* proteins. The amino acid is given in the single-letter code, and the symbols (vertical bar, :, and .) denote a decreasing order of matching similarity between each corresponding amino acid pair.

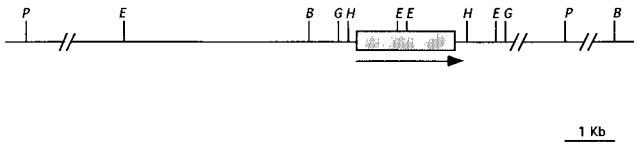

FIG. 2. Restriction map of the *CaHSP70* region (coding region boxed). Abbreviations of restriction endonuclease sites: E, *Eco*RI; P, *Pst*I; B, *Bam*HI; H, *Hin*dIII; G, *Bgl*II. The arrow indicates the direction of transcription.

Immune reactivity of CaHsp70 polypeptides. The approaches described in Materials and Methods allowed for a consistent expression-purification of the recombinant, 6xhis-CaHsp70 protein. In addition, segments of the *CaHSP70* coding region were also cloned in pDS56/RBSII6xhis/E$^-$ plasmid (pRLV198, pRLV197, and pRLV196 (Table 2). The efficiency and degree of CaHsp70 polypeptide purification (the whole protein, two N-terminal fragments, and one C-terminal fragment) are shown in Fig. 4.

The purified whole protein and its cloned fragments were immunoblotted with a monoclonal antibody recognizing an epitope common to all eukaryotic Hsp70s or with sera from normal, healthy subjects. As shown in the representative experiments in Fig. 5, the whole protein and the two N-terminal polypeptides reacted both with the monoclonal antibody (Fig. 5a) and with the human serum (Fig. 5b). (Three serum samples from different subjects were used with similar results). Moreover, an antiserum raised in a mouse against the recombinant CaHsp70 was used in immunoblots with whole-cell extracts from *C. albicans*. The presence of two reactive protein bands in the M_r region of around 70 kDa was clearly evident, and their reactivity was more intense when the fungal cell culture was shifted to 37°C (Fig. 6).

***CaHSP70* gene expression.** The expression of the gene coding for the CaHsp70 protein was studied in cells undergoing temperature shift (from 22°C to 37°C, 30 min) by Northern blotting with the *CaHSP70* cDNA or the Ca9 oligonucleotide used as a probe. Expression experiments were also done by RT-PCR with the Ca2 and Ca3 primers and the Ca9 probe, as detailed in Materials and Methods and Table 1.

The Northern blot with the cDNA (Fig. 7a) showed the presence of at least two clearly visible hybridization bands. Of the two, the 2.4-kb constituent apparently increased its expression in the time interval of the temperature shift, whereas the

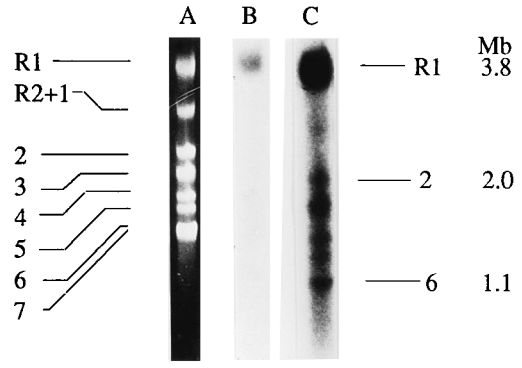

FIG. 3. Chromosome mapping of *CaHSP70*. Chromosome-sized bands of *C. albicans* DNA were separated by pulsed-field electrophoresis (TAFE) as described in Materials and Methods. (A) TAFE karyotype of *C. albicans* ATCC 20955. (B and C) Autoradiographs of the blot probed with *CaHSP70* under high (B)- or low (C)-stringency conditions. Chromosome designation is as defined by Chu et al. (4) and Magee et al. (25).

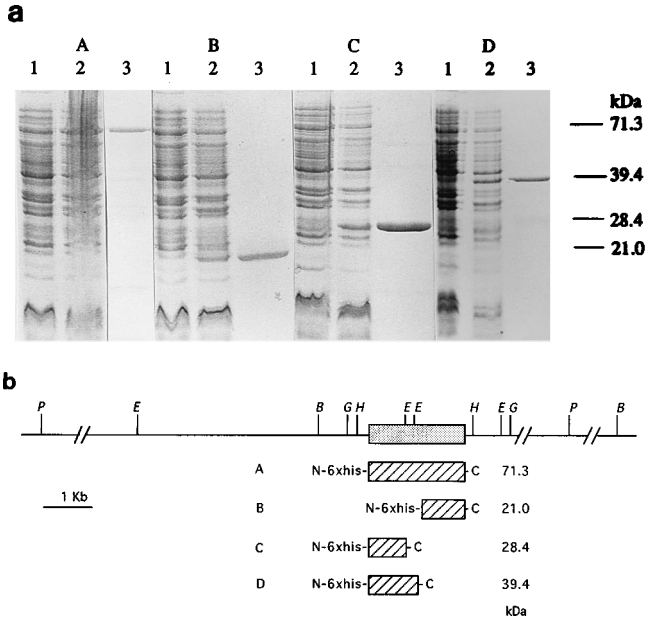

FIG. 4. Expression and purification of CaHsp70 and its cloned polypeptide fragments. (a) Cells of the strains M15(pUHA1, pRLV199) (A), M15(pUHA1, pRLV198) (B), M15 (pUHA1, pRLV197) (C), and M15 (pUHA1, pRLV196) (D), either noninduced (lanes 1) or induced with IPTG (lanes 2), were extracted and subjected to SDS-PAGE, together with the 6xhis-CaHsp70 Ni-NTA affinity chromatography-purified polypeptide (lanes 3). The gels were stained with Comassie blue. (b) Schematic representation of map position and size of the CaHsp70 polypeptides matched with the coding region and the whole restriction map of the *CaHSP70* region. For the meaning of the lettering, see the description of panel a and the legend to Fig. 2. The molecular masses of the cloned polypeptides are indicated by bars on the right side.

2.2-kb band was less expressed in the same time interval. In these experiments, the expression of the actin gene (*Act1*) of *C. albicans* was used as an internal transcriptional control (22). *CaHSP70* transcription was also confirmed by the Northern blot performed with the Ca9-specific primer used as a probe, showing a transcript with a size of 2.4 kb with increasing intensity between 0 and 30 min (Fig. 7).

The expression of *CaHSP70* was also demonstrated by RT-PCR, which identified a heat-inducible amplification product of the expected size (Fig. 7b) that specifically hybridized in Southern blotting with the Ca9 probe. In these experiments, RT-RNAs were compared to the messages of the *TEF2* gene (38) by using Ca5 and Ca6 oligonucleotide primers and Ca7 as a specific probe (Fig. 7b [also described in Materials and Methods and Table 1]). The comparison between *CaHSP70* gene expression and *TEF2* gene expression demonstrated a relative increase in the expression of the former gene when the temperature was shifted to 37°C.

DISCUSSION

In this paper, we describe cloning, molecular characterization and expression of a *C. albicans* gene (*CaHSP70*) belonging to the *HSP70* family and coding for a stress protein designated CaHSP70. Nucleotide analysis of the DNA insert from the cloned cDNA of *C. albicans* revealed a single open reading frame coding for a polypeptide with a size of 656 amino acids (around 70 kDa) which showed a significant similarity with *S. cerevisiae*, *Drosophila melanogaster*, human, and murine *HSP70* genes (23, 29).

The cloned cDNA reacted in Southern blots with digested

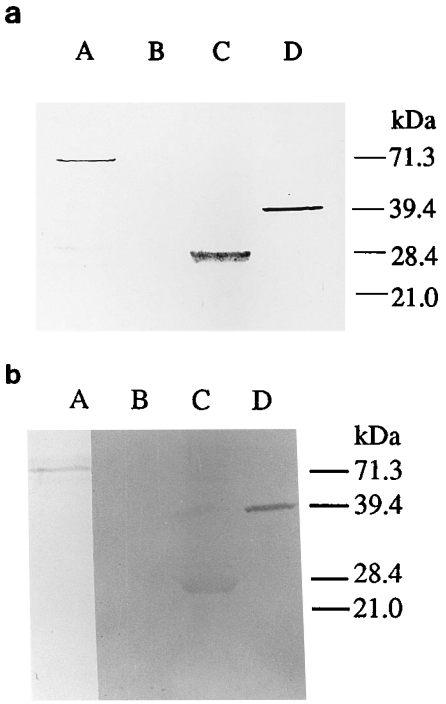

FIG. 5. Immunoblots of purified CaHsp70 polypeptides. Each polypeptide (1 μg) was run in SDS-PAGE and then electrotransferred to nitrocellulose membranes and immunoblotted with anti-Hsp70 monoclonal antibody (dilution, 1:5,000) (a) or human serum from a healthy, normal subject (dilution 1:200) (b). The reaction was developed by phosphatase-conjugated anti-mouse (1:7,500) (a) or anti-human (1:3,000) (b) immunoglobulin G. Lanes A to D are as described in the legend to Fig. 4, lane 3. The bars indicate the molecular masses of immunoblotted polypeptides. For other details, see Materials and Methods.

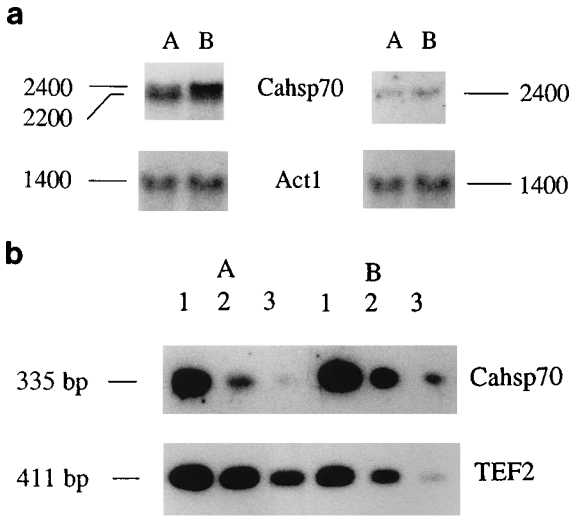

FIG. 7. *CaHSP70* gene expression in *C. albicans* cells exposed to a temperature upshift. RNA was extracted from exponentially growing cells in Lee's medium at 22°C before (A) and after (B) the shift of the temperature to 37°C for 30 min. (a) The nitrocellulose filter containing the blotted RNA was hybridized under high-stringency conditions with the whole *CaHSP70* gene probe (top left) and then dehybridized by being boiled in SDS prior to the subsequent hybridization with the Ca9 oligonucleotide probe (top right). The bottom of the panel (left and right) shows the actin message after hybridization of the same blot probed with the *Act1* gene. (b) RT-PCR of RNA from the samples in lanes A and B (as defined for panel a) performed with Ca2 and Ca3 oligonucleotide primers for the PCR of *CaHSP70* (top) and Ca5-Ca6 primers for the PCR of the control *TEF2* message (bottom). In both cases, the numbers denote decreasing amounts of RNA (250, 50, and 10 ng, approximately, for lanes 1, 2, and 3, respectively). The PCR products were Southern blotted with the Ca9 and Ca7 probes for the specific detection of the *CaHSP70* and *TEF2* gene messages, respectively. For other technical details, see Materials and Methods.

chromosomal DNA of *C. albicans*, and the corresponding gene was localized on the highest-M_r chromosome-sized band of *C. albicans*. Its nucleotide-amino acid sequence identification with a member of the Hsp70 family was strengthened by a clear positive reaction in immunoblots with a monoclonal antibody directed against a common Hsp70 epitope. Moreover, expression of *CaHSP70* was induced on transfer of *C. albicans* cells from 22°C to 37°C, as expected from an inducible Hsp, and a murine serum raised against the purified recombinant CaHsp70 protein clearly identified native, heat-inducible proteins of the expected molecular mass (70 to 71 kDa) in a whole-cell protein extract from *C. albicans.*

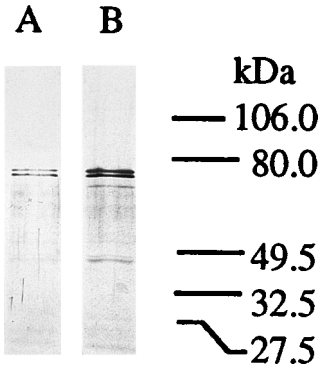

FIG. 6. Immunoblots of *C. albicans* protein extracts with mouse anti-CaHsp70 serum. The extract (10 μg of protein) from *C. albicans* grown at 22°C (lane A) or from the same culture shifted up to 37°C for 3 h (lane B) was run in SDS-PAGE and electrotransferred to nitrocellulose membrane. The reaction was performed with a mouse serum against CaHsp70 at a dilution of 1:800. The reaction was visualized by phosphatase-conjugated second antibody, as detailed in Materials and Methods. The bars indicate molecular mass standards.

Hsps are common and very abundant in all living organisms; thus, their presence in *C. albicans* is not surprising (5, 16, 27). In particular, Howard et al. (16) have shown induction of a 70- to 73-kDa cytoplasmic protein of *C. albicans* upon a shift in the temperature of growth from 23°C to 37°C. However, this is the first cloned *HSP70* gene in this organism. Hsps are induced by sudden increases in temperature as well as by a wide variety of other chemical and physical stresses (29). Thus, the more general term "stress protein" has been introduced, although some Hsps are also constitutively expressed in unstressed cells (9, 29).

The physiological role of CaHsp70, although not explored here, may be conceivably similar to that of other Hsps. For instance, they prevent the unfolding of proteins at high temperatures and serve important functions as housekeeping (molecular chaperone) proteins (7, 9, 28). *YSCSSA1 Hsp70* from *S. cerevisiae* (to which *CaHsp70* shows the highest similarity [i.e., around 84%]) has been suggested to allow for protein translocation across the microsomal membrane into the endoplasmic reticulum. In addition to the sequence similarity between *CaHSP70* and *YSCSSA1*, the expression of *CaHSP70* following temperature shift clearly resembles *SSA1* gene expression in *S. cerevisiae* (6, 18).

Although very extensive, and in some regions actually higher than 90%, the similarity in the deduced amino acid sequence between *YSCSSA1* and *CaHsp70* leaves 14 insertions in the latter and 101 amino acid substitutions between the two se-

quences. Most of the nonconservative substitutions are in the same region (amino acids 557 to 644), presenting the most numerous insertions. Overall, these sequence variations could confer to the CaHsp70 protein an individual immunogenicity. It is of interest that this variable region falls in the protein C terminus, where antigenicity of the molecule is usually dominant (20).

As a consequence of their abundance and the high level of homology among various microorganisms. Hsps are indeed major targets of host immune response (20). This is particularly true for the members of Hsp70 family, which are among the most immunogenic proteins of pathogenic microorganisms (20, 26). For instance, Hsp70s have been supposed to play an important immunogenic role in infections caused by *Plasmodium* (1) *Trypanosoma* (11), *Schistosoma* (14), *Leishmania* (24), and *Mycobacterium* (10) species. In the field of fungal pathogens, a member of the Hsp70 family of *Histoplasma capsulatum* was recognized as a target of cell-mediated, protective immunity (13). Hsp-derived peptides are presented by cells in the context of both major histocompatibility complex class I and class II molecules (19, 20, 34, 35). Since Hsp70 proteins are similar in all eukaryotic organisms, including humans, an immune response to CaHsp70 should be directed against nonhomologous epitopes (probably in the C-terminal residue) to be of any protective significance. In this context, Hsps are indeed likely to be involved also in autoimmune phenomena, if not autoimmune disease. In fact, self Hsp peptides, which are the target of the T-cell response, are homologous to microbial peptides (20), and the four Hsp-derived peptides eluted from human major histocompatibility complex II (19, 34) are conserved in CaHsp70.

In this context, our observations that CaHsp70 is recognized by sera from normal human subjects are relevant. Moreover, preliminary experiments with cultured peripheral blood mononuclear cells from the same subjects showed T-lymphocyte proliferation in response to the recombinant protein (unpublished data). Thus, CaHsp70 seems to be ordinarily expressed as both B-cell and T-cell immunogens in human subjects, and its role could be as important in infections caused by *C. albicans* as it has been demonstrated or supposed for other microorganisms (20, 26).

To our knowledge, the only *HSP* gene that has previously been cloned and characterized in *C. albicans* is *HSP90* (29). Matthews et al. suggested that antibody response to a 48-kDa protein, a breakdown product of Hsp90, protects against systemic candidiasis (27). A limited amino acid homology has also been demonstrated between Hsp70 and yeast enolase, another major immunogenic protein of *C. albicans* (37). The antibody response during systemic candidiasis in inbred CBA/H mice has been shown to include reactivity against a 75-kDa Hsp (5). The molecular cloning of the gene described here may open the way to the disclosure of the functional characteristics of CaHsp70 and to the assessment of its possible role in the pathogenesis of candidiasis and the immune response to *Candida* infection.

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