Sequence Elements Necessary for Transcriptional Activation of BAD1 in the Yeast Phase of Blastomyces dermatitidis

Peggy J. Rooney¹ and Bruce S. Klein¹,²,³*

Department of Medical Microbiology and Immunology,¹ Department of Pediatrics,² and Department of Internal Medicine and the Comprehensive Cancer Center,³ University of Wisconsin Medical School, Madison, Wisconsin 53792

Received 27 February 2004/Accepted 2 April 2004

Blastomyces dermatitidis is a dimorphic fungal pathogen that converts from mycelia or conidia to a host-adapted yeast morphology upon infection. Conversion to the yeast form is accompanied by the production of the virulence factor BAD1. Yeast-phase-specific expression of BAD1 is transcriptionally regulated, and its promoter shares homology with that of the yeast-phase-specific gene YPS3 of Histoplasma capsulatum. Serial truncations of the BAD1 upstream region were fused to the lacZ reporter to define functional areas in the promoter. Examination of P⁵BAD1-lacZ fusions in B. dermatitidis indicated that BAD1 transcription is upregulated in the yeast phase. The 63-nucleotide box A region conserved in the YPS3 upstream region was shown to be an essential component of the minimal BAD1 promoter. A matched P⁵YPS3-lacZ construct indicated that this same region was needed for minimal YPS3 promoter activity in B. dermatitidis transformants. Reporter activity in H. capsulatum transformants similarly showed a requirement for box A in the minimal BAD1 promoter. Several putative transcription factor binding sites were identified within box A of BAD1. Replacement of two of these predicted sites within box A—a cAMP responsive element and a Myb binding site—sharply reduced transcriptional activity, indicating that these regions are critical in dictating the yeast-phase-specific expression of this crucial virulence determinant of B. dermatitidis.

Most systemic fungal infections of immunocompetent humans are caused by dimorphic fungi. This group of pathogens includes Blastomyces dermatitidis, Histoplasma capsulatum, Paracoccidioides brasiliensis, Coccidioides immitis, and Sporothrix schenckii. These fungi share the unique ability to undergo reversible cellular differentiation, existing as an infectious form or morphotype in the environment but converting to a second, pathogenic form upon entry into the host. This morphological switch promotes host invasion and evasion (40). A change in fungal gene expression is one possible mechanism by which morphogenesis affects the outcome of infection. Factors required for colonization of the host or modulation of the host immune response may be upregulated, while other factors only necessary for growth in the environment may be downregulated. Identification of products whose expression is linked only to the pathogenic phase may uncover virulence factors of these fungi. Also, since prevention of this essential developmental event may hold the key to control of infections by these fungi, identification of molecules necessary for the morphological switch could lead to new drug or vaccine targets that block the earliest events in colonization or infection.

Several genes have been identified whose expression is specific to one morphotype, a subset of which have been shown to be required for virulence. For example, expression of SOWgp58, an extracellular matrix-binding protein of C. immitis, is limited to the endosporulating spherule form, which is the parasitic phase of this fungus (22). Deletion of SOWgp resulted in a reduction in virulence, and its product is presumed to play a role as an adhesin (23). In H. capsulatum, expression of the calcium-binding protein CBP1 was limited to the yeast phase, and a knockout strain lacking CBP1 was unable to destroy macrophages in vitro or colonize in a mouse model of pulmonary histoplasmosis (45). BAD1 of B. dermatitidis, like CBP1, was also shown to be a yeast-phase-specific gene and a virulence factor (41, 12). YPS1 of B. dermatitidis is a yeast-phase-specific gene of unknown function that contains 3’ signals found in message with regulated stability (13), but no such signals were found in the 3’ untranslated region of BAD1 (unpublished observation). Promoter analysis of CBP1 revealed that this gene is transcriptionally upregulated in the yeast and identified a stretch of DNA required for yeast-phase expression of this gene (35).

B. dermatitidis exists as a mold in the acidic soil found along the Mississippi and Ohio River valleys. Upon inhalation of conidia or mycelial fragments, this fungus converts to the yeast morphotype and colonizes the host alveoli (10). Infection can lead to an acute pulmonary disease or can disseminate to other sites, most notably bone and skin. Conversion to the yeast form induces the expression of BAD1, which becomes arrayed on the surface of the yeast and is released into the extracellular milieu (27, 41). A knockout strain lacking BAD1 was avirulent in a mouse model of infection, while a strain in which BAD1 expression was reconstituted was restored to virulence matching that of the parental strain (12). BAD1 has been shown to bind to complement receptor 3 and may serve as an adhesin, although recently it was shown that this property is dispensable for virulence (31, 11). Both cell-associated and secreted forms of the protein modulate the host inflammatory response in favor of the pathogen by downregulating production of the proinflammatory cytokine tumor necrosis factor alpha (17, 18).

Expression of BAD1 is limited to the yeast phase and regulated transcriptionally. Analysis of the promoter region iden-
A. Truncations of Bd BAD1 upstream

| Promoter designation | -1101 | -101 | -741 | -361 | -238 | -173 | -33 | +5 |
|----------------------|-------|------|------|------|------|------|-----|-----|
| B ivs A              |       |      |      |      |      |      |     |     |
| ivs-box A            |       |      |      |      |      |      |     |     |
| box A                |       |      |      |      |      |      |     |     |
| No BA                |       |      |      |      |      |      |     |     |
| No TATA              |       |      |      |      |      |      |     |     |
| No Start             |       |      |      |      |      |      |     |     |

B. Truncations of Hc YPS3 upstream

| Promoter designation | -741 | -378 | -344 | -253 | -214 | -188 | +162 | -741 |
|----------------------|------|------|------|------|------|------|------|------|
| B ivs A              |       |      |      |      |      |      |      |     |
| ivs-box A            |       |      |      |      |      |      |      |     |
| box A                |       |      |      |      |      |      |      |     |
| 1/2 box A            |       |      |      |      |      |      |      |     |
| No BA                |       |      |      |      |      |      |      |     |

FIG. 1. Serial truncations of the upstream regions of BAD1 and YPS3. Shown are schematic representations of amplified fragments that were fused to lacZ to assess the contributions of promoter regions to B. dermatitidis (Bd) BAD1 and H. capsulatum (Hc) YPS3 transcription (A and B, respectively). Transcriptional start sites are indicated by arrows. Numbering of the 5′ and 3′ (at the translation-initiating ATG) ends of the promoter fragments are indicated relative to the transcriptional start sites (+1). At the right are promoter fragment designations, based on either the distance upstream of the transcriptional start site or homology between the BAD1 and YPS3 promoters at boxes B and A, shown as shaded boxes.

Two regions of homology with that of another yeast-phase-specific gene, YPS3 of *H. capsulatum* (26). These regions were termed boxes B and A and shared 86 and 80% identity between the BAD1 gene of *B. dermatitidis* strain 26199 and the YPS3 gene of *H. capsulatum* strain G217B, respectively (see Fig. 1). An additional stretch of nucleotides upstream of boxes B and A in the YPS3 upstream region, which is able to bind protein present in a yeast nuclear extract via electrophoretic mobility shift assay (24), was also identified in the BAD1 upstream region. When the BAD1 promoter was expressed heterologously in *H. capsulatum*, its transcription was phase regulated, indicating that the regulatory machinery is conserved between these two fungi (41).

Herein, we explored the sequences required for regulating expression of BAD1. Serial truncations of upstream sequences fused to a β-galactosidase reporter were used to functionally analyze homologous regions of the BAD1 and YPS3 promoters in both *B. dermatitidis* and *H. capsulatum*. We observed that the conserved box A region is an essential component of the minimal BAD1 promoter. This same region is needed for minimal YPS3 promoter activity in *B. dermatitidis*, whereas additional upstream sequence 5′ to box A is required for YPS3 transcription in *H. capsulatum*. We also pinpointed two putative transcription factor binding sites within box A that are essential for upregulating BAD1 expression in the yeast phase.

**MATERIALS AND METHODS**

**Strains and growth.** *B. dermatitidis* strain 26199 and *Saccharomyces cerevisiae* strain 204509 were obtained from the American Type Culture Collection (Rockville, Md.). *H. capsulatum* G217/Ba65-23 (39) was provided by J. Woods (University of Wisconsin). *Agrobacterium tumefaciens* strain LBA1100 (4) was provided by C. van den Hondel (Leiden University, Leiden, The Netherlands) and grown at 28°C on Luria broth supplemented with 0.1% glucose. *S. cerevisiae* was grown on yeast extract-peptone-dextrose at 28°C. *B. dermatitidis* yeast was maintained on Middlebrook 7H10 medium containing oleic acid-albumin complex prior to transformation. For reporter assays, *B. dermatitidis* was grown as yeast at 37°C or as mycelia at 22°C in Histoplasma macrophage medium (HMM) (58). Conversion from yeast to mold was accomplished by transferring log-phase yeast cultures to 22 to 25°C. For studies of mycelia, conversion was allowed to proceed for approximately 8 weeks of growth at 22 to 25°C on an orbital shaker. *H. capsulatum* was grown on an orbital shaker in HMM or HMM supplemented with 100 μg of uracil/ml as yeast at 37°C in 5% CO2.

**Plasmid constructions.** A base vector to allow fusion of promoter fragments to the E. coli lacZ coding region was made by deletion of the BamHI site downstream of lacZ to create pWLTΔ3′Bam. Promoter fragments were amplified from BAD1 and YPS3 using the vectors pWLT (41) and pY1 (YPS3 of *H. capsulatum* strain G217B in the vector pGEM-Teasy), provided by J. Woods, University of Wisconsin) as templates, respectively. A fragment of the *S. cerevisiae ACT1* locus, representing bases 1940 to 2002 (GenBank accession number L100026.1), was amplified from genomic DNA. The URA5 promoter of *H. capsulatum* strain G184AS (from −311 to +87) was amplified from pWU20 (57), using primers N-XUA5ORF (5′-ATGCGGCGCTCTGAAAGCTTGATATAAATCCGCGAAGAG-3′) and URABAMREV (5′-TAGTAGCCATTGAGAGATG-3′). Amplification of BAD1 promoter fragments was performed using forward primers in Table 1, with added 5′ NotI and XbaI sites (5′-AAGCGGCCGCTCTAGA-3′) and reverse primer DELTAREV (5′-TAGTAGCCATTGAGAGATG-3′) to amplify the 5′ promoter region. Amplification of YPS3 promoter fragments was performed using forward primers in Table 1, including 5′ NotI and XbaI sites (5′-AAGCGGCCGCTCTAGA-3′) and reverse primer YPSREV (5′-TAGTAGCCATTGAGAGATG-3′), to amplify the 5′ promoter region. Modified BAD1 and YPS3 promoter fragments were created by amplification, either via splicing by overlap extension (SOE) or primer-induced sequence alterations (Table 2). Creation of the −741 ΔB:ACT1 and B ivs A ΔACT1 promoter modifications required two-step SOE, in which two products were fused, and these fusion products were then spliced to a third fragment prior to cloning. Amplifications were performed using Elongase (Invitrogen Corp., Carlsbad, Cali.), and the primers used are listed in Table 2. Those creating the 5′ end of the promoter included additional 5′ NotI and XbaI sites.

**PCR products** were digested with NotI and BamHI and cloned into pWLTΔ3′Bam in replacement of the large (−27.4 kb) BAD1 upstream region, and their sequences were confirmed. The 2.5-4 kb BAD1 upstream region fused to lacZ in T-DNA vector pBTS92 (41) was modified to receive promoter fragments. The Clal site outside of the lacZ coding region, and the XbaI site not immediately 5′ of the BAD1 promoter, were removed to create vector pBPR25. Promoters and a fused 5′ fragment of lacZ were removed by digestion of the

| Table 1. Primers for amplification of BAD1 and YPS3 promoter truncation fragments |
|-------------------------------|-------------------------------|-------------------------------|
| **Gene** | **Promoter** | **Forward primer sequence** |
| BAD1 | −1101 | ACCCTGGTTGGAATAATCCGAG |
| BAD1 | −741 | GAGAGGCGGTGCTGATATTT |
| BAD1 | B ivs A | CAGATGTTCGAGAAGCC |
| BAD1 | ivs-box A | TACATCCCGGTAGGATGTTAT |
| BAD1 | box A | ATTTTTGAGGATAGCTAGATA |
| BAD1 | No BA | AGGATATCCGCTAGCCGCTT |
| BAD1 | No TATA | CTCTCGTCCCTGCTCCTTT |
| BAD1 | No Start | GCCGCAGTCCCTGCTCCTTT |
| YPS3 | −741 | AACCTATGTCGCGAAAGA |
| YPS3 | B ivs A | TAGAGTTCGATAAGTCGAA |
| YPS3 | ivs-box A | ACACGCAGTGGACTGCAATA |
| YPS3 | box A | GTCCTTTCGAGACGCTT |
| YPS3 | YPS3 | TTAGCTCTTGGCCTGTTT |
| YPS3 | No BA | TAGCATGATGTTAGCTGGCA |

a Nomenclature based on areas of homology between the two promoters contained within the PCR product or the 5′ end of PCR product numbered relative to the transcriptional start site.

b Primers had 5′ extensions (see Materials and Methods). The YPS3 − 741 product was obtained by binding to a site containing a partial primer match.
TABLE 2. Primers for amplification of BAD1 and YPS3 modified promoter fragments

| Promoter | Primes
| --- | --- |
| -741 AΔ:ACTI | GAGAGTACGGGTCGTATATT/* |
|  B ivs AΔ:ACTI | CAGATGTTTTGGATGTCATATTTGACTACGTGCGGCTTAGGCTCTTGCCAATGT* |
|  Box A-PURAS | TTGTGTGTAGATAGCTATATATTTGGCTTAGGCTCTTGGCATA |
|  ACTI-PURAS | TCTGTTGGAGACTACGTGCATATTGACTACGTGCTTTTGGCTTAGGCTTCTTGCCA* |
|  A-CREΔ:sp | TTGTGTTGGAAGACTACGTGCTTTTGGCTTAGGCTTCTTGCCA* |
|  A-SOxΔ:sp | TTGTGTTGGAAGACTACGTGCTTTTGGCTTAGGCTTCTTGCCA* |
|  A-MybΔ:sp | TTGTGTTGGAAGACTACGTGCTTTTGGCTTAGGCTTCTTGCCA* |
|  A-NFΔ:sp | TTGTGTTGGAAGACTACGTGCTTTTGGCTTAGGCTTCTTGCCA* |
|  A-AREB6Δ:sp | TTGTGTTGGAAGACTACGTGCTTTTGGCTTAGGCTTCTTGCCA* |
|  A-CRE/MybΔ:sp | TTGTGTTGGAAGACTACGTGCTTTTGGCTTAGGCTTCTTGCCA* |

a All vectors contain modified Pbad1, unless otherwise indicated. Replacements of predicted transcription factor binding sites are indicated by the site (CRE) or the protein (Son5, Myb, NF-1, and AREB6) that may bind it. ACTI represents a 63-bp fragment of the S. cerevisiae actin locus (see Materials and Methods).

b Primers indicated by an asterisk had 5′ extensions (see Materials and Methods). Paired SOE primers are separated by a slash, and sets used together to generate the final promoter fragment are separated by a semicolon. The 10-base replacement sequences are underlined.
lowed by organic extraction (21). For each sample, 2.5 μg of genomic DNA was separately digested with EcoRI, isolating a fragment containing the \( P_{BAD1} \)- or \( P_{YP35} \)-lacZ fusion, and XbaI, which digests the T-DNA reporter vectors at a single site. Transforming plasmid DNAs digested with the same enzymes were included as controls. DNA analysis by Southern blot hybridization was performed using standard techniques (43). Blots were probed for lacZ using a BamHI fragment of pWLT (41). Probes for hybridizations were gel purified using the QIAquick gel extraction kit (QIAGEN, Inc., Valencia, Calif.) and radiolabeled using the Prime-a-Gene labeling system (Promega Corp., Madison, Wis.). Four transformants each for plasmids containing the No \( BA \)-lacZ and No Start-lacZ reporters (Table 1) were tested in this fashion to ensure that their relatively low level of \( β \)-galactosidase (LacZ) activity was not due to rearrangement of the promoter-reporter fusion. Results indicated that the fate of transforming DNA was integration at a single site, with multimerization of the input DNA. Hybridizing fusion-containing EcoRI fragments were of the expected sizes (data not shown).

Five other \( B. \ dermatitidis \) transformants whose \( β \)-galactosidase (LacZ) activity levels were dramatically lower than those of other transformants of the same vector were similarly analyzed (data not shown). Results indicated the partial transfer of a single copy of the T-DNA in three transformants, resulting in the truncation of the lacZ fusion. A fourth transformant with low LacZ activity gave a hybridizing \( P_{BAD1} \)-lacZ fragment of the expected size, but only a single (non-multimerized) copy of the transforming DNA was present in the genome. An additional transformant with low LacZ activity showed that transforming DNA was multimerized and the \( P_{YP35} \)-lacZ hybridizing fragment was of the expected size. In this case, the lack of LacZ activity may have resulted from point mutations or rearrangements not detected by Southern hybridization. In transformations of vectors containing partial \( BAD1 \) promoter replacements, three transformants showed low LacZ activity but were not analyzed by Southern hybridization. Since the fate of transforming DNA was not determined for all transformants, LacZ data from these eight aberrant transformants was included in Fig. 2 and 4 and in statistical analyses.

\( H. \ capsulatum \) transformants were screened by Southern hybridization to select clones in which the transforming DNA was not integrated into the genome. \( H. \ capsulatum \) genomic DNA was isolated according to the published protocol of Woods and Goldman (55). Undigested genomic DNA samples were probed with an EcoRI fragment of pWU56 containing the \( P_{URA5} \) gene. Episomal vectors were noted as appearing in multiple sizes, including the size of the linearized transforming DNA, a larger fragment that corresponds to the size of a linear, dimerized vector, and sometimes appearing as both forms in the same transformant. Transformants selected for analysis contained dimerized vectors, the predominant fate of the transforming DNA. To determine the relative copy number of episomes in \( H. \ capsulatum \) transformants, genomic DNA was serially diluted in reaction wells of a microtiter plate. DNA was denatured by NaOH (final concentration, 0.1 N), with incubation at 37°C for 5 min, followed by neutralization by addition of SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) to a final concentration of 6×. DNA was transferred to nitrocellulose using a 96-well vacuum blotting manifold (Schleicher & Schuell, Keene, N.H.). Samples to be compared (e.g., all \( P_{BAD1} \)-lacZ transformants) were included on the same membrane. Membranes were first hybridized with the \( P_{URA5} \) probe to determine the relative amounts of transforming DNA per well. Blots were then stripped and reprobed with a 700-bp XhoI-SmaI fragment of the \( H. \ capsulatum \) \( UR45 \) (or \( HcURA5 \)) gene (isolated from pWU20) to determine the relative amounts of genomic DNA. Hybridization signals were quantified using a Storm blot imaging system and ImageQuant for Macintosh software (Amersham Biosciences, Piscataway, N.J.). The \( P_{URA5} / HcURA5 \) hybridization signal was determined for each well. For each clone, this derived signal was averaged across wells of the dilution series within linearity. The average for each clone was divided by the lowest average value of a clone in its comparison group \( (P_{BAD1} \text{-lacZ or } P_{YP35} \text{-lacZ transformants}) \) to give the relative copy number factor, which varied from 1 to 3.5 (data not shown).

**Quantitation of \( β \)-galactosidase activity.** *Histoplasma* yeast and \( B. \ dermatitidis \) yeast and mycelial cultures were grown in HMM and assayed for \( β \)-galactosidase activity as previously described (41). \( β \)-Galactosidase activity was normalized to the protein content of extracts, which were quantified using 10 μl of sodium dodecyl sulfate in the BCA microtiter plate assay (Pierce Chemical Co., Rockford, III.). Activity from \( H. \ capsulatum \) transformants was then also normalized to the relative copy number of vector by dividing the protein-normalized value by the relative copy number factor. Pairwise comparisons of \( β \)-galactosidase activity values were performed using the two-sided Wilcoxon test.

**RESULTS**

**Activity of \( P_{BAD1} \)-lacZ fusions in \( B. \ dermatitidis \).** To map functional regions of the \( BAD1 \) promoter, a series of vectors containing nested deletions of the \( BAD1 \) promoter fused to the \( E. \ coli lacZ \) coding region were introduced into \( B. \ dermatitidis \) by *Agrobacterium*-mediated transformation. Constructs were designed to test the contributions of boxes B and A, as well as upstream sequences, to \( BAD1 \) transcription (Fig. 1A). Transformants were first analyzed for yeast-phase LacZ activity (Fig. 2A). Those harboring constructs containing \( BAD1 \) promoter sequence down to and including the 63-nucleotide box A sequence possessed robust yeast-phase reporter activity. Within this set, there were no significant differences in LacZ expression. In contrast, transformants with fusions lacking box A yielded LacZ activity consistent with those of control \( P_{BAD1} \)-lacZ vectors that lacked the TATA signal (No TATA-lacZ) or

**FIG. 2.** \( β \)-Galactosidase activity in \( B. \ dermatitidis \) \( P_{BAD1} \)-lacZ, \( P_{YP35} \)-lacZ, and control \( P_{URA5} \)-lacZ transformants. \( B. \ dermatitidis \) 26199 transformed with lacZ fusion vectors was analyzed in both the yeast and mycelial phase for \( β \)-galactosidase activity using an \( α \)-nitrophenyl \( β \)-galactopyranoside assay. Activity levels for yeast are shown as black bars, and the levels for mold are shown as shaded bars. Test promoters driving lacZ expression are indicated on the x axis according to the designations indicated in Table 1 and/or Fig. 2. Eight independent transformants of each vector were analyzed in three experiments, and the results were averaged. Values are reported in milliunits of \( β \)-galactosidase activity per milligram of protein and represent the mean ± standard error. (A) \( β \)-Galactosidase activity of transformants of \( BAD1 \) promoter truncations fused to lacZ. (B) \( β \)-Galactosidase activity of transformants of \( YPS3 \) promoter truncations fused to lacZ.
transcriptional start site (No Start-lacZ). This represents the background transcriptional activity in transformants of promoterless lacZ vectors. The sharp drop in LacZ activity between vectors containing box A and those lacking this region is statistically significant ($P < 0.01$).

Reporter activity was also quantified for each transformant grown as a mold. The level of mold-phase LacZ activity detected was similar for transformants of all constructs, including the No TATA-lacZ and No Start-lacZ controls (Fig. 2A). These data point to the presence of a positively acting region contributing to yeast-phase activity. Transformants of a control vector in which the H. capsulatum URA5 promoter was fused to lacZ showed similar levels of activity in yeast and mold phases.

**Activities of P$_{YPS3}$-lacZ fusions in B. dermatitidis.** The upstream region of the YPS3 gene of H. capsulatum was also analyzed in B. dermatitidis using a set of constructs containing nested YPS3 promoter deletions fused to lacZ. To determine if the areas of homology between the BAD1 and YPS3 promoters were functionally conserved, these constructs were designed to match the set of BAD1 promoter deletions (Fig. 1B). Deletions of the YPS3 promoter showed a slightly different trend than that seen for the BAD1 promoter. While transformants of the P$_{BAD1}$-lacZ fusions showed consistent LacZ activity from all constructs containing box A, those of the P$_{yps3}$-lacZ fusions displayed a stepwise decline in activity as the YPS3 promoter driving lacZ transcription was successively deleted from the 5′ end (Fig. 2B). For example, reporter activity was significantly greater from the construct containing both boxes B and A (B ivs A-lacZ) than from that containing box A alone (box A-lacZ, $P < 0.001$). Though less robust than the reporter activity of longer YPS3 upstream regions fused to lacZ, the activity of box A-lacZ transformants was still much greater than that of No NA-lacZ transformants ($P < 0.001$). As was the case with the BAD1 promoter, the loss of box A from the YPS3 promoter reduced reporter transcription to background levels. An additional similarity between transformants of the BAD1 and YPS3 reporter constructs was the lack of mold-phase LacZ activity. Thus, as with BAD1, a positively acting region is involved in the yeast-phase transcription of YPS3 in B. dermatitidis transformants.

An extra construct was included in the analysis of the YPS3 promoter in which the box A region was truncated. This vector lacked the 5′ half of box A, leaving only the 3′ half and downstream sequence fused to lacZ (1/2 box A-lacZ) (Fig. 1B). Strains harboring this fusion gave yeast-phase LacZ activity between that of box A-lacZ and that of No NA-lacZ transformants (Fig. 2B). The decrease in activity of transformants of box A-lacZ vectors versus that of 1/2 box A-lacZ vectors was highly significant ($P < 0.001$). Although the level of yeast-phase LacZ activity of 1/2 box A-lacZ was greater than that of the No NA-lacZ fusion ($P < 0.05$), it had returned to the background levels seen in mold-phase transformants. This suggests that box A is an essential component of the minimal promoter for YPS3.

**β-Galactosidase activities of BAD1 and YPS3 promoter truncations in H. capsulatum.** A subset of the BAD1 and YPS3-nested promoter deletions fused to lacZ was tested in H. capsulatum to determine if the upstream regions of these genes function in a manner similar to that seen in B. dermatitidis. In H. capsulatum, positional effects necessitating the screening of multiple transformants for each reporter construct can be avoided by the use of vectors that can remain episomal (56). To this end, reporter constructs were cloned into telomeric vectors. Yeast-phase H. capsulatum transformants in which the transforming vectors remained episomal were analyzed for LacZ activity. Although quality genomic DNA could not be isolated from mycelium-phase cultures, cells which had undergone transition to mold were converted back to yeast and reexamined to ensure that the transforming DNA remained episomal. In nearly all samples tested, the transforming DNA had integrated into the genome after conversion, potentially affecting the level of reporter transcription (data not shown). As such, LacZ activity was quantified exclusively from yeast-phase cultures.

Overall, transcriptional activity from P$_{BAD1}$-lacZ fusions demonstrated a similar requirement for box A in yeast-phase H. capsulatum, but some fusions gave unexpected results. Transformants containing the longest BAD1 upstream regions (−1101-lacZ and −741-lacZ) expressed background levels of LacZ activity, equivalent to that in untransformed H. capsulatum (Fig. 3A; data not shown). Those with BAD1 upstream regions from box B (B ivs A-lacZ) to box A (box A-lacZ) possessed LacZ activity, though at a much lower level than that of matched B. dermatitidis transformants. As with the matched integrated constructs in B. dermatitidis, however, there was no significant difference in LacZ activity between these constructs. Similarly, the No BA-lacZ transformant did not express LacZ activity above background levels, indicating that the minimal promoter region for BAD1 in H. capsulatum is the same as that in B. dermatitidis and requires box A.

H. capsulatum P$_{yps3}$-lacZ activity differed in some aspects from P$_{BAD1}$-lacZ activity in this fungus. The longest P$_{yps3}$-lacZ construct (−741-lacZ) produced a high level of LacZ activity (2,200 relative mU/mg). Also, while transformants of vectors containing B ivs A-lacZ and ivs A-lacZ expressed LacZ activity, LacZ activity was reduced to background levels in the box A-lacZ transformant (Fig. 3B).

**Box A is necessary but not sufficient for transcriptional activation in B. dermatitidis.** Serial truncations of the BAD1 promoter fused to lacZ demonstrated that inclusion of box A was necessary for minimal BAD1 promoter activity. To determine whether sequences upstream of box A could compensate for a loss of this region, the 63-bp box A sequence was replaced with a 63-bp fragment amplified from the coding region of the S. cerevisiae actin gene. Resulting constructs containing −741 AΔ:ACT1-lacZ and B ivs AΔ: ACT1-lacZ, and −741-lacZ and B ivs A-lacZ matched controls, were introduced into B. dermatitidis, and transformants were analyzed for yeast-phase LacZ activity. Replacement of box A in either promoter background resulted in a loss of LacZ activity, indicating that this region is necessary for transcription from the BAD1 promoter (Fig. 4A).

Since box A is required for activating transcription from the BAD1 promoter in the yeast phase, this sequence was analyzed for its ability to confer transcriptional activation on a foreign promoter in B. dermatitidis. For this purpose, we employed the H. capsulatum URA5 promoter, which produces a low level of activity in B. dermatitidis (Fig. 2A). Box A, or a 63-bp fragment
of the *S. cerevisiae* actin gene, was fused upstream of a *URA5* promoter fragment containing 175 bp upstream of the transcriptional start site, driving *lacZ* transcription. The placement of box A, or the actin fragment, relative to the transcriptional start site was conserved between these constructs and the *BAD1* box A-*lacZ* vector. No statistically significant increase in reporter activity was observed from yeast-phase *B. dermatitidis* transformants of the box A-P*URA5*-lacZ vector over that for those harboring the *ACT1*-P*URA5*-lacZ vector (Fig. 4B). Both groups possessed relatively low levels of promoter activity compared to that of the *BAD1* box A-*lacZ* transformants. These data indicate that the box A region of the *BAD1* promoter is not sufficient for transcriptional activation of the *H. capsulatum UR45* promoter.

**Analysis of *BAD1* upstream region for transcription factor binding sites.** Since the minimal *BAD1* promoter region in *B. dermatitidis* requires box A, we sought to determine if this region contained any potential binding sites for known transcription factors. To this end, the box A sequence was subjected to analysis by MatInspector (v3.0) (http://www.genomatix.de) (38), comparing it to both the fungal and vertebrate transcription factor binding site libraries. The most probable matches for transcription factors binding this region include a cAMP-responsive element (CRE) and binding sites for the mammalian transcription factors Sox-5, v-Myb, nuclear factor 1 (NF-1), and...
AREB6 (Fig. 5A). Although fungal homologs to a subset of these factors have been identified, the presence of consensus binding sites may simply suggest that transcription factors with similar DNA binding domains could interact at these locations. In S. cerevisiae, three ATF/CREB proteins that associate with CRE have been identified, including both repressors and activators of transcription (51, 19). Sox-5 is a member of a protein family involved in governing cell fate decisions during embryogenesis. These proteins contain a high-mobility group (HMG)-type DNA binding domain that associates with the minor groove and bends DNA, allowing establishment of a local chromatin structure favorable to transcription (36). HMG-box proteins have been identified in fungi, including some factors regulating mating in Schizosaccharomyces pombe (20). Overlapping the Sox-5 binding site was a match with the consensus v-Myb binding site (6). The Myb family of proteins includes transcriptional activators found in many eukaryotes, including plants, mammals, fungi, and Drosophila. Myb family members share a repeated helix-turn-helix DNA binding domain (50). Although the ubiquitous nuclear factor 1 has not been identified in any fungi, its binding site has been noted in another fungal promoter (34, 47). AREB6 is a transcription factor possessing two zinc-finger DNA binding domains and is important for tissue-specific expression and development in vertebrates (25). It can act as either a positive or a negative regulator of gene expression, depending on the concentration and promoter context. A match to the consensus binding site for the N-terminal zinc finger is present in box A, but AREB6 is proposed to only bind this sequence when in conjunction with a second consensus site (CACCTGT) and in such context act as a negative regulator. This second consensus site was not identified in or adjacent to the box A region of the BAD1 promoter. The box A region of YPS3 contains several identical binding elements, including the CRE and a partial AREB6 site.

Numerous putative transcription factor binding sites were identified elsewhere within the upstream region of the BAD1 promoter (Fig. 6). From the 5' end of box B (−361) to 741 bp upstream of the transcriptional start site are additional consensus matches for v-Myb and CRE binding sites. Also of note, a stress response element (STRE) (16) and a MADS box factor binding site similar to those which bind Rlm1 and Mcm1 of S. cerevisiae and the human myocyte enhancer factor 2 (Mef2a) were identified in this same region (2, 32, 53). Box B contains a GATA site (44), and the intervening sequence between boxes B and A contains a second GATA site and a heat shock element (HSE) homologous to those of S. cerevisiae (14). Downstream of box A, additional Sox, GATA, and MADS box factor binding sites were identified, as well as binding sites for the A. nidulans conidiation-regulating transcription factors AbaA and StuA (3, 15).

Two regions influence transcriptional activation by box A. The impact of putative transcription factor binding sites within box A on transcription from the BAD1 promoter was assessed by modification of the BAD1 box A-lacZ construct, in which a 10-bp sequence encompassing each putative binding site was replaced. B. dermatitidis transformants of five modified constructs and the control box A-lacZ vector were analyzed for LacZ activity in the yeast phase (Fig. 5B). Removal of the putative Sox-5, NF-1, or AREB6 binding sites (replacing −217 to −208, −194 to −185, and −186 to −177, respectively) did not significantly affect reporter activity compared to the box

![FIG. 5. Possible transcription factor binding sites within the box A region and their effect on transcription from the BAD1 promoter. (A) Transcription factor binding sites within the box A region. (B) β-Galactosidase activities of yeast-phase B. dermatitidis transformants of BAD1 wild-type and modified box A-lacZ vectors. On the x axes, modified box A promoters, in which a 10-bp region is replaced, are indicated by a delta symbol, followed by the predicted transcription factor binding site (CRE), or the protein (Sox-5, Myb, NF-1, and AREB6) that may bind the site found within the altered region. Eight independent transformants of each vector were analyzed in three experiments, and the results were averaged. Values are reported as milliunits of β-galactosidase activity per milligram of protein and represent the mean ± standard error. Panels B and C represent analyses of transformant sets generated in separate experiments.](image-url)
A-\textit{lacZ} control. However, transformants in which \(-228\) to \(-219\) or \(-213\) to \(-204\), encompassing the predicted CRE and the Myb binding site, were replaced displayed a significant reduction in LacZ activity relative to that with box A-\textit{lacZ} \((P < 0.005)\). Although showing reduced transcriptional activity, neither region alone was essential for the transcriptional activation conferred by box A on the \textit{BAD1} promoter in \textit{B. dermatitidis}. However, replacement of both the CRE and the Myb binding site within box A resulted in a dramatic reduction in transformant LacZ activity compared to that of the wild-type box A-\textit{lacZ} or either single site replacement reporter \((P < 0.001)\) (Fig. 5C). Thus, the upstream sequences from \(-228\) to \(-219\) and \(-213\) to \(-204\), containing predicted CRE and Myb binding sites, together are critical in transcriptional activation of the \textit{BAD1} promoter.

**DISCUSSION**

Herein, we demonstrate the presence of a region conferring transcriptional activation of both \textit{BAD1} and \textit{YPS3} in the yeast phase. Transcription of the \textit{H. capsulatum} yeast-phase-specific factor \textit{CBP1} is also activated in the yeast phase, although it does not show sequence similarity to the upstream regions of \textit{YPS3} or \textit{BAD1}. Although both \textit{CBP1} and \textit{YPS3} are upregulated in the yeast phase, \textit{H. capsulatum} may possess at least two distinct mechanisms for regulating these phase-specific genes.

We found no evidence for repression of \textit{BAD1} transcription in the mold phase. The \textit{BAD1} promoter contains p30 M binding sites (also present in the \textit{YPS3} promoter) at positions \(-20\), \(+19\), and \(+47\) relative to the transcriptional start site \((41)\). In studies of \textit{H. capsulatum}, Abidi et al. detected a protein which bound these sites that is present only in nuclear extracts of mycelia, which led them to hypothesize that \textit{YPS3} expression is negatively regulated in the mycelial phase \((1)\). None of the \textit{BAD1} promoter truncations fused to \textit{lacZ} yielded activity in mold-phase cells, as one might expect from deletion of a repression. However, since box A is upstream of the p30 M binding sites and is required for activation of transcription, our approach of serially truncating the \textit{BAD1} promoter 5’ to 3’ may not have been suitable for uncovering deregulated, mold-phase reporter expression. Determining whether the TCC motif regulates \textit{BAD1} transcription may require deletions of p30 M sites within the context of a longer promoter including box A. Thus, while box A is required for transcriptional activation, p30 M sites could still contribute to regulation of \textit{BAD1} production. Activation of transcription in yeast and repression in mold would likely result in tightly regulated expression of this essential virulence factor.

Perhaps the most significant finding from these studies is that the box A region conserved between yeast-phase-specific genes \textit{BAD1} and \textit{YPS3} is a required element of the minimal promoter region for \textit{BAD1} expression in \textit{B. dermatitidis} and its heterologous expression in \textit{H. capsulatum}. This 63-nucleotide box A region is also essential to the minimal promoter region for \textit{YPS3} in \textit{B. dermatitidis}. Interestingly, the \textit{YPS3} box A-\textit{lacZ} fusion was inactive in \textit{H. capsulatum} transformants.

DNA sequence analysis identified putative transcription factor binding sites within box A, which were tested functionally, and other sites outside of this region. Replacement of the sequence encompassing the CRE within the \textit{BAD1} box A-\textit{lacZ} vector resulted in a reduction in transcriptional activity in \textit{B. dermatitidis} transformants. CRE-binding protein (CREB) is ubiquitously expressed. In eukaryotes, protein kinases phosphorylate CREB in response to an increase in cAMP levels, enabling transactivation functions \((7)\). CREB proteins in \textit{S. pombe} are targets of signal transduction pathways for handling osmotic stress and sexual development \((8)\). Phosphorylated CREB protein recruits CREB-binding protein (CBP), which is a general coactivator molecule able to associate with several transcription factors. One mode by which CBP is thought to mediate CREB-dependent transcriptional activation is by acting as a bridging molecule, linking the DNA-bound CREB to the general transcription factor TFIIB \((46)\). CBP is also an acetyltransferase. This activity can assist in chromatin remodeling to enhance transcriptional activity or can affect the function of other transcription factors by their direct acetylation \((7)\).

Removal of the predicted Myb binding site from the box A-\textit{lacZ} vector similarly resulted in a loss in transcriptional activation. The Myb protein family members share a DNA binding motif made up of two to three repeats, each forming a helix-turn-helix structure \((33)\). Vertebrate Myb proteins can also associate with the transactivation domain of CBP, using the same region that binds CREB \((46)\). Transcriptional activation by Myb proteins may be mediated in much the same way as CREB-induced transactivation. Although no homolog of CBP has been identified in \textit{S. cerevisiae}, tests of v-Myb molecules mutated in the activation domain which binds CBP showed an effect on transcriptional activity in a yeast model. This finding suggests the possibility of a functional yeast CBP
homolog that is involved in Myb-mediated transactivation (52). Recently, a Myb-like protein was identified in Giardia lamblia. Giardia, like dimorphic fungi, requires cellular differentiation for its success as a pathogen. In this protozoan, gMyb2 was shown to be important for the expression of the encystation-specific gene gbpi-b, encoding glucosamine-6-phosphate isomerase B, the first enzyme in the biosynthetic pathway for synthesis of cyst wall polysaccharides (49). The binding site for gMyb2 was found in the promoters of four encystation-induced genes. The fungus A. nidulans also utilizes a Myb domain containing transcription factor for inducing genes involved in a cellular shape change. In this case, the transcription factor FlbD is required for early conidiophore production (54). Since Myb family members are involved in regulation of differentiation events in diverse systems, from vertebrates (hematopoiesis) (33) and plants (hair cell formation in Arabidopsis) (28) to fungi and protozoans, it is not difficult to envision a role for a Myb-like protein of B. dermatitidis in the modulation of the yeast-phase-specific transcription of BAD1.

Loss of both the CRE and Myb binding site within box A resulted in a substantial reduction in reporter activity compared to replacement of either single site, indicating that these two regions together are critical for transcriptional activation of the BAD1 locus. It is of note that the YPS3 1/2 box A-lacZ construct, which gave equivalent levels of LacZ activity in yeast- and mold-phase transformants (Fig. 2B), is lacking the regions corresponding to the CREB, Sox-5, and v-Myb binding sites within box A of BAD1. Although both CREs and Myb binding sites were found upstream of box A, these sites were unable to compensate for the loss of box A in the −741 AΔ::ACT1-lacZ and B ivs AΔ::ACT1-lacZ vectors.

The binding sites of other transcription factors implicated in governing gene expression during changes in cellular morphology were identified outside of box A (Fig. 6). MADS-box transcription factor binding sites found in the BAD1 promoter shared homology to the consensus binding site for the S. cerevisiae protein Mcm1p, whose C. albicans homolog was shown to be important for morphogenesis (42). Binding sites for the A. nidulans condensation regulators AbaA and StuA were also identified in the BAD1 upstream. The processes of conidia generation and yeast formation from fungi of a filamentous morphotype share similarities. Both require the coupling of nuclear division with cytokinesis to go from a multicellular mycelium to single cells. Conidiation requires the initial step of generating unicellular forms (metulae and phialides) that give rise to conidia. The formation of these structures is governed by several transcription factors, including AbaA and StuA (30, 29). In the dimorphic fungus Penicillium marneffei, we see the linkage between condiation and yeast formation, since AbaA was shown to be required for both processes (9). MADS-box transcription factor, AbaA, and StuA binding sites were found downstream of box A. Since this region was included in the BAD1 box A-lacZ construct, these sites could contribute to the level of transcriptional activity seen in its transformants. The inability of box A alone to stimulate transcriptional activation of the H. capsulatum URA5 promoter suggests that elements downstream of box A may contribute to transcriptional activation from the BAD1 promoter.

The identification of HSE in the BAD1 upstream was surprising, since heat shock responses are generally thought of as reactions to a stress on the cells, rather than a condition for which an organism has a specially adapted morphotype. It is known, however, that the heat shock factor inducing transcription of heat shock proteins in S. cerevisiae is encoded by an essential gene, indicating that heat shock proteins are necessary for survival under normal growth conditions. STRE is found in nearly 200 potential STRE-regulated genes in yeast. Differing patterns of expression of stress-induced genes containing STRE are thought to be due to promoter context. The combination of the HSE with the STRE is found in at least two yeast genes, SSA3, which encodes an Hsp70 involved in prevention of protein aggregation, and HSP104, which has been shown to rescue heat-inactivated proteins (16). The HSE and STRE are both found in the BAD1 upstream region. Although these elements were found upstream of the minimal promoter, they may still play a role in regulation of BAD1 expression. Utilization of these elements may be responsible for the rapid onset of BAD1 gene expression after morphogenesis is stimulated by switching the cells from 25 to 37°C, while other factors may be involved in maintenance of BAD1 transcription in stable yeast-phase cells.

ACKNOWLEDGMENTS

This work was supported by NIH grants AI035681 and AI50882 from the USPHS and by a Burroughs Wellcome Fund Scholar Award in Molecular Pathogenic Mycology (B.S.K.).

We thank Jens Eckhoff of the Department of Biostatistics and Medical Informatics, University of Wisconsin, for assistance with statistical analyses.

REFERENCES

1. Abidi, F. E., H. Roh, and E. J. Keath. 1998. Identification and characterization of a phase-specific, nuclear DNA binding protein from the dimorphic pathogenic fungus Histoplasma capsulatum. Infect. Immun. 66:3867–3873.
2. Acton, T. B., H. Zhong, and A. K. Vershon. 1997. DNA-binding specificity of Mcm1: operator mutations that alter DNA-bending and transcriptional activities by a MADS box protein. Mol. Cell. Biol. 17:1881–1889.
3. Andriamopoulos, A., and W. E. Timerlake. 1994. The Aspergillus nidulans abac gene encodes a transcriptional activator that acts as a genetic switch to control development. Mol. Cell. Biol. 14:2503–2515.
4. Beijersbergen, A., A. den Dulk-Ras, R. A. Schilperoort, and P. J. J. Hooi-kaas. 1992. Conjugative transfer by the virulence system of Agrobacterium tumefaciens. Science 256:1324–1327.
5. Bevan, M. 1984. Binary agrobacterium vectors for plant transformation. Nucleic Acids Res. 12:8711–8721.
6. Biedenkapp, H., U. Borgmeyer, A. E. Sippel, and K. H. Klempnauer. 1988. Viral myb oncogene encodes a sequence-specific DNA-binding activity. Nature 335:835–837.
7. Blobel, G. A. 2000. CREB-binding protein and p300: molecular integrators of hematopoietic transcription. Blood 95:745–755.
8. Borgnes-Walsmey, M. L., and A. R. Walsmey. 2000. cAMP signaling in pathogenic fungi: control of dimorphic switching and pathogenicity. Trends Microbiol. 8:133–141.
9. Borneman, A. R., M. J. Hynes, and A. Andriamopoulos. 2000. The abad homologue of Penicillium marneffes participates in two developmental pro- grammes: condiation and dimorphic growth. Mol. Microbiol. 38:1034–1047.
10. Bradsher, R. W. 1996. Histoplasmosis and blastomycosis. Clin. Infect. Dis. 22:S102–S111.
11. Brandhorst, T., M. Wüthrich, B. Finkel-Jimenez, and B. Klein. 2003. A C-terminal EGF-like domain governs BAD1 localization to the yeast surface and fungal adherence to phagocytes, but is dispensable in immune modulation and pathogenicity of Blastomyces dermatitidis. Mol. Microbiol. 48:53–65.
12. Brandhorst, T. T., M. Wüthrich, T. Warmer, and B. S. Klein. 1999. Targeted gene disruption reveals an adhesin indispensable for pathogenicity of Blastomyces dermatitidis. J. Exp. Med. 189:1207–1216.
13. Burg, E. F., III, and L. H. Smith, Jr. 1994. Cloning and characterization of the yox, a temperature-dependent cDNA specific to the yeast phase of the pathogenic dimorphic fungus Blastomyces dermatitidis. Infect. Immun. 62: 2521–2528.
14. Chen, J., and D. S. Pederson. 1993. A distal heat shock element promotes the rapid response to heat shock of the HSP26 gene in the yeast Saccharomyces cerevisiae. J. Biol. Chem. 268:7442–7448.
