Role of Negative Regulation in Promoter Specificity of the Homologous Transcriptional Activators Ace2p and Swi5p

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The Ace2p and Swi5p zinc finger proteins have nearly identical DNA-binding domains, yet in vivo they activate transcription of different genes, *CTS1* and *HO*. We now demonstrate that Ace2p and Swi5p recognize sites in the *CTS1* and *HO* promoters with the same affinities, raising the question of how promoter specificity is achieved by these proteins with similar DNA-binding domains. It has been previously shown that Swi5p binds to the *HO* promoter cooperatively with the Pho2p (Bas2p/Grf10p) homeodomain protein, and we now show that Ace2p does not interact with Pho2p. Analysis of *CTS1* promoter fragments inserted into a heterologous promoter identify a sequence 90 bp away from the Ace2p binding sites which is required to prevent activation by Swi5p through these binding sites. These results suggest that a regulatory protein bound to the *CTS1* promoter is needed to prevent Swi5p from activating *CTS1* expression. A genetic screen was conducted to identify suppressor mutations which allow *CTS1* expression in the absence of the Ace2p activator. The *nce3* mutation suppresses the *ace2* defect in *CTS1* expression only if the strain contains a functional *SWI5* gene, suggesting that *NCE3* normally functions to prevent Swi5p from activating *CTS1*. The role of negative regulators such as *NCE3*, as well as the previously described *SIN5* gene, in determining the promoter specificity of homologous activators is discussed.

The yeast *ACE2* and *SWI5* genes encode DNA-binding proteins which, despite many similarities, activate transcription of different genes. The zinc finger DNA-binding domains of Ace2p and Swi5p are highly conserved, with 83% identity and 95% similarity with conservative substitutions (8, 14). On the basis of the Zif268 zinc finger protein-DNA cocrystal (32), the amino acids predicted to make nucleotide specific contacts with the DNA are absolutely conserved between Ace2p and Swi5p, leading to the prediction that these two proteins would recognize the same DNA sequences. *ACE2* and *SWI5* display the same precise pattern of cell cycle-regulated transcription and nuclear localization. *ACE2* and *SWI5* are transcribed during the G₂ portion of the cell cycle, and the translated Ace2p and Swi5p remain in the cytoplasm during G₂ and M. Ace2p and Swi5p enter the nucleus as the cells complete mitosis and then disappear rapidly (14, 31, 40).

Despite these similarities in DNA-binding domains and in cell cycle regulation, *ACE2* and *SWI5* regulate distinct genes in vivo (14). *ACE2* is required for expression of the *CTS1* gene encoding chitinase, which degrades the chitin septum between mother and daughter cells after the completion of cell division. Cells with either an *ace2* or a *cts1* mutation are defective in cell separation. Swi5p does not normally function as an activator of *CTS1*, although overexpression of *SWI5* partially suppresses the defect in *CTS1* expression due to an *ace2* mutation. *SWI5* is a transcriptional activator of the *HO* gene, which encodes an endonuclease that initiates mating-type interconversion. *HO* is not expressed in a *swi5* mutant, and mating-type switching does not occur. Although *ACE2* does not activate *HO* expression when present normally in a single copy, *ACE2* overproduction

from a multicopy plasmid can stimulate *HO* transcription. Additionally, *CTS1* and *HO* are identically cell cycle regulated, expressed at the same time in G₁.

Thus, there are two parallel pathways of transcriptional regulation. Ace2p and Swi5p, with nearly identical DNA-binding domains, enter the nucleus at the same time and activate transcription of two different genes. In this report, we demonstrate that Ace2p and Swi5p can bind to the same sequences in vitro. This result raises the question of how activation of both genes by either transcription factor is prevented. One model for achieving promoter specificity requires combinatorial control, with an additional factor(s) promoting binding of Ace2p or Swi5p to only one of the two promoters. We have previously shown that Swi5p binding to the *HO* promoter is enhanced by the Pho2p (Bas2p/Grf10p) homeodomain factor (4, 5). We now demonstrate that *ACE2* fails to interact with *PHO2* by both genetic and biochemical criteria, which may contribute to the inability of *ACE2* to activate *HO* transcription. A second model for the promoter specificity of these homologous transcription factors invokes specific negative regulation. Here, we show that point mutations or deletions of a negative regulatory region within the *CTS1* promoter allow Swi5p to activate *CTS1* expression. This finding suggests that a specific repressor protein binds to this region and prevents Swi5p from activating *CTS1*. A screen for *trans*-acting negative regulatory mutants has identified genes that may encode this proposed Swi5p-specific repressor protein binding to the *CTS1* promoter.

MATERIALS AND METHODS

Strains and media. Yeast strains used in this study are listed in Table 1. All strains are isogenic in the W303 background (41). Standard genetic methods were used for strain construction and tetrad analysis (36). Preparation of strains with *ace2::HIS3* and *pho2::LEU2* gene disruptions using plasmids M1227 and M1876, respectively, has been described previously (5, 14). Plasmid M1225 digested with *EcoRI* was used to replace the *ACE2* gene with an *ace2::TRP1* disruption allele by the one-step gene replacement method (34). Plasmid M596 digested with *EcoRI* and *PvuII* and plasmid M1476 digested with *BamHI* were

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TABLE 1. Strains used

Strain	Genotype
DY150.....	<i>MATa ade2 can1 his3 leu2 trp1 ura3</i>
DY411.....	<i>MATa swi5::hisG ade2 can1 his3 leu2 trp1 ura3</i>
DY1143.....	<i>MATa ace2::HIS3 swi5::hisG ade2 can1 his3 leu2 trp1 ura3</i>
DY1159.....	<i>MATa ace2::TRP1 ade2 can1 his3 leu2 trp1 ura3</i>
DY1921.....	<i>MATa pho2::LEU2 ade2 can1 his3 leu2 trp1 ura3</i>
DY1923.....	<i>MATa ace2::TRP1 pho2::LEU2 ade2 can1 his3 leu2 trp1 ura3</i>
DY1938.....	<i>MATa pho2::LEU2 swi5::hisG ade2 can1 his3 leu2 trp1 ura3</i>
DY1985.....	<i>MATa ace2::HIS3 pho2::LEU2 swi5::hisG ade2 can1 his3 leu2 trp1 ura3</i>
DY1636.....	<i>MATα LEU2::CTS1-lacZ ade2 can1 his3 trp1 ura3</i>
DY1653.....	<i>MATa LEU2::CTS1-lacZ swi5::URA3 ade2 can1 his3 trp1 ura3</i>
DY1159.....	<i>MATa ace2::HIS3 ade2 can1 his3 trp1 ura3</i>
DY1656.....	<i>MATa LEU2::CTS1-lacZ ace2::HIS3 swi5::URA3 ade2 can1 his3 trp1 ura3</i>
DY2051.....	<i>MATa LEU2::CTS1-lacZ ace2::HIS3 ade6 can1 his3 trp1 ura3</i>
DY2052.....	<i>MATα LEU2::CTS1-lacZ ace2::HIS3 ade2 can1 his3 trp1 ura3</i>
WY101.....	<i>MATa LEU2::CTS1-lacZ ace2::HIS3 swi5::URA3 nce2-1 can1 his3 trp1 ura3</i>
WY103.....	<i>MATa LEU2::CTS1-lacZ ace2::HIS3 nce2-1 ade6 can1 his3 trp1 ura3</i>
WY105.....	<i>MATa LEU2::CTS1-lacZ ace2::HIS3 swi5::URA3 nce3-1 ade6 can1 his3 trp1 ura3</i>
WY110.....	<i>MATα LEU2::CTS1-lacZ ace2::HIS3 nce3-1 ade2 can1 his3 trp1 ura3</i>
WY147.....	<i>MATa LEU2::CTS1-lacZ ace2::HIS3 swi5::URA3 nce1-1 can1 his3 trp1 ura3</i>
WY194.....	<i>MATa LEU2::CTS1-lacZ ace2::HIS3 nce1-1 ade2 can1 his3 trp1 ura3</i>
WY289.....	<i>MATα LEU2::CTS1-lacZ ace2::HIS3 swi5::URA3 nce3-1 ade2 can1 his3 trp1 ura3</i>
WY290.....	<i>MATa LEU2::CTS1-lacZ ace2::HIS3 swi5::URA3 nce2-1 ade6 can1 his3 trp1 ura3</i>
WY291.....	<i>MATa LEU2::CTS1-lacZ ace2::HIS3 swi5::URA3 nce1-1 ade2 can1 his3 trp1 ura3</i>
WY306.....	<i>MATα ace2::HIS3 nce3-1 ade2 can1 his3 leu2 trp1 ura3</i>
WY308.....	<i>MATα ace2::TRP1 nce2-1 ade2 ade6 can1 his3 leu2 trp1 ura3</i>
WY309.....	<i>MATα ace2::TRP1 nce1-1 ade2 can1 his3 leu2 trp1 ura3</i>

used to disrupt the *SWI5* and *CTS1* genes with a *hisG-URA3-hisG* allele (1), and then the strains bearing the *swi5::hisG-URA3-hisG* or *cts1::hisG-URA3-hisG* allele were passaged over 5-fluoro-orotic acid medium (3) to select for loss of the *URA3* marker, resulting in strains with a *swi5::hisG* or a *cts1::hisG* allele. Similarly, plasmid pNKY85 (1) was used to convert the *pho2::LEU2* allele to a *pho2::hisG* allele. Gene disruptions were confirmed by Southern blotting. Media and cell cultivation conditions are indicated in the text or as described previously (20, 21). Cells were grown at 30°C in synthetic complete medium (35) containing 2% glucose supplemented with adenine, uracil, and amino acids, as appropriate, but lacking essential components to select for plasmids. YEPD and 5-fluoro-orotic acid media and plates were prepared as described (35, 37).

Ethyl methanesulfonate mutagenesis (26) of strains DY2051 and DY2052 was performed under conditions that yielded a survival rate of 60%. Mutagenized cells were plated onto nitrocellulose filters resting on YEPD plates, and colonies were screened for *lacZ* activity on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) after freezing in liquid nitrogen. From 40,000 colonies screened, 84 blue colonies were identified after 45 to 90 min of incubation with X-Gal at room temperature. The number of potential mutants was reduced to 28 by rescreening, and 3 mutants showed a dominant phenotype when mated to an *NCE*⁺ parent. Complementation analysis demonstrated that there were three alleles for *nce1*, two alleles for *nce2*, and two alleles for *nce3*. Complementation analysis of the remaining 18 mutants is still in progress.

Plasmids. Table 2 lists all plasmids used. In many cases, multiple steps were involved in plasmid construction, and details are available upon request. Plasmid M596, containing a *swi5::hisG-URA3-hisG* allele, was constructed by replacing the *Bgl*II (−875 to +1702) fragment in *SWI5* cloned in pUC19 with a 3.8 kb *Bam*HI-*Bgl*II fragment of pNKY51 (1). Similarly, the *Bgl*II fragment (−177 to +2032) of *CTS1* was deleted in the construction of plasmid M1475, containing a *cts1::hisG-URA3-hisG* allele. Plasmid M1225 was designed for gamma disruption (38) of *ACE2*, deleting sequences from the *Bgl*II site to the *Kpn*I site (+212 to +1764), and is derived from plasmid pRS304 (38).

Plasmid M1659 contains a 3.5-kb *Bam*HI-*Eco*RI fragment with *CTS1* subcloned into pTF63 (29). The *CTS1-lacZ* reporter plasmid M1591 (*URA3* marker)

is derived from YEp353 (30) and contains 1.4 kb of the *CTS1* promoter driving expression of the *lacZ* gene.

The *CTS1-CYC1-lacZ* reporters listed in Table 2 contain *CTS1* promoter fragments inserted upstream of the *CYC1* TATA element in plasmid pLGΔ-178 (17) or a derivative with additional restriction sites. M2570 is identical to M1820, in which the *CTS1* promoter region from −651 to −394 is driving expression of *CTS1-lacZ*, except that an *Xho*I site was introduced between the two Ace2p binding sites by changing two nucleotides by site-directed mutagenesis as described below. Plasmids M2337 and M3121 contain *CTS1* promoter fragments inserted between the *CYC1* upstream activation sequence (UAS) and TATA elements in plasmid pLGΔ-312 (16).

Bacterial expression plasmids M1462 and M1642 direct expression of glutathione *S*-transferase (GST)–Ace2p (amino acids 491 to 771) and GST–Swi5p (amino acids 384 to 709) fusion proteins, respectively. These plasmids are derived from pGEX-3X (Pharmacia), and bacterial expression is under control of the *tac* promoter. Plasmid M2025 (5) expresses His-tagged Pho2p from plasmid pET21b (Novagen). Plasmid M1402 (CS169 [4]) contains the Swi5p and Pho2p binding sites from the *HO* promoter in pIC19R. Plasmid M1818 contains a *CTS1* promoter fragment from −651 to −394, including the two Ace2p binding sites, cloned into pIC19H.

Plasmid YCpALTER-*URA3* (M2437) was constructed by replacing the 1.47-kb *Pvu*I fragment of pALTER-1 (Promega) with the 2.97-kb *Pvu*I fragment from pRS316 (38), which contains a centromere, an autonomously replicating sequence, and the *URA3* gene. pALTER-1 is designed for efficient site-directed mutagenesis, and the YCpALTER-*URA3* derivative is a yeast shuttle vector. Plasmid M2457, which was used for random mutagenesis of a region of the *CTS1* promoter, was constructed in two steps. First, a 4.68-kb *Bam*HI-*Nsi*I *CTS1-lacZ* fragment from M1591 was inserted into YCpALTER-*URA3* that had been digested partially with *Pst*I and to completion with *Bam*HI, and then an *Xho*I site was introduced between the two Ace2p binding sites by changing two nucleotides by site-directed mutagenesis with oligonucleotide F230 (5′ ATGCTGGTCCCTc GAgAAAATAACCAGC 3′) and the Altered Sites mutagenesis kit (Promega).

Directed mutagenesis of the negative regulatory region. The following oligomer was designed to introduce mutations in the negative regulatory site at the *CTS1* promoter: 5′-GTGTTTGTGTTTATTTGTTTATGTTTCAGATTGAG TAAACATTAGCATTGGTGTAAGAA-3′. The letters in boldface indicate residues in the sequence that were doped with other nucleotides at a concentration of 8.1%, which should yield an average of three mutations per oligonucleotide (18, 27).

Site-directed mutagenesis was performed according to the instructions for the Promega Altered Sites mutagenesis kit, with the following exceptions. After annealing of both the ampicillin repair oligomer and the mutagenic oligomer, T4 gene 32 protein (Pharmacia) or single-stranded binding protein was added at a final concentration of 0.2 μg/μl. The gene 32 protein was added to prevent annealing within the inverted repeat formed by *lacZ* in the *CTS1-lacZ* reporter and the *lacZ*′ in the vector, thus reducing the formation of deletions by increasing the fidelity of the subsequent synthesis reaction. After a 5-min incubation at room temperature, the microcentrifuge tube was placed on ice for 5 min to stimulate the binding of the gene 32 protein. The components of the synthesis reaction were then added to the reaction mixture on ice. *Escherichia coli* BMH71-18 mutS (Promega) was made competent by the procedure of Chung and Miller (10) on the day of the mutagenesis experiment. DNA was prepared from this strain by a Triton lysis procedure (2).

In vitro binding assays. Gel retardation assays were performed as described previously (4). All binding reaction mixtures (20-μl volume) used in gel retardation experiments contained the following components: 15 mM Tris (pH 8.0), 75 mM NaCl, 750 μg of bovine serum albumin (Sigma) per ml, 12.5 mM dithiothreitol, 7.5% glycerol, 50 μg of poly(dI-dC) · poly(dI-dC) per ml, and approximately 1 ng of labeled probe (about 30,000 cpm). The same binding conditions for gel retardation assays were used for DNase I footprint analysis except that reaction volumes were doubled (40 μl) and did not contain poly(dI-dC) · poly(dI-dC).

End labeling of the 258-bp *CTS1* promoter fragment (−651 to −394 and containing both Ace2p binding sites) at either the *Bam*HI or the *Eco*RI site of M1818 was followed by digestion with the other restriction enzyme. End labeling of the *HO* site B probe at either the *Hind*III or the *Asp* 718 site of M1402 was followed by digestion with the other restriction enzyme. All probes were end labeled with T4 polynucleotide kinase and [γ-³²P]ATP (9,000 Ci/mmol).

The GST-Ace2 and GST-Swi5 fusion proteins were purified as described previously (4) except for the following changes. Upon addition of isopropyl-β-D-thiogalactoside (IPTG) to induce fusion protein expression, Zn(C₂H₃O₂)₂ · 2H₂O was added to the culture at a final concentration of 0.1 mM. Sodium dodecyl sulfate-gel electrophoresis demonstrated that the concentrations of the purified GST-Ace2 and GST-Swi5 fusion proteins are equivalent (data not shown). The Pho2-HIS fusion protein was purified as described previously (4). The purified proteins were not subjected to a secondary DNA affinity purification step. The purified proteins were dialyzed against AN100 buffer (4).

Miscellaneous methods. Primer extension analysis was performed essentially as described previously (43). The *CTS1* (5′-TTCTGCTGAGTTTGTACCC-3′) and *URA3* (5′-CGTGCATGATATTAATAGC-3′) primer oligonucleotides were labeled with [γ-³²P]ATP and T4 polynucleotide kinase. The labeled oligonucleotide was hybridized to 20 μg of total yeast RNA and treated with reverse

TABLE 2. Plasmids used

Plasmid	Description
M1227	<i>ace2::HIS3</i> disruption plasmid
M1876	<i>pho2::LEU2</i> disruption plasmid
M1225	<i>ace2::TRP1</i> disruption plasmid
M596	<i>swi5::hisG-URA3-hisG</i> disruption plasmid
M1476	<i>cts1::hisG-URA3-hisG</i> disruption plasmid
pNKY85	<i>leu2::hisG-URA3-hisG</i> disruption plasmid
pTF63	YE _p vector with <i>URA3</i> marker
M1659	<i>CTS1</i> (to -1397) subcloned into pTF63
M1659(-1067)	Exonuclease III subclone of M1659 to -1067
M1659(-687)	Exonuclease III subclone of M1659 to -687
M1659(-525)	Exonuclease III subclone of M1659 to -525
M1659(-122)	Exonuclease III subclone of M1659 to -122
M1659(+60)	Exonuclease III subclone of M1659 to +60
M1591	<i>CTS1-lacZ</i> reporter plasmid; YE _p plasmid with <i>URA3</i> marker
pLGΔ-178	<i>CYC1</i> promoter without UAS driving <i>lacZ</i> expression
M1820	<i>CTS1</i> promoter (-651 to -394) inserted into <i>CYC1-lacZ</i> (pLGΔ-178)
M1912	<i>CTS1</i> promoter (-560 to -515) inserted into <i>CYC1-lacZ</i> (pLGΔ-178)
M2255	<i>CTS1</i> promoter (-551 to -394) inserted into <i>CYC1-lacZ</i> (pLGΔ-178)
M2257	<i>CTS1</i> promoter (-539 to -394) inserted into <i>CYC1-lacZ</i> (pLGΔ-178)
M2258	<i>CTS1</i> promoter (-520 to -394) inserted into <i>CYC1-lacZ</i> (pLGΔ-178)
M2260	<i>CTS1</i> promoter (-651 to -508) inserted into <i>CYC1-lacZ</i> (pLGΔ-178)
M2261	<i>CTS1</i> promoter (-596 to -394) inserted into <i>CYC1-lacZ</i> (pLGΔ-178)
M2272	<i>CTS1</i> promoter (-651 to -470) inserted into <i>CYC1-lacZ</i> (pLGΔ-178)
M2333	<i>CTS1</i> promoter (-651 to -418) inserted into <i>CYC1-lacZ</i> (pLGΔ-178)
M2334	<i>CTS1</i> promoter (-651 to -444) inserted into <i>CYC1-lacZ</i> (pLGΔ-178)
M2570	M1820, but with <i>XhoI</i> site between Ace2p binding sites
M2571	M2570, but with point mutations in negative regulatory site
pLGΔ-312	<i>CYC1</i> UAS and promoter driving <i>lacZ</i> expression
M2337	<i>CTS1</i> promoter (-521 to -394) inserted between <i>CYC1</i> UAS and TATA in pLGΔ-312
M3121	<i>CTS1</i> promoter (-470 to -404) inserted between <i>CYC1</i> UAS and TATA in pLGΔ-312
M1462	Expresses GST-Ace2 (amino acids 491-771) fusion protein in bacteria
M1642	Expresses GST-Swi5 (amino acids 384-709) fusion protein in bacteria
M2025	Expresses Pho2-HIS (amino acids 35-528) protein fusion in bacteria
M1402	Swi5p and Pho2p binding sites from the <i>HO</i> promoter
M1818	Ace2p binding sites from the <i>CTS1</i> promoter
YCpALTER- <i>URA3</i>	Yeast shuttle vector for site-directed mutagenesis (Amp ^r Tet ^r)
M2457	<i>CTS1-lacZ</i> (with <i>XhoI</i> site between Ace2p binding sites) in YCpALTER- <i>URA3</i>
M2569	M2457, but with point mutations in negative regulatory site

transcriptase. The primer extension products were electrophoretically separated on a 14.5-cm 6% polyacrylamide-8 M urea gel and autoradiographed. Northern (RNA) analysis was performed as described previously (14). *lacZ* color colony assays were performed by the procedure of Breeden and Nasmyth (6), using 8.26-cm-diameter Whatman grade 5 filters instead of nitrocellulose. Quantitative β-galactosidase activity assays were performed as described previously (7). Computer-generated images of autoradiographs were generated with Adobe Photoshop.

RESULTS

***CTS1* promoter.** We demonstrated previously that *ACE2* functions as a transcriptional regulator of the *CTS1* gene (14). The published sequence upstream of the *CTS1* gene (24), extending 450 bp upstream of the initiating methionine codon, did not contain any sequence homologous to the Swi5p binding site in the *HO* promoter. We therefore isolated a clone which includes DNA extending approximately 1,400 bp upstream of *CTS1*, constructed exonuclease III deletions, and determined the DNA sequence of this region. As shown in Fig. 1, there is an upstream open reading frame, and thus the *CTS1* promoter region can extend as far as -856. The promoter contains several potential Ace2p binding sites, which are indicated in Fig. 1. These potential binding sites contain a characteristic ACCAGC or the complementary GCTGGT sequence which is found in the core of the site B Swi5p binding site in the *HO* promoter (see below). While this report was in preparation, the sequence of this region from chromosome XII was submit-

ted to GenBank (accession number U17243) as part of the yeast genome sequencing project. The two sequences are identical.

The exonuclease III deletion clones generated for sequencing were used in a complementation experiment to identify promoter regions required for normal regulation of the *CTS1* gene. In this experiment, promoter deletion clones were transformed into strain DY1595, which contains a *CTS1* gene deletion. RNA was isolated from logarithmically growing cells, and *CTS1* mRNA levels were determined by primer extension analysis (Fig. 2). A control primer extension experiment was also conducted with a *URA3*-specific primer, and the results show that the RNA is not degraded in any of the samples (data not shown). The major transcription start site is located at -238 ± 10 bp from the ATG, and thus *CTS1* has a rather long 5' untranslated region by yeast standards. A perfect TATAAA element is present 70 bp upstream of the transcription start site. The primer extension analysis demonstrates that clones extending 1396, 1067, or 687 bp upstream of the ATG promoter direct full *CTS1* expression (Fig. 2, lanes 4 to 6). In contrast, a clone which contains sequences 525 bp upstream of the ATG showed a reduced level of the normal primer extension product, and some transcripts were longer, indicating abnormal start site selection (lane 7). Importantly, all but one of the potential Ace2p binding sites have been deleted in this promoter construct. Finally, clones deleted to 122 bp upstream

FIG. 1. Complete sequence of the *CTS1* promoter. (A) The DNA sequence was determined to the *Bam*HI site at -1396, where +1 represents the initiator codon ATG of *CTS1*. Only the sequence up to the upstream open reading frame is shown, but the sequence of this entire region is available from GenBank under accession number U17243. The amino acid sequences of the upstream open reading frame and of *CTS1* are given below the nucleotide sequence, using the single-letter abbreviations. The 5' end of the mRNA, mapped by primer extension, is indicated, and a TATAAA element approximately 70 bp upstream is boxed. The 46-bp fragment extending from -560 to -515, which functions as a UAS, is boxed, and the cores of the two Ace2p binding sites in this region are highlighted. Just upstream of the 46-bp boxed region is a weak Ace2p binding site (underlined) discussed in the text. The endpoints of the 258-bp *Hpa*I-*Taq*I fragment (-651 to -394) used in some experiments are indicated with brackets. The point mutations at -428 and -426 in the putative negative regulatory element are indicated. (B) Fragments of the *CTS1* promoter used in heterologous promoter constructs. The location of the Ace2p binding sites and the point mutations in the putative negative regulatory element are indicated.

or to 60 bp downstream of the ATG did not show any normal primer extension products (lanes 8 and 9), as expected, since these clones lack the transcription start site. On the basis of the primer extension data, the sequences sufficient for normal expression of a plasmid-borne *CTS1* promoter are located within the 687-bp region upstream of the ATG.

Binding sites for Ace2p and Swi5p. We predicted that Ace2p and Swi5p should recognize the same DNA sequences (14),

given the strong similarity of their DNA-binding domains. We have studied the binding of Swi5p to the *HO* promoter (site B) in some detail, using DNase I footprinting, methylation interference, and hydroxyl radical interference methods (4, 5). A summary of these contacts between Swi5p and the *HO* promoters site is shown in Fig. 3. The core of this binding site, ACCAGC, is present three times in the *CTS1* promoter (indicated in Fig. 1). Two of the three potential Ace2p binding sites from the *CTS1* promoter demonstrated a strong similarity to the site B Swi5p binding site at the *HO* promoter (Fig. 3). The left binding site or *CTS1* site L shows a 10-of-16-nucleotide (nt) match with the Swi5p binding site, while the right hand binding site or *CTS1* Site R shows a remarkable 14-of-16 match.

Given the similarity of the Swi5p binding site at the *HO* promoter with the potential Ace2p binding sites at the *CTS1* promoter and given the similarity of the DNA-binding domains of the Ace2p and Swi5p activators, we decided to test the hypothesis that both proteins would bind to the same sequences in vitro. Ace2p and Swi5p were expressed in *E. coli* as GST fusion proteins and purified by using a glutathione-agarose affinity resin. In the GST-Ace2p construct, the GST domain was fused to the carboxy-terminal 281 amino acids of Ace2p, which contains the proposed zinc finger DNA-binding

FIG. 2. Primer extension analysis of *CTS1* promoter deletion constructs. A map of the different *CTS1* deletion constructs, and the Ace2p binding sites, is presented at the top. Yeast strain DY1595, containing a *cts1Δ::hisG* gene disruption, was transformed with the indicated *CTS1* deletion construct. Transformants were grown under selective conditions to maintain the plasmid, and RNA isolated from log-phase cells was used for primer extension reactions with a *CTS1*-specific primer (lanes 4 to 9). Control reactions were also performed with tRNA (lane 1), with mRNA isolated from a *cts1Δ::hisG* strain not bearing any plasmid (lane 2), and with mRNA isolated from a *cts1Δ::hisG* strain bearing the parental vector pTF63 (lane 3). The major primer extension product is indicated. A control primer extension experiment was also conducted with a *URA3*-specific primer, and the results show that the RNA is not degraded in any of the samples (data not shown).

FIG. 5. DNase I protection on the *CTS1* promoter by GST-Ace2p and GST-Swi5p. A DNase I footprinting experiment was performed with a *CTS1* promoter –651 to –394 fragment labeled at the 5' end and with and GST-Ace2 and GST-Swi5 fusion proteins purified from *E. coli*. Binding reaction mixtures contained the following amounts of fusion protein: lane 1, no added protein; lane 2, 1 μ l of GST-Ace2p; lane 3, 2 μ l of GST-Ace2p; lane 4, no added protein; lane 5, 1 μ l of GST-Swi5p; lane 6, 2 μ l of GST-Swi5p; and lane 7, no added protein. The regions of protection from DNase I cleavage are indicated by brackets.

teract with Pho2p and what role *PHO2* plays in the transcriptional activation by *ACE2* and *SWI5*.

A *lacZ* reporter plasmid driven by 1.4 kb of the *CTS1* promoter was used to determine whether *PHO2* is required for *CTS1* expression. This *CTS1-lacZ* reporter plasmid was transformed into eight isogenic strains that differ only at the *ACE2*, *SWI5*, and *PHO2* loci. Transformants were grown under selection to maintain the *CTS1-lacZ* reporter plasmid, and extracts were prepared from log-phase cells for quantitative β -galactosidase measurements. The results in Table 3 confirm that *CTS1* expression requires *ACE2* but not *SWI5*. Importantly, a *pho2* mutation does not lead to a reduction in *CTS1* expression in any of the four genotypes tested.

Gel shift experiments were performed with purified GST-Ace2p and GST-Swi5p in the absence or presence of added Pho2p. A labeled DNA probe containing sequences from the *HO* promoter was used for the experiment shown in Fig. 4A. The cooperative binding of Swi5p and Pho2p at *HO* can be seen by comparing Swi5p binding in the absence (lanes 11 to 14) and presence (lanes 16 to 19) of Pho2p. Pho2p addition results in the conversion of the Swi5p-DNA binary complex into the more slowly migrating Swi5p-Pho2p-DNA ternary complex. Similarly, the Pho2p-DNA binary complex (lane 15) disappears upon addition of Swi5p (lanes 16 to 19). In contrast, addition of Pho2p (lanes 7 to 10) causes no apparent increase in affinity of Ace2p for the *HO* promoter compared with Ace2p alone (lanes 2 to 5). Both Pho2p and Ace2p can bind to the *HO* promoter, but no ternary complexes containing both proteins can be seen, suggesting that binding by both Ace2p and Pho2p is independent and not cooperative.

The analogous gel shift experiment performed with a *CTS1* promoter probe is shown in Fig. 4B. First, Pho2p does bind to the *CTS1* promoter fragment (lanes 6 and 15). In vitro binding by homeodomain proteins shows low sequence selectivity (25), with the common sequence TAAT being a primary binding determinant. We suggest that the in vitro binding of Pho2p to

CTS1 results from this type of low selectivity (TAAT motifs are present in the *CTS1* probe), since *CTS1* expression is not reduced by a *pho2* mutation (Table 3).

Comparison of Ace2p binding in the absence (Fig. 4, lanes 2 to 5) and presence (lanes 7 to 10) of Pho2p demonstrates that Pho2p does not enhance the affinity of Ace2p for the *CTS1* promoter. Moreover, there is no evidence for a protein-DNA complex that contains both Ace2p and Pho2p. Different results were obtained when Swi5p binding to the *CTS1* promoter was measured in the absence (lanes 11 to 14) and presence (lanes 16 to 19) of added Pho2p. First, incubation of both Swi5p and Pho2p (lanes 16 to 19) results in a sharp increase in the amount of ternary complex and the appearance of an additional species (quaternary complex) which may contain DNA bound by two molecules of Swi5p and one molecule of Pho2p. Second, the amount of the Pho2p-DNA binary complex (lane 15) decreases upon addition of Swi5p (lanes 16 to 19). However, the Swi5p-dependent decrease in the Pho2p-DNA binary complex with the *CTS1* probe (Fig. 4B, lanes 15 to 19) is slight compared with the highly cooperative interactions seen at the *HO* promoter (Fig. 4A, lanes 15 to 19).

The analysis of binding at *CTS1* is complicated by several features. First, since the *CTS1* probe contains two binding sites for Ace2p or Swi5p, it is not clear if a more slowly migrating ternary complex (Fig. 4B, lanes 15 to 19) contains two molecules of Swi5p or one molecule of Swi5p and one molecule of Pho2p. Second, because the Swi5p-DNA and the Pho2p-DNA binary complexes run with the same mobility on the *CTS1* promoter, the disappearance of the Pho2p-DNA complex is difficult to monitor. Nevertheless, these results demonstrate that the interaction of Swi5p with Pho2p is specific because Ace2p fails to interact with Pho2p.

The in vitro DNA-binding experiments suggested that Swi5p and Pho2p may interact at the *CTS1* promoter. To determine whether this observation had any relevance for regulation in vivo, genetic experiments using a *CTS1-lacZ* reporter were performed. Although *CTS1* expression is normally independent of *SWI5* (14), *SWI5* overexpression promotes expression of *CTS1* in an *ace2* mutant (14). We reasoned that if Pho2p is required for efficient Swi5p binding to the *CTS1* promoter in vivo, then a *pho2* mutation should abrogate the ability of overexpressed Swi5 to activate *CTS1*. However, suppression of the *ace2* defect in *CTS1* expression by *SWI5* overexpression does not require *PHO2* (data not shown). This finding indicates that in vivo binding of Swi5p to the *CTS1* promoter does not require Pho2p. Similarly, *ACE2* overexpression can suppress a *swi5* defect in *HO* expression (14), and this *ACE2* suppression is independent of *PHO2* (data not shown). We conclude that the *ACE2* suppression of the *swi5* defect at *HO* does not require *PHO2*.

In summary, the in vitro DNA-binding experiments demon-

TABLE 3. *PHO2* is not required for *CTS1* expression

Strain	Genotype	<i>CTS1-lacZ</i> activity	
		U	%
DY150	Wild type	12,800 $\pm$ 950	100
DY411	<i>swi5</i>	7,700 $\pm$ 1,050	60
DY1159	<i>ace2</i>	750 $\pm$ 50	6
DY1143	<i>ace2 swi5</i>	200 $\pm$ 65	2
DY1921	<i>pho2</i>	15,500 $\pm$ 400	121
DY1938	<i>swi5 pho2</i>	6,900 $\pm$ 650	54
DY1923	<i>ace2 pho2</i>	1,500 $\pm$ 300	12
DY1985	<i>ace2 swi5 pho2</i>	200 $\pm$ 90	2

TABLE 4. Expression of *CTS1-lacZ* reporters in isogenic strains^a

Strain	Genotype	Activity					
		<i>CTS1-lacZ</i>		<i>CTS1(258)-CYC1-lacZ</i>		<i>CTS1(46)-CYC1-lacZ</i>	
		U	%	U	%	U	%
DY150	Wild type	12,800 ± 950	100	3,200 ± 200	100	2,800 ± 270	100
DY411	<i>swi5</i>	7,700 ± 1,050	60	2,400 ± 270	73	1,200 ± 90	42
DY1159	<i>ace2</i>	750 ± 50	6	33 ± 7	1	1,000 ± 30	38
DY1143	<i>ace2 swi5</i>	200 ± 65	2	19 ± 3	1	32 ± 2	1

^a Expression of the *CTS1(258)-CYC1-lacZ* and *CTS1(46)-CYC1-lacZ* reporters is independent of *PHO2*, as for the complete *CTS1-lacZ* reporter (Table 3).

strate that Swi5p binds cooperatively with Pho2p at *HO* and that there may be some interaction between Swi5p and Pho2p at *CTS1*, at least in vitro. Ace2p does not show any in vitro interaction with Pho2p. However, the in vivo experiments clearly show that *PHO2* does not play any role in the activation of *CTS1*, either by Ace2p or by overexpression of Swi5p. Pho2p does not stimulate the binding of Ace2p at either the *CTS1* or the *HO* promoter.

Identification of a UAS within the *CTS1* promoter. To demonstrate in vivo that *ACE2* activates *CTS1* transcription through the binding sites mapped by sequencing and DNA-binding experiments, analysis of the *CTS1* cis-acting promoter sequences in a heterologous context was undertaken. Plasmid pLGΔ178 contains the *CYC1* TATA box fused to the *lacZ* gene but lacks a UAS, and thus this promoter is transcriptionally inactive (17). A fragment containing a UAS can be inserted into this vector, and the resulting transcriptional activity can be measured quantitatively by performing β-galactosidase assays. A 258-bp fragment extending from nt -651 to -394 of the *CTS1* promoter, which binds Ace2p in gel shift assays (Fig. 5), was cloned into pLGΔ178 to test it for UAS activity. As shown in Table 4, the 258-bp fragment in plasmid *CTS1(258)-CYC1-lacZ* contained UAS activity, exhibiting 3,200 U of β-galactosidase activity in a wild-type strain. As a control, the pLGΔ178 vector, which lacks a UAS, showed less than 10 U of β-galactosidase activity. The *CTS1(258)-CYC1-lacZ* plasmid was subsequently transformed into a series of isogenic strains bearing null alleles of *ACE2* and/or *SWI5* (Table 4). When the β-galactosidase levels were measured from either the *ace2* single or the *ace2 swi5* double mutant, the level of activity dropped to 1% of the wild-type level. On the other hand, the level of β-galactosidase activity in a *swi5* single mutant was close to normal, at 70% of wild-type activity. Thus, one can conclude that the region of the *CTS1* promoter extending from nt -651 to -394 contains a UAS which is dependent on the presence of *ACE2*, but not *SWI5*, for activation. Sequences from the *CTS1* promoter downstream of -394, when cloned into pLGΔ178, failed to demonstrate any UAS activity (data not shown).

For comparison, plasmid YEp*CTS1-lacZ*, bearing the full-length promoter (including the *CTS1* TATA box) driving expression of the *lacZ* reporter gene, was transformed into the same isogenic four strains. Although the level of activation from the full-length promoter is significantly higher than that from the *CTS1(258)-CYC1-lacZ* construct (13,000 U, compared with 3,200), the normalized levels of activation show a profile similar to that of the 258-bp construct. The level of activation from the YEp*CTS1-lacZ* reporter is significantly reduced in the *ace2* single-mutant and the *ace2 swi5* double-mutant strains. We attribute the increased level of activation in the YEp*CTS1-lacZ* construct to sequences extending beyond the 258-bp fragment. Additionally, these experiments show that *CTS1* is a very strong promoter, as these β-galactosidase

levels are very high compared with those for other *lacZ* promoter fusions or other *CYC1-lacZ* promoter constructs.

A 46-bp fragment from the *CTS1* promoter demonstrates UAS activity. The DNase I footprinting experiments identified two high-affinity binding sites for Ace2p within the 258-bp *CTS1* DNA promoter fragment. On the basis of the DNase I protection data, the boundary of the two regions of protection could be localized to a 46-bp segment of DNA. We decided to address whether this smaller region of 46 bp was sufficient for UAS activity by cloning this region into the pLGΔ178 reporter plasmid and transforming this *CTS1(46)-CYC1-lacZ* plasmid into a set of isogenic strains bearing null alleles of *ACE2* and/or *SWI5*. The 46-bp fragment in *CTS1(46)-CYC1-lacZ* directed levels of β-galactosidase similar to those seen with the 258-bp fragment (Table 4). However, the 46-bp fragment provided 38% of wild-type UAS activity in the *ace2* mutant, while the 258-bp fragment exhibited only 1% activity. In addition, UAS activity was completely absent in the *ace2 swi5* double mutant. We conclude that the 46-bp UAS can be activated by either *ACE2* or *SWI5*, while only *ACE2* can activate the 258-bp UAS. This finding suggests that there is a negative regulatory sequence present in the 258-bp fragment that prevents *SWI5* activation. When this negative sequence element is removed, as in the case of the 46-bp fragment, then both *SWI5* and *ACE2* can activate transcription to approximately equal extents.

Identification of a negative element within the *CTS1* promoter. We further investigated the role of negative regulation in determining promoter specificity of *ACE2* at *CTS1*. The different responses of the 46- and 258-bp *CTS1* promoter fragments to activation by *SWI5* suggested that the 258-bp fragment contains a negative sequence element that prevents activation by Swi5p but still allows Ace2p to activate. The 46-bp element, containing the Ace2p binding sites, is roughly centered within the 258-bp *CTS1* promoter fragment; we constructed deletions from the 5' and 3' sides of the 258-bp fragment and cloned these deletion fragments into a *lacZ* reporter plasmid (Fig. 6). These constructs were then tested for UAS activity by quantitative β-galactosidase assays in a wild-type strain and in an *ace2* mutant strain. We expected two types of results. First, a deletion construct, if it contained Ace2p binding sites, would show a level of activity comparable to that of the full-length 258-bp fragment in the wild-type strain. More importantly, a deletion construct lacking the negative sequence element would show increased activation in an *ace2* mutant strain over that seen with the full 258-bp UAS. This would indicate that the negative element is removed, suggesting that *SWI5* can activate transcription through this deletion construct.

Deletions made from the 5' end of the 258-bp promoter fragment are shown in Fig. 6B. Deletion construct -539 to -394, in which one of the high-affinity Ace2p binding sites is deleted, has 13% of wild-type UAS activity. Likewise, deletion

FIG. 6. A negative regulatory site within the *CTS1* promoter. Various *CTS1* promoter fragments were cloned upstream of the *CYC1* promoter driving expression of the *lacZ* gene. The *CYC1-lacZ* reporter lacks a UAS element, and transcriptional activation is dependent on UAS activity in the inserted fragment; activity of the *CTS1*(258)-*CYC1-lacZ* reporter is 1,000 times greater than that of the parent pLGA178 vector (see Fig. 7B). The *CTS1-CYC1-lacZ* constructs were transformed into isogenic strains DY150 (wild type [WT]) and DY1159 (*ace2*) and grown under selection to maintain the plasmids, and extracts were prepared for quantitative β -galactosidase measurements. The results given are the averages of at least three independent transformants, and the standard error for the β -galactosidase assays was under 25%. The values given are expressed as a percentage of the value for the *CTS1*(258)-*CYC1-lacZ* construct transformed into the wild-type strain. All promoter constructs were tested in an *ace2 swi5* double mutant, and all had <1% of the activity of the *CTS1*(258)-*CYC1-lacZ* reporter in the *ACE2 SWI5* strain (data not shown), demonstrating that *SWI5* is responsible for the activity seen in *ace2* mutants. All constructs in panels A, C, and D were assayed on the same day. In this case, *CTS1*(258)-*CYC1-lacZ* produced $2,000 \pm 510$ U of activity. The constructs in panel B were assayed on a different day, but activities are normalized to that for a *CTS1*(258)-*CYC1-lacZ* control in DY150 assayed simultaneously.

construct -520 to -394, in which the left Ace2p binding site and the first A residue in the conserved ACCAGC core of the right Ace2p binding site are deleted, does not activate transcription at all. The right Ace2p binding site is probably compromised by loss of this residue, since β -galactosidase activity was completely abrogated. Thus, deletion of one or both of the Ace2p binding sites severely lowered the normal level of UAS activity in the wild-type strain, consistent with the fact that the 46-bp element containing the sites is an *ACE2*-dependent UAS. Finally, none of the 5' deletion constructs activates transcription in the *ace2* strain, suggesting that the negative sequence element is located downstream of the two high-affinity binding sites.

Constructs with deletions of the 3' region of the 258-bp fragment define a negative regulatory sequence that blocks *CTS1* activation by Swi5p (Fig. 6C). One deletion construct, -651 to -418, showed essentially the same regulation as the full-length 258-bp fragment, demonstrating that the negative element sequences are not located between nt -418 and -394. When an additional 26 bp were deleted from the promoter

fragment in deletion construct -651 to -444, the level of activity of the promoter fragment in the *ace2* mutant increased to 7% (normalized to the value for the 258-bp fragment in the wild type). Thus, at least part of the negative sequence element has been removed, allowing activation by *SWI5*. All further deletion constructs allowed increased expression in the *ace2* mutant, providing approximately 15 to 20% of wild-type activity. It should be noted that none of the 3' deletion constructs ever reached 100% of wild-type activity. This is not surprising because the 46-bp fragment showed only 36% of wild-type activity in an *ace2* mutant (Fig. 6A), indicating the maximum level of activation by *SWI5* in an *ace2* mutant strain. All of the deletion constructs are transcriptionally silent in an *ace2 swi5* double mutant (data not shown), and thus the activation of the deletion constructs in the *ace2* mutant is dependent on *SWI5*. From these results, one can conclude that a negative sequence element must be located between nt -470 and -418 within the promoter.

To determine whether this region can negatively regulate a heterologous promoter, we cloned segments of the *CTS1* pro-

FIG. 7. An *nce3* mutation relieves repression by the *CTS1* negative regulatory element. Quantitative measurements of β -galactosidase activity were made from extracts prepared from isogenic strains that contain the a plasmid reporter. Strains DY150 (*ACE2 NCE*), DY1159 (*ace2 NCE*), WY309 (*ace2 nce1*), WY308 (*ace2 nce2*), and WY306 (*ace2 nce3*) were used. (A) Plasmid reporters. Line 1, pLGΔ-312, containing the *CYC1* UAS; line 2, M2337, containing the *CTS1* promoter region from –521 to –394 inserted between the *CYC1* UAS and TATA elements; line 3, M3121, containing the *CTS1* promoter region from –470 to –404 inserted between the *CYC1* UAS and TATA elements. (B) Plasmid reporters. Line 4, pLGΔ-178, a *CYC1-lacZ* reporter lacking a UAS element; line 5, M1820, the *CTS1(258)-CYC1-lacZ* reporter; line 6, M1912, the *CTS1(46)-CYC1-lacZ* reporter. The ratio of expression of the *CTS1(258)-CYC1-lacZ* and *CTS1(46)-CYC1-lacZ* reporters is given as a percentage.

motor downstream of the *CYC1* UAS. *CTS1* promoter fragments of 126 and 67 bp, corresponding to nt –521 to –394 and –470 to –404, respectively, were cloned into plasmid pLGΔ312, which contains the *CYC1* promoter driving expression of a *lacZ* reporter. Placing this region of the *CTS1* promoter downstream of the *CYC1* UAS causes a 10- to 20-fold drop in *lacZ* expression (Fig. 7A). These fragments also repress the *CYC1* promoter when inserted in the opposite orientation (data not shown). Insertion of similar-size DNA fragments, such as *lexA* or *MATα2* operators (23, 44) or fragments from the *HO* promoter (unpublished observations), into pLGΔ312 reduces *lacZ* expression up to twofold. We therefore suggest that the decreased expression is not simply due to increasing the distance between the *CYC1* UAS and TATA elements. Thus, the *CTS1* negative element can repress expression of a heterologous promoter.

Negative regulatory sequence. A genetic screen was undertaken to more closely define the nucleotide residues that constitute the negative sequence element. A site-directed mutagenesis procedure was used to target mutations to the negative regulatory region of the *CTS1* promoter as mapped by deletion analysis. We converted plasmid pALTER-1 (Promega), which is designed for oligonucleotide-directed mutagenesis, to a yeast shuttle vector called YCpALTER-URA3 by inserting a yeast *URA3* marker, a yeast origin of replication, and a yeast centromere. We then inserted the full-length *CTS1* promoter (with an *XhoI* site between the two Ace2p binding sites) driving expression of the *lacZ* reporter gene into YCpALTER-URA3 to construct plasmid M2457, which was used

as a substrate for site-directed mutagenesis. A doped oligonucleotide, containing on average three mutations per oligonucleotide (18), was used to construct a plasmid library containing the reporter with mutations in the region from –454 to –418, which was then transformed into an *ace2* mutant strain. In an *ace2* mutant, the parental *CTS1-lacZ* reporter is expressed at a very low level and yeast colonies remain white on the chromogenic substrate X-Gal because *SWI5* fails to activate transcription. On the other hand, mutations in the negative regulatory sequence should allow *SWI5* to function at the *CTS1* promoter and thus cause the colonies that harbor these mutant plasmids to turn blue in the presence of X-Gal. Of a total of 6,000 colonies screened, 5 colonies exhibited a slightly more blue phenotype in the *ace2* strain. All five colonies contained the same two mutations (described below); the five plasmids may not reflect independent mutations, since the library had been amplified in *E. coli*. Plasmid M2569 containing the mutant promoter was isolated and transformed into an *ace2 swi5* double-mutant strain, and no transcriptional activation was seen (data not shown). Thus, we conclude that activation of this mutant promoter requires *SWI5*.

The 258-bp *HpaI-TaqI* fragment from plasmid M2569 containing the two point mutations in the *CTS1* promoter was cloned into pLGΔ178 for direct comparison with the wild-type 258-bp fragment in the heterologous *CTS1* promoter, constructing *CTS1(258-mutant)-CYC1-lacZ*. As shown in Fig. 6D, the 258-bp fragment containing this negative site mutation showed an increase in transcription in the *ace2* mutant strain to 6% of wild-type activity. The activation did not reach the 15 to

20% level seen in the -651 to -470 deletion, suggesting that the negative site had not been completely destroyed. Expression from the *CTS1*(258-mutant)-*CYC1-lacZ* construct is comparable to that from the -651 to -444 deletion construct, which may lack only part of the negative regulatory element. It is possible that additional mutations in another part of the negative regulatory element are required to increase the activity of the *CTS1*(258-mutant)-*CYC1-lacZ* construct to 15 to 20%. We suggest that the deletion construct -651 to -470 exhibits 16% activity because the second element may have been eliminated. Finally, the activity of the *CTS1*(258-mutant)-*CYC1-lacZ* reporter in *ace2 swi5* double mutant is less than that seen for the control plasmid pLGA178, which lacks a UAS (data not shown). Thus, we conclude that *SWI5* is required for the transcriptional activation seen in the *CTS1* mutant promoter construct.

DNA sequence analysis revealed that two mutations were present in the *CTS1* promoter: an A residue at position -428 changed to a T, and an A residue at position -426 changed to a G (Fig. 1). Interestingly, these mutations lie in a 15-bp region, ATGTTGAGTAAACAT (mutated residues are underlined), that demonstrate imperfect dyad symmetry, since the ATGTTNAN sequence appears on opposite strands of the symmetry, with the center of the dyad at the second N residue. DNA sequences with dyad symmetry are often sites that can be recognized by dimeric DNA-binding proteins. Perhaps a specific repressor protein recognizes this sequence and blocks *SWI5* activation of *CTS1*.

The data in Fig. 7A demonstrates that the *CTS1* negative element can repress a heterologous UAS. A reporter plasmid identical to M3121 (Fig. 7A, line 3) but with the TAAACAT-to-TTAGCAT substitutions shown in Fig. 1 was constructed, and its expression was assayed in wild-type strains. The plasmid with the substitutions had essentially the same level of expression as M3121, indicating that these mutations do not alleviate repression in the context of this heterologous *CYC1* UAS (data not shown). Although the TAAACAT region does not appear to contribute to repression of the heterologous *CYC1* UAS, this experiment does not eliminate a role of this sequence element in blocking activation by *SWI5*.

Negative regulators of *CTS1* expression. The promoter analysis suggests that a negative regulatory element present in the *CTS1* promoter prevents Swi5p from functioning as a transcriptional activator of *CTS1*. We reasoned that if there were a specific repressor protein recognizing this promoter element, then a mutation in the gene encoding this repressor should allow Swi5p to activate *CTS1* expression in the absence of Ace2p. We performed a genetic screen to look for such a mutation. Our criteria were that the mutation should permit *CTS1* to be expressed in an *ace2* mutant and that *SWI5* must be required for the *CTS1* expression.

A *CTS1-lacZ* reporter was integrated at the *LEU2* locus of yeast strains bearing an *ace2* gene disruption; this strain displays a white colony color under appropriate assay conditions using X-Gal. This strain was mutagenized with ethyl methane-sulfonate, and 28 blue colonies were identified as containing potential mutations in *NCE* (negative regulator of *CTS1* expression) genes. Genetic analysis demonstrated that three mutations were dominant, and these mutations were not characterized further. Seven of the remaining recessive mutations could be sorted into three complementation groups, which we call *NCE1*, *NCE2*, and *NCE3*. We decided to focus on the complementation groups with multiple alleles. Strains carrying *nce1*, *nce2*, or *nce3* were backcrossed to wild-type strains. Each of the *nce* mutations segregated 2:2, demonstrating that single mutations are responsible.

TABLE 5. Quantitative measurements of β -galactosidase activity in extracts prepared from isogenic strains that contain an integrated *CTS1-lacZ* reporter

Strain	Relevant genotype	<i>CTS1-lacZ</i> activity (U)
DY1636	<i>ACE2⁺ NCE⁺ SWI5⁺</i>	1,970 $\pm$ 410
DY1654	<i>ace2 NCE⁺ SWI5⁺</i>	56 $\pm$ 2
WY194	<i>ace2 nce1 SWI5⁺</i>	200 $\pm$ 6
WY147	<i>ace2 nce1 swi5</i>	110 $\pm$ 8
WY103	<i>ace2 nce2 SWI5⁺</i>	290 $\pm$ 24
WY101	<i>ace2 nce2 swi5</i>	60 $\pm$ 15
WY110	<i>ace2 nce3 SWI5⁺</i>	380 $\pm$ 43
WY105	<i>ace2 nce3 swi5</i>	27 $\pm$ 18

FIG. 8. *nce* mutations allow *CTS1* expression in an *ace2* mutant. A Northern blot containing RNA prepared from isogenic strains was probed with a *CTS1* probe and an actin probe as an internal control. The following strains were used: DY1636 (lane 1), DY1653 (lane 2), DY1654 (lane 3), DY1656 (lane 4), WY194 (lane 5), WY291 (lane 6), WY103 (lane 7), WY290 (lane 8), WY110 (lane 9), and WY289 (lane 10).

All of the *nce* mutations allow the *CTS1* gene to be expressed in the absence of the Ace2p activator. We were interested in identifying mutations which permitted Swi5p to activate *CTS1*. We therefore constructed *ace2 swi5 nce* strains carrying the integrated *CTS1-lacZ* reporter. To quantitate the promoter activity of the *CTS1-lacZ* reporter, extracts were prepared from log-phase cells and β -galactosidase activity assays were performed (Table 5). Suppression by the *nce1* and *nce2* mutations is largely *SWI5* independent. Less than a two-fold difference is seen when the *ace2 nce1 SWI5* strain is compared with the *ace2 nce1 swi5* strain, and the *nce2* strain shows a four- to fivefold difference when *SWI5* is present. In contrast, the *nce3* mutation shows a substantial dependence on *SWI5*. The *ace2 nce3 SWI5* strain has 14-fold more *CTS1-lacZ* activity than the *ace2 nce3 swi5* strain (compare WY110 [*ace2 nce3 SWI5*] with WY105 [*ace2 nce3 swi5*]). To verify that the *nce* mutations suppressed the *ace2* defect in *CTS1* transcription at the native *CTS1* locus, RNA was prepared from isogenic strains and analyzed by Northern blotting. As shown in Fig. 8, an *ace2* mutation sharply reduces *CTS1* mRNA levels (lanes 1 to 4). The *nce1*, *nce2*, and *nce3* mutations partially suppress the *ace2* defect in *CTS1* expression (lanes 5, 7, and 9). The *nce1* (lanes 5 and 6) and *nce2* (lanes 7 and 8) mutations are largely *SWI5* independent. The *nce3* mutation, however, is strongly *SWI5* dependent (lanes 9 and 10). Both the *CTS1-lacZ* expression studies and the Northern analysis show that *ace2* suppression of *CTS1* expression by *nce3* requires the presence of a functional *SWI5* gene.

Two experiments were performed to examine whether the *NCE* genes act via the negative regulatory element in the *CTS1* promoter. First, we used plasmid constructs with *CTS1* promoter fragments inserted downstream of the *CYC1* UAS. These *CTS1* promoter fragments were able to reduce tran-

scription from the heterologous *CYC1* UAS (Fig. 7A). The *nce1* and *nce3* mutations partially relieved the repression imposed by insertion of the *CTS1* promoter fragment, suggesting that the *NCE1* and *NCE3* gene products act via these promoter sequences. We provide two explanations as to why these mutations did not completely abrogate the repression mediated by the inserted fragments. First, these mutations may be missense mutations that still retain partial function. Second, there may be multiple, partially redundant pathways contributing to repression.

Next, we determined the effects of the *nce* mutations on the *CTS1*(258)-*CYC1-lacZ* and *CTS1*(46)-*CYC1-lacZ* reporters. The negative element is present in the construct with the 258-bp *CTS1* fragment but absent from the reporter with the 46-bp fragment, and *SWI5* can activate only the *CTS1*(46)-*CYC1-lacZ* reporter (Fig. 6). The activity of each of these promoters was determined in *ace2* mutant strains that also had an *nce1*, *nce2*, or *nce3* mutation (Fig. 7B). The *nce3* mutation permits expression of *CTS1*(46)-*CYC1-lacZ* to 10% of the level seen with the *CTS1*(258)-*CYC1-lacZ* reporter. Thus, *NCE1* and *NCE3* are candidates for specific repressor(s) that bind to the *CTS1* promoter and prevent *SWI5* from acting as a functional activator.

DISCUSSION

Our results demonstrate that the Ace2p and Swi5p transcriptional activators bind to the same DNA sequences in vitro. Both proteins recognize sites within the *CTS1* and *HO* promoters, and these binding sites show considerable similarity. This result is not surprising, because the DNA-binding domains in the Ace2 and Swi5 zinc finger proteins are nearly identical. Given the fact that both activators can bind to the same sequences in vitro, why does *ACE2* not activate *HO*, nor *SWI5* activate *CTS1*? How do these two transcriptional pathways remain distinct in vivo?

Our data suggest that promoter specificity is achieved by at least two different mechanisms. First, specificity of activation by *SWI5* at the *HO* promoter is due, in part, to combinatorial control. The specificity of binding of Swi5p to the *HO* promoter is enhanced by an additional protein factor called Pho2p. Our data further support this notion because *ACE2* fails to interact with *PHO2*, as demonstrated by both biochemical and genetic criteria, at either *CTS1* or *HO*. Because there are two adjacent binding sites for Ace2p at the *CTS1* promoter, cooperative DNA-binding interactions between two Ace2 proteins could provide a mechanism for promoter specificity, assuming that Swi5p was unable to bind cooperatively at these sites. However, in vitro binding experiments have failed to detect any cooperativity in Ace2p binding at the *CTS1* promoter (data not shown and reference 13). It is possible that an as yet unidentified factor promotes cooperative binding at *CTS1* by Ace2p and thus enhances *CTS1* promoter specificity for Ace2p but not for Swi5p.

Second, our results illustrate the importance of negative regulation as a mechanism for determining promoter specificity via the Ace2p and Swi5p activators. Deletion or mutation of sequences in a *cis*-acting negative regulatory region within the *CTS1* promoter allow *SWI5* to partially function at *CTS1*, a gene that *SWI5* does not normally activate. Likewise, a mutation in the *NCE3* gene results in *SWI5*-dependent up-regulation of *CTS1* expression in the absence of the Ace2p activator. These results, taken together, suggest that there is a protein factor capable of mediating its negative effects through the *CTS1* negative regulatory site. Whether an *NCE* gene encodes

this factor, and whether it interacts through the negative regulatory site, remains to be determined.

Given the observation that negative regulation occurs at *CTS1*, it is notable that negative regulation also plays a role in the specific activation of the *HO* gene by Swi5p. Our data indicate that negative regulation plays a role in preventing *ACE2* from activating *HO* (39). *SIN5* was identified by a suppressor mutation that allows *HO* expression in the absence of the Swi5p activator, and thus *SIN5* is a negative regulator of *HO*. Importantly, the *sin5* mutation was shown to be unable to suppress the *swi5* defect in *HO* expression when the strain also lacked Ace2p. One interpretation of this result is that the normal function of *SIN5* is to prevent *ACE2* from activating *HO* transcription. Thus, the *SIN5* gene functions at *HO* in a fashion reciprocal to that seen for *NCE3* at *CTS1*.

Negative regulation could contribute to promoter specificity of Ace2p by multiple mechanisms (12, 19, 28, 33). First, a negative regulatory protein could compete with the activator for its binding site. In this case, the negative regulatory protein would selectively interfere with Swi5p binding. Given that the negative regulatory region of the *CTS1* promoter is situated 80 to 90 bp downstream from the Ace2p binding sites, we feel that it is unlikely that such a protein would prevent Ace2p or Swi5p from binding, although this possibility still exists. Second, negative regulation could contribute to promoter specificity by interfering with the abundance, level of activity, or cell cycle-regulated expression of each activator. Similarly, the DNA binding of either activator could be blocked via sequestration by interaction with a particular negative regulator. We think that both of these mechanisms are unlikely, given the facts that *ACE2* and *SWI5* show identical patterns of cell cycle-regulated mRNA expression and nuclear localization and that *CTS1* and *HO* show identical cell cycle regulation (14). However, it is possible that the *nce3* mutation affects the stability, localization, or abundance of Swi5p and thus allows Swi5p to overcome the negative regulation at *CTS1*. Finally, negative regulation could be achieved by interfering with the DNA-bound activator, analogous to Gal80p, which binds to Gal4p and inhibits the Gal4p activation domain. For example, a *CTS1*-specific repressor could interfere with the activity of the Swi5p activator at the *CTS1* promoter but not with the Ace2p activator. It is not entirely clear how such a regulator would achieve this selective interference at *CTS1*, because it should similarly interfere with activation of *HO* by Swi5p. As described below, we believe that the negative regulation occurs at the promoter, thus providing promoter specificity.

There are a number of observations supporting the role of negative regulation in determining promoter specificity of Ace2p and Swi5p. First, point mutations or deletions of the negative regulatory element within the *CTS1* promoter allow *SWI5* to partially activate the *CTS1* promoter. Second, an *nce3* mutation also allows the *CTS1* gene to be activated by *SWI5*. These results are consistent with, but do not prove, the existence of a DNA-binding negative regulator that binds to the *CTS1* promoter and prevents Swi5p activator function. At present, we cannot distinguish between a model in which the negative regulator blocks Swi5p binding and one in which the negative regulator simply interferes with the activation domain of Swi5p. Additionally, we have demonstrated that overexpression of either *ACE2* or *SWI5* allows inappropriate activation of the opposite target gene. This observation is consistent with the negative regulation model, as increased abundance of the Swi5p activator may overcome the negative regulator simply by mass action. Not surprisingly, *CTS1* or *HO* expression never achieves wild-type levels in response to overexpression of the incorrect activator.

Negative regulation has recently been shown to play an important role in the tissue specificity of activation of mammalian genes (9, 45). Analogous to our situation with Ace2p and Swi5p, the two basic helix-loop-helix proteins MyoD and E12 can recognize the same DNA-binding sites in vitro (46). MyoD is responsible for activating myogenic genes, whereas E12 is responsible for activating the immunoglobulin heavy-chain enhancer. Although both activators can bind to the same sequences in vitro, one reason that MyoD does not activate the nonmuscle enhancers is that these enhancers contain *cis*-acting repression elements (45). It appears, analogous to our situation, that these *cis*-acting elements prevent MyoD from functioning as an activator. Weintraub et al. (45) also propose the existence of a specific repressor protein, but they note that other mechanisms of negative regulation could also be responsible. Chong et al. (9) identified a zinc finger DNA-binding protein, called REST, that acts to repress gene expression in a tissue-specific manner. Thus, negative regulation is emerging as an important mechanism for determining promoter specificity of transcriptional activators.

The question then arises as to why *ACE2* and *SWI5* did not evolve to recognize unique DNA sequences, or alternatively, why does *ACE2* (or *SWI5*) not activate both the *CTS1* and *HO* genes in vivo? *ACE2* and *SWI5* can be considered genetically redundant in that they bind to the same DNA sequences in vitro and show a high degree of homology. At this point, the selective value of maintaining both genes is not directly apparent. Thomas (42) discussed several mechanisms by which selection could act to maintain this genetic redundancy. One potential mechanism that applies is the selection of divergent function. Thus, although both genes evolved divergently (i.e., *ACE2* for *CTS1* expression and *SWI5* for *HO* expression), if the two activators have overlapping but nonidentical functions, selective pressure will maintain their redundant regions. Our preliminary observations suggest that either *ACE2* or *SWI5* is required for the transcriptional activation of other yeast genes (unpublished observations), and thus these two activators are redundant for this function. We propose that *SWI5* and *ACE2* arose by duplication of a single precursor gene followed by divergence. At the same time, selective pressure acted to maintain the very similar DNA-binding domains required for the activation of genes that can respond to either *ACE2* or *SWI5*. Logically, the regions of divergence between Ace2p and Swi5p must be responsible for the specificity in activation of either *CTS1* or *HO*. It is clear from domain-swapping experiments with chimeric activators that the specificity domain lies outside the DNA-binding domains of Ace2p and Swi5p (14). In fact, we have been able to construct a chimeric protein capable of activating both *CTS1* and *HO* genes (28a), and we would suggest that this hybrid protein contains the specificity domains from both Ace2p and Swi5p.

In this report, we have focused on the role of negative regulation in determining the promoter specificities of transcriptional activators. Clearly, there are many examples of situations in which combinatorial control participates in promoter selectivity of DNA-binding proteins. For example, Oct-1 and Oct-2 homeodomain proteins recognize the same *cis*-regulatory DNA sequences in vitro, but association with other proteins confers a new DNA-binding specificity upon Oct-1, leading to recognition of specific promoters (11). The MAT α 2 protein can bind DNA cooperatively with different partner proteins, and thus it can recognize distinct sites and regulate different sets of genes (15, 22). At the *HO* promoter, Swi5p binds cooperatively with the Pho2 homeodomain protein (4, 5) whereas Ace2p cannot, and this combinatorial control probably contributes to the *HO* promoter specificity of Swi5p. On the

basis of the presence of two Ace2p binding sites at the *CTS1* promoter, we proposed that two Ace2p molecules (but not Swi5p) may bind cooperatively with an additional protein and thus contribute to promoter specificity. Finally, we note that the negative regulation and combinatorial control models are not mutually exclusive, and thus both mechanisms can contribute to promoter specificity in vivo.

Presumably, the specificity domains of each transcription factor are regions involved in protein-protein interactions. We believe that the role of these domains is to increase the specificity of activation in two ways: first, at the level of DNA binding through the cooperative interaction with a second protein, and second, by overcoming the repressive influences of specific negative regulators. Combining these two mechanisms increases the likelihood that activation by the inappropriate transcription factor does not occur. Clearly, a detailed analysis of the regions involved will be required to understand the promoter specificity of transcriptional activation of Ace2p and Swi5p.

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REFERENCES

- Alani, E., L. Cao, and N. Kleckner. 1987. A method for gene disruption that allows repeated use of URA3 selection in the construction of multiply disrupted yeast strains. *Genetics* **116**:541-545.
- Ausubel, F. M., R. Brent, R. E. Kingston, D. E. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.). 1987. *Current protocols in molecular biology*. John Wiley & Sons, New York.
- Boeke, J. D., F. LaCrute, and G. R. Fink. 1984. A positive selection for mutants lacking orotidine-5'-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. *Mol. Gen. Genet.* **197**:345-346.
- Brazas, R. M., and D. J. Stillman. 1993. Identification and purification of a protein that binds DNA cooperatively with the yeast SWI5 protein. *Mol. Cell. Biol.* **13**:5524-5537.
- Brazas, R. M., and D. J. Stillman. 1993. The Swi5 zinc finger and the Grf10 homeodomain proteins bind DNA cooperatively at the yeast HO promoter. *Proc. Natl. Acad. Sci. USA* **90**:11237-11241.
- Breedeen, L., and K. Nasmyth. 1985. Regulation of the yeast *HO* gene. *Cold Spring Harbor Symp. Quant. Biol.* **50**:643-650.
- Breedeen, L., and K. Nasmyth. 1987. Cell cycle control of the yeast *HO* gene: cis- and trans-acting regulators. *Cell* **48**:389-397.
- Butler, G., and D. J. Thiele. 1991. *ACE2*, an activator of yeast metallothionein expression which is homologous to *SWI5*. *Mol. Cell. Biol.* **11**:476-485.
- Chong, J. A., J. Tapia-Ramirez, S. Kim, J. J. Toledo-Aral, Y. Zheng, M. C. Boutros, Y. M. Altshuler, M. A. Frohman, S. D. Kraner, and G. Mandel. 1995. REST: a mammalian silencer protein that restricts sodium channel gene expression to neurons. *Cell* **80**:949-957.
- Chung, C. T., and R. H. Miller. 1988. A rapid and convenient method for the preparation and storage of competent bacterial cells. *Nucleic Acids Res.* **16**:3580.
- Cleary, M. A., S. Stern, M. Tanaka, and W. Herr. 1993. Differential positive control by Oct-1 and Oct-2: activation of a transcriptionally silent motif through Oct-1 and VP16 corecruitment. *Genes Dev.* **7**:72-83.
- Cowell, I. G. 1994. Repression versus activation in the control of gene transcription. *Trends Biochem. Sci.* **19**:38-42.
- Dohrmann, P. R. 1994. Transcriptional regulation by the *ACE2* and *SWI5* activators in yeast. Ph.D. dissertation, University of Utah, Salt Lake City.
- Dohrmann, P. R., G. Butler, K. Tamai, S. Dorland, J. R. Greene, D. J. Thiele, and D. J. Stillman. 1992. Parallel pathways of gene regulation: the homologous regulators *SWI5* and *ACE2* differentially control transcription of *HO* and chitinase. *Genes Dev.* **6**:93-104.
- Goutte, C., and A. D. Johnson. 1993. Yeast α 1 and α 2 homeodomain proteins form a DNA-binding activity with properties distinct from those of either protein. *J. Mol. Biol.* **233**:359-371.
- Guarente, L., and E. Hoar. 1984. Upstream activation sites of the *CYC1* gene of *Saccharomyces cerevisiae* are active when inverted but not when

- placed downstream of the "TATA box." *Proc. Natl. Acad. Sci. USA* **81**:7860–7864.
17. **Guarente, L., B. Lalonde, P. Gifford, and E. Alani.** 1984. Distinctly regulated tandem upstream activation sites mediate catabolite repression of the *CYC1* gene of *S. cerevisiae*. *Cell* **36**:503–511.
 18. **Hermes, J. D., S. M. Parekh, S. C. Blackow, H. Koster, and J. R. Knowles.** 1989. A reliable method for random mutagenesis: the generation of mutant libraries using spiked oligo-deoxyribonucleotide primers. *Gene* **84**:143–151.
 19. **Herschbach, B. M., and A. D. Johnson.** 1993. Transcriptional repression in eukaryotes. *Annu. Rev. Cell Biol.* **9**:479–509.
 20. **Jiang, Y. W., and D. J. Stillman.** 1992. Involvement of the *SIN4* global transcriptional regulator in the chromatin structure of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **12**:4503–4514.
 21. **Jiang, Y. W., and D. J. Stillman.** 1995. Regulation of *HIS4* expression by the *Saccharomyces cerevisiae* *SIN4* transcriptional regulator. *Genetics* **140**:103–114.
 22. **Keleher, C. A., S. Passmore, and A. D. Johnson.** 1989. Yeast repressor $\alpha 2$ binds to its operator cooperatively with yeast protein Mcm1. *Mol. Cell. Biol.* **9**:5228–5230.
 23. **Keleher, C. A., M. J. Redd, J. Schultz, M. Carlson, and A. D. Johnson.** 1992. Ssn6/Tup1 is a general repressor of transcription in yeast. *Cell* **68**:709–719.
 24. **Kuranda, M. J., and P. W. Robbins.** 1991. Chitinase is required for cell separation during growth of *Saccharomyces cerevisiae*. *J. Biol. Chem.* **266**:19758–19767.
 25. **Laughon, A.** 1991. DNA binding specificity of homeodomains. *Biochemistry* **30**:11357–11372.
 26. **Lawrence, C. W.** 1991. Classical mutagenesis techniques. *Methods Enzymol.* **194**:273–281.
 27. **Leuther, K. K., J. M. Salmeron, and S. A. Johnston.** 1993. Genetic evidence that an activation domain of GAL4 does not require acidity and may form a β sheet. *Cell* **72**:575–585.
 28. **Levine, M., and J. L. Manley.** 1989. Transcriptional repression of eukaryotic promoters. *Cell* **59**:405–408.
 - 28a. **McBride, H., and D. J. Stillman.** Unpublished observations.
 29. **Miles, J., and T. Formosa.** 1992. Evidence that POB1, a *Saccharomyces cerevisiae* protein that binds to DNA polymerase α , acts in DNA metabolism in vivo. *Mol. Cell. Biol.* **12**:5724–5735.
 30. **Myers, A. M., A. Tzagoloff, D. M. Kinney, and C. J. Lusty.** 1986. Yeast shuttle and integrative vectors with multiple cloning sites suitable for construction of lacZ fusions. *Gene* **45**:299–310.
 31. **Nasmyth, K., G. Adolf, D. Lydall, and A. Seddon.** 1990. The identification of a second cell cycle control on the *HO* promoter in yeast: cell cycle regulation of SWI5 nuclear entry. *Cell* **62**:631–647.
 32. **Pavletich, N. P., and C. O. Pabo.** 1991. Zinc finger-DNA recognition: Crystal structure of a Zif268-DNA complex at 2.1 Å. *Science* **252**:809–817.
 33. **Renkawitz, R.** 1990. Transcriptional repression in eukaryotes. *Trends Genet.* **6**:192–197.
 34. **Rothstein, R.** 1991. Targeting, disruption, replacement, and allele rescue: integrative DNA transformation in yeast. *Methods Enzymol.* **194**:281–302.
 35. **Sherman, F.** 1991. Getting started with yeast. *Methods Enzymol.* **194**:1–21.
 36. **Sherman, F., G. R. Fink, and J. B. Hicks.** 1986. *Methods in yeast genetics.* Cold Spring Harbor Laboratory, Plainview, N.Y.
 37. **Sikorski, R. S., and J. D. Boeke.** 1991. *In vitro* mutagenesis and plasmid shuffling: from cloned gene to mutant yeast. *Methods Enzymol.* **194**:302–318.
 38. **Sikorski, R. S., and P. Hieter.** 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* **122**:19–27.
 39. **Stillman, D. J., S. Dorland, and Y. Yu.** 1994. Epistasis analysis of suppressor mutations that allow *HO* expression in the absence of the yeast *SWI5* transcriptional activator. *Genetics* **136**:781–788.
 40. **Tebb, G., T. Moll, C. Dowser, and K. Nasmyth.** 1993. SWI5 instability may be necessary but is not sufficient for asymmetric *HO* expression in yeast. *Genes Dev.* **7**:517–528.
 41. **Thomas, B. J., and R. Rothstein.** 1989. Elevated recombination rates in transcriptionally active DNA. *Cell* **56**:619–630.
 42. **Thomas, J. H.** 1993. Thinking about genetic redundancy. *Trends Genet.* **9**:395–399.
 43. **Wang, H., I. Clark, P. R. Nicholson, I. Herskowitz, and D. J. Stillman.** 1990. The *Saccharomyces cerevisiae* *SIN3* gene, a negative regulator of *HO*, contains four paired amphipathic helix motifs. *Mol. Cell. Biol.* **10**:5927–5936.
 44. **Wang, H., and D. J. Stillman.** 1993. Transcriptional repression in *Saccharomyces cerevisiae* by a SIN3-lexA fusion protein. *Mol. Cell. Biol.* **13**:1805–1814.
 45. **Weintraub, H., T. Genetta, and T. Kadesch.** 1994. Tissue-specific gene activation by MyoD: determination of specificity by cis-acting repression elements. *Genes Dev.* **8**:2203–2211.
 46. **Wright, W. E.** 1992. Muscle basic helix-loop-helix proteins and the regulation of myogenesis. *Curr. Opin. Gen. Dev.* **2**:243–248.