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(21) International Application Number: PCT/US00/01184 (22) International Filing Date: 18 January 2000 (18.01.00) (30) Priority Data: 09/258,634 26 February 1999 (26.02.99) US (71) Applicant: THE GOVERNMENT OF THE UNITED STATES OF AMERICA as represented by THE SECRETARY, DEPARTMENT OF HEALTH AND HUMAN SERVICES [US/US]; Office of Technology Transfer, National Institutes of Health, Suite 325, 6011 Executive Boulevard, Rockville, MD 20852 (US). (72) Inventors: ROBERTS, David, D.; 6808 Persimmon Tree Road, Bethesda, MD 20817 (US). YAN, Sizhuang; 4998 Battery Lane #519, Bethesda, MD 20814 (US). (74) Agents: FEILER, William, S. et al.; Morgan & Finnegan, L.L.P., 345 Park Avenue, New York, NY 10154 (US).		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>Without international search report and to be republished upon receipt of that report.</i>
(54) Title: NUCLEIC ACID AND AMINO ACID SEQUENCES OF HEMOGLOBIN-RESPONSE GENES IN CANDIDA ALBICANS AND THE USE OF REAGENTS DERIVED FROM THESE SEQUENCES IN THE DIAGNOSIS OF DISSEMINATED CANDIDA ALBICANS INFECTION (57) Abstract Three hemoglobin-response genes in the pathogenic yeast <i>Candida albicans</i> are disclosed. The expression of these genes is specifically induced when the organism is exposed to hemoglobin during disseminated infections. The invention relates to the nucleic acid and amino acid sequences of these hemoglobin-response genes. The invention also relates to diagnostic methods, kits and compositions which employ the nucleic acid and amino acid sequences of the invention.		

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TITLE OF THE INVENTION

NUCLEIC ACID AND AMINO ACID SEQUENCES OF
HEMOGLOBIN-RESPONSE GENES IN CANDIDA ALBICANS
AND THE USE OF REAGENTS DERIVED FROM THESE SEQUENCES
IN THE DIAGNOSIS OF DISSEMINATED CANDIDA ALBICANS INFECTION

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FIELD OF THE INVENTION

The present invention relates to the nucleic acid and amino acid sequences of hemoglobin-response genes in the pathogenic yeast *Candida albicans*. More specifically, as these hemoglobin-response genes are specifically induced when the organism initiates disseminated infections, the invention relates to diagnostic methods, kits and compositions which employ the nucleic acid and amino acid sequences of the invention.

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BACKGROUND OF THE INVENTION

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Candida albicans is a pathogenic yeast commonly found in the intestinal digestive tract of healthy individuals. *Candida albicans* is usually limited to the intestines of the digestive tract, where it causes harmless commensal colonization. However, *Candida albicans* can also infect various parts of the body such as the skin, nails, mouth, vagina, airways and bloodstream, causing both superficial and disseminated infections. Candidiasis refers to chronic infections by *Candida albicans*. Candidiasis is often fatal among patients with compromised immune system such as organ and bone marrow transplant recipients, human immunodeficiency virus carriers, and neonates. Candidiasis has become more common due to its increasing incidence in immunocompromised patients (5-10) and its development of resistance toward the limited range of available antifungal agents (11-15).

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Diagnostic kits for the detection of commensal colonization by *Candida albicans* in humans are currently available. However, as approximately 60% of healthy individuals harbor *Candida albicans* in their gastrointestinal tract, it is important to identify specific marker genes which can differentiate a harmless commensal colonization from a life-threatening blood-borne disseminated infection.

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Recently, hemoglobin, a component of blood, has been identified as a host factor that specifically induces increased adhesion of *C. albicans* blastoconidia to fibronectin (20, 21). Fibronectin is a major component of the host extracellular matrix that may play an important role in the initiation and dissemination of *C. albicans* infection. Hemoglobin has also been shown to induce changes in expression of several proteins exposed on the yeast cell wall, including a 55 kDa protein that binds fibronectin (21, 22). Thus, the identification and characterization of hemoglobin-response genes may be useful in differentiating a harmless commensal colonization from a life-threatening blood-borne disseminated infection.

Recognition of host factors by pathogens may also initiate adaptive responses that facilitate infection. Successful pathogens have developed bidirectional signal transduction pathways to achieve environments in their hosts that are optimal for colonization and growth of the invading microorganisms (1-3). Following contact with the host, pathogens produce factors that alter gene expression or function in host cells to facilitate infection (2). Conversely, pathogenic microorganisms must also alter their gene expression in response to specific signals from the host (1, 3, 4). Thus, identification of host factor-response genes could lead to new therapeutic approaches to prevent or treat these infections.

SUMMARY OF THE INVENTION

The present invention relates to the isolation and characterization of the nucleic acid sequences of three hemoglobin-response genes in the pathogenic yeast *Candida albicans*. The expression of these genes is specifically induced when the organism is exposed to hemoglobin during disseminated infections. In particular, the present invention relates to these nucleic acid sequences and to the encoded amino acid sequences.

The invention also relates to vectors comprising the nucleic acids of this invention and to cultured host cells comprising these vectors.

The invention also relates to proteins or peptide fragments thereof encoded by the nucleic acids of this invention.

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The invention further relates to methods for producing the proteins or peptide fragments of the invention.

The invention also relates to antibodies which have specific binding affinity to the proteins or peptide fragments encoded by the hemoglobin-response genes of the invention.

The invention also relates to the use of the nucleic acid sequences, proteins or peptide fragments or antibodies of the invention in methods for diagnosing disseminated *Candida albicans* infection in a mammal. In one embodiment, the method utilizes the proteins or peptide fragments of the invention to detect antibodies which have specific binding affinity to the proteins or peptide fragments of this invention in a mammal. In another embodiment, the method uses the antibodies of this invention to detect the proteins or peptide fragments encoded by the hemoglobin-response genes. In yet another embodiment, the method comprising analyzing the nucleic acid of a mammal for the presence of hemoglobin-response gene sequences using the nucleic acid sequences of this invention.

The invention also provides kits for the diagnosis of disseminated *Candida albicans* infection in a mammal. In one embodiment, the kit contains at least one proteins or peptide fragment of this invention useful for the detection of antibodies specific for the proteins or peptide fragments of the invention. The invention also relates to kits for detecting the proteins or peptide fragments of this invention, wherein the kit comprises at least one antibody of this invention. In another embodiment, the kit comprises nucleic acid molecules having sequences useful as hybridization probes in determining the presence or absence of the nucleic acids of the hemoglobin-response genes of this invention. In yet another embodiment, the kit comprises nucleic acids having sequences useful as primers for nucleic acid sequence-based amplification (NASBA) analysis of RNA for the presence of the hemoglobin-response genes in a mammal.

The invention further relates to isolated and substantially purified nucleic acids, proteins, peptide fragments and antibodies of the invention.

The invention also relates to compositions comprising one or more of the nucleic acids, proteins, peptide fragments and antibodies of the invention.

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A-E. Differential display polymerase chain reaction (PCR) identification of iron- and hemoglobin-induced genes. (1A) Hemoglobin-induced gene transcripts (H) were compared to cDNA from non-induced controls (C) and ferrous sulfate-induced cultures (F) amplified using three arbitrary primers (lanes 1-3). (1B) Specificity of *HBR1* mRNA induction by hemoglobin. Total RNA from cells grown in yeast nitrogen base (YNB), YNB + ferrous sulfate (iron), YNB + 1 mg/ml hemoglobin (Hb), or induced to form hyphae were hybridized with DNA probes for *HBR1* or pyruvate carboxylase 2 (control) in a Northern analysis. (1C) Kinetics of induction of mRNAs by hemoglobin. *HBR1*, *HBR2*, *HBR3*, actin, or 25S ribosomal RNA were hybridized with the respective probes. Temperature-dependence for *HBR3* (1D) and *HBR1* induction (1E). Total RNA was analyzed by Northern blots and quantified by PhosphorImager analysis using ImageQuant (Molecular Dynamics, Sunnyvale, CA). The fold-induction in a representative experiment is presented for iron-(Fe) or hemoglobin-induced cultures (Hb) relative to uninduced control cultures grown at the respective temperatures.

Figures 2A-B. Induction of hemoglobin response genes in whole blood. (2A). Cells from *C. albicans* cultures were resuspended in heparinized rabbit whole blood and allowed to grow at 26⁰ C for 5 h. Total RNA was extracted from control and treated cells, and expression was determined by Northern analyses. (2B) Hemolytic activity of *C. albicans* strains 44807 and 32354, which is hemolytic positive control (2, 3) was determined by growing on a Sabouraud dextrose blood plate. Clear zones surrounding the colonies indicate areas of hemolysis.

Figures 3A-B. Induction of *HBR1* and *HBR2* expression *in vivo* in rabbits. *C. albicans* blastoconidia were injected into Silastic venous catheters implanted into the anterior vena cava of 3 New Zealand white rabbits (26) and incubated *in vivo* for 5 h before withdrawal for RNA preparation and Northern blots. Representative PhosphorImager analyses of Northern blots show the fold-induction of *HBR1* (3A) and *HBR3* mRNA expression (3B) for cells recovered from the catheter (*in vivo*) or parallel cultures maintained in YNB or exposed to 0.1% hemoglobin (Hb) in YNB *in vitro*.

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Figures 4A-B. Induction of tyrosine phosphorylation by hemoglobin.

(4A) *C. albicans* 44807 cultures growing in YNB were supplemented with ferrous sulfate (Fe) or hemoglobin (Hb) and incubated with rocking at 26⁰ C. At the indicated times, cells lysates were prepared and analyzed by Western blotting using a phosphotyrosine antibody. (4B) Tyrosine phosphorylation induced by hemoglobin in the *ura3* mutant CAI4 at 60 min. was compared to that of wild type *C. albicans* (44807) by Western blotting using the anti-phosphotyrosine antibody RC20.

Figures 5A-B. Hemoglobin responses in *C. albicans* mutants

impaired in hyphal formation. (5A) ¹²⁵I-Fibronectin binding (mean ± SD) was measured (20) to each strain after growth in YNB (striped bars) or YNB containing 0.1% hemoglobin (solid bars) for the wild type isolates, ATCC 44807 and SC5314, the *URA3*+ homozygous mutants *cph1/cph1*, *hst7/hst7* and *cst20/cst20*, the *ura3* mutant CAI4, and the corresponding *ura3/URA3* parent strain CAF2 (29). (5B) Induction of *HBR1* expression by hemoglobin (solid bars) in the *ura3* mutant CAI4 was compared to that of wild type *C. albicans* 44807 and SC5314 by Northern blot analysis. Expression in control cultures without hemoglobin (striped bars) was normalized to 1 and is presented as mean ± SD.

Figure 6. Identification of HBR gene copy number from genomic DNA by Southern blot. Genomic DNA isolated from *Candida albicans* were either undigested (control), or digested with EcoRI, HindIII and BamHI, and HindIII and EcoRI, separated by electrophoresis, and immobilized onto membrane. Hybridization was performed using HBR1 gene as a probe in QuikHyb solution (Stratagene, La Jolla, CA) at 68⁰ overnight.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to the isolation and characterization of three hemoglobin-response genes in the pathogenic yeast *Candida albicans*. The expression of these genes is specifically induced when the organism is exposed to hemoglobin during disseminated infections. These hemoglobin-response genes, designated HBR1, HBR2 and HBR3, were identified by screening a *Candida albicans* genomic library using DNA from EST clones (SEQ ID NOS: 11-13). The EST clones comprise sequences which exhibited increased expression in *Candida*

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albicans cultures containing hemoglobin and were obtained using random arbitrary primer-polymerase chain reaction.

5 The present invention therefore relates to nucleic acid molecules having the sequences of any one of the hemoglobin-response genes of this invention (nucleic acid sequences are shown in SEQ ID NOS:1-3; amino acid sequences shown in SEQ ID NOS: 4 and 5 are encoded by SEQ ID NOS: 1 and 2, respectively) as well as fragments (or partial sequences) thereof. Partial sequences may be obtained by various methods, including restriction digestion, PCR
10 amplification and direct synthesis.

The invention also relates to nucleic acids having complementary (or antisense) sequences of the sequence shown in SEQ ID NOS:1-3, as well as fragments thereof.

15 The present invention also relates to nucleic acids derived from the nucleic acid sequences shown in SEQ ID NOS:1-3.

The present invention also relates to vectors comprising the nucleic acids of the invention, where suitable vectors include, but are not limited to, any vector that is capable of carrying and expressing the nucleic acids of the invention in
20 prokaryotic or eukaryotic host cells.

The present invention therefore also relates to host cells comprising the vectors of the invention.

25 The expression of the hemoglobin-response genes of the invention is specifically induced when *Candida albicans* is exposed to hemoglobin during disseminated infections. Therefore, these hemoglobin-response genes provide a useful tool for the diagnosis of disseminated *Candida albicans* infection in a mammal.

30 The present invention therefore also relates to a method for diagnosing disseminated *Candida albicans* infection in a biological sample using the nucleic acids of this invention.

35 Biological samples appropriate for the diagnostic assays of this invention include, but are not limited to, serum, and tissue samples from a biopsy obtained from a mammal.

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The word “mammal” as used throughout the specification, includes, but is not limited to humans.

In one embodiment, the diagnostic method involves analyzing DNA of a biological sample. DNA can be isolated by methods well known in the art.

5 The methods for analyzing DNA for diagnosing disseminated *Candida albicans* infection include Southern blotting, and dot and slot hybridization.

10 The nucleic acid sequences used in the detection methods set forth above are derived from any one of the nucleic acid sequences shown in SEQ ID NOS:1-3. When used as a hybridization probe, the size of the sequence used is usually at least 20 bases long, preferably 200-500 bases long.

15 The nucleic acid probes of this invention may be DNA or RNA. Nucleic acids can be synthesized using any of the known methods of nucleotide synthesis, or they can be isolated fragments of naturally occurring or cloned DNA.

 The nucleic acid probes can be labeled using methods known to one skilled in the art. Such labeling techniques can include radioactive labels, biotin, avidin, enzymes and fluorescent molecules.

20 The nucleic acid probes used in the detection methods set forth above are derived from sequences substantially homologous to any one of the sequences shown in SEQ ID NOS:1-3, or its complementary sequence. By “substantially homologous”, as used throughout the specification to describe the nucleic acid sequences of the present invention, is meant a high level of homology between the
25 nucleic acid sequence and any one of the sequences of SEQ ID NOS:1-3, or its complementary sequence. Preferably, the level of homology is in excess of 80%, more preferably in excess of 90%, with a preferred nucleic acid sequence being in excess of 95% homologous with a portion of any one of the sequences of SEQ ID
30 NOS:1-3, or its complement.

 In another embodiment of the invention, the diagnostic method comprises analyzing the RNA isolated from a biological sample for detecting the presence of disseminated *Candida albicans* infection. RNA can be isolated by
35 methods well known in the art.

The methods for analyzing the RNA for diagnosing disseminated *Candida albicans* infection include, but are not limited to, Northern blotting, dot and slot hybridization, filter hybridization, RNase protection, reverse-transcription polymerase chain reaction (RT-PCR), and nucleic acid sequence-based amplification (NASBA). A preferred method is NASBA. This method utilizes AMV reverse transcriptase, Rnase H and T7 RNA polymerase. In this method, the RNA can be transcribed to a first strand cDNA using AMV reverse transcriptase and a first nucleic acid probe. The first nucleic acid probe typically comprises a T7 polymerase promoter sequence in addition to a nucleic acid sequence derived from the nucleic acids of this invention. A second strand cDNA is then synthesized with a second nucleic acid probe derived from the nucleic acids of this invention utilizing the DNA-dependent DNA polymerase activity of the AMV reverse transcriptase. Once the double-stranded DNAs are synthesized, they serve as templates for the synthesis of antisense and sense RNAs in a reaction utilizes T7 RNA polymerase. The "cyclic phase" of the reaction begins as the newly synthesized RNA molecules serve as templates for the synthesis of additional first and second strand cDNA molecules.

The size of the nucleic acid probes used as primers in the amplification methods set forth above is usually at least 7 bases long, preferably 20-40 bases long.

The amplification products obtained from the amplification methods can be detected either directly or indirectly. In one embodiment, direct detection of the amplification products is carried out via labeling of the nucleic acid probes. Labels suitable for labeling the nucleic acid probes of the present invention are known to one skilled in the art and include radioactive labels, biotin, avidin, enzymes and fluorescent molecules.

In another embodiment, unlabelled amplification products can be detected via hybridization with labeled nucleic acid probes in methods known to one skilled in the art, such as dot or slot blot hybridization or filter hybridization.

The present invention also relates to diagnostic kits comprising the nucleic acid probes of this invention. The diagnostic kits may include other

reagents and materials required for the hybridization or amplification protocols in addition to the probes.

The present invention also relates to recombinant methods of using the nucleic acids of the invention to produce proteins or peptide fragments.

5 In one embodiment, the method comprises:

(a) preparing of a nucleic acid capable of directing a host cell to produce a protein or peptide fragment encoded by any one of the sequences shown in SEQ ID NOS:1-3;

10 (b) cloning the nucleic acid into a vector capable of being transferred into and replicated in a host cell;

(c) transferring the vector containing the nucleic acid into a host cell capable of expressing the protein or peptide fragment;

15 (d) growing the host under conditions appropriate for expression of the protein or peptide fragment; and

(e) harvesting the protein or peptide fragment.

The vectors contemplated for use in the present invention include any vectors into which a nucleic acid sequence as described above can be inserted, along with any preferred or required sequences, and which vector can then be
20 subsequently transferred into a host cell and, preferably, replicated in such cell.

The present invention also relates to proteins or peptide fragments encoded by and/or derived from the nucleic acids of this invention. These proteins or peptide fragments may be natural, synthetic or produced by recombinant
25 methods. They can be obtained as a crude lysate or can be purified by standard protein purification procedures known in the art which may include differential precipitation, molecular sieve chromatography, ion-exchange chromatography, isoelectric focusing, gel electrophoresis and affinity and immunoaffinity
30 chromatography. The present invention also relates to compositions comprising one or more of the proteins or peptide fragments of the invention.

A recombinant or derived proteins or peptide fragments is not necessarily translated from a designated nucleic acid sequence; it may be generated

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in any manner, including for example, chemical synthesis, or expression of a recombinant expression system.

It should be noted that the nucleotide sequences described herein represent one embodiment of the present invention. Due to the degeneracy of the genetic code, it is to be understood that numerous choices of nucleotides may be made that will lead to a sequence capable of directing production of the proteins or peptide fragments set forth above. As such, nucleic acid sequences which are functionally equivalent to the sequences described herein are intended to be encompassed within the present invention. For example, preferred codons which are appropriate to the host cell may be used.

The present invention further relates to the use of the proteins or peptide fragments of the invention as diagnostic agents.

In one embodiment, the proteins or peptide fragments of the invention can be used in immunoassays for detecting the presence of antibodies against proteins or peptide fragments encoded by any one of the hemoglobin-response genes in a biological sample and for diagnosing disseminated *Candida albicans* infection in a biological sample.

In a preferred embodiment, test serum is reacted with a solid phase reagent having a surface-bound proteins or peptide fragments of this invention as an antigen. The solid surface reagent can be prepared by known techniques for attaching proteins or peptide fragments to solid support material. These attachment methods include non-specific adsorption of the proteins or peptide fragments to the support or covalent attachment of the proteins or peptide fragments to a reactive group on the support. Unbound serum components are removed by washing and the antigen-antibody complex is reacted with a secondary antibody such as labeled anti-human antibody. The label may be an enzyme which is detected by incubating the solid support in the presence of a suitable fluorimetric or colorimetric reagent. Other detectable labels may also be used, such as radiolabels or colloidal gold, and the like.

Immunoassays of the present invention may be a radioimmunoassay, Western blot assay, immunofluorescent assay, enzyme immunoassay,

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chemiluminescent assay, immunohistochemical assay and the like. Standard techniques for ELISA are well known in the art.

The present invention further relates to diagnostic kits comprising the proteins or peptide fragments of this invention. Such diagnostic kits may include other reagents and materials required for the immunoassays.

In another embodiment, the proteins or peptide fragments of the invention can be used to prepare antibodies against proteins or peptide fragments encoded by any one of the hemoglobin-response genes that are useful in diagnosis.

The term "antibodies" is used herein to refer to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules. Exemplary antibody molecules are intact immunoglobulin molecules, substantially intact immunoglobulin molecules and portions of an immunoglobulin molecule, including those portions known in the art as Fab, Fab', F(ab')₂ and F(v) as well as chimeric antibody molecules.

An antibody of the present invention is typically produced by immunizing a mammal with an immunogen. In one embodiment, the immunogen contains one or more proteins or peptide fragments of the invention, or a structurally and/or antigenically related molecule, to induce, in the mammal, antibody molecules having immunospecificity for the immunizing peptide or peptides. The peptide(s) or related molecule(s) may be monomeric, polymeric, conjugated to a carrier, and/or administered in the presence of an adjuvant. In another embodiment, the immunogen contains one or more nucleic acids encoding one or more proteins or peptide fragments of the invention, or one or more nucleic acids encoding structurally and/or antigenically related molecules, to induce, in the mammal, the production of the immunizing proteins or peptides.

The antibody molecules of the present invention may be polyclonal or monoclonal. Monoclonal antibodies may be produced by methods known in the art. Portions of immunoglobulin molecules may also be produced by methods known in the art.

The presence of the antibodies of the invention can be determined by, but are not limited to, the various immunoassays described above.

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The present invention also relates to the use of the antibodies of the invention for diagnosing disseminated *Candida albicans* infection in a biological sample. The antibodies can be used as an *in vitro* diagnostic agent in biological samples in standard immunoassay protocols. Preferably, the assays which use the antibodies to diagnosing disseminated *Candida albicans* infection in a sample involve contacting the sample with at least one of the antibodies under conditions which will allow the formation of an immunological complex between the antibody and the *Candida albicans* antigen that may be present in the sample. The formation of an immunological complex if any, is then detected and measured by suitable means. Such assays include, but are not limited to, radioimmunoassays (RIA), ELISA, indirect immunofluorescence assay, Western blot and the like. The antibodies may be labeled or unlabeled depending on the type of assay used. Labels which may be coupled to the antibodies include those known in the art and include, but are not limited to, enzymes, radionucleotides, fluorogenic and chromogenic substrates, cofactors, biotin/avidin, colloidal gold and magnetic particles. Modification of the antibodies allows for coupling by any known means to carrier proteins or peptides or to known supports, for example, polystyrene or polyvinyl microtiter plates, glass tubes or glass beads and chromatographic supports, such as paper, cellulose and cellulose derivatives, and silica.

The present invention further relates to diagnostic kits comprising the antibodies of this invention. Such diagnostic kits may include other reagents and materials required for the immunoassays.

The present invention further relates to antisense nucleic acids designed to inhibit the translation of proteins or peptide fragments encoded by the hemoglobin-response genes of the invention. The antisense nucleic acids are complementary to *Candida albicans* mRNAs encoding the proteins or peptides fragments of this invention. The antisense nucleic acids may be in the form of synthetic nucleic acids or they may be encoded by a nucleotide construct.

As homologous of the hemoglobin-response genes of the invention can be readily obtained using hybridization conditions such as the one described in Figure 6, it is to be understood that the invention also encompasses the homologous

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of these hemoglobin-response genes in other pathogenic species of *Candida* such as *Candida glabrata*, *Candida guilleirmondii*, *Candida kruzei*, *Candida tropicalis*, and *Candida trachomatis*.

5 The present invention will now be described by way of examples, which are meant to illustrate, but not limit, the scope of the invention.

All of the references cited herein are hereby incorporated by reference.

Examples

10 Materials and Methods

Cell culture

The wildtype *C. albicans* strain 44807 (American Type Culture Collection) was inoculated into 4 x yeast nitrogen base (YNB) medium with or without 62 μ M (expressed as Fe equivalents) hemoglobin or ferrous sulfate and
15 grown at 26° C for 48 hr (20-22, 24). Under these growth conditions, no germination was found upon microscopic examination. For induction of the hyphal form of *C. albicans*, cells grown in YNB were resuspended into RPMI 1640 supplemented with 2 mM glutamine in the absence of hemoglobin and incubated 2 h
20 at 37° C with shaking at 250 rpm. A two hour incubation converted nearly 100% of candidal cells to hyphae or pseudohyphae by microscopic examination.

The wildtype *C. albicans* strain SC5314 (wildtype parental isolate for all mutant strains), and the *URA3*+ homozygous mutants *cph1/cph1* (Can16, *ura3::imm434/ura3::imm434 cph1::hisG/cph1::hisG-URA3-hisG*), *hst7/hst7* (Can18, *ura3::imm434/ura3::imm434 hst7::hisG/hst7::hisG-URA3-hisG*), *cst20/cst20* (Can 26, *ura3::imm434/ura3::imm434 cst20::hisG/cst20::hisG-URA3-hisG*), the *ura3* mutant CAI4, and the corresponding *ura3/URA3* parent strain CAF2
25 (29) were also used in the experiments.

30 *Total RNA preparation and differential expression display*

Cells were collected, washed with TE buffer, pH 7.4, and mixed with RNAzol or Ultraspec RNA (Biotecx Laboratories, Houston, TX) in the presence of baked glass beads on ice. The cells were disrupted using a mini Bead-Beater for an
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accumulated period of 5 min, with cooling on ice at 1 min intervals, followed by briefly vortexing in the presence of chloroform (20% of total volume). The extracted total RNA was precipitated and dried. Differentially expressed genes were identified by RNA arbitrarily primed PCR (RAP-PCR) (Stratagene, La Jolla, CA). The first strand cDNA was synthesized with MMLV-reverse transcriptase using arbitrary primers, followed by a PCR with a higher stringency in the presence of [α -³²P]dATP according to the manufacturer's instructions. Five distinct arbitrary primers were used: AATCTAGAGCTCCTCCTC (SEQ ID NO:6); AATCTAGAGCTCCA-GCAG (SEQ ID NO:7); AATCTAGAGCTCTCCTGG (SEQ ID NO:8); AATCTAGAG-CTCTCCAGC (SEQ ID NO:9); and AATCTAGAGCTCCCTCCA (SEQ ID NO:10).

Nucleotide sequencing, Northern hybridization and genomic library screening

DNA from positive RAP-PCR bands were extracted, amplified and subcloned into a TA cloning vector (Invitrogen, Carlsbad, CA). Expressed sequence tag (EST) sequences were determined by automated sequencing on an ABI Prism sequencer, model 377, using fluorescent terminators. The sequences were verified by multiple sequencing reactions on each strand. Differential expression of the genes induced by hemoglobin was confirmed by Northern hybridization with DNA from each clone as probe. Probe DNA was labeled with Prime-It RmT (Stratagene, La Jolla, CA). Total RNAs (25 μ g/lane) from *C. albicans* prepared under various conditions were separated on 1% agarose gels containing 6.2% formaldehyde and transferred to GeneScreen Plus membrane (NEN Research Products, Boston, MA). Hybridization was performed with the corresponding probes in a buffer containing 0.5% BSA, 0.1% SDS, 1 mM EDTA and 0.5 M NaH₂PO₄, pH 7.4 at 65⁰ C overnight.

The *C. albicans* C9 genomic library was obtained from the National Institutes of Health AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. *E. coli* harboring the C9 genomic library were diluted and plated onto ampicillin-containing plates. Colonies were lifted onto nitrocellulose membranes, followed by in-situ hybridization using standard procedures. The

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genomic clones for *HBR1*, *HBR2*, and *HBR3* were obtained by screening the library using DNA fragments acquired from differential display.

Chromosomal mapping

For chromosome mapping, *HpaI* fragments of *HBR1* (4kb) and *HBR2* (5kb) and the *HindIII/Scal* fragment of *HBR3* (1.8kb) from the gene inserts in plasmid 1041 were labeled with ^{32}P dATP and probed to chromosome separations of *Candida albicans* strains 1006 and WO-1, and to *SfiI* digested chromosomal DNA of strain 1006 (25).

In vivo induction of hemoglobin-response genes

C. albicans (ATCC 44807) organisms from stock isolates were streaked onto Sabouraud glucose agar plates, and incubated at 37°C for 24 h. Three to five discrete colonies were then inoculated into 50 ml of Emmon's modified Sabouraud broth, pH 7.0 and incubated at 37°C for 16 h on a shaking incubator at 80 rpm. The *Candida* suspension was then centrifuged, washed three times in sterile 0.9% NaCl, counted by hemacytometer, and diluted to 6.4×10^8 CFU/mL concentration of blastoconidia. Inoculum size was confirmed by plating serial dilutions onto SGA check plates. A volume of 1000 μl of this suspension was mixed with 1000 μl of heparinized whole blood from each rabbit. The final inoculum was 1×10^8 CFU/ml.

Three New Zealand white rabbits weighing 2.5 to 3.5 kg were individually housed and provided food and water in accordance with National Institutes of Health and American Association for the Accreditation of Laboratory Animal Care guidelines on care and use of laboratory animals. A silastic venous catheter was inserted under sterile operative conditions, as previously described in each rabbit (26). The central venous catheter permitted *C. albicans* to be incubated with heparinized whole blood within the anterior vena cava of the rabbit. Following incubation for five hours, 1.0 ml of blood was withdrawn through the catheter in order to retrieve the *C. albicans* for extraction of fungal RNA. This incubation process was repeated with a new inoculum of *C. albicans* once each day for three consecutive days. *Candida* cells were recovered from whole blood by hypotonic

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lysis of the erythrocytes followed by centrifugation. Total RNA was prepared as above and analyzed by Northern blotting.

Tyrosine phosphorylation assays

Equal amounts of *Candida* cells grown in the presence or absence of 0.1% hemoglobin for 30 to 180 min were washed, suspended in RIPA buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1 mM EGTA, 1 mM NaF, 1 mM sodium orthovanadate, 10 µg/ml antipain, pepstatin, chymostatin, leupeptin, aprotinin, soybean trypsin inhibitor, 1 mM PMSF) with 0.5 mm glass beads, and broken using a Bead-Beater as described above. Clarified lysates from equal cell numbers were separated by SDS gel electrophoresis on a 4-15% gradient acrylamide gel (BioRad) and transferred to nitrocellulose. Tyrosine phosphorylation was detected using RC-20 antibody (Transduction Laboratories), peroxidase-conjugated anti-mouse IgG, and ECL reagent (Amersham).

Example 1

Hemoglobin specifically alters expression of multiple genes

To understand the basis for the altered expression of cell surface proteins in *C. albicans* cells grown in the presence of hemoglobin (22), random arbitrary primed-PCR was used to examine changes in mRNA expression in *C. albicans* induced by hemoglobin. Expression patterns were compared to untreated control cells or cells treated with equivalent concentrations of ferrous sulfate to exclude those mRNAs induced by iron that may be released by degradation of the added hemoglobin (Fig.1A). Some of the differentially expressed PCR products were induced by either inorganic iron or hemoglobin (Fig.1A, **), whereas others were specifically induced (*) or inhibited (-) by hemoglobin.

cDNAs for the differentially expressed genes were amplified, subcloned into a TA cloning vector, and sequenced. A total of 33 ESTs that exhibited increased expression in cultures supplemented with hemoglobin but not in cultures supplemented with an equivalent molar concentration of an inorganic ferrous salt were cloned and sequenced. By sequence comparison and homology searches against GenBank and the *Saccharomyces* genome database, 7 of the 33

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ESTs were identified as ESTs from carboxypeptidase Y, and the remainder represented ESTs from 21 discreet mRNAs.

The latter clones and representative carboxypeptidase Y clones were screened by Northern blotting for differential induction by hemoglobin, inorganic iron, or hyphal differentiation. Carboxypeptidase Y is induced both late in hyphal differentiation (27) and by hemoglobin (Table 1). Of the remaining clones, 5 were validated by Northern blots to be induced preferentially by hemoglobin compared to mRNAs from cells treated with inorganic iron or cells induced to form hyphae in RPMI 1640 supplemented with glutamine at 37° C (Fig.1B and Table 1).

The kinetics of induction of these mRNAs varied both in the time required for maximal induction and the duration of the induced expression (Fig.1C). *HBR1* and *HBR2* were transiently induced, but *HBR3* expression was sustained beyond 72 h. Although genes that showed reduced expression by RAP-PCR were not systematically examined, we noted that actin expression was suppressed following 24 h growth in hemoglobin (Fig.1C). Induction of expression was observed at physiological temperatures. Hemoglobin elicited a similar increase in *HBR3* expression at either 26° or 37° C (Fig.1D). Induction of *HBR1* expression, however, was 5-fold greater at 37° than at 26° C (Fig. 1E).

Example 2

Characterization of hemoglobin-response genes

A *C. albicans* genomic library was screened using probes derived from three cloned ESTs (SEQ ID NOS:11-13) that were confirmed to be positive by Northern blots. *HBR1*, *HBR2*, and *HBR3* (SEQ ID NOS:1-3) are novel genes, each of which showed limited homology to hypothetical proteins of *Saccharomyces cerevisiae* (Table 1). These genomic clones were mapped to distinct chromosomes (Table 1). Therefore, the hemoglobin-response genes are not physically associated, in contrast to the host-response genes of some pathogenic bacteria which are frequently clustered in pathogenicity islands (3).

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Table 1

Properties of Hemoglobin-Induced Genes¹

Gene	Hemoglobin response	Hyphal response	<i>C. albicans</i> Chromosome	<i>S. cerevisiae</i> homologs (% DNA homology)	ORF Features
<i>HBR1</i>	+++	+	1 <i>SfiI</i> frag S	<i>YDL166C</i> (62%)	ATP/GTP-binding site motif (P loop); phosphorylation sites for PKC, PKA, CK2
<i>HBR2</i>	++	o	6 <i>SfiI</i> frag C	<i>YFL030W</i> (72%)	pyridoxal phosphate motif
<i>HBR3</i>	+++	o	1 <i>SfiI</i> frag E	<i>YOR056C</i> (34%)	
<i>HBR4</i>	++	o			
<i>HBR5</i>	++	o			
<i>CPY1</i>	+	+	7 <i>SfiI</i> frag G		carboxypeptidase Y
Actin	-		1 <i>SfiI</i> frag L		actin

Example 3Induction of hemoglobin-response genes by whole blood

Pathogenic isolates of *C. albicans* express a hemolysin that could release hemoglobin from erythrocytes when the pathogen gains access to the vascular compartment (23). As predicted from this observation, exposure of *C. albicans* to whole blood *in vitro* at 26 or 37° C for 5 h resulted in rapid induction of *HBR1* and *HBR2* expression (Fig. 2A). The ATCC 44807 strain used for these experiments was verified to express hemolytic activity following growth on Sabouraud dextrose agar containing rabbit blood and 3% glucose (Fig. 2B). Strain ATCC 32354 was reported previously to produce hemolytic factor (23) and used here as a positive control.

¹ Properties of 5 novel hemoglobin-inducible ESTs (*HBR1-5*) and *CPY1* are summarized. Induction (+), inhibition (-), or lack of response (o) following growth in 0.1% hemoglobin or hyphal differentiation medium were assessed by Northern blots. Chromosome localization for *HBR1*, *HBR2*, and *HBR3* was performed using fragments from genomic clones. ESTs for *HBR1-HBR5* were searched against the *Saccharomyces cerevisiae* genome database (<http://genome-www.stanford.edu/Saccharomyces/>) using FASTA version 3.0t77 (34). Open reading frame motifs were identified using MotifFinder (www.genome.ad.jp).

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Example 4Induction of HBR 1 and HBR 3 expression in vivo

The hemoglobin response genes may also play a role during *in vivo* pathogenesis of *C. albicans*, because expression of *HBR1* and *HBR3* was rapidly induced in a rabbit *in vivo* model of disseminated candidiasis. *C. albicans* blastoconidia were incubated for 5 h with heparinized whole blood within the anterior vena cava of rabbits. Induction of mRNAs for both genes was detected following recovery of the cells from the implanted central venous catheter (Fig. 3A and 3B).

Example 5Hemoglobin induction alters phosphorylation of Candida proteins

The effect of hemoglobin induction on tyrosine phosphorylation in *C. albicans* was also examined. Two proteins with apparent molecular masses of 85 And 160-180 kDa were phosphorylated in control cells, but increased phosphorylation of several proteins at 25-50 kDa was detected at 30 min and reached a maximal level at 60 min. As was seen for the changes in gene expression, the hemoglobin response could not be replicated by exposure of the cells to the same medium containing inorganic iron (Fig. 4A). Therefore tyrosine phosphorylation may be an early event in signaling exposure to hemoglobin following invasion into the vascular compartment.

Example 6Differentiation induced by hemoglobin is distinct from hyphal differentiation

To further define the relationship between hyphal differentiation and differentiation induced by hemoglobin, the responses of several *C. albicans* mutants deficient in hyphal formation were examined. Signal transduction through Cst20p, a homolog of the Ste20p/p65^{PAK} kinases, can trigger hyphal formation in *C. albicans* (28) and mutants affecting the Cst20 MAP kinase signaling pathway in *C. albicans* are defective in hyphal formation (19). Three *C. albicans* mutants defective in this pathway, however, displayed comparable induction of fibronectin binding to their parental isolate SC5314 following growth in medium containing

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hemoglobin (Fig. 5A). Therefore, the Cst20p/Hst7p pathway is not required for the adhesion response of *C. albicans* to hemoglobin.

The *C. albicans ura3/ura3* mutant strain CAI4 (29) is also derived from SC5314 and is impaired in hyphal formation (19). When maintained serially in Sabouraud broth cultures, this strain failed to respond to hemoglobin by increasing fibronectin binding activity (results no shown), but cultures grown in broth inoculated directly with the CAI4 strain or the *ura3/URA3* parent strain (CAF2) grown on Sabouraud agar showed induction of fibronectin binding by hemoglobin (Fig. 5A). The defect in hemoglobin response in the CAI4 mutant when grown serially in broth probably does not involve early events in signaling, since this mutant, when grown serially in broth, showed a similar increase in protein tyrosine phosphorylation as the 44807 isolate following transfer to medium containing hemoglobin (Fig. 4B). Northern blot analyses showed that this strain, when maintained as a serial broth culture, failed to induce *HBR1* expression following growth in the presence of hemoglobin (results not shown) but the CAI4 mutant and its parent strain SC5314 both induced *HBR1* expression when grown in the same medium inoculated from colonies on solid medium (Fig. 5B). Thus, the induction of *HBR1* expression by hemoglobin in strain CAI4 correlates with induction of fibronectin binding.

Discussion

Opportunistic pathogens must recognize specific environments in the host. Some virulence genes remain silent during commensal colonization of environments such as the gastrointestinal tract but become highly expressed when the pathogen reaches a tissue where opportunistic infections can be established. Thus, tissue-specific host factors may be important regulators of virulence gene expression in these organisms. Based on its rapid and pleiotropic effects on protein phosphorylation, cell surface protein expression (22) and gene expression, hemoglobin is a host factor that profoundly alters the phenotype of *C. albicans*.

Contact of *C. albicans* with host epithelial cells results in altered tyrosine phosphorylation of several *Candida* proteins (4). The specific host factors on epithelial cells responsible for this response have not been identified. The

phosphorylated proteins induced by hemoglobin differ in molecular weight from those reported for the response of *C. albicans* to epithelial cell contact (4). These distinct signaling responses suggest that *C. albicans* can differentially respond to contact with the host epithelia during commensal colonization and exposure to the vascular space in disseminated infections.

The hemolytic factor expressed by pathogenic isolates of *C. albicans* may enable the cells to acquire iron for maintaining growth (23). The above data demonstrates that expression of this hemolysin can release hemoglobin from whole blood, which in turn alters gene expression and the adhesive phenotype of *C. albicans*. Although the ability to acquire iron has long been considered an important adaptive response for microbial pathogenesis (30, 31) exposure of *Candida* to hemoglobin appears to have additional effects on this opportunistic pathogen. The above Examples demonstrate that hemoglobin but not inorganic iron dramatically enhances *Candida* binding to fibronectin (20) and differentially induces binding to several other extracellular matrix proteins (22). Although iron regulates growth of *C. albicans* (32), supplementing the growth medium with inorganic iron has no effect on adhesion (20). In addition, hemoglobin induces expression of several surface proteins in the cells grown in defined medium supplemented with hemoglobin. Among these induced surface proteins, fibronectin recognizes a protein with a molecular mass of 55 kDa that is not present in cells grown in the absence of hemoglobin (21, 22). Enhancement of fibronectin binding by hemoglobin is conserved among several pathogenic species in the *Candida* genus but was not observed in nonpathogenic yeasts or *Trichosporon beigelii*, which rarely causes disseminated infections (33). Therefore, one role of hemoglobin in the pathogenesis of *C. albicans* and related species is to regulate interactions with host adhesion molecules. Defining the function of the hemoglobin-response genes may reveal additional pathogenic processes that are induced by hemoglobin.

The above Examples describe the identification of five genes that are preferentially induced by hemoglobin but not by hyphal differentiation. The three genes for which genomic clones were obtained all appear to encode cytoplasmic proteins. Although their functions are unknown, none of the cloned hemoglobin-

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response genes appears to be the hemoglobin-induced extracellular matrix receptor. The presence of a nucleotide binding P loop in *HBR1* suggest that it could be a regulatory protein that mediates some of the phenotypic responses of *C. albicans* to hemoglobin. Unlike the host-response genes in some bacteria which are clustered in pathogenicity islands, the hemoglobin-response genes in *C. albicans* appear to be dispersed in the genome. Thus hemoglobin-induced transcription factors and signaling pathways may coordinately regulate the induction of these genes by hemoglobin. Based on the disparate kinetics for their induction, however, a complex signaling pathway may be required to mediate this hemoglobin response.

The process of hyphal differentiation is associated with altered expression of several genes (16, 17). Hyphal differentiation is now recognized as an essential response for pathogenesis of *C. albicans*, and disruption of several genes required for hyphal differentiation produces avirulent organisms (18, 19). However, the *C. albicans* MAP kinase mutants that are impaired in hyphal differentiation examined here are not defective in responses to hemoglobin. Our data demonstrate that hemoglobin induces expression of some genes in the hyphal differentiation pathway but does not induce hyphae. However, hemoglobin also induces a second set of genes that are not involved in hyphal differentiation and are rapidly induced both *in vitro* and *in vivo*. These genes define a second differentiation pathway that is triggered when *C. albicans* gains access to the vascular compartment of its host. Future studies will examine the function and expression of these genes during the transition from commensal colonization to disseminated candidiasis.

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CLAIMS

1. A nucleic acid molecule which encodes a protein having SEQ ID NO:4.
2. The nucleic acid molecule of claim 1 comprising a nucleotide sequence according to SEQ ID NO:1.
3. A nucleic acid molecule which encodes a protein having SEQ ID NO:5.
4. The nucleic acid molecule of claim 3 comprising a nucleotide sequence according to SEQ ID NO: 2.
5. A nucleic acid molecule comprising a nucleotide sequence according to SEQ ID NO: 3.
6. A vector comprising the nucleic acid molecule of claim 1.
7. A vector comprising the nucleic acid of molecule of claim 3.
8. A vector comprising the nucleic acid of molecule of claim 5.
9. A cell comprising the vector of claim 6.
10. A cell comprising the vector of claim 7.
11. A cell comprising the vector of claim 8.
12. A composition comprising the nucleic acid molecule of claim 1.
13. A composition comprising the nucleic acid molecule of claim 3.
14. A composition comprising the nucleic acid molecule of claim 5.
15. A protein encoded by the nucleic acid molecule according to claim 1 or a peptide fragment thereof.

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16. A protein encoded by the nucleic acid molecule according to claim 3 or a peptide fragment thereof.

17. A protein encoded by the nucleic acid molecule according to claim 5 or a peptide fragment thereof.

18. A composition comprising the protein or the peptide fragment of claim 15, and a physiologically acceptable carrier.

19. A composition comprising the protein or the peptide fragment of claim 16, and a physiologically acceptable carrier.

20. A composition comprising the protein or the peptide fragment of claim 17, and a physiologically acceptable carrier.

21. A method for producing the protein or the peptide fragment of claim 15, said method comprising growing the cell of claim 9 under conditions such that the encoded protein or peptide fragment is produced.

22. A method for producing the protein or the peptide fragment of claim 16, said method comprising growing the cell of claim 10 under conditions such that the encoded protein or peptide fragment is produced.

23. A method for producing the protein or the peptide fragment of claim 17, said method comprising growing the cell of claim 11 under conditions such that the encoded protein or peptide fragment is produced.

24. An antibody having specific binding affinity to the protein or the peptide fragment according to claim 15.

25. An antibody having specific binding affinity to the protein or the peptide fragment according to claim 16.

26. An antibody having specific binding affinity to the protein or the peptide fragment according to claim 17.

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27. A method for diagnosing disseminated *Candida albicans* infection in a sample comprising contacting said sample with the antibody of claim 24 under conditions that allow the formation of a complex between said antibody and proteins or peptide fragments encoded by *Candida albicans*, wherein the presence of said complex indicates the presence of disseminated *Candida albicans* infection.

28. A kit for diagnosing disseminated *Candida albicans* infections in a sample comprising an antibody of claim 24.

29. A method for diagnosing disseminated *Candida albicans* infection in a sample comprising contacting said sample with the antibody of claim 25 under conditions that allow the formation of a complex between said antibody and proteins or peptide fragments encoded by *Candida albicans*, wherein the presence of said complex indicates the presence of disseminated *Candida albicans* infection.

30. A kit for diagnosing disseminated *Candida albicans* infections in a sample comprising an antibody of claim 25.

31. A method for diagnosing disseminated *Candida albicans* infections in a sample comprising contacting said sample with the antibody of claim 26 under conditions that allow the formation of a complex between said antibody and proteins or peptide fragments encoded by *Candida albicans*, wherein the presence of said complex indicates the presence of disseminated *Candida albicans* infection.

32. A kit for diagnosing disseminated *Candida albicans* infections in a sample comprising an antibody of claim 26.

33. A method for diagnosing disseminated *Candida albicans* infection in a sample comprising contacting said sample with a protein or the peptide fragment according to claim 15 under conditions that allow the formation of a complex between said protein or peptide fragment and an antibody to a *Candida*

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albicans epitope, wherein the presence of said complex indicates the presence of disseminated *Candida albicans* infection.

5 34. A method for diagnosing disseminated *Candida albicans* infection in a sample comprising contacting said sample with a protein or the peptide fragment according to claim 16 under conditions that allow the formation of a complex between said protein or peptide fragment and an antibody to a *Candida albicans* epitope, wherein the presence of said complex indicates the presence of disseminated *Candida albicans* infection.

10 35. A method for diagnosing disseminated *Candida albicans* infection in a sample comprising contacting said sample with a protein or the peptide fragment according to claim 17 under conditions that allow the formation of a complex between said protein or peptide fragment and an antibody to a *Candida albicans* epitope, wherein the presence of said complex indicates the presence of disseminated *Candida albicans* infection.

 36. A nucleic acid probe comprising a sequence derived from the nucleic acid molecule of claim 1.

20 37. A nucleic acid probe comprising a sequence derived from the nucleic acid molecule of claim 3.

 38. A nucleic acid probe comprising a sequence derived from the nucleic acid of claim 5.

25 39. A method of diagnosing disseminated *Candida albicans* infection in a biological sample comprising:

 (a) contacting nucleic acid of the biological sample with the nucleic acid probe of claim 36; and

30 (b) detecting the presence or absence of complexes formed between said nucleic acid of the biological sample and said nucleic acid probe, wherein the presence of said complexes indicate the presence of disseminated *Candida albicans* infection.

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40. A method of diagnosing disseminated *Candida albicans* infection in a biological sample comprising:

(a) contacting nucleic acid of the biological sample with the nucleic acid probe of claim 37; and

(b) detecting the presence or absence of complexes formed between said nucleic acid of the biological sample and said nucleic acid probe, wherein the presence of said complexes indicate the presence of disseminated *Candida albicans* infection.

41. A method of diagnosing disseminated *Candida albicans* infection in a biological sample comprising:

(a) contacting nucleic acid of the biological sample with the nucleic acid probe of claim 38; and

(b) detecting the presence or absence of complexes formed between said nucleic acid of the biological sample and said nucleic acid probe, wherein the presence of said complexes indicate the presence of disseminated *Candida albicans* infection.

42. A method of diagnosing disseminated *Candida albicans* infection in a biological sample comprising:

(a) amplifying the RNA of the biological sample with at least two nucleic acid probes of 36 to produce amplification products; and

(b) detecting the presence or absence of amplification products, wherein the presence of said amplification products indicate the presence of disseminated *Candida albicans* infection.

43. A method of diagnosing disseminated *Candida albicans* infection in a biological sample comprising:

(a) amplifying the RNA of the biological sample with at least two nucleic acid probes of 37 to produce amplification products; and

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(b) detecting the presence or absence of amplification products, wherein the presence of said amplification products indicate the presence of disseminated *Candida albicans* infection.

5 44. A method of diagnosing disseminated *Candida albicans* infection in a biological sample comprising:

(a) amplifying the RNA of the biological sample with at least two nucleic acid probes of 38 to produce amplification products; and

10 (b) detecting the presence or absence of amplification products, wherein the presence of said amplification products indicate the presence of disseminated *Candida albicans* infection.

15 45. A kit for diagnosing disseminated *Candida albicans* infections in a sample comprising a nucleic acid probe of claim 36.

46. A kit for diagnosing disseminated *Candida albicans* infections in a sample comprising a nucleic acid probe of claim 37.

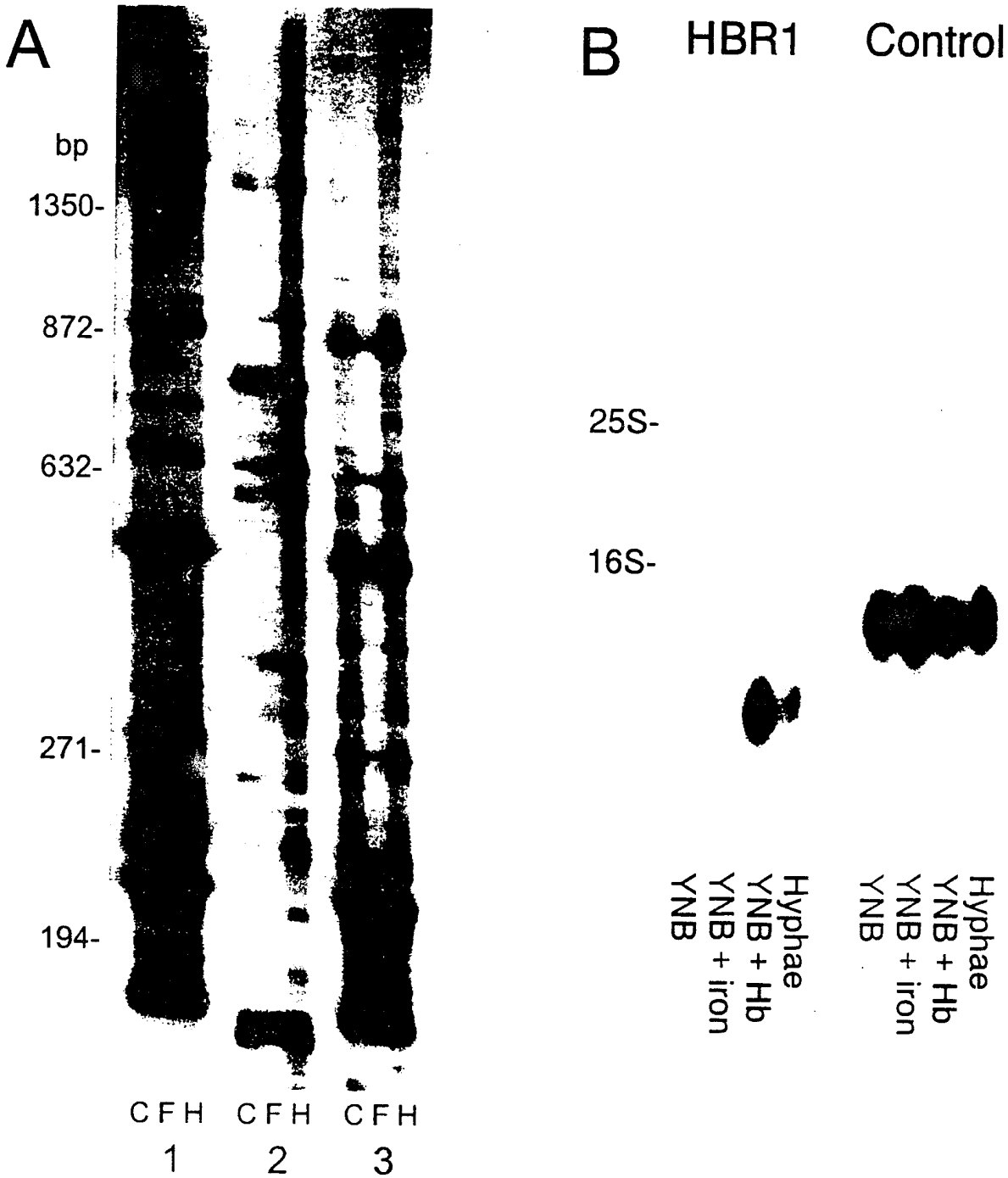
20 47. A kit for diagnosing disseminated *Candida albicans* infections in a sample comprising a nucleic acid probe of claim 38.

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FIG. 1A-B



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FIG. 1C

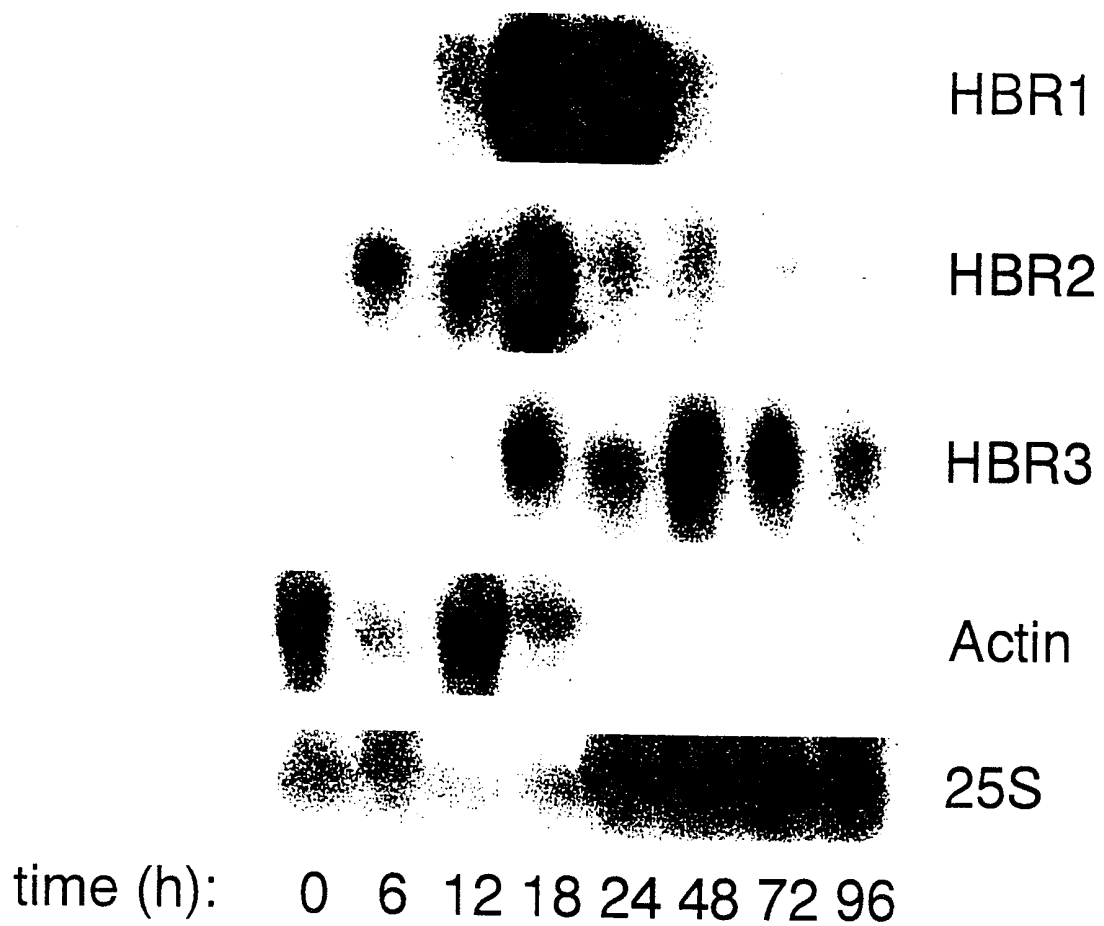


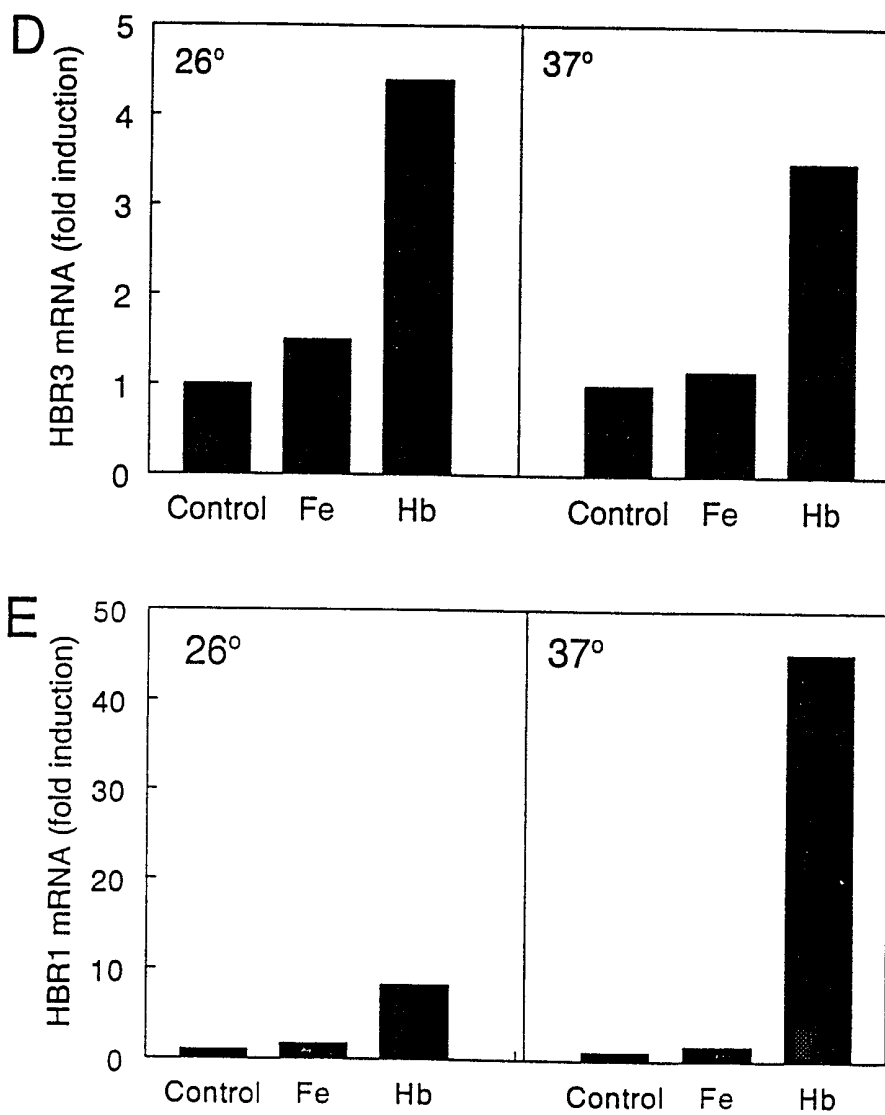
FIG. 1D-E

FIG. 2A-B

A

HBR1

HBR2



Blood:

-

+

-

+

B

32354

44807



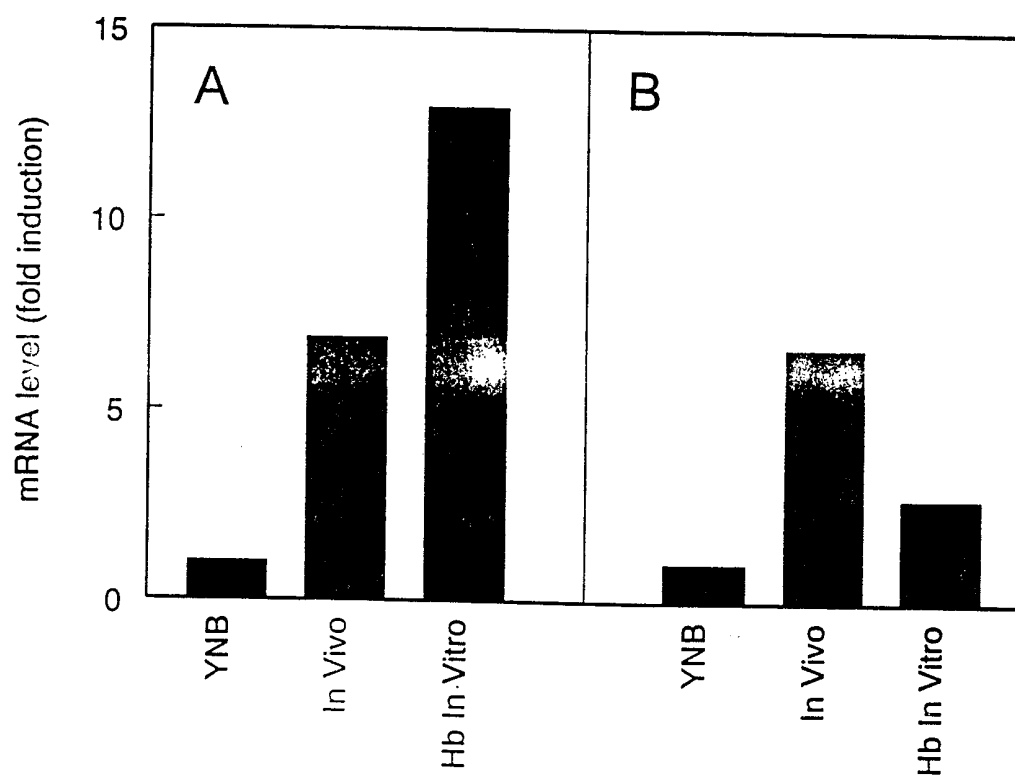
FIG. 3A-B

FIG. 4A

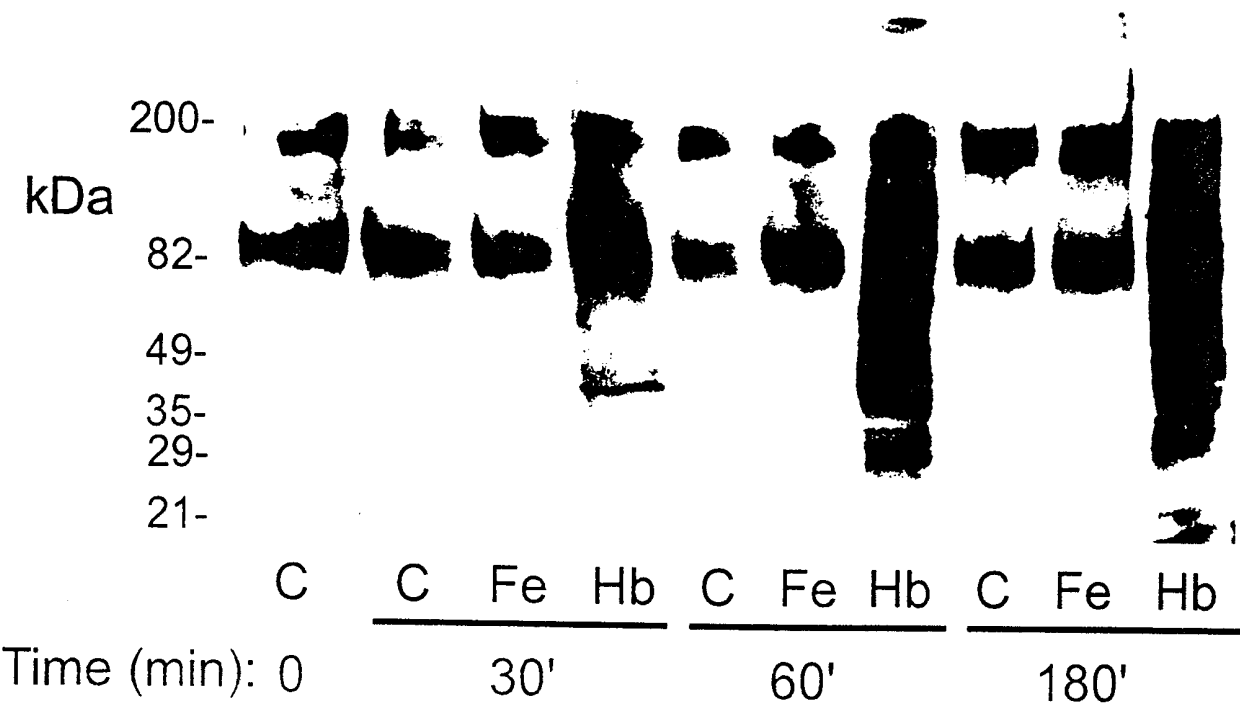


FIG. 4B

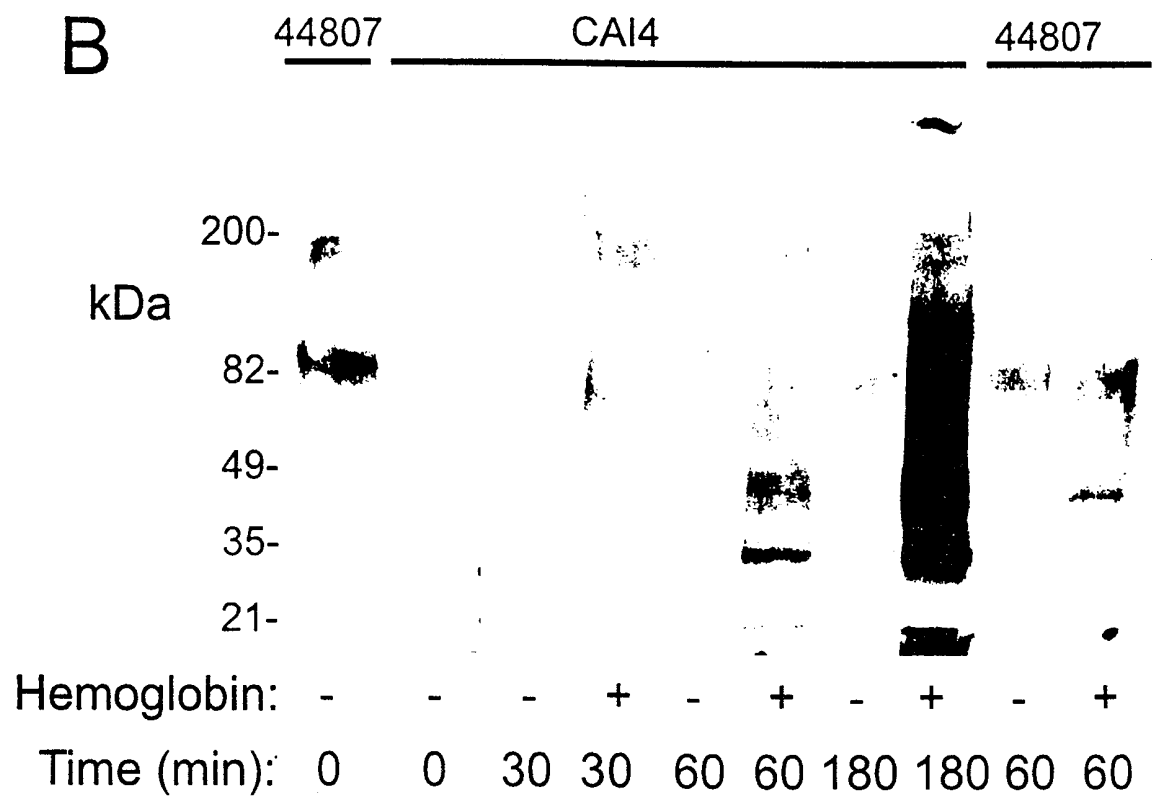
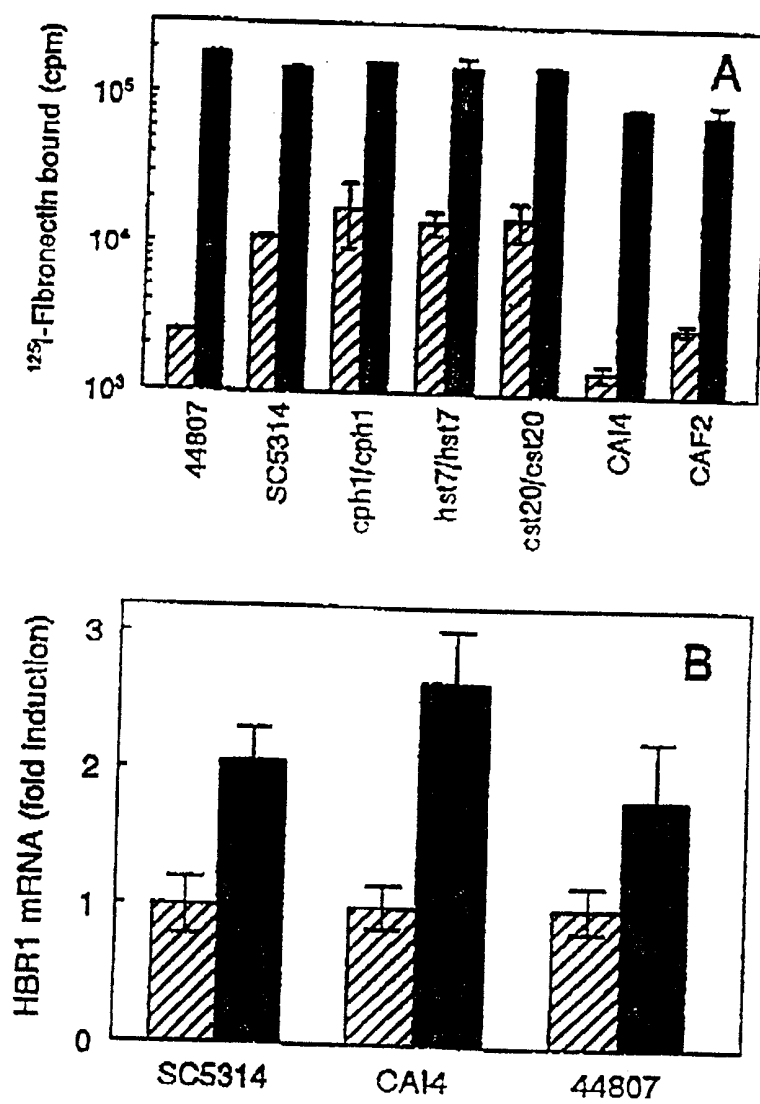


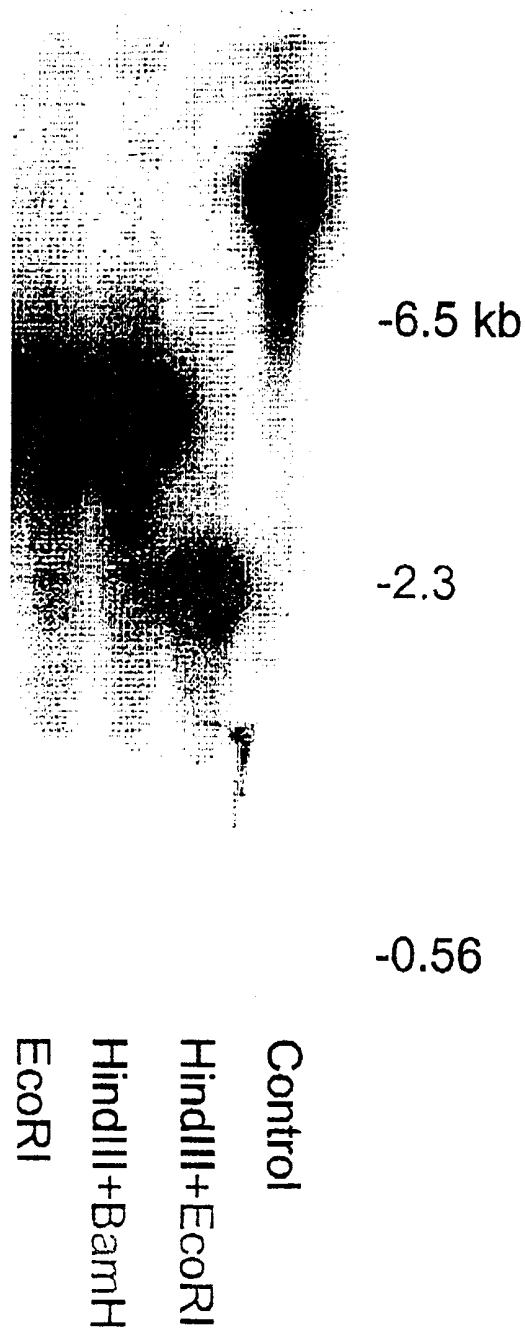
FIG. 5A-B



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FIG. 6

HBR1 Southern blot



SEQUENCE LISTING

<110> Roberts, David
Yan, Sizhuang

<120> Nucleic Acid And Amino Acid Sequences Of
Hemoglobin-Response Genes In Candida Albicans And The
Use Of Reagents Derived From These Sequences In The
Diagnosis Of disseminated Candida Albicans Infection

<130> 20264301PC

<140> TBA

<141> 2000-01-18

<150> 09/258,634

<151> 1999-02-26

<160> 13

<170> PatentIn Ver. 2.1

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<213> Candida albicans

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gatgattctt ctgatgaagg tgatgacaac agcgattccg atgaatatga acttgaagaa 960
gacgaacaag aagaagagga ggaaagagaa gaggatgatg aagagaccaa tgaagagatg 1020
gagcatcacg aagatatattg acaatagaaa aggcataatat tatctaaatt aatgtacaga 1080
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aaatatgtcc aagtacccga aagtttgtcc atgttgtggt tttctaattc agccacacat 1260
 ttaacgtcat gttcgaaaat gtattttgat ttttcaatga tttccaactt ttgttcttcc 1320
 gtcaaaccat ttcttgtcac caactcg 1347

<210> 2

<211> 3250

<212> DNA

<213> Candida albicans

<220>

<221> unsure

<222> (14)

<223> The "n" at position 14 can be either A, T, G or C.

<220>

<221> unsure

<222> (55)

<223> The "n" at position 55 can be either A, T, G, or C.

<220>

<221> unsure

<222> (163)

<223> The "n" at position 163 can be either A, T, G, or C.

<220>

<221> unsure

<222> (436)

<223> The "n" at position 436 can be either A, T, G, or C.

<220>

<221> unsure

<222> (3210)

<223> The "n" at position 3210 can be either A, T, G, or C.

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 aaatccatat ttaataattt aacaaatgta ttataagcaa ttngaggaat tgccagtttg 180
 gcaacaacta gcaaaaatgg cattaacgta attctccaaa taataacagc accaaatcgg 240
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 ttctwracca twttgkyamm aamkratwry swtaatttgg cmcaykatww stmgmakttt 420
 gmrattwara ttawtntttc ggccaaatgg ttttaaataa tgatttcamc ataaatgatt 480

```

cactatttkc gctactacta ctaccactac tagtagtatac attatcttgt attggtgggt 540
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tcgaagtcga agtcgatttt aatgatgatg atgatgaagt tgatttgaaa ttagaaggtt 660
atttattagt gaaatatcta atacttgata ttttaattga tctagaaata ggaaaactaa 720
aaggtattgg tgatataaac ctacacgggag atgggttttat caatttaaca tttctaataa 780
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tttcggaagc gacttcttaa tatgttatcc catatatgtc attagtagtca tcaatgctag 1200
aaaagattaa taaacctact ggttgagtag ttttagagta aattaatttg caacagctct 1260
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taaaaaattg ataaacctgc tggaacacca atagcctttt gagatgcagt caaagcaaaa 2040
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ggaaagtctg gagaaacgga acttttctct ttttctttct ttttttcttt ctttttggtg 3060
gctcttttga ttgcttcaaa aactctctct ctctctctct tctccccct ccccgccacc 3120
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atcgctccgc 3250

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<210> 3
 <211> 1428
 <212> DNA
 <213> *Candida albicans*

<220>
 <221> unsure
 <222> (1053)
 <223> The "n" at position 1053 can be either A, T, G or C.

<220>
 <221> unsure
 <222> (1314)
 <223> The "n" at position 1314 can be either A, T, G or C.

<400> 3
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 taaggaagca ggaaggaatc atttagttca acgggataaa attcgtgtgc aactcaaatt 180
 ggcggggaaa ttgtgcagca atggaaaaaa ggatctcata ctagatttac actcacttta 240
 acaaactaac aaacaattga gtgttggtwa catttttttt ttgttttttg gtctttgtgt 300
 ttagtttctt ttgttggtta tctttgtaaa aaatgcttat caggaattaa acaatatgaa 360
 actttacaaa ctggggagag ttcaaaaaaa ctgatataaa tggggaaggt ttttgctcca 420
 aatgtttttt gaattgattt ttgtttttgc tttttgtttt tactttttcc tccagctatt 480
 ataacaattt taatatcact ttctactact attcaaaata atgtgtaaaa ctgaaatttg 540
 cggaatttgt cgtatgtgat tgctcctagc ttttattgaa ttgattcact ttttaactgtt 600
 caatgatact aacatcaaga atttttaatt tagaacataa atcttggacc ggttggtgta 660
 aacatgtcca cgaaataatg gatatcagtt ctaaagataa ttggtgtacc tgtgagccat 720
 tggataaaga tgaagaagtt ttcattgaag gtcattgggt ttatccacca aaagcagggtc 780
 aaggtttgag aagaggcagt ggttgcggtt accagagttc ttccccaaga taaataagaa 840
 cgtctgcgat gaatcgtgas gttagggtgt tgttggtttt tttttattgc aaaattaaaa 900
 agttaattta ttggttttgt gctggtattc caaatagtta gtattgtttt acatagaaaa 960
 tataattaaa catattcagg ttttttggtg ggtgttgatt tgtggacact acatgtadra 1020
 gcgttggtga tattgggggt atattcgga ganaactaaa attgcaaaac gtaactgttg 1080
 ttattgagca cttgccagtt gatactctta cctacagttg accttttaac gctagggttag 1140
 tttatgacct ttttccaatc tacaagtga agataataat ttaaagacga attcttggag 1200
 ttgagcaaaa gcttttttgt agctgttgaa atttgctatt gcccttgtgc cgcaaataca 1260
 ascctacaaa ctcaagggtg twagcccaat aacgtgttgt cttaaagctt tganctagtc 1320
 ttctatttag aatgcctgac aattaagtta aattttgatt gtgagctcaa cagatgttct 1380
 atcctactca catggcccgat atattgttgc gaatttgca atgttcac 1428

<210> 4
 <211> 248
 <212> PRT

<213> Candida albicans

<400> 4

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Met Thr Thr Met Ser Arg Arg Tyr Thr Pro Asn Ile Ile Ile Thr Gly
  1                      5                      10                      15

Thr Pro Gly Cys Gly Lys Ser Ser His Ser Leu Ser Leu Val Ser Gln
                20                      25                      30

Leu Asn Gln Thr Leu Gly Lys Glu Thr Thr Ile His Phe Lys His Phe
      35                      40                      45

Asn Ile Ser Glu Ile Ala Lys Glu Arg Asp Cys Ile Glu Ser Tyr Asp
      50                      55                      60

Ala Lys Leu Asp Thr Ser Ile Val Asp Glu Asp Lys Leu Leu Asp Ser
      65                      70                      75                      80

Leu Glu Pro Asp Leu Glu Lys Gly Gly Val Val Val Asp Trp His Cys
      85                      90                      95

Cys Asp Ile Phe Pro Glu Arg Leu Ile Asp Leu Val Val Val Leu Arg
      100                      105                      110

Thr Asp Asn Ser Asn Leu Phe Asp Arg Leu Lys Thr Arg Asn Tyr Asn
      115                      120                      125

Asp Leu Lys Leu Gln Glu Asn Leu Asp Cys Glu Ile Met Glu Val Ile
      130                      135                      140

Leu Gln Glu Ala Lys Asp Ser Tyr Ile Pro Asp Ile Val Ile Glu Leu
      145                      150                      155                      160

Arg Ser Asp Thr Ala Glu Glu Met Asp Glu Asn Val Asp Arg Ile Ser
      165                      170                      175

Ser Trp Val Glu Thr Trp Ile Glu Asp His Pro Asp Gly Val Ser Asn
      180                      185                      190

Glu Leu Asn Lys Gln Tyr Asn Pro Asp Asp Ser Ser Asp Glu Gly Asp
      195                      200                      205

Asp Asn Ser Asp Ser Asp Glu Tyr Glu Leu Glu Glu Asp Glu Gln Glu
      210                      215                      220

Glu Glu Glu Glu Arg Glu Glu Tyr Asp Glu Glu Thr Asn Glu Glu Met
      225                      230                      235                      240

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Glu His Thr Glu Asp Ile Ala Gln
245

<210> 5

<211> 287

<212> PRT

<213> Candida albicans

<400> 5

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1 5 10 15

Gly Pro Ile Asn Phe Ser Asp Asp Val Leu Ala Ser Met Ala Thr Pro
20 25 30

Ser Gln Ala His Thr Ser Pro Glu Phe Ile Ser Thr Phe Gln Ser Val
35 40 45

Leu Lys Ser Leu Arg Lys Val Phe Lys Ser Thr Thr Asn Thr Gln Gly
50 55 60

Tyr Val Leu Ser Gly Ser Gly Thr Leu Gly Trp Asp Val Ala Ser Ser
65 70 75 80

Asn Leu Leu Asn Pro Gly Glu Lys Val Leu Val Leu Ser Thr Gly Phe
85 90 95

Phe Ser Asp Ser Phe Ala Gly Met Ser Lys Val Tyr Gly Ala Asp Val
100 105 110

Asp Val Leu Thr Ala Glu Val Gly Asp Val Val Pro Leu Asp Lys Ile
115 120 125

Glu Gln Gln Leu Gln Arg Glu Lys Tyr Ser Ala Ile Thr Ile Thr His
130 135 140

Val Asp Thr Ser Thr Ser Val Val Ser Asp Val Glu Ala Ile Lys Val
145 150 155 160

Val Lys Lys Val Ser Pro Asp Thr Leu Ile Ile Val Asp Gly Val Cys
165 170 175

Ser Ile Ala Val Glu Asp Leu Glu Phe Asp Lys Trp Gly Ile Asp Phe
180 185 190

Ala Leu Thr Ala Ser Gln Lys Ala Ile Gly Val Pro Ala Gly Leu Ser
195 200 205

Ile Phe Tyr Ala Ser Glu Arg Ala Leu Ala Lys Ala Leu Asn Lys Glu
 210 215 220

Lys Glu Ser Thr Phe Phe Ala Ser Leu Lys Arg Trp Thr Pro Ile Met
 225 230 235 240

Lys Ala Tyr Glu Asn Gly Asn Gly Ala Tyr Phe Ala Thr Pro Ala Val
 245 250 255

Gln Thr Ile Thr Ala Leu Lys Thr Ser Leu Asp Glu Ile Leu Ser Gln
 260 265 270

Pro Ile Asp Glu Arg Phe Ala Lys His Ala Gln Val Ser Asp Lys
 275 280 285

<210> 6

<211> 18

<212> DNA

<213> Candida albicans

<400> 6

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<210> 7

<211> 18

<212> DNA

<213> Candida albicans

<400> 7

aatctagagc tccagcag 18

<210> 8

<211> 18

<212> DNA

<213> Candida albicans

<400> 8

aatctagagc tctcctgg 18

<210> 9

<211> 18

<212> DNA

<213> Candida albicans

<400> 9

aatctagagc tctccagc

18

<210> 10

<211> 18

<212> DNA

<213> Candida albicans

<400> 10

aatctagagc tccctcca

18

<210> 11

<211> 399

<212> DNA

<213> Candida albicans

<220>

<221> unsure

<222> (204)

<223> The "n" at position 204 can be either A, T, G or C.

<220>

<221> unsure

<222> (225)

<223> The "n" at position 225 can be either A, T, G or C.

<400> 11

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atggagcata cagaagatat tgcacaatag aaaaggcata tattatctaa attaatgtac 120
agaagttttc ataagtgttt ttattttttt atttaggtta atttattgac aactcttctt 180
aagtagatca aagccaacaa aganaaaagt accaaggcag catantagcc tcttgctact 240
aagaaatatg tccaagtacc cgaaagtgtg tccatgttgt gtttttctaa ttcagccaca 300
catttaacgt catgttcgaa aatgtatttt gattcttcaa tgatttccaa cttttgttct 360
tccgtgtgcg ttgtgatttg ttcttcccgt gtggtgtaa 399

<210> 12

<211> 270

<212> DNA

<213> Candida albicans

<220>

<221> unsure

<222> (158)

<223> The "n" at position 158 can be either A, T, G or C.

<220>

<221> unsure

<222> (173)

<223> The "n" at position 173 can be either A, T, G or C.

<220>

<221> unsure

<222> (236)

<223> The "n" at position 236 can be either A, T, G or C.

<220>

<221> unsure

<222> (249)

<223> The "n" at position 249 can be either A, T, G or C.

<220>

<221> unsure

<222> (251)

<223> The "n" at position 251 can be either A, T, G or C.

<400> 12

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tccattgatt ccatcaggga aataaactgc tgттаатсса tgagcagcaa ctttatgac 120
aacaggcaag atttttaaac ccaacttttc aacattanat ttgaatttat canaaacttg 180
agcatgttta gcaaattcttt catcaattgg ttgactcaaa atttcatcta atgaantttt 240
ttaaagcant natggttttg aacacctgga 270
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<210> 13

<211> 351

<212> DNA

<213> Candida albicans

<220>

<221> unsure

<222> (124)

<223> The "n" at position 124 can be either A, T, G or C.

<400> 13

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aaaaaaaaaaca acaacaacct aacgtcacga ttcatcgag acgttcttat ttatcttggg 120
gaanaactct ggtaaccgca accactgcct cttctcaaac cttgacctgc ttttggtgga 180
taaaccatcat gaccttcaat gaaaacttct tcattcttat ccaatggctc acaggtacac 240
caattatctt tagaactgat atccattatt tccgtggaca tgtttaccac aaccggtcca 300
agatttatgt ttgacaaatt cccgcaaatt tcaagtttta cacattgttt t 351
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