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Structure and Regulation of the *HSP90* Gene from the Pathogenic Fungus *Candida albicans*

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Candida albicans HSP90 **sequences were isolated by screening cDNA and genomic libraries with a probe derived from the *Saccharomyces cerevisiae* homolog, *HSP82*, which encodes a member of the heat shock protein 90 family of molecular chaperones. Identical sequences were obtained for the 2,197-bp overlap of the cDNA and gene sequences, which were derived from *C. albicans* 3153A and ATCC 10261, respectively. The *C. albicans HSP90* gene contained no introns, and it showed strong homology (61 to 79% identity) to *HSP90* sequences from other fungi, vertebrates, and plants. The C-terminal portion of the predicted Hsp90 amino acid sequence was identical to the 47-kDa protein which is thought to be immunoprotective during *C. albicans* infections (R. C. Matthews, J. Med. Microbiol. 36:367–370, 1992), confirming that this protein represents the C-terminal portion of the 81-kDa Hsp90 protein. Quantitative Northern (RNA) analyses revealed that *C. albicans HSP90* mRNA was heat shock inducible and that its levels changed during batch growth, with its maximum levels being reached during the mid-exponential growth phase. *HSP90* mRNA levels increased transiently during the yeast-to-hyphal transition but did not correlate directly with germ tube production per se. These data do not exclude a role for Hsp90 in the dimorphic transition. Southern blotting revealed only one *HSP90* locus in the diploid *C. albicans* genome. Repeated attempts to disrupt both alleles and generate a homozygous *C. albicans* Δ*hsp90*/Δ*hsp90* null mutant were unsuccessful. These observations suggest the existence of a single *HSP90* locus which is essential for viability in *C. albicans.***

Candida albicans is a major fungal pathogen in humans (35). It is carried as a commensal by many individuals, sometimes causing irritating but superficial infections of the mucus epithelia. However, in immunocompromised patients, infections can become deep-seated or systemic (35). A number of factors are thought to contribute to the virulence of *C. albicans* (11, 35), but definitive experiments which establish the relative importance of these factors have yet to be performed. One potential virulence factor is the ability of this fungus to undergo a dimorphic transition from a yeast to a hyphal form (35, 46), but the importance of this transition in pathogenicity remains unclear (11, 35, 38). Although both the yeast and hyphal forms are found in infected tissues, *C. albicans* hyphae appear better adapted to penetrate tissue (44), and hence this form might play a role in the development of serious, deep-seated infections.

A variety of in vitro conditions can be used to stimulate the yeast-to-hyphal transition in *C. albicans* (35). These conditions frequently include the imposition of a stress, for example, a heat shock. It has been suggested that a heat shock or some other form of stress and possibly the heat shock proteins themselves may be important in the morphological changes observed in other systems, including fungi, protozoa, and mammalian cells (7, 22, 28, 43, 52). Heat shock proteins play a key role in protein biogenesis: they include essential components of the chaperone apparatus that promotes protein folding in vivo (9, 18, 19). Heat shock protein 90 (Hsp90), in particular, has a number of different functions (20), some of which may be involved in the regulation of the morphogenesis. Hsp90 interacts with steroid hormone receptors (36, 40), possibly influences the regulation of protein synthesis (37), and might modulate tyrosine kinase activity (10), for example, by governing the activity of $pp60^{v\text{-}src}$ kinase (54).

Heat shock proteins are major immunogens during a variety of different types of infection (21), including candidiasis (31, 51). An immunodominant 47-kDa protein from *C. albicans* has been identified (31). Antibodies against this 47-kDa protein appear to provide some protection against candidiasis. In animal models, the presence of these antibodies enhances the survival of the host against a *Candida* infection (32), and these antibodies are always present in humans who have survived systemic candidiasis (33). Partial sequencing of the gene encoding the 47-kDa protein revealed homology to Hsp90 sequences, and on this basis it was suggested that the 47-kDa protein is derived from Hsp90 (31). Antibodies against Hsp90 are known to be protective in *Plasmodium falciparum* infections (2). Hence, there is considerable interest in the potential diagnostic and therapeutic value of Hsp90-related products (29, 30).

In this study, we report the isolation and characterization of the *C. albicans HSP90* gene. In addition, we describe its regulation during growth, dimorphism, and heat shock. Ultimately, we hope to understand the importance of Hsp90 for the dimorphism and pathogenesis of *C. albicans.*

MATERIALS AND METHODS

Strains. *C. albicans* (Robin) Berkhout 3153A from the London Mycological Reference Laboratory was used throughout (49), except for the *HSP90* gene disruption experiments, in which *C. albicans* CAI-4 (Δ*ura3*::*imm434*/Δ*ura3*::*imm434*) was used (17). The *C. albicans* genomic library was derived from strain ATCC 10261 (45), and the *C. albicans* cDNA library was derived from strain 3153A (49). The cDNA clone, genomic library, and *HSP90* subclones were maintained in *Escherichia coli* XL-1 Blue (*supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac*$^-$

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F[*proAB*$^+$ *lacI*^{q} *lacZ* ΔM15Tn*10*(Tet^r)]) (Stratagene Ltd., Cambridge, United Kingdom).

Culture conditions. The yeast-to-hyphal transition was induced in vitro by using two alternative experimental conditions. *C. albicans* was grown in the yeast form to the late-exponential growth phase at 25°C with shaking at 200 rpm in YPD (2% glucose, 2% bacteriological peptone, 1% yeast extract). To induce hyphal growth, 10 ml of this culture was then used to inoculate 100 ml of YPD containing 10% bovine calf serum at 37°C (approximately 2×10^7 cells per ml) and samples were removed at various times up to 120 min. Control cultures were grown in fresh YPD at 25°C without serum, 25°C with serum, and 37°C without serum. Alternatively, *C. albicans* was grown in the yeast form to the late-exponential growth phase in Soll's medium at 25°C and pH 4.5 with shaking at 200 rpm, hyphal growth was induced by inoculating 10 ml of this culture into 100 ml of Soll's medium at 37°C and pH 6.5 (approximately 2×10^7 cells per ml) (46), and samples were removed at various times up to 180 min. Control cultures were grown in fresh medium at 25°C and pH 4.5, 25°C and pH 6.5, and 37°C and pH 4.5. Soll's medium refers to the defined medium of Lee et al. (23) as modified by Buffo et al. (6). Cell numbers and morphology were monitored by light microscopy.

To impose heat shock, *C. albicans* yeast cells were grown in YPD at 25°C to the mid-exponential growth phase with shaking at 200 rpm. Cells were diluted 1 to 10 (vol/vol) into fresh YPD preheated to 45°C and left at this temperature for 10 min. Cells were then returned to 25°C and harvested at various times up to 60 min. Control cells were added to medium prewarmed to 25 and 37°C.

To compare the growth of the *C. albicans* Δ*hsp90*/*HSP90* heterozygote with that of the parental strain CAI-4, overnight cultures were grown in SD minimal medium [2% glucose, 0.17% yeast nitrogen base without amino acids, 0.5% $(NH_4)_2SO_4$] containing uridine (100 μg ml^{-1}). Equal numbers of cells from these cultures were then added either to fresh minimal medium containing uridine or to YPD at 25°C, and cell numbers and optical density at 600 nm were measured at different times. At the same time, the dimorphic transition was induced by inoculating cells into YPD containing 10% serum at 37°C, whereupon cell numbers and morphology were monitored by light microscopy.

Library screening. To isolate the *C. albicans HSP90* cDNA, the cDNA library (49) was incubated with 100 μl of XL-1 Blue cells at 37°C for 20 min. This mixture was plated with 8 ml of NZYM agarose onto an NZYM agar plate (NZYM is 1% NZ medium, 0.5% NaCl, 0.5% Bacto extract, and 0.2% $MgSO_4$) and incubated at 37°C until plaques were clearly visible (8 to 10 h). Nitrocellulose filters were laid onto the plates for 5 min. Filters were soaked in 1.5 M NaCl and 0.5 M NaOH for 5 min and then in 1.5 M NaCl and 1 M Tris-HCl (pH 7.5) for 5 min, and this was followed by a short wash in 2× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7]). DNA was bound to the filters by baking at 80°C. The filters were probed with a purified 750-bp *Xba*I fragment derived from the 5′ end of the *Saccharomyces cerevisiae HSP90* gene (15, 27) radioactively labelled by random priming (16). Hybridization was performed at 37°C in solutions containing 50% formamide (34), and the filters were washed with 2× SSPE containing 0.1% sodium dodecyl sulfate (SDS) at 55°C. Positive phage were purified by a second round of plaque hybridization and were stored in SM (39) with chloroform at 4°C.

To isolate the *C. albicans HSP90* gene, approximately 20,000 colonies of the genomic library (45) were subjected to colony hybridization by using standard procedures (39). The filters were probed with the purified 2.2-kbp *Apa*I-*Sma*I *HSP90* cDNA fragment labelled by random priming (16). Hybridization was performed at 42°C in solutions containing 50% formamide (34), and the filters were washed in 2× SSPE containing 0.1% SDS at 60°C. Positive clones were purified by a second round of screening.

DNA sequencing. The *HSP90* cDNA and gene were sequenced by dideoxy procedures (41) with synthetic oligonucleotide primers (Oswel, Edinburgh, United Kingdom). Double-stranded DNA templates were sequenced with Sequenase (Amersham) by using ^{35}S-dATP or with *Taq* polymerase (Promega, Southampton, United Kingdom) by using ^{32}P-end-labelled primers. Both strands of the cDNA and genomic clones were sequenced to completion. The DNA sequences were analyzed by using the Genetics Computer Group programs (13) on the BBSRC computer at the Daresbury Laboratory.

Preparation and analysis of nucleic acids from *C. albicans*. Genomic DNA (50) and total RNA (5, 24) were prepared from *C. albicans* as described previously. Southern blotting (42) and Northern (RNA) analysis (34) were performed with radiolabelled probes prepared by random priming (16) the purified *Apa*I-*Sma*I *HSP90* cDNA fragment. For Northern blotting, yields of total RNA were measured accurately by spectrophotometry at 260 nm, and 20 μg of RNA was loaded in each lane. Following electrophoresis on formaldehyde-containing gels, the RNA was transferred to nylon membranes by vacuum blotting (34). Hybridizations were performed under conditions of probe excess (34) with approximately 5×10^8 dpm of labelled probe, and signals were quantified directly by two-dimensional radioimaging with an AMBIS radioanalytic system (LabLogic; Sheffield, United Kingdom) (34). To date, there has been no report of a *C. albicans* mRNA that remains at sufficiently constant levels during the dimorphic transition to use as an internal loading control on Northern blots (12, 47, 48, 50). Therefore, mRNA levels were measured relative to those of the rRNAs by loading equal amounts of total RNA in each lane of the Northern gels (12, 47, 48, 50).

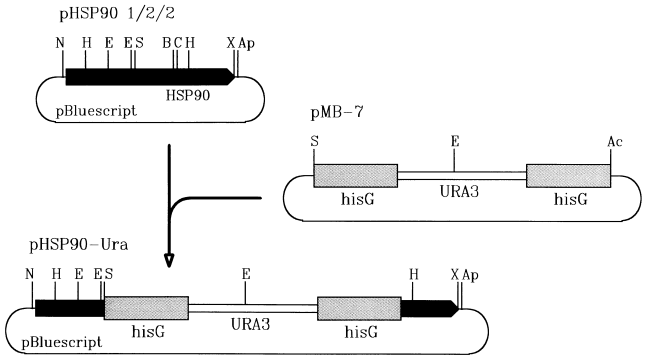

FIG. 1. Construction of pHSP90-Ura for disruption of the *HSP90* gene. The *Sac*I-*Apa*I fragment containing the *hisG*::*URA3*::*hisG* cassette was purified from pMB-7 and ligated between the *Sac*I and *Cla*I sites in the *HSP90* cDNA clone 1/2/2 to construct pHSP-Ura. Solid arrow, *HSP90* cDNA; stippled boxes, *hisG* sequences; white rectangle, *URA3*; thin line, pBluescript sequences; Ac, *Acc*I; Ap, *Apa*I; B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; N, *Not*I; S, *Sac*I; X, *Xba*I. The diagram is not to scale.

RNA 5′ mapping. Primer extension was carried out by following standard procedures (39). In short, 1 μg of primer 5′-CCTTTCTTTCCTTTGGAC-3′ (Oswel) was end labelled by a standard procedure (53) with 150 μCi of [γ-^{32}P]ATP. A total of 500 ng of the labelled primer was precipitated with 220 μg of total RNA prepared from heat-shocked *C. albicans* yeast cells. (These cells were grown overnight in YPD at 25°C, diluted twofold into fresh YPD medium prewarmed at 45°C, and incubated for 10 min at 45°C and then for 10 min at 25°C.) After the mixture was denatured and annealed, primer extension was performed with 48 U of avian myeloblastosis virus reverse transcriptase (Boehringer, Mannheim, Germany) in a total volume of 20 μl. At the same time, the other half of the end-labelled oligonucleotide was used to prime a DNA sequencing reaction on the genomic *HSP90* clone by using *Taq* polymerase. The end-labelled oligonucleotide, the control sequencing reactions, and the primer extension reaction were analyzed on a 6% polyacrylamide-urea gel.

Disruption of *HSP90*. The *hisG*::*URA3*::*hisG* Ura-blaster cassette from pMB7 (17) was isolated by digestion with *Sac*I and *Acc*I and cloned in a two-step ligation into the *HSP90* cDNA, thereby replacing an internal 0.54-kbp *Sac*I-*Cla*I fragment, to generate pHSP90URA (Fig. 1). The 6-kbp *hsp90*::*hisG*::*URA3* fragment in pHSP90URA was released by digestion with *Not*I and *Apa*I and used to transform CAI-4 cells by electroporation. CAI-4 cells were grown overnight in 200 ml of SD medium containing uridine at 25°C with shaking at 200 rpm to the late-logarithmic growth phase. The cells were washed three times in STE (1 M sorbitol, 1 mM EDTA, 10 mM Tris-HCl [pH 7.5]) at room temperature, resuspended in STE containing 50 mM dithiothreitol, and incubated for 10 min at room temperature. The cells were then washed twice in SSE (1 M sorbitol, 10 mM EDTA, 0.1 M sodium citrate [pH 5.8]), resuspended in 5 ml of SSE containing 600 μl of β-glucuronidase (Boehringer), and incubated for 1 h at 30°C. The cells were then washed three times with STE at 4°C and resuspended in about 200 μl of STE. About 10 μg of *hsp90*::*hisG*::*URA3* fragment was added to 100 μl of cell suspension, and electroporation was performed with 0.2-cm cuvettes in a Gene Pulser (Bio-Rad) at a setting of 1.5 kV, 100 Ω, and 25 μF. The cells were resuspended in SD medium containing 1 M sorbitol and plated onto SD plates without uridine. The plates were incubated at 25°C until colonies were visible after about 3 days. Two to five transformants per microgram of DNA were obtained.

Nucleotide sequence accession number. The accession number (EMBL) for the *HSP90* sequence is X81025 CAHSP90.

RESULTS

Isolation of *HSP90* cDNA and genomic clones. The *C. albicans HSP90* cDNA clone was isolated from our cDNA expression library (49) by plaque hybridization, with an *Xba*I fragment with a size of about 750 bp from the *S. cerevisiae HSP90* gene being used as a probe (15, 27). This probe, which was derived from the 5′ end of the *HSP90* gene, increased the likelihood of isolating *C. albicans HSP90* cDNA clones that were close to full length. Two positive plaques were isolated from the 200,000 plaques screened. The cDNA with the longer insert (2.2 kbp) was used in all subsequent experiments. DNA sequencing suggested that, by comparison with other Hsp90

FIG. 2. Sequence of the *C. albicans HSP90* gene. The cDNA sequence extends from nucleotides 681 (arrow) to 2877. The predicted amino acid sequence of the open reading frame is shown. Asterisks above the sequence show the positions of major (641 and 642) and minor (625, 638, 647, 652, and 654) transcriptional start sites (Fig. 4) and 3′ cleavage and polyadenylation sites (2877 to 2881). The two putative heat shock elements are underlined. Significant homologies to other gene sequences are shown in Table 1.

gene sequences (see below), this *C. albicans HSP90* cDNA lacked the 5′ end of its cognate mRNA. Therefore, the full-length *HSP90* gene was isolated by screening a *C. albicans* genomic library (45) by colony hybridization, with the cDNA clone being used as a probe. One positive genomic clone was isolated.

DNA sequencing of *HSP90*. The *HSP90* cDNA and genomic clones were sequenced on both strands (accession number X81025 CAHSP90) (Fig. 2). The cDNA library was generated from *C. albicans* 3153A (49), and the genomic library was generated from ATCC 10261 (45). No differences between the two sequences were observed in the 2,197-bp overlap. The sequence revealed an open reading frame of 707 codons which is predicted to encode a protein with a mass of 81 kDa and a pI of 4.63. The *C. albicans* sequence showed strong homology to Hsp90 sequences from other organisms, with the *S. cerevisiae HSC82* and *HSP82* genes showing the best matches (Tables 1 and 2 and Fig. 3).

Residues 313 to 707 of the predicted Hsp90 amino acid sequence were identical to the sequence of the 47-kDa protein from *C. albicans* described by Matthews et al. (31) (Fig. 3). This suggested strongly that this immunogenic protein represents a Hsp90 degradation product and that we had isolated a full-length version of the gene encoding this 47-kDa protein.

Mapping the *HSP90* transcription unit. The transcriptional start sites on the *HSP90* gene were mapped by primer extension (Fig. 4). Major transcriptional start sites were observed at −29 and −30 with respect to the predicted translational start codon, which represents the 5′-proximal ATG on these *HSP90* mRNAs (Fig. 2). A TATA-like sequence (4, 14) exists 79 bases upstream from the major transcriptional start sites, and two putative heat shock elements (8, 14) lie in the sequenced part of the 5′ flanking region (Fig. 2). A comparison of the cDNA and genomic sequences revealed that the gene carried no introns and that 3′ polyadenylation occurred between nucleotides 2877 and 2881. It was not possible to determine the exact

TABLE 1. Homologies to the *C. albicans HSP90* gene sequence[a]

Gene	Identity (%)	Length of overlap (bp)
Saccharomyces cerevisiae hsc82	79.0	2,262
Saccharomyces cerevisiae hsp82	78.1	1,568
Histoplasma capsulatum hsp82	67.0	1,153
Lycopersicon esculentum hsc80	64.8	1,894
Arabidopsis thalia hsp83	64.5	2,204
Pharbitis nil hsp83	64.1	1,617
Arabidopsis thalia hsp81	63.8	1,662
Chicken *hsp90*	63.7	1,608
Human *hsp90*	63.3	1,545
Rat *hsp90*	61.5	1,510
Mouse *hsp84*	61.0	1,507

[a] Sequence comparisons were achieved with the FASTA program and the EMBL and GenBank databases at the SERC Daresbury Computer facility. Only significant sequence homologies (>50%) are shown.

position of 3′ processing, because a stretch of five A residues exists in this region of the genomic sequence (Fig. 2).

***HSP90* expression under heat shock conditions.** To confirm that we had isolated a heat shock gene, we examined *HSP90* mRNA levels under heat shock conditions. Quantitative Northern analysis was performed on RNA prepared from *C. albicans* yeast cells following treatments at 25, 37, or 45°C, with the *HSP90* cDNA being used as a probe (Fig. 5). Ethidium bromide staining of the Northern gel confirmed that, with the exception of the 30-min timepoint at 37°C, similar amounts of RNA were loaded in each lane (data not shown). The *HSP90* probe detected a single band of about 2.5 kb in length on Northern blots. This length is consistent with that expected from the RNA mapping data (Fig. 2 and 4).

The *HSP90* mRNA was strongly induced by heat shock at 45°C (about ninefold), returning to normal levels after about 60 min. A shift in temperature to 37°C or the addition of fresh medium at 25°C had little effect upon the level of *HSP90* mRNA. Under identical experimental conditions, ribosomal protein 10 (Fig. 5) and pyruvate kinase mRNAs (48) were repressed by heat shock. All three mRNAs are abundant under normal growth conditions. Therefore, the *HSP90* gene is heat shock inducible.

***HSP90* mRNA levels during morphogenesis.** It has been suggested that stress proteins may play a role in dimorphism (22, 28, 43, 52). Therefore, we measured *HSP90* mRNA levels during the yeast-to-hyphal transition under different experimental conditions. The dimorphic transition was induced by the addition of serum combined with an increase in temperature (Fig. 6), by an increase in medium pH combined with a temperature upshift (Fig. 7), or by the use of a medium containing *N*-acetylglucosamine (data not shown). The observed patterns of *HSP90* expression were different from those observed previously for other *C. albicans* mRNAs. In contrast to that of the *HSP90* mRNA, the levels of glycolytic mRNAs decrease transiently during the yeast-to-hyphal transition (48). Also, although translation elongation factor 3 (48), actin (48), and ribosomal protein 10 mRNAs (50) increase transiently during the transition, they display kinetics different from those of *HSP90* mRNA.

A transient increase in the *HSP90* mRNA level was observed when dimorphism was induced by serum addition combined with a temperature upshift. However, a similar increase was observed in the culture receiving the temperature upshift alone (Fig. 6). Cells undergoing the pH- and temperature-induced transition did not show such a dramatic increase in *HSP90* mRNA abundance, although a large increase was observed in cells treated by the temperature upshift alone (Fig. 7). Therefore, increased *HSP90* mRNA levels did not correlate directly with the elevation in temperature from 25 to 37°C, although the mRNA did appear to respond to a mild heat shock under some conditions (compare Fig. 5 to 7). Also, *HSP90* mRNA levels did not correlate closely with germ tube formation. Dimorphism was induced by using serum and temperature (Fig. 6), pH and temperature (Fig. 7), or *N*-acetylglucosamine (data not shown). No consistent relationship between the number of cells forming germ tubes and the level of the *HSP90* mRNA was observed.

***HSP90* mRNA levels during growth.** The experiments described above indicated that *HSP90* expression does not respond to a temperature increase in all cases or to dimorphism per se. To test the possibility that this gene is regulated during growth, we measured *HSP90* mRNA levels in *C. albicans* yeast cells during growth and starvation in YPD medium at 25°C. Approximately equal amounts of RNA isolated at different times during growth were analyzed by Northern analysis (Fig. 8). Maximal levels of *HSP90* mRNA were observed during the early logarithmic growth phase, and these levels declined during the transition phase to relatively low levels in stationary cultures. Therefore, the expression of the *HSP90* gene is regulated during batch growth. However, *HSP90* mRNA was present under all experimental conditions analyzed (Fig. 5 to 8).

Number of *HSP90* loci. *S. cerevisiae* has two homologous loci, *HSC82* and *HSP82*, which encode members of the Hsp90 family (8). To investigate the possible existence of several *HSP90*-like loci in *C. albicans*, genomic DNA was subjected to Southern analysis by using the cDNA as a probe and with the filters washed at medium stringencies (2× SSPE containing 0.1% SDS at 65°C) (Fig. 9). We reasoned that genes with a high degree of homology in their coding regions are likely to show restriction polymorphisms in their flanking regions. Therefore, a range of different restriction digests were performed with enzymes which digest within (for example, *Bam*HI, *Eco*RI, and *Hin*dIII) or without (for example, *Pst*I and

TABLE 2. Homologies to the *C. albicans* Hsp90 amino acid sequence[a]

Protein	Identity (%)	Length of overlap (aa)[b]
Saccharomyces cerevisiae Hsc82	84.2	704
Saccharomyces cerevisiae Hsp82	84.2	709
Histoplasma capsulatum Hsp82	74.7	617
Arabidopsis thaliana Hsp83	65.9	706
Rice Hsp82	64.4	705
Trypanosoma cruzi heat shock-like 85-kDa protein	63.0	705
Leishmania amazonensis Hsp83	62.7	703
Trypanosoma brucei subsp. *brucei* Hsp83	62.4	705
Chicken Hsp90-alpha	62.3	718
Human Hsp90-alpha	62.0	723
Theileria parva Hsp90	61.7	716
Mouse Hsp86	61.5	724
Drosophila melanogaster Hsp82	61.2	714
Chicken Hsp90-beta	61.0	721
Human Hsp90-beta	60.9	719
Mouse Hsp84	60.9	719

[a] Sequence comparisons were achieved with the FASTA program and the Swissprot database at the SERC Daresbury Computer facility. Only significant sequence homologies (>50%) are shown.

[b] aa, amino acids.

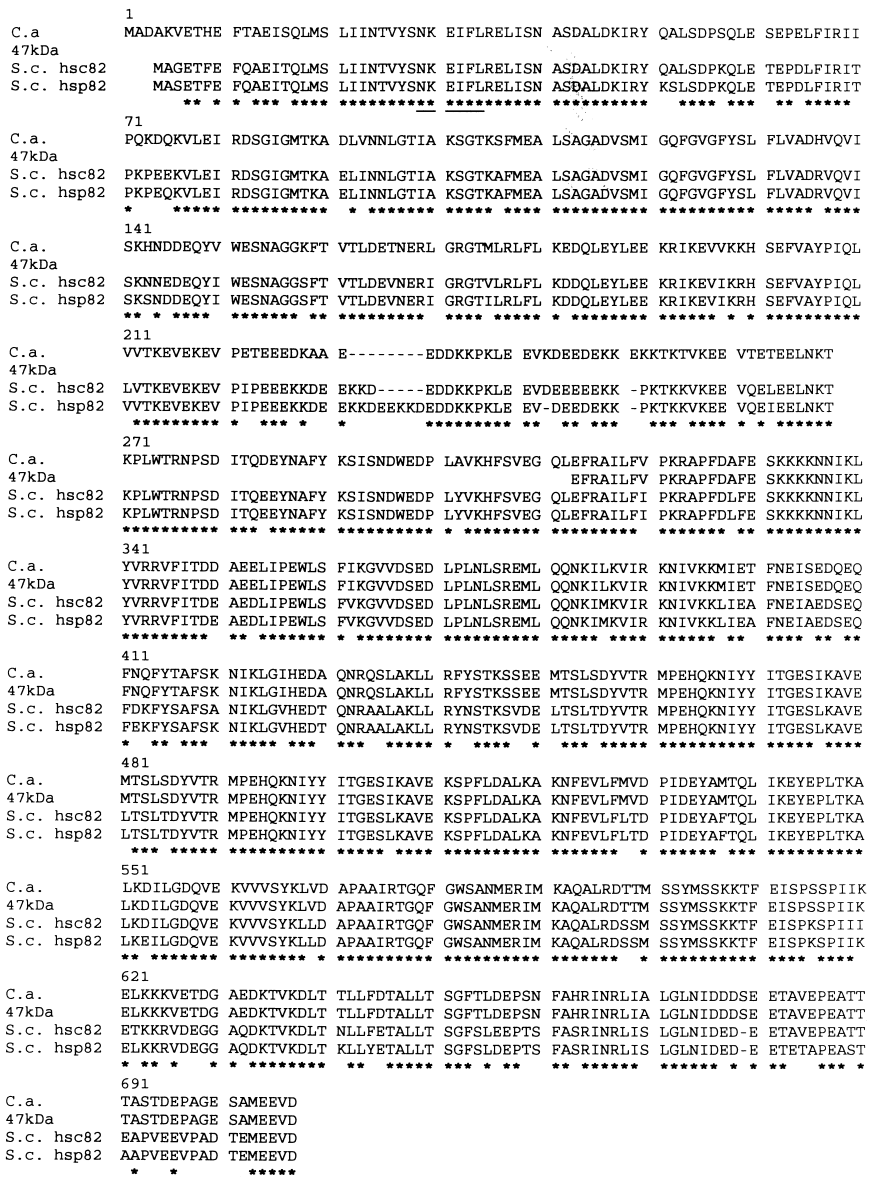

FIG. 3. Alignment of the predicted amino acid sequence of the Hsp90 protein (C.a.) with those of the *C. albicans* 47-kDa protein (47kDa) and Hsc82 (s.c. hsc82) and Hsp82 (s.c. hsp82) from *S. cerevisiae*. The percentages of identity to the two *S. cerevisiae* proteins and to other Hsp90 proteins are shown in Table 2. Asterisks indicate identical residues in all proteins shown. The Hsp90 protein family motif, NKEIFL, is underlined.

*Xba*I) the *HSP90* cDNA. The patterns of hybridization suggested that, as in *Drosophila melanogaster* and *E. coli* (25), a single *HSP90* locus exists in *C. albicans*. The number of bands in each lane could be accounted for by a single *HSP90* locus. No heterozygosity was observed for this locus with respect to the enzymes studied.

***HSP90* gene disruption.** To investigate the roles of Hsp90 in the diploid yeast *C. albicans*, we have attempted to create a Δ*hsp90* null mutant by gene disruption by the Ura-blasting procedure (17) first developed in *S. cerevisiae* (1). Plasmid pHSP90-Ura was created by inserting the *hisG*::*URA3*::*hisG* cassette from pMB-7 between the *Sac*I and *Cla*I sites in the *HSP90* cDNA, thereby deleting 0.54 kbp of the coding region (Fig. 1). The *Pst*I-*Apa*I fragment carrying the resulting Δ*hsp90*::*hisG*::*URA3*::*hisG* cassette was transformed into *C. albicans* CAI-4 cells by electroporation, selecting for Ura^+ transformants. Of 25 transformants obtained, 7 were subjected to Southern analysis to test for the insertion of the *hisG*::*URA3*::*hisG* cassette at the correct locus. All seven transformants showed the expected hybridization pattern for the *HSP90*/Δ*hsp90*::*hisG*::*URA3*::*hisG* heterozygote. One of these transformants was streaked onto plates containing 5′-fluoroorotic acid (FOA), and seven of the resulting $Ura3^-$ colonies were selected for Southern analysis. Five of the seven FOA-resistant segregants had reverted to the wild type (*HSP90*/*HSP90*), but two had undergone the desired *hisG* recombination to generate genotype *HSP90*/Δ*hsp90*::*hisG* with the concomitant loss of *URA3* (Fig. 10).

Five unsuccessful attempts to generate a homozygous null mutant by transforming the *HSP90*/Δ*hsp90*::*hisG* heterozygote with the Δ*hsp90*::*hisG*::*URA3*::*hisG* cassette were made. Our inability to isolate the homozygous null mutant was consistent with the notion that a single *HSP90* locus exists in *C. albicans* and that Hsp90 is essential for viability.

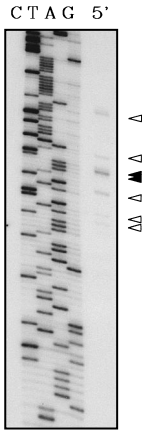

FIG. 4. Transcriptional start sites on the *C. albicans HSP90* gene. The transcriptional start sites were mapped by primer extension (5′) and electrophoresed alongside an *HSP90* sequencing reaction (CTAG) performed with the same primer. The positions of the major start sites are shown on the *HSP90* sequence (Fig. 2). Solid arrowheads, major start sites; open arrowheads, minor start sites.

The phenotype of the *HSP90*/Δ*hsp90*::*hisG* heterozygote was compared with that of parental strain CAI-4 during growth and dimorphism. The mutant showed normal growth rates in both minimal and rich media and normal rates of germ tube formation following induction with serum and a temperature upshift (Fig. 11).

DISCUSSION

Heat shock proteins have been associated with morphological changes in pathogenic fungi and protozoa (28, 43, 52). Also, it has been suggested that antibodies against the 47-kDa protein play a major role in the immunological defense against systemic *C. albicans* infections (32, 33) and that this 47-kDa protein is derived from Hsp90 (31). For these reasons, we isolated and characterized cDNA and genomic versions of *HSP90* from the dimorphic fungus *C. albicans.*

Two types of data showed that we were successful in cloning *C. albicans HSP90*. The first was the strong sequence homology

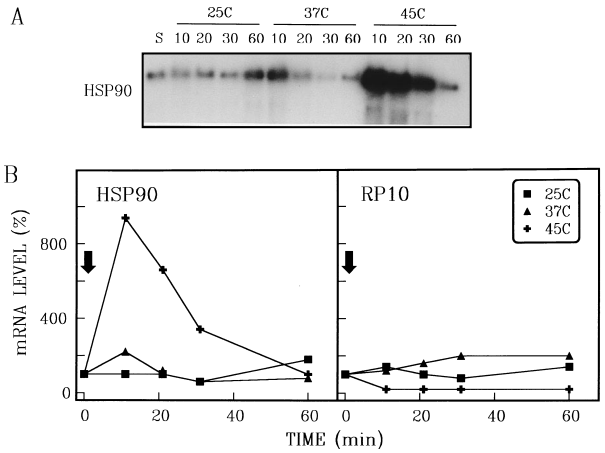

FIG. 5. *HSP90* mRNA is heat shock inducible. An early exponential culture of *C. albicans* grown in YPD at 25°C (S, start) was diluted fourfold in fresh YPD at 25, 37, or 45°C; the cultures were held at these temperatures for 10 min; and then they were cooled to 25°C (time = 0 [arrow]). RNA from each culture was prepared at various times thereafter, and the levels of *HSP90* mRNA were determined by Northern analysis (A), with two-dimensional radioimaging being used to quantify signals directly on the filter (B). mRNA levels were measured as a percentage of those at time 0. Cells grew in the yeast form throughout the experiment. Data on ribosomal protein 10 (RP10) mRNA, obtained under identical conditions, were taken from reference 50.

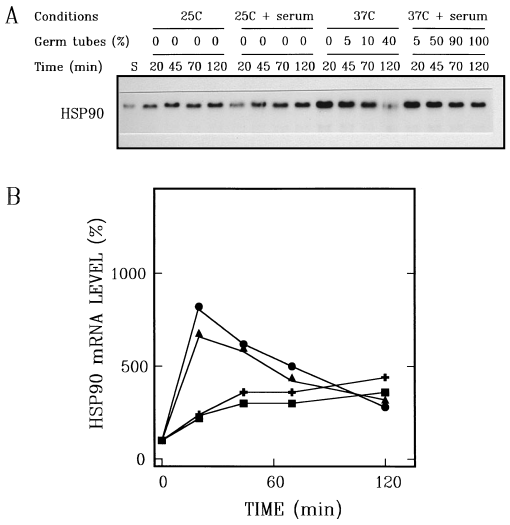

FIG. 6. *HSP90* mRNA levels during the yeast-to-hyphal transition in YPD medium containing serum. An overnight starter culture of *C. albicans* (S) grown in YPD at 25°C was used to inoculate flasks containing either YPD at 25°C (A, 25C; B, ■), YPD containing serum at 25°C (A, 25C + serum; B, ✚), YPD at 37°C (A, 37C; B, ●), or YPD containing serum at 37°C (A, 37C + serum; B, ▲), and the proportion of cells forming germ tubes in each culture was determined by light microscopy. The levels of the *HSP90* mRNA were measured at various times thereafter by quantitative Northern analysis. The radioactive signals obtained on the filter (A) were quantified directly by two-dimensional radioimaging (B). The mRNA levels presented are relative to those in the starter culture immediately before dilution into fresh medium (100%).

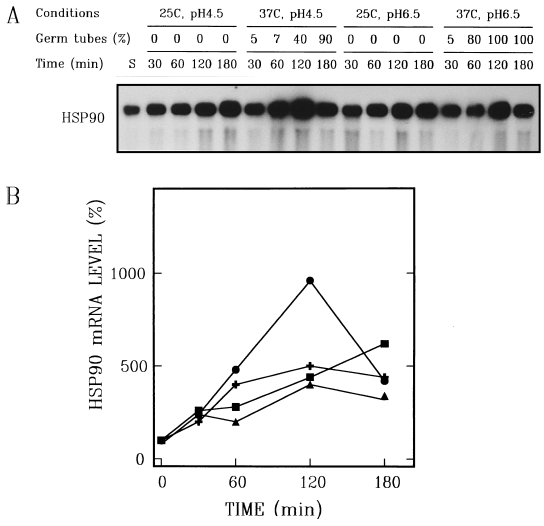

FIG. 7. *HSP90* mRNA levels during the yeast-to-hyphal transition in Soll's medium. An overnight starter culture of *C. albicans* (S) grown in Soll's medium at pH 4.5 and 25°C was used to inoculate flasks containing Soll's medium at pH 4.5 and 25°C (A, 25C, pH 4.5; B, ■), pH 4.5 and 37°C (A, 37C, pH 4.5; B, ●), pH 6.5 and 25°C (A, 25C, pH 6.5; B, ✚), or pH 6.5 and 37°C (A, 37C, pH 6.5; B, ▲). The proportion of cells forming germ tubes in each culture was determined by light microscopy. The levels of *HSP90* mRNA were measured at various times thereafter by quantitative Northern analysis. The radioactive signals obtained on the filter (A) were quantified directly by two-dimensional radioimaging (B). The mRNA levels are presented relative to those in the starter culture immediately before dilution into fresh medium (100%).

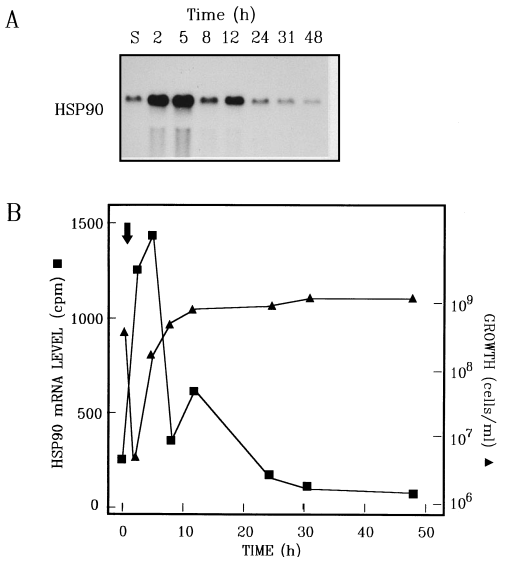

FIG. 8. *HSP90* mRNA levels during growth of the yeast form in YPD at 25°C. An overnight starter culture of *C. albicans* 3153A was used to inoculate a fresh culture of YPD (arrow). The culture was incubated at 25°C with shaking (200 rpm). Northern analysis was performed on RNA prepared from the starter culture (S) and from cells harvested at various times during growth. Cell numbers were monitored by light microscopy. Radioactive signals on this filter (A) were quantified by two-dimensional radioimaging (B).

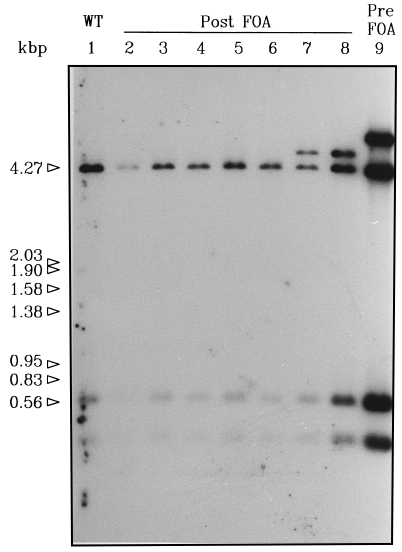

FIG. 10. Southern analysis of *C. albicans HSP90* mutants. DNAs from parental strain CAI-4 (WT [wild type], lane 1), a Ura3$^+$ transformant (*HSP90*/Δ*hsp90*::*hisG*::*URA3*::*hisG*) (Pre FOA, lane 9), and seven FOA-resistant segregants (*HSP90*/Δ*hsp90*::*hisG*) (Post FOA, lanes 2 to 8) were digested with *Eco*RI and probed with the radiolabelled *Apa*I-*Sma*I fragment of *HSP90* cDNA.

with fungal, plant, and vertebrate Hsp90s (Tables 1 and 2). Not surprisingly, the predicted amino acid sequence of *C. albicans* Hsp90 was most homologous to those of *S. cerevisiae* Hsc82 and Hsp82 (84% identity) (Fig. 3). In addition, *C. albicans* Hsp90 contained the sequence NKEIFL, which is characteristic of the Hsp90 family, as well as the C-terminal sequence EEVD common to all eukaryotic cytosolic Hsp90s and Hsp70s (26). The predicted mass of *C. albicans* Hsp90 (81 kDa) was smaller than the size (92 kDa) observed on SDS-polyacrylamide gel electrophoresis gels (31). This was also the case for the *S. cerevisiae* homolog Hsp82 (15).

The heat shock inducibility of the *HSP90* gene also supported the idea that a bona fide heat shock gene had been cloned. Quantitative Northern analysis revealed that, in contrast to those of the ribosomal protein 10 and pyruvate kinase mRNAs (48, 50), the level of *C. albicans HSP90* mRNA increased about ninefold following a temperature shift from 25 to 45°C (Fig. 5). This heat shock inducibility was consistent with the identification of two sequences with homology to heat shock elements in the sequenced region of the *HSP90* promoter (Fig. 2), and it is possible that additional elements lie further upstream. Such elements, which consist of three or more GAA repeats in alternating orientation, have been shown to play a role in the transcriptional induction of genes in response to heat shock (8). Although the location of these putative heat shock elements in the *HSP90* promoter region would be consistent with such a role, their functional significance remains to be tested experimentally.

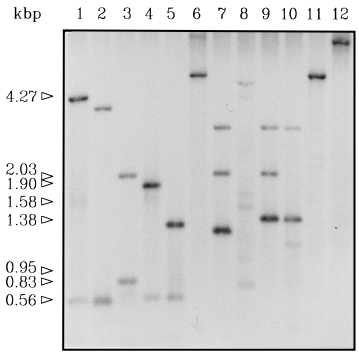

FIG. 9. Southern analysis of *HSP90* sequences in genomic DNA from *C. albicans* 3153A. Filters were probed with the *Apa*I-*Sma*I fragment of the *HSP90* cDNA at medium stringencies. Enzymes used to digest genomic DNA: lane 1, *Eco*RI; lane 2, *Eco*RI plus *Bam*HI; lane 3, *Eco*RI plus *Hin*dIII; lane 4, *Eco*RI plus *Pst*I; lane 5, *Eco*RI plus *Xba*I; lane 6, *Bam*HI; lane 7, *Bam*HI plus *Hin*dIII; lane 8, *Bam*HI plus *Xba*I; lane 9, *Hin*dIII; lane 10, *Hin*dIII plus *Xba*I; lane 11, *Xba*I; lane 12, *Pst*I.

Under some experimental conditions, a mild heat shock from 25 to 37°C correlated with an increase in the level of *HSP90* mRNA (Fig. 6). Hence, the protein coded for by *HSP90* might correspond to the heat shock-inducible protein with a size of 84 to 85 kDa described by Zeuthen and Howard (55). However, an increase in the level of *HSP90* mRNA did not follow the imposition of a mild heat shock under all experimental conditions (Fig. 5 and 7). Hence, although *HSP90* was clearly heat shock inducible (Fig. 5), the gene might not be capable of responding to a mild heat shock under all growth conditions.

Our data suggest that Hsp90 might be coded for by a single locus in *C. albicans*. Only one *HSP90*-like locus was revealed by Southern analysis under conditions which detect closely related genes, such as *S. cerevisiae HSC82* and *HSP82* (Fig. 9). In *S. cerevisiae*, both of these Hsp90-encoding loci must be disrupted before viability is lost (3). However, we were unable to construct a homozygous *C. albicans* Δ*hsp90*/Δ*hsp90* null mutant even after five attempts, although the generation of the heterozygous Δ*hsp90*/*HSP90* strain was relatively straightforward. Obviously, the existence of a single essential *HSP90* locus could account for these negative observations. However, formal proof of this idea is dependent upon the generation of a conditional mutation in the *C. albicans HSP90* gene or in a strain in which Hsp90 synthesis is driven by a regulatable pro-

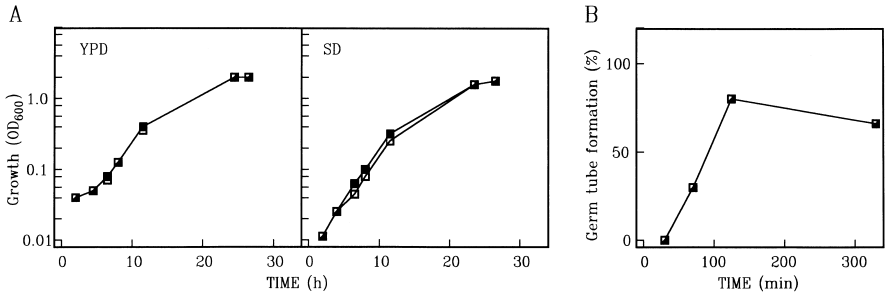

FIG. 11. Growth and dimorphism of *HSP90* mutant HSP-FOA 23. The phenotypes of HSP-FOA23 (open squares) and CAI-4 (solid squares) were compared. (A) Growth in rich medium (YPD) or minimal medium containing uridine (SD). (B) Kinetics of germ tube formation in YPD and serum. OD_{600}, optical density at 600 nm.

moter (such as the *GAL1* promoter in *S. cerevisiae*). So far, there are no reports describing a regulatable promoter that has been developed for use in *C. albicans.*

Hsp90s are thought to be essential for viability (3). Hence, the existence of a single *HSP90* locus would have implications for the provision of this family of molecular chaperones in *C. albicans*. A single *HSP90* locus would presumably have to provide this function under all growth conditions. Interestingly, *C. albicans HSP90* mRNA was detected under all of the growth conditions studied, for example, in heat-shocked and non-heat-shocked cells (Fig. 5), in yeast and hypal cells (Fig. 6 and 7), during exponential growth and stationary phase (Fig. 8), and in both rich and defined media (Fig. 6 and 7). With respect to the two Hsp90-encoding loci in *S. cerevisiae*, a division of labor has evolved: *HSP82* expression is induced in response to stress, whereas *HSC82* is expressed constitutively (3, 15).

HSP90 mRNA levels were not induced during the yeast-to-hyphal transition under all of the experimental conditions tested, and when an induction was observed, similar increases were observed in control cultures. Therefore, *HSP90* expression did not appear to be regulated specifically in response to the dimorphic transition. Also, the kinetics of germ tube induction were not affected by the disruption of one *HSP90* allele (Fig. 11). These data, however, do not exclude a possible role for Hsp90 in hyphal development. Rather, Hsp90 probably fulfills numerous important roles during growth, stress, and morphogenesis.

The C-terminal portion of the Hsp90 sequence (Fig. 3) was identical to the published sequence for the immunoprotective 47-kDa protein (31). Since our data suggest that only one *HSP90* locus exists in *C. albicans*, the 47-kDa protein would appear to be an Hsp90 degradation product. Given the complete identity of their amino acid sequences (Fig. 3), the detection of a single *HSP90*-like mRNA on Northern blots, and the lack of other *HSP90*-like loci on the Southern blots (Fig. 9), the existence of a second locus encoding the 47-kDa protein is unlikely (29). However, the availability of the complete *HSP90* gene sequence might now present new opportunities for the development of anti-*Candida* therapies. For example, like the 47-kDa protein (29, 30, 32, 33), the N-terminal region of the *HSP90* gene product might prove immunoprotective against *Candida* infections.

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