Molecular cloning and sequence analysis of yps-3, a yeast-phase-specific gene in the dimorphic fungal pathogen *Histoplasma capsulatum*

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Genes specifically expressed in the parasitic yeast phase of *Histoplasma capsulatum* have been cloned to clarify the mechanisms underlying both pathogenesis and morphogenesis in this human dimorphic fungal pathogen. Previous studies have determined that the yeast-phase-specific gene, yps-3, is expressed differentially in two non-isogenic strains which differ in their thermostolerance and virulence. We have cloned the yps-3 homologues from the high virulence (G217B) and low virulence (Downs) strains, and obtained a partial cDNA clone representing the expressed gene from *H. capsulatum* G217B. The Downs clone harbours a 287 bp insertion sequence that disrupts a long ORF defined by the yps-3 G217B cDNA. Although the insertion sequence contains features reminiscent of mobile genetic elements, including 15 bp direct repeats of flanking sequence, it is not randomly distributed in the *H. capsulatum* genome. S1 nuclease analysis was utilized to map the 5' end of the expressed yps-3 gene in G217B to potential regulatory regions which are largely homologous in both strains. This finding may point to a deficiency in a temperature inducible regulatory protein in the low virulence, temperature-sensitive Downs strain. The nucleotide sequence of the yps-3 gene and the predicted amino acid sequence of its product represents the first report of phase-specific gene and protein sequences in this widely distributed fungal pathogen. Further analysis of the product encoded by the yps-3 gene may provide significant insight into the pathogenic repertoire of *H. capsulatum.*

**Keywords**: *Histoplasma capsulatum*, yps-3, yeast-phase-specific genes, cloning

**INTRODUCTION**

Yeast-phase-specific (yps) genes which are potentially related to virulence or morphogenesis, or both, have been isolated and cloned from *Histoplasma capsulatum*, a dimorphic human fungal pathogen (Keath *et al.*, 1989a). The organism exists as a multicellular mycelium in rich soils and organic matter in temperate environments worldwide, and proliferates as a unicellular yeast in infected host tissues (Wheat, 1988; Maresca & Kobayashi, 1989). Clinically inapparent or mild disease can result from limited, primary site infection of *H. capsulatum* in the lungs, but an often life-threatening disseminated form of histoplasmosis can occur in immunodeficient patients, particularly the elderly and groups with acquired immunodeficiency syndrome (AIDS) (Graybill, 1988; Wheat *et al.*, 1990).

Since the yeast phase of the organism is the parasitic form, one approach to understanding virulence and/or morphogenesis has been to clone genes expressed specifically in the yeast phase at 37 °C. Expression of the previously cloned yps-3 gene appears to correlate with virulence, temperature sensitivity, and the speed of the hyphal-to-yeast transition in three non-isogenic *H. capsulatum* strains (Medoff *et al.*, 1986b; Keath *et al.*, 1989a). For example, the yps-3 gene is expressed exclusively in the yeast phase of virulent strains, such as G217B, but is not transcribed in either the mycelial or yeast phases of low virulence strains, such as Downs or several similar isolates recently obtained from patients with AIDS (Spitzer *et al.*, 1990). In G217B or other class 2 strains defined by analysis of various
restriction fragment length polymorphisms in mitochondrial (Vincent et al., 1986), ribosomal (Spitzer et al., 1989), or nuclear genes (Keath et al., 1989b, 1992), the yps-3 gene is expressed 1 d following an in vitro transition when mycelia grown initially at 25 °C are induced to form yeast by shifting the temperature of incubation to 37 °C (Keath et al., 1989a).

Virulence in H. capsulatum, and its relation to morphogenesis is of particular interest. Although conversion to the yeast phase is most probably required for progression in vitro, phases in 41 °C (Medoff et al., 1987). Genes essential for early adaptation to elevated temperatures or for the conversion to, and maintenance of, the parasitic phase are likely to represent significant virulence determinants. Adaptation to the macrophage or tissue environment of the host and transformation to the yeast state may entail the activation of temperature-sensitive promoters or control elements which may, in turn, be strongly influenced by alteration in temperature.

To further understand the genetic and regulatory processes in H. capsulatum, the genomic yps-3 gene from G217B and the non-expressed homologue from the Downs strain have been cloned and sequenced. The Downs strain contains a repetitive 287 bp sequence which may be a major determinant. Adaptation to the macrophage or tissue environment of the host and transformation to the yeast state may entail the activation of temperature-sensitive promoters or control elements which may, in turn, be strongly influenced by alteration in temperature.

METHODS

Organisms and culture conditions. Stock cultures of H. capsulatum G217B (ATCC 26032) and the temperature-sensitive Downs strain (ATCC 38904) were maintained in liquid medium containing 2% (w/v) glucose and 1% (w/v) yeast extract at 25 °C or 37 °C for hyphal and yeast phases, respectively. For temperature sensitivity experiments, mycelia were induced to form yeast cells at 34 °C in liquid culture.

Isolation of mRNA for Northern blot analysis and cDNA library construction. Total cellular RNA was isolated from the Downs and G217B strains of H. capsulatum by mechanical disruption in UNSET buffer (8 M urea, 2%, w/v, SDS, 150 mM NaCl, 100 mM Tris/HCl, pH 7-5, and 1 mM EDTA) by Braun homogenization. Nucleic acids were repeatedly extracted in phenol–chloroform–isoamyl alcohol (PCI), followed by ethanol precipitation. Following recovery, the RNA was resuspended in NSET buffer (UNSET buffer lacking urea), and digested with proteinase K (35 µg ml⁻¹, Boehringer Mannheim) for 30 min at 37 °C. The RNA was re-extracted with PCI and precipitated in ethanol. PolyA RNA was obtained from both strains and phases by oligoT cellulose chromatography (Ono et al., 1980), electrophoresed with commercial RNA markers (Gibco BRL) in 1:1% (v/v) formaldehyde agarose (Rave et al., 1979) and transferred to Nitran (Schleicher and Schuell) as previously described (Keath et al., 1992). The blot was hybridized under aqueous conditions (7% SDS, 1% BSA, 1 mM EDTA, 0.5 M sodium phosphate buffer) with 10³ c.p.m. of probe per ml of hybridization solution (Church & Gilbert, 1984). Probes were prepared by nick-translation (Rigby et al., 1977) and included the previously described 1.85 kb HindIII fragment representing the G217B yps-3 gene (Keath et al., 1989a), and the 1.4 kb BamHI–HindIII fragment containing the cloned H. capsulatum actin gene (Spitzer et al., 1990). For CDNA library construction, SDS was omitted from the isolation procedure during the initial breakage and in the subsequent steps, since SDS appeared to inhibit the extent of first strand synthesis (unpublished observations).

Generation of a yeast-phase-specific subtraction library from G217B. A subtraction library enriched for G217B yeast-phase-specific sequences was prepared in the ampicillin resistant phagemid, pTZ (Invitrogen). Individual double-stranded cDNA libraries from the yeast and mycelial phases of G217B were generated by a modified Gubler–Hoffmann protocol (Gubler & Hoffman, 1983) from 50 µg polyA⁺ RNA, and inserted into the phagemid vector in the non-palindromic BstXI site. Following transformation, single-stranded (ss) phagemid DNA from each library was rescued by infection with R408 helper phage. Photo-biotinylated ss phagemid DNA from the mycelial phase was hybridized with ss yeast phagemid DNA; hybrid cDNAs common to both phases were removed with streptavidin, followed by PCI extraction. The organic phase was back-extracted three times with TE buffer (50 mM Tris/HCl, pH 7.5, 1 mM EDTA) and the aqueous phases were pooled prior to ethanol precipitation. The yps subtraction library was recovered following reverse transcription and transformation in Escherichia coli DH5α. Recombinants harboring the yps-3 gene were identified by colony filter hybridization (Maniatis et al., 1982), and the insert sizes of five clones were determined by gel electrophoresis of Xbal-digested mini-prep DNAs.

Dideoxy DNA sequencing of the genomic yps-3 homologues from the G217B and Downs strains of H. capsulatum. The Downs and G217B yps-3 genes were sequenced by the dideoxy chain termination method (Sanger et al., 1977) using Sequenase version 2.0 (USB). Clones were sequenced in both directions by a combined approach using ligation of appropriate restriction fragments into M13mp18 and M13mp19 vectors, and by the formation of a series of unidirectional nucleic acid deletions (Maniatis et al., 1982) from both the Downs and G217B clones. The pTZ yps-3 cDNA from strain G217B was sequenced in both directions using the Bla31 deletion strategy.

S1 nuclelease analysis of the 5' end of the yps-3 gene in G217B. A 1.35 kb HindIII–SmaI fragment from the genomic yps-3 sequence was subcloned in both orientations in M13mp18 and M13mp19 vectors. Preliminary studies (data not shown) demonstrated that only the M13mp19 construct hybridized to yps-3 transcripts in Northern blot analysis, and this construct was used in the protection experiments. The 1.35 kb HindIII–SmaI probe was prepared by primer extension using [³²P]dATP and the Klenow fragment of DNA polymerase I (New England Biolabs). The probe was purified by separation on a urea polyacrylamide gel, and the radio-labelled fragment removed by electrophoresis in 1 x TBE buffer. The probe was hybridized (25000 c.p.m. per reaction) at 55 °C overnight in 20 mM Tris/HCl, pH 7.4, 0.4 M NaCl, 100 mM EDTA and 10 mM dithiothreitol to 2 µg polyA⁺ RNA from the yeast and mycelial phases of Downs and G217B. 51 nuclelease digestion was performed at 37 °C for 1 h in 1 x S1 buffer (40 units S1 nuclease, 0.3 M NaCl, 30 mM sodium acetate, pH 4.6, 1 mM ZnSO₄, 20 µg salmon sperm DNA ml⁻¹). Samples were electrophoresed on 6% urea polyacrylamide gels followed by autoradiography. Labelled pBR322 markers prepared by digestion with EcoRI and HindIII (Maniatis et al., 1982) were electrophoresed in parallel to determine the size of the protected fragment(s).

Field inversion gel electrophoresis (FIGE) of chromosomal sized DNA molecules from H. capsulatum. Chromosomal sized
DNAs from the 217B and Downs strains of *H. capsulatum* were fractionated on 0.4% agarose gels by FIGE (Carle et al., 1986) using an embedded lysis technique modified for *Histoplasma* (Steele et al., 1989). *Histoplasma* DNAs were electrophoresed in parallel with chromosomal sized DNA obtained from *Saccharomyces cerevisiae* obtained by the method of Carle & Olsen (1985). FIGE was performed under ramping conditions for 140 h at 35 V. Initial conditions were established at 1200 s forward, 200 s reverse to a final setting of 3600 s forward, 1800 s reverse. The gel was treated with 0.1 M HCl, neutralized and then transferred to Nytran (Southern, 1975). The hybridization conditions were as described above.

**RESULTS AND DISCUSSION**

Expression of the *yps-3* gene in the virulent G217B and the low virulence Downs strain of *H. capsulatum*

Previous studies (Keath et al., 1989a) had suggested that expression of the *yps-3* gene correlated with thermotolerance and virulence in the yeast phases of three non-isogenic *H. capsulatum* strains cultured at 37 °C. It was possible, however, that the absence of *yps-3* gene expression in the Downs strain was due to temperature stress in this thermosensitive strain. As a result, gene expression was evaluated in the mycelial phase and in the yeast phase achieved following a temperature-induced transition at 34 °C. As seen in Fig. 1(b), yeast phase organisms of the G217B strain cultured at 34 °C express the *yps-3* gene as three transcripts ranging in size from 0.9 to 1.3 kb. In contrast, the gene is not transcribed in either yeast (grown at 34 °C) or in mycelial forms of the Downs strain. The absence of phase-specific gene expression in Downs was not due to RNA degradation or transfer artifacts, since the staining in Fig. 1(a) is roughly equivalent for all tested RNAs, and since the 1.4 kb BamHI-HindIII fragment containing the *Histoplasma* actin gene probe hybridizes to transcripts of 1.4 kb in all lanes (Fig. 1c).

DNA sequence analysis and comparison of the Downs and G217B genomic homologues of the *yps-3* gene

Although the *yps-3* gene was not expressed in the yeast phase of the low virulence Downs strain at either 34 °C or 37 °C, previous studies have demonstrated that the gene was not deleted from the genome but was carried on a larger 2.1 kb HindIII fragment (Keath et al., 1989a,b). The Downs *yps-3* clone was obtained by screening a genomic

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**Fig. 2.** Restriction analysis and DNA sequencing strategy for the genomic 1.85 kb HindIII G217B (a) and the 2.1 kb HindIII Downs (b) *yps-3* gene homologues. The restriction maps of the G217B (a) and Downs (b) clones were determined by Southern analysis. The Downs sequence carries a repetitive element designated by the black box. The G217B *yps-3* gene was sequenced by both the restriction fragment strategy [a(i)] and by Bal31 unidirectional deletions [a(ii)]. The genomic Downs sequence was determined as shown in [b(i)] and [b(ii)] by the restriction fragment and deletion methods, respectively. The extent and direction of the determined sequences are indicated by arrows. A, AvaI; Bg, BglII; H, HindIII; Ha, HaeII; P, PstI; Pv, PvuII; Sma, SmaI.
library generated from this strain (Harris et al., 1989) using the 1-85 kb G217B HindIII fragment as a probe. Detailed restriction endonuclease analysis was performed on both the G217B and Downs clones as described in Fig. 2(a) and (b), respectively. The HindIII fragments from the two strains differed with respect to several restriction endonuclease cleavage sites: the 1-85 kb G217B HindIII fragment harboured sites for PvuII and a single BglII site, while the Downsyps-3 homologue had two BglII sites, but had recognition sites for PstI. The greatest restriction fragment length polymorphisms mapped to a HaeIII-AvaI fragment which was 295 bp and approximately 780 bp in the G217B and Downs homologues, respectively (Fig. 2a and b, data not shown). These preliminary findings suggested that the Downs clone might carry an insertion sequence which was absent in the G217Byps-3 gene.

Based on these observations, a sequencing strategy was developed for the HindIII fragments from both strains as detailed in Fig. 2. A series of overlapping deletions were generated for both clones by nuclease Bal31 digestion followed by linker ligation and insertion into M13mp18 or mp19 series vectors [Fig. 2a(ii) and b(ii)]. Additional sequence was confirmed by fragment subcloning in the same vectors [Fig. 2a(i) and b(i)].

Comparative DNA sequence analysis of the Downs and G217B yps-3 homologues (Fig. 3) indicated that the two genes were over 92% homologous in the first 1370 bp, mapping from the HindIII site (at position 1); the majority of differences with respect to endonuclease cleavage sites were due to point mutations which disrupted or created novel sites when the two clones were evaluated (Fig. 3).

A unique sequence was observed in the Downsjyps-3 homologue at position 1376, where an internally repetitive 15 bp element disrupted the homology shared between the two sequences. A CCCACCGACAAATA(C/T) motif was reiterated 13 times within this region. Moreover, the unique Downs sequence was flanked at the right-hand end by a 14 bp direct repeat of the G217B sequence at this region. Based on these observations, a sequencing strategy was developed for the HindIII fragments from both strains as detailed in Fig. 2. A series of overlapping deletions were generated for both clones by nuclease Bal31 digestion followed by linker ligation and insertion into M13mp18 or mp19 series vectors [Fig. 2a(ii) and b(ii)], additional sequence was confirmed by fragment subcloning in the same vectors [Fig. 2a(i) and b(i)].

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yps-3 gene in *Histoplasma capsulatum*

Fig. 3. Comparative DNA sequence analysis of the Downs (upper line) and G217B (lower line) yps-3 homologues. Both sequences are numbered from the first nucleotide of the upstream HindIII recognition sequence. There is extensive homology (greater than 92 %) in the initial 1370 bp of the sequences. A 287 repetitive element interrupts the Downs and G217B homology at position 1376 of the non-expressed sequence. The observed left-hand and right-hand direct repeats are indicated by the arrows. A 123 bp HindIII fragment was subsequently utilized as a repetitive-sequence-specific probe. A conserved TATA box (underlined) is found in both the Downs and G217B sequences at positions 1058 and 1067, respectively. The most significant difference in the Downs strain was an additional series of nine T residues (positions 1102–1110, Fig. 3) which localized to the region immediately prior to the start site observed in G217B (see below). Several deletions (T) and point mutations (CC in G217B replaced by TT in Downs) were also apparent in this region. These differences, although slight, may be sufficient for strain-specific yps-3 gene expression. Alternatively, the Downs strain may be deficient in the synthesis of a temperature or stress inducible transcription factor which may recognize potential phase-specific promoters in both strains. Regulation of the expression or the levels of such transcription factors might play a significant role in the phase transition and in the morphogenic process. The discovery of additional yeast-phase-specific genes in the G217B strain which failed to be expressed in either phase of the Downs strain (unpublished observations) provides some preliminary support to this contention. However, the upstream regions of the Downs and G217B sequences may provide in vitro target binding sites for phase-specific transcription factors, and should be invaluable tools in the analysis of morphogenesis in *H. capsulatum*.

The unique Downs element within the yps-3 sequence fails to function as a mobile element

The unusual features of the Downs sequence with respect to redundancy and duplication of flanking sequence DNA suggested that the region might function as a mobile genetic element. An internal 123 bp HindIII fragment (Fig. 3) contained within the novel sequence region of Downs was used as a probe for chromosomal sized DNA molecules separated by FIGE. Recent studies by Steele et al. (1989) have demonstrated the presence of three large chromosomal-like bands ranging from 2 to 7 Mb in the G217B strain inferred from parallel electrophoresis of *Schiprachymye J pombe* and *Saccharomyces cerevisiae*. In contrast, the Downs strain harbours five chromosomes visualized by ethidium bromide staining and chromosome specific probes (Steele et al., 1989). In Fig. 4(a), the blot
additional Southern blot experiments with EcoRI, BamHI, and XhoI (data not shown) suggested that the 287 bp sequence found in the Downs strain was unique to this low virulence isolate, and that the element was not distributed randomly in the H. capsulatum genome.

The origin of this repetitive element in the Downs strain is unclear. The sequence does not function as a mobile genetic element, and the redundant nature of the region would have minimal coding capacity. One tempting derivation for such a repetitive sequence might be as a telomere (Blackburn, 1991). Healing of chromosomes by telomerase is known to play a role in the DNA sequence rearrangements described for developmentally regulated organisms such as Tetrabymena (Yu & Blackburn, 1991). Native telomeric sequences have been cloned recently and described for Histoplasma (Woods & Goldman, 1993) and contain reiterations of a GGTTTA sequence dissimilar from the 15 bp motif observed in the Downs sequence.

**DNA sequence analysis of a partial yrs-3 cDNA clone from the G217B strain**

It was of interest to determine if the unusual sequence uncovered by genomic sequence and chromosome blotting techniques in the Downs strain contributed to the absence of yeast-phase-specific gene expression observed in the temperature-sensitive strain. A cDNA subtraction library enriched for yeast-phase-specific sequences from G217B in the pTZ phagemid was screened using the 185 kb HindIII fragment. Subclones of the largest pTZ yrs-3.3 cDNA were generated in M13mp18/M13mp19 vectors at relevant restriction endonuclease sites, and by a bidirectional Bal31 deletion series. The 3' end of the yrs-3 gene of G217B was defined by a consensus poly-AUUAAA adenylation signal and a polyA tail which extended approximately 200 bases beyond the HindIII site (Fig. 5) of the genomic clone. The sequence of the 773 bp pTZ yrs-3 cDNA was aligned with the genomic sequence of the G217B clone (Fig. 5). The long ORF of this cDNA was in-frame with an upstream ATG beginning at position 1280 of the G217B genomic sequence and boxed in Fig. 5. A UGA stop codon would terminate the yrs-3 mRNA. To determine the precise end of the gene and, if expressed, would have significantly disrupted the coding capacity of the yrs-3 mRNA.

**5' end analysis of the expressed yrs-3 gene in G217B**

The larger sizes (1.3, 1.1, and 0.9 kb) of the authentic yrs-3 transcripts detected in Northern blots (Fig. 1b) suggested that the pTZ yrs-3.3 cDNA was a partial clone which lacked the 5' end of the mRNA. To determine the precise end of the gene and thus to localize potential upstream promoter sequences in the G217B and Downs clones, S1 nuclease analysis was performed to map the 5'
The predicted transcription start site inferred from 51 analysis is designated by an arrow; a TATAA element, and a sequence. The long ORF of the 773 bp cDNA was oriented with respect to the genomic sequence, beginning at position 1328. The translated product was in-frame with the boxed ATG start codon at position 1280 of the genomic sequence.

![Nucleotide and predicted amino acid sequence of the partial pTZyps-3.3 cDNA aligned with the genomic G2178 sequence. The long ORF of the 773 bp cDNA was oriented with respect to the genomic sequence, beginning at position 1328. The translated product was in-frame with the boxed ATG start codon at position 1280 of the genomic sequence. The predicted transcription start site is inferred from S1 analysis is designated by an arrow; a TATAA element, and a putative ribosome binding site are found at positions 1067 and 1207, respectively. A consensus polyadenylation signal located downstream of the 3' HindII sequence is underlined and the putative stop codon is designated by asterisks.](image-url)
E. J. KEATH and F. E. ABIDI

nor the predicted protein product of the cDNA are homologous to sequences currently in GenBank.

The yps-3 gene itself, expressed at high levels in the virulent G217B strain, may represent an important virulence determinant. Although the gene is not required for the transition from hyphal to yeast forms, it may play a role in facilitating recovery of the virulent strains from environmental or thermal stress. Further analysis of the yps-3 gene product will be necessary to ascertain its role in virulence in G217B and other H. capsulatum strains, since only the yeast phase is known to survive within tissues or within macrophages (Eissenberg & Goldman, 1991).

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**Fig. 6.** S1 nuclease analysis of the 5′ end of the yps-3 gene. A 1.35 kb HindIII–SmaI fragment was subcloned in M13mp19 for the nuclease analysis (see Fig. 2a). The single-stranded probe was hybridized to mycelial and yeast polyA RNAs from the Downs and G217B strains. A major protected fragment of 230 nucleotides was obtained following S1 digestion in the G217B yeast sample. The full length probe fragment was observed in the control lane.
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