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Functional Analysis of the Promoter of the Phase-Specific *WH11* Gene of *Candida albicans*

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Candida albicans **WO-1 switches spontaneously, frequently, and reversibly between a hemispherical white and a flat gray (opaque) colony-forming phenotype. This transition affects a number of morphological and physiological parameters and involves the activation and deactivation of phase-specific genes. The *WH11* gene is transcribed in the white but not the opaque phase. A chimeric *WH11*-firefly luciferase gene containing the 5′ upstream region of *WH11* was demonstrated to be under phase regulation regardless of the site of integration, and a series of promoter deletion constructs was used to delineate two white-phase-specific transcription activation domains. Gel retardation experiments with the individual distal or proximal domain and white-phase or opaque-phase protein extract demonstrated the formation of one distal white-phase-specific complex and two proximal white-phase-specific complexes. Specific subfragments were tested for their ability to compete with the entire domain in the formation of complexes with white-phase protein extract in order to map the proximal domain sequence involved in white-phase-specific complex formation. Our results indicate that white-phase-specific transcription of *WH11* is positively regulated by *trans*-acting factors interacting with two *cis*-acting activation sequences in the *WH11* promoter.**

As is the case for a number of microbial pathogens (4, 11, 12, 36), *Candida albicans* switches spontaneously, reversibly, and at high frequencies between a number of general phenotypes, distinguishable by colony morphology (21, 24, 26). However, switching in *C. albicans* differs from switching in other microbial pathogens because of its pleiotropic consequences (26). In the white-opaque transition in strain WO-1 (25), a switch between a hemispherical white and a flat grey (opaque) colony morphology affects cell size, cell mass, wall morphology, budding pattern, sugar assimilation pattern, adhesion, drug susceptibility, sensitivity to oxidants, and patterns of tissue invasion in systemic mouse models (reviewed in references 26 and 28). The pleiotropy of this transition suggested that it involved the coordinate regulation of phase-specific genes, and differences in the in vitro translation products of white-phase and opaque-phase RNAs supported this hypothesis (27). The hypothesis was verified by the isolation and characterization of white-phase-specific and opaque-phase-specific genes. In the opaque phase, cells differentially express the opaque-phase-specific genes *PEP1* (19), which encodes a secreted aspartyl proteinase (13) and is also referred to as *SAP1* (40); *SAP3*, which encodes a second secreted aspartyl proteinase (40); and *Op4* (18), which encodes a unique protein which is selectively expressed not only in the opaque phase of strain WO-1 but also in the variant phenotypes of strain 3153A (17). In the white phase, cells differentially express the white-phase-specific gene *WH11* (33), which encodes a protein homologous to the glucose-lipid-regulated *GLP1* protein of *Saccharomyces cerevisiae* (34). Since these four phase-specific genes probably represent only a fraction of the phase-specific genes regulated by switching, and since *PEP1*, *Op4*, and *WH11* have been demonstrated to reside on different chromosomes (18, 19, 33), it seemed likely that the coordinate activation and deactivation of unlinked phase-specific genes accompanying switching in *C. albicans* would be regulated by phase-specific *trans*-acting factors in the white-opaque transition (30, 31).

To test this hypothesis, we analyzed the upstream region of the white-phase-specific gene *WH11* for *cis*-acting regulatory sequences and tested cell extracts for phase-specific *trans*-acting factors by gel retardation assays. The results suggest that white-phase-specific transcription of *WH11* is positively regulated by phase-specific *trans*-acting factors which interact with two *cis*-acting sequences in the *WH11* promoter.

MATERIALS AND METHODS

Maintenance of stock cultures. *C. albicans* WO-1 was maintained in liquid nitrogen. For experimental purposes, cells were plated on agar containing Lee's medium (15) supplemented with 70 μg of arginine per ml and 0.1 μM $ZnSO_4$ (modified Lee's medium) (5). To obtain cells in the white or opaque phase, cells from a white or opaque colony, respectively, were inoculated into 25 ml of supplemented Lee's medium in a 125-ml Erlenmeyer flask, which was then rotated at 25°C as previously described (5, 29). When white or opaque cells were used for biochemical purposes, an aliquot of the experimental culture was plated and the proportions of white and opaque colony morphologies were determined (25) in order to assess the purity of the analyzed cultures. The *ade2* derivative Red 3/6 of strain WO-1 was maintained on adenine-supplemented agar by previously described methods (32).

Plasmids and chimeric gene constructs. Cloning of the *WH11* gene and the 5′ upstream flanking sequence and construction of the reporter plasmid pWf20 have been previously described (32, 33). A diagram of pWf20 with the major features relevant to this study is presented below (see Fig. 2A). pWf20 contains the *C. albicans ADE2* gene, a *C. albicans* autonomously replicating sequence (7), and a *WH11*-firefly luciferase (*WH11*-flux) chimeric gene containing the 1,200-bp upstream region of *WH11* (see Fig. 2B).

Transformation of spheroplasts. The methods for generating spheroplasts in the *ade2* derivative Red 3/6 and transformation with pWf20 or derivative plasmids have been described in detail in a previous report (32). In brief, cells were pretreated with a sorbitol-EDTA-dithiothreitol solution, incubated with a sorbitol solution containing zymolyase (Seikagaku America Inc., Rockville, Md.), and washed. A total of 2×10^8 spheroplasts was then transformed with 10 to 25 μg of linearized plasmid in 200 μl of a solution containing 1 M sorbitol, 0.01 M $CaCl_2$, 0.01 M Tris-HCl (pH 7.5), and 10 μg of denatured calf thymus DNA. This treatment was followed by the addition of 1 ml of a solution containing 20% polyethylene glycol 4000, 10 mM $CaCl_2$, and 10 mM Tris-HCl (pH 7.4) and incubation for 20 min at 30°C and by the addition of 1 ml of SOS (1 M sorbitol, 0.35% yeast extract, 0.7% Bacto Peptone, 6.5 mM $CaCl_2$) and incubation for 40 min at 30°C. Spheroplasts from white or opaque cultures were then plated on agar containing modified Lee's medium supplemented with 1 M sorbitol and incubated for 7 days. This procedure allowed us to verify by colony morphology

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whether spheroplasts made from white or opaque cell populations maintained their original phenotype.

Northern (RNA) blot analysis. Methods for isolation of total cellular RNA and Northern blot hybridization were described in an earlier publication (33). To assess the level of the chimeric *WH11*-flux transcript, Northern blots were probed with an antisense flux RNA synthesized in vitro by using the pGEM-Luc plasmid according to the specifications of the manufacturer (Promega Corp., Madison, Wis.). Prehybridization and hybridization procedures were performed by the method of Church and Gilbert (10) with the addition of 100 μg of denatured calf thymus DNA per ml to quench nonspecific hybridization. Blots were stripped and rehybridized with a *WH11* cDNA (33). Autoradiogram images were digitized and band intensities were analyzed in the linear range with the Dendron software program (2) to obtain quantitative estimates of intensity.

Preparation of cell extracts for gel retardation experiments. Cells from a clonal colony in the white or opaque phase were grown to late log phase in modified Lee's medium in 1-liter cultures. A total of 2×10^{10} white cells and 8×10^9 opaque cells were harvested and washed once with cold water and then twice with cold extraction buffer [180 mM Tris-HCl, 350 mM $(NH_4)_2SO_4$, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 7 mM 2-mercaptoethanol] containing the following protease inhibitors: 1 mM phenylmethylsulfonyl fluoride, 2 mM ε-amino caproic acid, 2 mM pepstatin, 0.6 μM leupeptin, 2 μg of chymostatin per ml, and 50 μg of aprotinin per ml. Washed cell pellets were then resuspended in equal volumes of extraction buffer containing protease inhibitors, mixed with an equal volume of glass beads (0.45-mm diameter), and disrupted with a Bead Beater (Biospec Products, Bartlesville, Okla.) for 10 cycles of 15-s duration at 4°C. Extracts were cleared by centrifugation for 2 h at 150,000 × *g*. The supernatants were dialyzed against a solution containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.9), 5 mM EDTA, 20% glycerol, and 1 mM phenylmethylsulfonyl fluoride. The extracts from white and opaque cells each contained between 5 and 12 mg of protein per ml as determined by the Bradford method (Bio-Rad, Hercules, Calif.).

Probes for gel retardation experiments. The fragment used for gel retardation assays to analyze the distal domain contained 171 bp spanning positions −475 to −304 of the upstream region of *WH11*. The fragment used to analyze the proximal domain contained 370 bp spanning positions −310 to +60 of the *WH11* gene and upstream region. The following fragments of the *WH11* promoter (see Fig. 1C) were generated by PCR using specific oligonucleotide primer pairs: A, bp −305 to −83; B, bp −305 to −230; C, bp −245 to −83; D, bp −305 to −148; E, bp −164 to −83; and F, bp −83 to +60. Two fragments, G (bp −310 to −271) and H (bp −270 to −231), were individually derived by annealing two specific oligonucleotide primer pairs in each case. For gel retardation assays, the distal domain fragment was isolated from plasmid subclones by cutting with *Eco*RI and *Xba*I. The remaining PCR and oligonucleotide fragments were purified by agarose gel electrophoresis and used directly. The precise concentration of each fragment was estimated with Hoechst 33258 by fluorometric methods. Fifty to 100 ng of each fragment was end labeled with [γ-^{32}P]ATP by using polynucleotide kinase according to standard methods (16).

Gel retardation assays. Basic binding reactions were performed at 25°C with 15 μl of a solution containing 25 mM HEPES (pH 7.9), 50 mM NaCl, 1 mM EDTA, 0.05% Nonidet P-40, 1 mM dithiothreitol, 5% glycerol, 5 μg of poly(dG-dC), and various amounts of protein extract. This mixture was first preincubated for 10 min. Radiolabeled DNA probe (100 to 300 pg) was then added, and the mixture was incubated for 20 min at 25°C. The binding buffer containing xylene-cyanol and bromophenol blue was added, and the reaction mixture was loaded immediately onto a nondenaturing polyacrylamide gel (39:1, acrylamide to bisacrylamide) in 0.5× TBE (44.5 mM Tris-HCl [pH 8.1], 44.5 mM H_3BO_3, 1 mM EDTA). Gel electrophoresis was performed at a constant voltage of 100 V at 4°C until the marker had migrated through 90% of the gel. Gels were then transferred to Whatman 3MM paper, dried, and autoradiographed.

Sequence analysis. Sequences were analyzed with the Geneworks software (IntelliGenetics Inc., Mountain View, Calif.).

RESULTS

The white-opaque phase transition in the *ade2* derivative Red 3/6. Since all transformations for the functional dissection of the *WH11* promoter were performed in strain Red 3/6, an adenine-requiring auxotroph derived from strain WO-1, it was first necessary to demonstrate that Red 3/6 underwent the white-opaque transition in a normal fashion. Therefore, cells from individual Red 3/6 white colonies were plated at low density on agar and incubated at 25°C for 7 days, and the proportions of opaque colonies and white colonies with opaque sectors were determined. An example of a white colony with an opaque sector is presented in Fig. 1A. The proportion was 2×10^{-2} for 4,897 colonies examined. In the reverse direction, cells from individual Red 3/6 opaque colonies were plated at low density on agar and incubated at 25°C for 7 days, and the proportions of white colonies and opaque colonies with white sectors were determined. An example of a predominantly white colony emanating from opaque cells is presented in Fig. 1B. The frequency was 10^{-2} for 756 colonies examined. These frequencies are close to those obtained for the parent strain WO-1 (3, 25). In addition, Red 3/6 opaque and white budding cells exhibited the same differences in cellular morphology demonstrated for the parental WO-1 strain (3, 25).

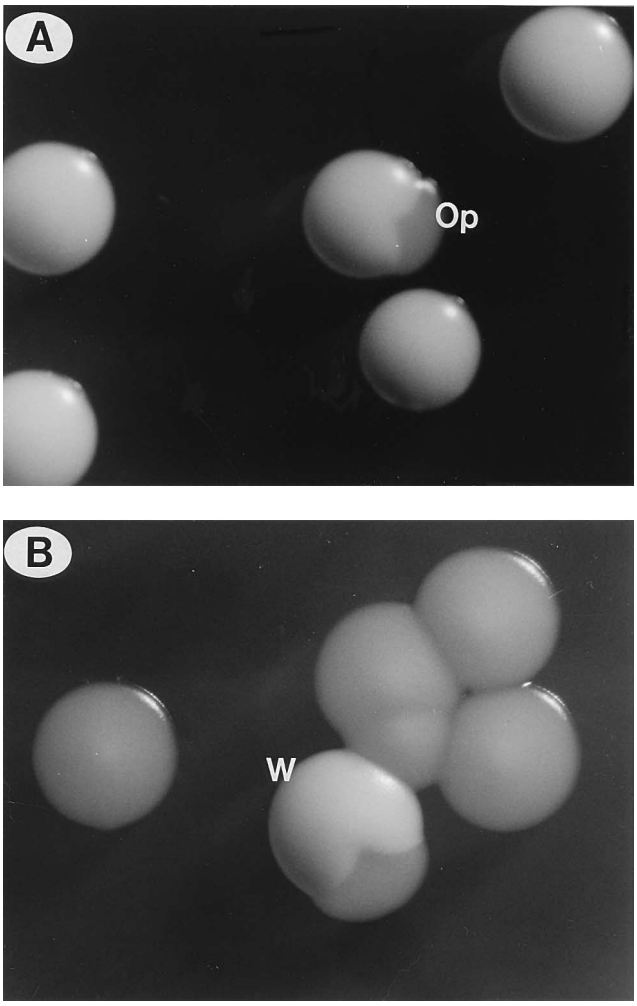

FIG. 1. White-opaque transition in strain Red 3/6, an *ade2* derivative of *C. albicans* WO-1. (A) Switch from white to opaque. Cells from a single white colony were plated on nutrient agar and examined after 7 days at 25°C. A white colony formed an opaque sector (Op). (B) Switch from opaque to white. Cells from an opaque colony were plated on nutrient agar and examined after 7 days at 25°C. A single white colony (W) with an opaque sector formed in the predominantly opaque population.

Expression of the integrated chimeric *WH11*-flux gene is under regulation of the phase transition. The expression plasmid pWf20, diagrammed in Fig. 2A, contained in frame the 1,200 bp of the 5′-flanking sequence of *WH11*, the entire open reading frame of *WH11*, the firefly luciferase gene, and the 180-bp transcription termination control sequence of *WH11* (32). This construct was originally developed to assess chimeric gene expression by chemiluminescence of the gene product. Since the gene product was undetectable, the chimeric gene transcript was employed as a reporter of gene transcription. Site-specific integration at the *WH11* gene was achieved by

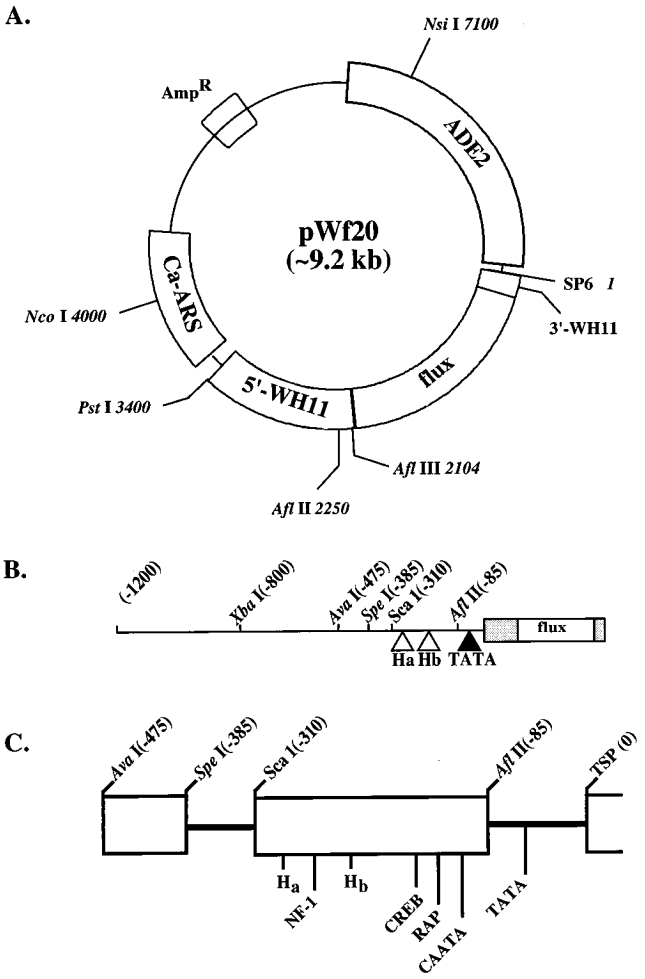

FIG. 2. Basic transforming vector pWf20 used for the functional dissection of the promoter region of *WH11*. (A) The entire fusion construct, with the relative locations (not to scale) of the *C. albicans ADE2* gene, the ampicillin resistance gene (Amp^R), the *C. albicans* autonomously replicating sequence (Ca-ARS), the upstream region and the 5′ end of the *WH11* gene (5′-*WH11*), the firefly luciferase gene (flux), and a 3′ region of *WH11* necessary for transcription termination (3′-*WH11*) indicated. Relevant restriction sites for *Nsi*I, *Afl*III, *Afl*II, *Pst*I, and *Nco*I and the location of the SP6 promoter binding site are noted. Numbers next to restriction sites denote base pairs. (B) The region spanning *Pst*I-*Afl*III (in panel A) represented in greater detail. Ha and Hb, heat shock consensus sequences; TATA, canonical TATA box. Region 5′-*WH11* in panel A includes 1,200 bp of the 5′ upstream flanking region of the *WH11* gene plus the entire open reading frame. Region 3′-*WH11* in panel A includes 180 bp of the 3′ downstream region of the *WH11* gene. (C) The region spanning the *Ava*I site at bp −475 and the transcription start site shown in greater detail. Sequences homologous to known regulatory elements in other organisms are noted. Numbers represent nucleotide positions relative to the transcription start site.

linearizing pWf20 at a unique *Afl*II site (at bp −85) in the *WH11* gene (32). Alternatively, site-specific integration at the *ade2* gene was achieved by linearizing pWf20 at a unique *Nsi*I site in the *ADE2* gene of pWf20 (32). Site-specific integration was confirmed in all cases by Southern analysis (32).

When pWf20 integration was targeted at either the *ade2* (*Nsi*I site) or the *WH11* (*Afl*II site) locus of Red 3/6, transcription of the chimeric *WH11*-flux sequence was under the regulation of the white-opaque transition (Fig. 3). At both integration sites, there were strong and moderate hybridization bands with molecular sizes of approximately 1.7 and 1.5 kb, respectively, in Northern blots of white-phase cellular RNA probed

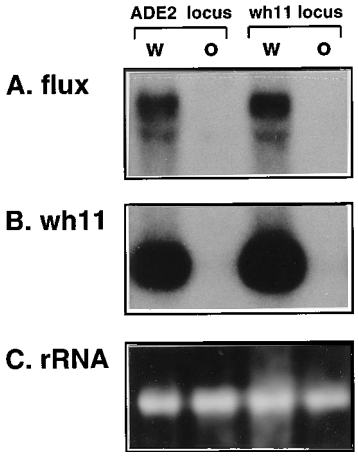

FIG. 3. The site of integration has no effect on the phase specificity of transcription of the chimeric *WH11*-flux gene under the control of the upstream regulatory region of *WH11*. pWf20 was linearized at either the *ADE2* locus (*Nsi*I) or *WH11* (*Afl*II). In the former case, site-specific integration occurred at the *ADE2* locus, and in the latter case, site-specific integration occurred at the *WH11* locus (32). White (w) and opaque (o) colonies from the alternative transformants were grown to late log phase. Total RNA was extracted and Northern blots of each were first hybridized with a flux probe (A) to assess the level of *WH11*-flux transcript and then with a *WH11* probe (B) to assess the level of the endogenous *WH11* transcript. The ethidium bromide-stained 28S rRNA band is shown for each lane (C) to demonstrate comparable loading.

with the firefly luciferase antisense probe but a negligible signal in Northern blots of opaque-phase cellular RNA (Fig. 3). The 1.5-kb band appears to be a truncated transcript. When the same Northern blots were stripped and reprobed with the *WH11* cDNA, a strong hybridization signal was observed at 0.5 kb, the molecular size of the *WH11* transcript (33), in the white phase but not in the opaque phase (Fig. 3). A chimeric transcript was also observed in both cases, with a molecular size of 1.7 kb in the white phase only (data not shown). Therefore, phase-specific transcription of the chimeric *WH11*-flux sequence is under regulation of the *WH11* promoter regardless of the site of integration, and phase-regulated expression of the chimeric sequence has no effect on the expression of the endogenous *WH11* gene, even when integration occurs at one of the two *WH11* homologs.

The region between bp −475 and −388 of the 5′ upstream region of *WH11* contains a distal white-phase-specific transcription activation domain. Deletion of the first distal 400 bp, between bp −1200 and −800, of the 5′ upstream region of the *WH11* gene in pWf20Δ3 had no significant effect on the level of the chimeric *WH11*-flux transcript and did not affect phase specificity (Fig. 4). Deletion of the subsequent 325 bp, between bp −800 and −475, of the 5′ upstream region in pWf20Δ6, resulting in a total deletion of bp −1200 to −475, again had no significant effect on the level of the *WH11*-flux transcript and did not affect phase specificity (Fig. 4). These results suggest that the region spanning bp −1200 to −475 contains no *cis*-acting sequences necessary for white-phase-specific *WH11* transcription. However, deletion of the subsequent 88 bp, between positions −475 and −388, of the 5′ upstream region in pWf20Δ7, resulting in a total deletion of bp −1200 to −388, caused an approximately sixfold reduction in the level of the *WH11*-flux transcript but did not affect phase specificity (Fig. 4). This latter result suggests that the 88-bp region between positions −475 and −388 contains one or more *cis*-acting sequences which enhance *WH11* transcription in the white phase. However, the low level of *WH11*-flux transcript present in the

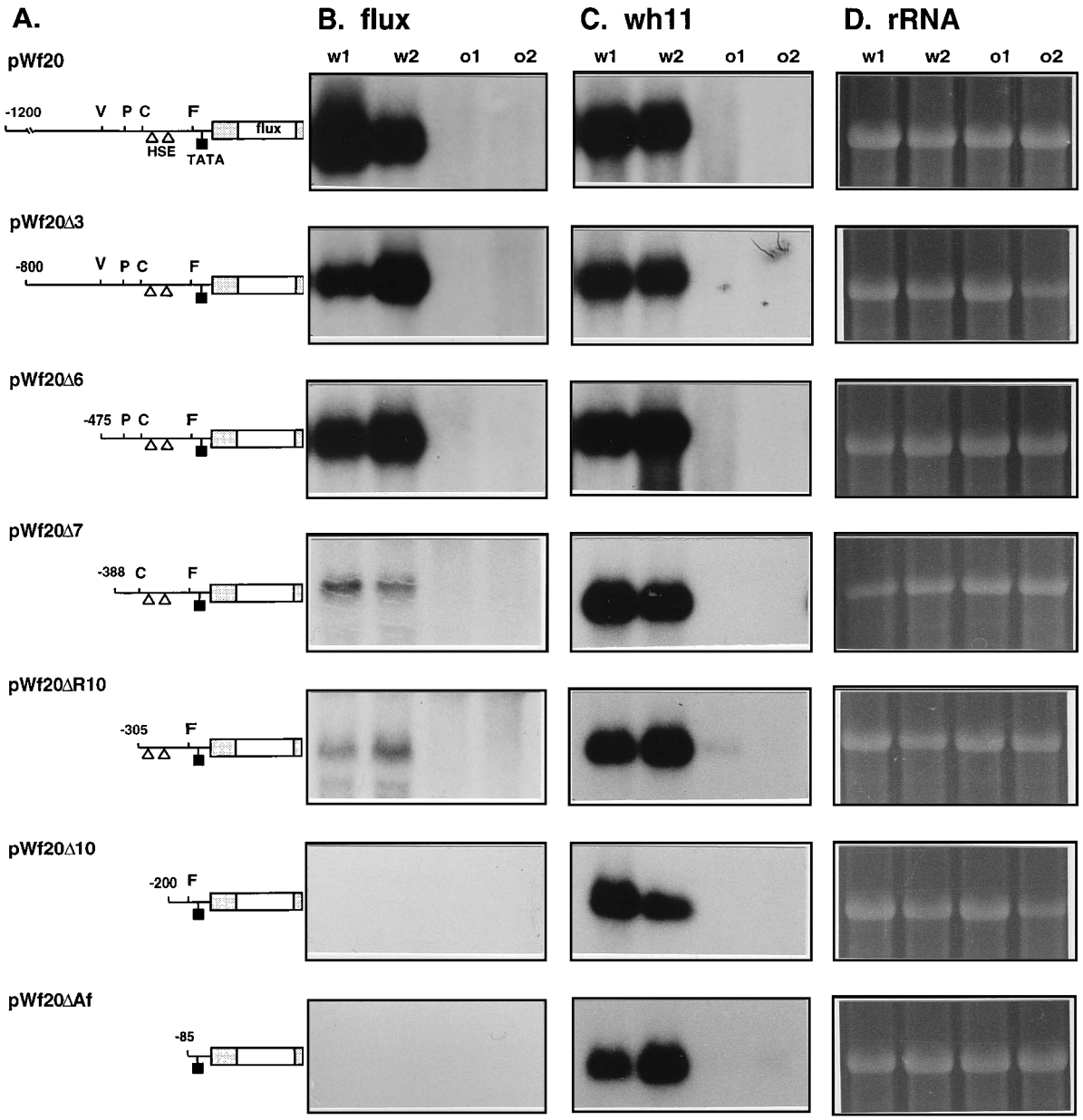

FIG. 4. Phase-specific transcription of the chimeric *WH11*-flux gene and the endogenous *WH11* gene in transformants containing various deletion constructs of the upstream regulatory region of *WH11*. (A) Schematic representations of the original pWf20 construct and deletion constructs. V, *Ava*I; P, *Spe*I; C, *Sca*I; F, *Afl*II; unfilled triangles, heat shock elements (HSE); filled square, TATA box; w1 and w2, two independent white-phase clones; o1 and o2, two independent opaque-phase clones. Numbers to the left of each construct represent the nucleotide positions of the 5′ ends relative to the transcription start site. (B) Northern blots of white cell (w1 and w2) and opaque cell (o1 and o2) RNA of each transformant hybridized with the antisense flux probe. (C) Northern blots hybridized with the *WH11* probe (in this case, the position of endogenous *WH11* RNA is shown). (D) The ethidium bromide-stained 28S rRNA band is shown for each lane to demonstrate comparable loading.

white phase of the construct with the deletion from bp −1200 to −388 suggests that the distal domain does not contain an exclusive activator of white-phase-specific transcription.

The region from bp −305 to −200 of the 5′ upstream region of *WH11* contains a proximal phase-specific transcription activation domain. Deletion of 83 bp, between positions −388 and −305, of the 5′ upstream region of *WH11* in pWf20ΔR10, resulting in a total deletion of bp −1200 to −305, caused no further reduction in the level of the *WH11*-flux transcript (Fig. 4). However, deletion of the next 105 bp, between positions −305 and −200, in pWf20Δ10, generating a total deletion of bp −1200 to −200, resulted in the complete loss of detectable *WH11*-flux transcript in the white phase (Fig. 4). This latter result suggests that the sequence between bp −305 and −200 contains one or more *cis*-acting sequences involved in transcriptional activation. Deletion of the next 115 bp, between positions −200 and −85 of the 5′ upstream region of *WH11*, in pWf20ΔAf had no reverse effect on the absence of the *WH11*-flux transcript in the white phase or the opaque phase (Fig. 4). When the sequence between bp −388 and −85 in pWf20Δ7 was inverted, in pWf20Δ1, the level of the *WH11*-flux transcript was again abolished (data not shown), demonstrating that the

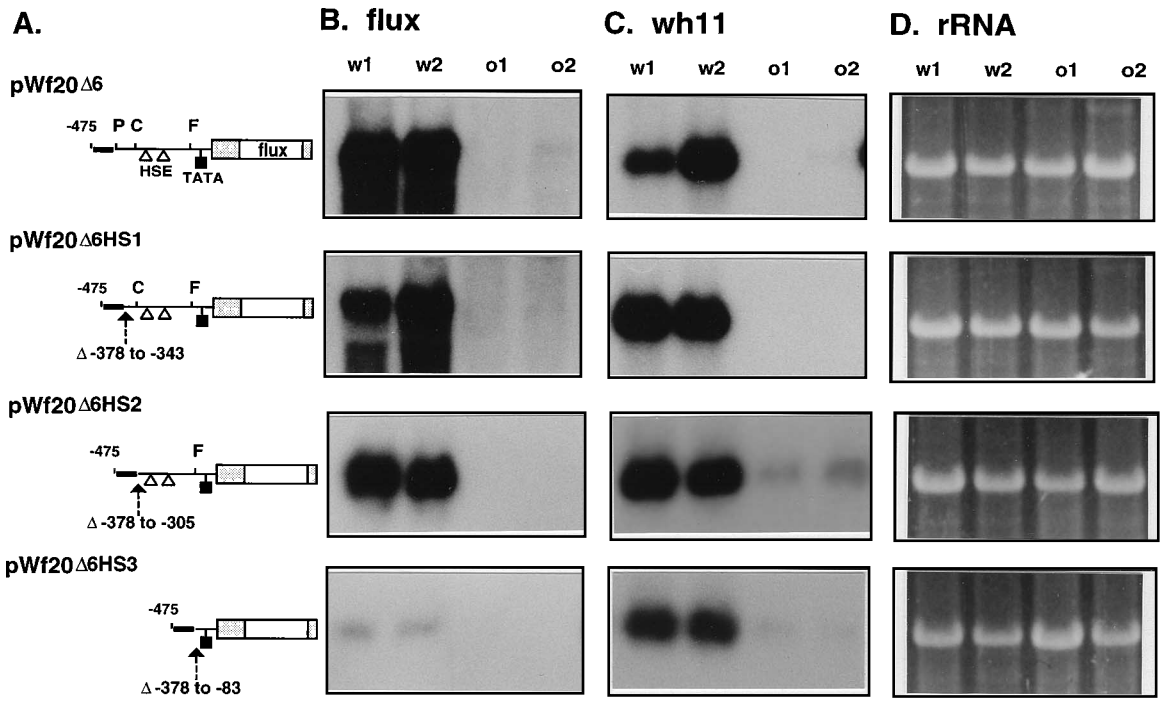

FIG. 5. Phase-specific transcription of the chimeric *WH11*-flux gene and the endogenous *WH11* gene in transformants containing various deletion constructs of the upstream regulatory region of *WH11*. In all of these constructs, the deletions (dashed arrows) are proximal to the distal domain (thick line spanning bp −475 to −378). See the legend to Fig. 4 for an explanation of panels A through D. The low level of endogenous *WH11* transcript observed for pWf20Δ6HS2 and pWf20Δ6HS3 in opaque clones o1 and o2 probed with *WH11* cDNA reflects the somewhat higher level of contaminating white cells in these opaque cell populations due to spontaneous switching.

proximal transcription activation sequence(s) in the proximal domain must be in the proper orientation for activation.

The distal domain alone can activate white-phase-specific transcription. The functional dissection of the *WH11* promoter diagrammed in Fig. 4 suggested that there were two transcription activation domains, a distal domain between bp −475 and −388 and a proximal domain between bp −305 and −200. In addition, these results demonstrated that the proximal domain could activate white-phase-specific transcription of the *WH11*-flux gene in the absence of the distal domain. To test whether the distal activation domain could activate white-phase-specific transcription of the *WH11*-flux gene in the absence of the proximal domain, the 88-bp sequence between positions −475 and −388 was transposed downstream by a series of proximal deletions of variable length which left the distal domain intact (Fig. 5). A deletion between bp −378 and −343 in pWf20Δ 6HS1 had no significant effect on the level of the *WH11*-flux transcript and did not affect phase specificity (Fig. 5). A subsequent deletion between bp −378 and −305 in pWf20Δ6HS2 resulted in no detectable change in the level of the *WH11*-flux transcript and had no effect on phase specificity (Fig. 5). However, a deletion between bp −378 and −83 in pWf20Δ6HS3, which removed the proximal domain, resulted in an approximately 15-fold reduction in the level of the *WH11*-flux transcript but did not affect the phase specificity of this low level of transcript (Fig. 5). Therefore, the distal domain can activate white-phase-specific transcription of the *WH11*-flux gene in the absence of the proximal domain but at a substantially reduced level.

Demonstration of white-phase-specific factors which bind to the distal domain. To test whether the distal domain binds to white-phase-specific factors, gel mobility shift assays were performed with an end-labeled 172-bp fragment from positions −475 to −305, containing the distal domain, and increasing concentrations of protein extract from either white or opaque cells. Two complexes, dI and dII, were formed with white-phase protein extract, and the levels of both increased with increasing concentrations of extract (Fig. 6A, lanes 3 to 6). Neither complex was formed in the absence of extract or in the presence of bovine serum albumin (BSA) alone (Fig. 6A, lanes 1 and 2). When binding assays were performed with opaque-phase protein extract, complex dI still formed, and its level increased with increasing concentrations of opaque-phase protein extract (Fig. 6A, lanes 7 to 10), demonstrating that formation of complex dI was not phase specific; however, complex dII did not form at any of the tested concentrations of opaque-phase protein extract, demonstrating that formation of complex dII was white phase specific. When the formation of radiolabeled complexes between the end-labeled 172-bp fragment containing the distal domain and white-phase protein extract was challenged with 50- and 100-fold (50× and 100×, respectively) molar excesses of the unlabeled 172-bp fragment, the formation of radiolabeled complex dII was abolished and the formation of complex dI was diminished (Fig. 6B). When challenged with a 100× molar excess of an unlabeled nonspecific competitor, the *Hae*III fragment of ϕX174, there was no significant reduction in the formation of either complex (Fig. 6B, lane 6). When the formation of radiolabeled complexes between the end-labeled 172-bp fragment and opaque-phase protein extract was challenged with 50× and 100× molar excesses of the unlabeled 172-bp fragment, the formation of complex dI was diminished (Fig. 6B). However, when challenged with a 50× or 100× molar excess of unlabeled nonspe-

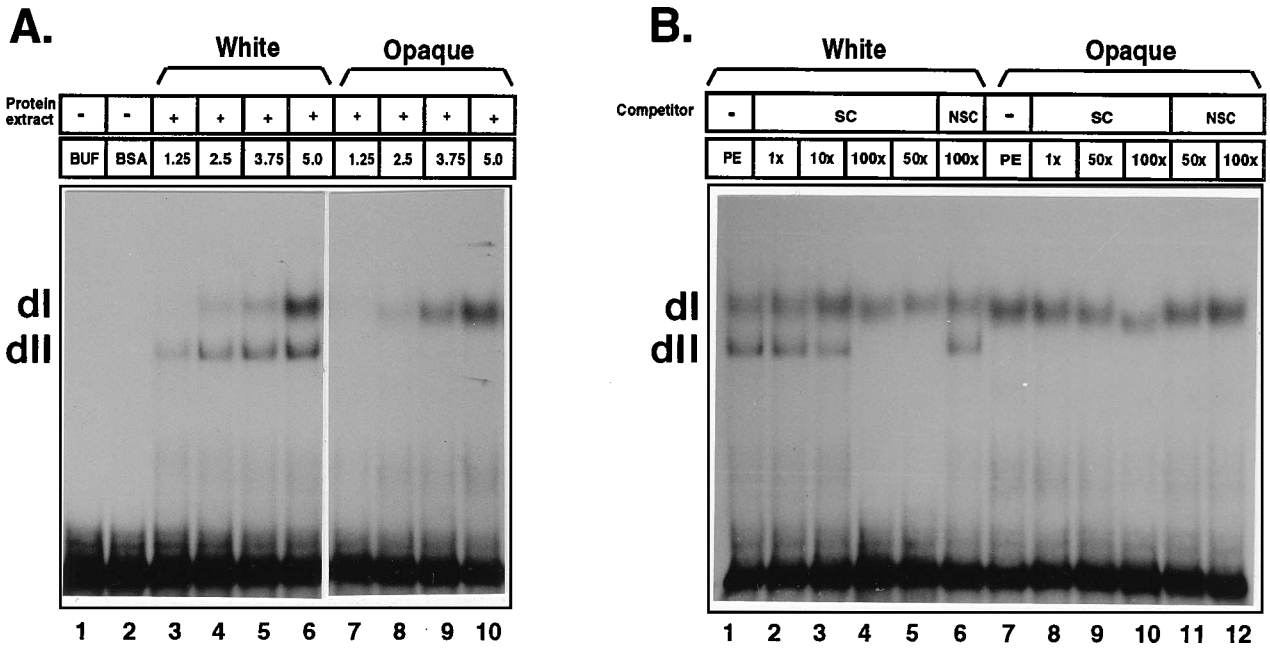

FIG. 6. Gel retardation and competition assays with the 172-bp distal transcription activation domain (bp −475 to −305) and phase-specific protein extracts. (A) Gel retardation with a fixed concentration of end-labeled distal domain fragment and increasing concentrations of white- or opaque-cell protein extracts. BUF, binding buffer with no extract; BSA, 5 μg of BSA alone. Numbers above the lanes indicate micrograms of white- or opaque-cell protein in the binding reaction mixture. (B) Competition experiment. An end-labeled fragment containing the distal domain was challenged with specific competitor DNA (unlabeled homologous fragment) (SC) or nonspecific competitor DNA (194-bp *Hae*III fragment of ϕX174) (NSC). A fixed concentration of end-labeled fragment plus 5.0 μg of either white- or opaque-cell protein extract was incubated in binding buffer with the indicated molar ratios of SC or molar excesses of NSC. dI, nonspecific complex; dII, white-phase-specific complex.

cific competitor, there was no significant reduction in the formation of complex dI (Fig. 6B).

Demonstration of white-phase-specific factors which bind to the proximal domain. To test whether the proximal transcription activation domain binds to white-phase-specific factors, electrophoretic gel mobility shift assays were performed with an end-labeled 370-bp fragment from positions −310 to +60, containing the proximal domain, and increasing concentrations of protein extract from either white or opaque cells. Two discrete complexes, pI and pIII, and one diffuse complex, pII, were formed with white-phase protein extract, and the levels of all three complexes increased with increasing concentrations of

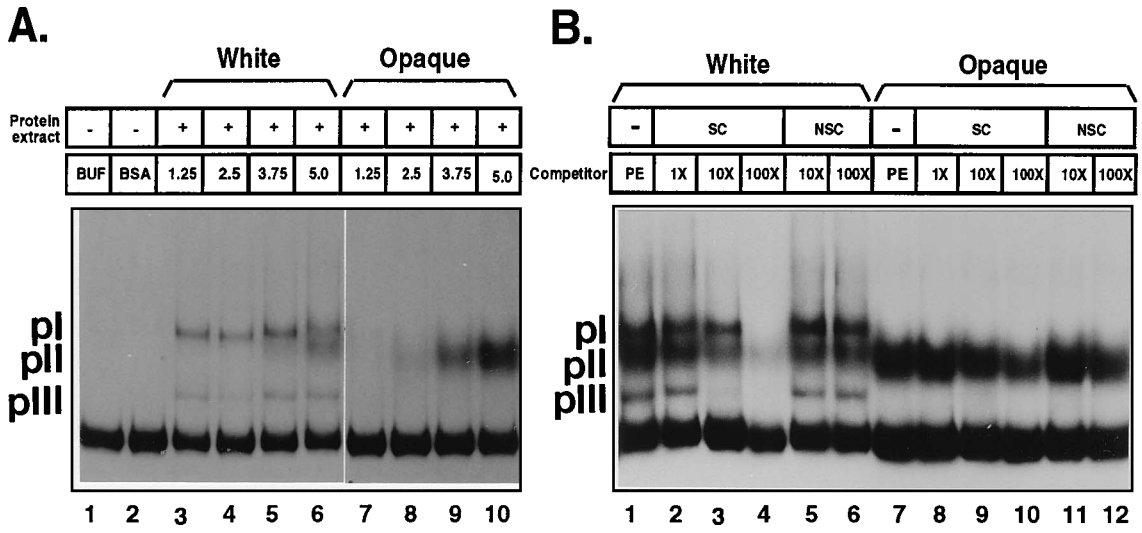

FIG. 7. Gel retardation and competition assays with the 370-bp proximal transcription activation domain (bp −310 to +60) and phase-specific protein extracts. (A) Gel retardation with increasing concentrations of white- or opaque-cell protein extracts. BUF, binding buffer with no extract; BSA, 5 μg of BSA alone. Numbers above the lanes indicate micrograms of white- or opaque-cell protein in the binding reaction mixture. (B) Competition experiment. An end-labeled fragment containing the proximal domain was challenged with specific competitor DNA (unlabeled homologous fragment) (SC) or nonspecific competitor DNA (194-bp *Hae*III fragment of ϕX174) (NSC) for complex formation. The fixed concentration of end-labeled proximal domain fragment plus 5.0 μg of either white- or opaque-cell protein extract was incubated in binding buffer with the indicated molar excesses of SC or NSC. pI and pIII, white-phase-specific complexes; pII, nonspecific complex.

white-phase protein (Fig. 7A, lanes 3 to 6). None of the three complexes were observed in the absence of protein or in the presence of BSA (Fig. 7A, lanes 1 and 2). When binding assays were performed with opaque-phase protein extract, only complex pII formed and its level increased with increasing concentrations of protein extract (Fig. 7A, lanes 7 to 10), demonstrating that it was not phase specific; however, complexes pI and pIII did not form, demonstrating that both were white phase specific. When the formation of radiolabeled complexes between the end-labeled 370-bp fragment containing the proximal domain and white-phase protein extract was challenged with a 100× molar excess of the unlabeled 370-bp fragment, the levels of radiolabeled complexes pI, pII, and pIII were dramatically reduced (Fig. 7B, lane 4). When formation was challenged with a 100× molar excess of an unlabeled nonspecific competitor, the *Hae*III fragment of ϕX174, there was no reduction in the levels of the three radiolabeled complexes (Fig. 7B, lane 6). When the formation of radiolabeled complex pII between the end-labeled 370-bp fragment containing the proximal domain and the opaque-phase protein extract was challenged with a 100× molar excess of the unlabeled 370-bp fragment, there was no reduction (Fig. 7B, lane 10).

Resolution of the *cis*-acting sequence of the proximal domain by gel mobility shift assays. An analysis of the base sequence of the distal domain (Fig. 8A) revealed no significant homology to a known regulatory sequence. However, a similar analysis of the fragment containing the proximal domain (Fig. 8B) revealed sequences homologous to a heat shock regulatory element (38), Ha, between bp −270 and −260; the NF-1 regulatory element (20), between bp −239 and −235; and the sequence TTGCTA (HR), between positions −302 and −297, which is repeated three more times in the region between the proximal domain and the transcription start point (Fig. 8A). In addition, the region between the proximal domain and the transcription start point of *WH11* contains sequences homologous to a heat shock regulatory element (38), Hb, between bp −210 and −203; the CREB regulatory element (39), between bp −158 and −154; the RAP1 regulatory element (23), between bp −137 and −129; a CAATA box (9), between bp −121 and −117; and a TATA box (8), between bp −36 and −29 (Fig. 8B). We therefore determined the site(s) in the proximal domain forming the two white-phase-specific complexes by a competition assay. Complex formation between the end-labeled 370-bp fragment from bp −310 to +60, containing the proximal domain, and white-phase protein extract was challenged with 10× and 100× molar excesses of various subfragments of the proximal domain and the region between the proximal domain and the transcription start point, diagrammed in Fig. 8C. The results of these competition experiments are presented in Fig. 9.

Molar excesses (10× and 100×) of fragment A, between bp −305 and −83 (Fig. 8C), effectively competed with the formation of radiolabeled complexes pI and pIII, demonstrating that a competing sequence resided between bp −305 and −83 (Fig. 9). Fragments B and D, between bp −305 and −230 and bp −305 and −148, respectively (Fig. 8C), also effectively competed (Fig. 9A, lanes 6 and 7 and lanes 10 and 11, respectively), narrowing the location of the competing sequence to between bp −305 and −230. As expected, fragments C, E, and F, between bp −245 and −83, −164 and −83, and −83 and +60, respectively (Fig. 8C), did not compete at 100× molar excesses (Fig. 9A, lanes 9, 13, and 14, respectively), narrowing the location of the competing sequence to a region between bp −304 and −245. This region contained one of two sequences homologous to a heat shock element, Ha (Fig. 8B). To test whether the region containing the Ha sequence or the region upstream from the Ha sequence contained the competing sequence, we tested an oligonucleotide (H) spanning bp −270 to −231, which included the Ha element, and an oligonucleotide (G) spanning bp −310 to −271. Oligonucleotide G, but not H, effectively competed at 10× and 100× molar excesses (Fig. 9B, lanes 2 and 3 and lanes 4 and 5, respectively), demonstrating that the proximal competing sequence(s) resides between bp −305 and −271, which does not include Ha.

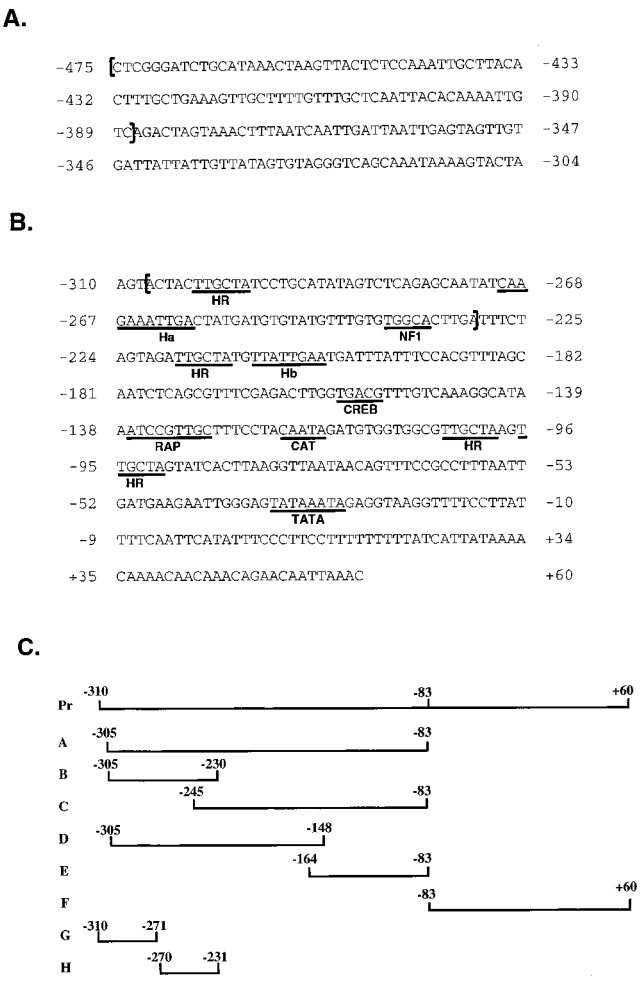

FIG. 8. Upstream region of *WH11*, including the distal and proximal transcription activation domains, and the subfragments of the proximal domain used in competition experiments. (A) Upstream region between bp −475 and −304. The distal domain (bracketed) plus intervening sequence (bp −387 to −304) is shown. (B) Upstream region and first 60 bp of the *WH11*, between bp −304 and +60. The proximal domain (bp −310 to −229; bracketed) plus flanking sequence (bp −228 to +60) is shown. Sequences homologous to heat shock regulatory elements Ha and Hb, the NF-1 element, the CREB element, the RAP1 binding site, a CAAT box, and a TATA box are noted. Four repeats of the hexameric sequence TTGCTA (HR) are indicated. (C) Subfragments of the proximal domain used in competition experiments for white-phase-specific complex formation between the proximal domain and white-phase-specific protein extracts in Fig. 9. Fragments were generated from the upstream region and first 60 bp of *WH11*, between bp −304 and +60.

DISCUSSION

Phenotypic switching in most microbial pathogens results in the replacement of cell surface antigens, which is believed to be a strategy either for evading the immune system (4, 11, 12, 36) or for altering adherence to mucosa (12). In most of these

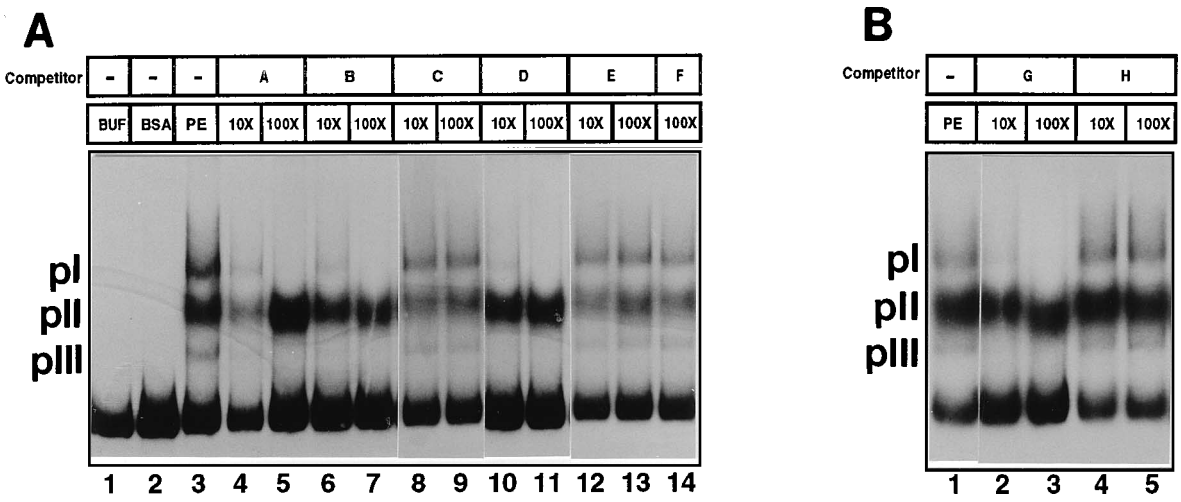

FIG. 9. Competition of white-phase-specific proximal domain–white-cell protein extract complexes with subfragments of the proximal domain. The basic binding mixture contained an end-labeled fragment spanning bp −310 to +60 of the *WH11* locus and 5 μg of white-cell protein extract (in all lanes but BUF and BSA). Unlabeled subfragments (see Fig. 8C) were added at the indicated molar excesses of the end-labeled fragment. BUF, buffer only (no protein extract); PE, basic reaction mixture with white-cell protein extract; BSA, 5 μg of BSA alone (no protein extract); A, 230-bp fragment spanning bp −305 to −83; B, 76-bp fragment spanning bp −305 to −230; C, 163-bp fragment spanning bp −245 to −83; D, 158-bp fragment spanning bp −305 to −148; E, 83-bp fragment spanning bp −164 to −83; F, 141-bp fragment spanning bp −83 to +60; G, 40-bp fragment spanning bp −310 to −271; H, 40-bp fragment spanning bp −270 to −231. pI and pIII, white-phase-specific complexes; pII, nonspecific complex. The experiments shown in panels A and B were performed separately; therefore, the panels contain repeated PE controls.

systems, it has been demonstrated that the regulation of gene expression in the alternative states involves DNA reorganization. Switching in *C. albicans* can also affect antigenicity at the cell surface (1, 3) but differs from the switching systems in other pathogens in the pleiotropic effect it exerts at all levels of cellular phenotype (26, 28). For this reason, it was not surprising to discover in the white-opaque transition in strain WO-1 that there are at least three opaque-phase-specific genes (18, 19, 40) and at least one white-phase-specific gene (33), that at least three of these genes are unlinked, and that there probably exist a number of additional phase-specific genes. These phase-specific genes are coordinately activated or deactivated at the same point in temperature-induced synchronous mass conversion from the opaque to the white phase (18, 31, 33). For these reasons, we hypothesized that coordinate activation and deactivation of phase-specific genes are regulated by phase-specific, *trans*-acting factors which in turn are regulated by a pivotal switch event (30, 31).

We first tested the 5′ upstream region of the *WH11* gene for white-phase-specific, *cis*-acting sequences. The chimeric *WH11*-flux gene containing the 5′ upstream region of *WH11* was demonstrated to be under the regulation of the phase transition regardless of the site of integration, and a series of promoter deletion constructs were used to delineate the domains containing *cis*-acting sequences. Two transcription activation domains were first delineated, a distal 88-bp domain, between bp −475 and −388 of the 5′ upstream region, and a proximal 78-bp domain, between bp −307 and −230 of the 5′ upstream region. Selective deletion of the distal domain resulted in an approximately sixfold reduction in the level of the chimeric gene transcript, and selective deletion of the proximal domain resulted in an approximately 15-fold reduction in the level of the chimeric gene transcript. However, deletion of either domain did not abolish the phase specificity of the low level of activation by the remaining domain. Therefore, each domain alone is capable of phase-specific activation but at significantly reduced levels, suggesting that the two domains act synergistically in the activation of *WH11* transcription. Other promoters, such as those of the testis-specific H2B histone gene (14), several genes regulated by steroid hormone receptors (22, 35), the *XPR2* gene of the yeast *Yarrowia lipolytica* (6), and the ε-globin gene (37), also contain two positive regulatory elements which function additively or synergistically.

Gel retardation assays with the two activation domains resulted in the formation of white-phase-specific complexes in both cases but not opaque-phase-specific complexes. The distal domain formed one white-phase-specific complex, and the proximal domain formed two white-phase-specific complexes. Both domains formed an additional nonspecific complex with both white- and opaque-phase protein extracts. These results suggest that phase-specific, *trans*-acting factors positively regulate *WH11* transcription by interacting with sequences in two activation domains in the promoter.

The distal activation domain of the *WH11* gene contains no sequences with significant homology to known consensus sequences of promoter regulatory elements. However, the proximal domain contains two sequences homologous to other known regulatory elements, the first (Ha) to a heat shock regulatory element (38) and the second to the NF-1 regulatory element (20). To test whether the sequences homologous to Ha and NF-1 represented transcription activation sequence in the proximal domain, we employed gel retardation assays to test different fragments of the domain for their capacity to compete with complex formation between the entire proximal domain and white-phase-specific factors. Our results suggest that neither Ha nor the NF-1 sequence is involved in the formation of complexes containing the proximal domain. The *cis*-acting sequence(s) in the proximal domain is therefore located between bp −271 and −305. This region contains a sequence, HR, which is repeated several times in the *WH11* promoter. Because of its redundancy downstream from the proximal domain, it seems unlikely that alone it represents a phase-specific activation sequence.

Finally, it should be noted that the construct which removed the two activation domains but left intact the 200 bp immediately upstream of the transcription start point was not transcribed in either the opaque or the white phase, even though it contained sequences homologous to the CREB element, the

RAP1 binding site, a CAAT box, two HR sites, and a TATA box. When the upstream region of the chimeric gene was reduced to 85 bp containing only a putative TATA box, there was again no transcription in either phase. These results further support the conclusion that *WH11* is positively regulated by phase-specific, *trans*-acting factors interacting with the two activation domains. The transition from the white to the opaque phase must result in the depletion or inactivation of these *trans*-acting factors, and the reverse transition from the opaque to the white phase must result in their reappearance or activation. However, there is a second phenotypic transition which also inactivates the *WH11* gene, the differentiation from the white budding to the white hypha phenotype (33). This dimorphic transition is distinct from the more general white-opaque switch event (26). We are now testing whether inactivation of *WH11* transcription in the bud-hypha transition involves depletion or inactivation of the same complex-forming factors as the white-opaque transition. If it involves the same mechanism, it will reinforce the suggestion that the white-opaque transition and the bud-hypha transition share some regulatory mechanisms (26).

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