Parallel pathways of gene regulation: homologous regulators SWI5 and ACE2 differentially control transcription of HO and chitinase

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Two independent pathways of transcriptional regulation that show functional homology have been identified in yeast. It has been demonstrated previously that SWI5 encodes a zinc finger DNA-binding protein whose transcription and cellular localization both are cell cycle regulated. We show that ACE2, whose zinc finger region is nearly identical to that of SWI5, shows patterns of cell cycle-regulated transcription and nuclear localization similar to those seen previously for SWI5. Despite their similarities, SWI5 and ACE2 function in separate pathways of transcriptional regulation. SWI5 is a transcriptional activator of the HO endonuclease gene, whereas ACE2 is not. In contrast, ACE2 is a transcriptional activator of the CTS1 gene (which encodes chitinase), whereas SWI5 is not. An additional parallel between the SWI5/HO pathway and the ACE2/CTS1 pathway is that HO and CTS1 both are cell cycle regulated in the same way, and HO and CTS1 both require the SWI4 and SWI6 transcriptional activators. Overproduction of either SWI5 or ACE2 permits transcriptional activation of the target gene from the other pathway, suggesting that the DNA-binding proteins are capable of binding in vivo to promoters that they do not usually activate. Chimeric SWI5/ACE2 protein fusion experiments suggest that promoter specificity resides in a domain distinct from the zinc finger domain.

[Key Words: Transcription; cell cycle; nuclear localization; zinc finger; CTS1]

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One of the major questions in molecular biology is how transcription is regulated. It is generally believed that transcription is controlled by site-specific DNA-binding proteins that interact with control regions of promoters. These transcriptional regulators are often classified by DNA-binding motif, such as zinc finger, helix–loop–helix, or homeo domain [Johnson and McKnight 1989; Gutman and Wasylyk 1991]. For many genes the pattern of gene regulation is determined simply by the promoter sequences and the proteins that bind there. However, the situation becomes more complicated because several proteins containing the same DNA-binding motif can recognize the same DNA sequence in vitro. This is particularly striking for homeo-domain proteins, which show similar DNA-binding specificity in vitro even though they regulate different genes in vivo [Hayashi and Scott 1990]. It is becoming increasingly clear that in vitro interactions between a DNA-binding protein and a promoter do not always reflect the transcriptional regulation seen in vivo. Recently, two transcriptional activators from Saccharomyces cerevisiae with similar DNA-binding domains, ACE2 and SWI5, have been described [Butler and Thiele 1991].

The ACE2 gene is homologous to the SWI5 gene, which encodes a zinc finger protein that functions as an activator of HO gene transcription [Stillman et al. 1988; Butler and Thiele 1991]. The two genes show 37% similarity over their entire length. The zinc finger DNA-binding domains of these two proteins are very highly conserved, with 83% identity in amino acid sequence. The similarity of the two DNA-binding domains rises to 95% if one includes conservative substitutions. More importantly, the amino acid residues predicted to make nucleotide-specific contacts with DNA, based on the zinc finger–DNA cocystal [Pavletich and Pabo 1991], are the same for these two proteins [Fig. 1]. We would therefore predict that SWI5 and ACE2 bind to the same DNA sequences.

The ACE2 gene was isolated as a multicopy suppressor of an ace1 defect, based on its ability to permit growth in

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the presence of high environmental concentrations of copper (Butler and Thiele 1991). ACE1 is required for activation of the CUP1 locus, which encodes metallothionein, and an ace1 mutant strain is therefore copper sensitive (Thiele 1988).

SWI5 was identified as a transcriptional activator of the yeast HO gene (Stern et al. 1984). HO encodes an endonuclease that initiates mating-type interconversion, and in a swi5 mutant HO is not expressed and mating-type switching does not occur (Nasmyth and Shore 1987; Herskowitz 1988). HO is expressed in one of the two cells resulting from mitotic division, the mother cell, but it is not expressed in daughter cells (Strathern and Herskowitz 1979). SWI5 encodes a protein that binds in vitro to a site in the HO promoter and plays a role in determining the differential expression of HO in mothers and daughters (Nasmyth 1987; Nasmyth et al. 1987; Stillman et al. 1988).

Nasmyth et al. (1987, 1990) have shown that SWI5 is cell cycle regulated in two distinct ways. First, it is transcriptionally regulated such that maximal mRNA levels accumulate in the G2 phase of the cell cycle (Nasmyth et al. 1987). Second, the entry of Swi5 protein into the nucleus is regulated in the cell cycle (Nasmyth et al. 1990): Swi5 protein is cytoplasmic when synthesized in G2; at mitosis, it moves to the nucleus and then rapidly disappears.

In this report we show that ACE2 is a transcriptional activator of theCTS1 gene. TheCTS1 gene encodes chitinase, which is needed for separation of mother and daughter cells during mitotic growth (Kuranda and Robbins 1991). We also show that ACE2 displays a pattern of cell cycle-regulated transcription and cellular localization identical to that seen for SWI5, and thatCTS1 and HO display a similar pattern of cell cycle-regulated transcription. The structural similarity of the two transcriptional activators prompted us to determine whether they share functions in vivo. Despite their similarities in cell cycle regulation and DNA-binding domains, SWI5 and ACE2 regulate the transcription of different genes.

**Results**

**Transcriptional regulation of ACE2**

ACE2 and SWI5 encode homologous zinc finger proteins. Because SWI5 expression is cell cycle regulated, we determined whether ACE2 is differentially expressed during the cell cycle. Yeast cells were arrested with the mating pheromone α-factor, inoculated into fresh media lacking α-factor, and allowed to grow synchronously through several cell cycles. Samples were taken at 10-min intervals, RNA was extracted, and ACE2 mRNA levels were assayed by slot hybridization (Fig. 2). ACE2 RNA is absent from α-factor-arrested cells and from cells in G1, with maximal RNA accumulation late in the cell cycle, identical to that seen for SWI5. It is worth noting that both ACE2 and SWI5 are very low-abundance transcripts.

Because ACE2 and SWI5 show identical patterns of mRNA accumulation, we compared the DNA sequences within their promoter regions. An exact 12 of 12 nucleotide match was seen [Fig. 3]. Recently, two genes encoding G2 or mitotic cyclins were cloned from S. cerevisiae, and the pattern of cell cycle regulation of these genes is identical to that seen for SWI5 (Ghiara et al. 1991; Surana et al. 1991). We therefore compared the putative promoter regions of these mitotic cyclin genes, CLB1/SCB1 andCLB2, with the ACE2/SWI5 match, and significant homology was seen. It should be noted that the two 9/12 matches identified for theSCB2 gene are actually in tandem. This high degree of similarity seen in
promoters of G2-expressed genes suggests that this sequence plays a functional role in transcriptional regulation. Functional studies will be required to determine the relevance of these promoter sequences in gene regulation.

Subcellular localization of Ace2

Because Ace2 shows sequence similarity to Swi5, and because the subcellular localization of Swi5 varies within the cell cycle [Nasmyth et al. 1990], we decided to examine the localization of Ace2. The localization of Ace2 in the cell was determined using a fusion between the entire Ace2 open reading frame (ORF) and the Escherichia coli β-galactosidase gene [lacZ] on a multicopy plasmid. As in the case of Ace2, this large Ace2-LacZ fusion is biologically active as a multicopy suppressor of the copper-sensitive phenotype of an ace1 deletion [data not shown, Butler and Thiele 1991]. The fusion protein was detected by indirect immunofluorescence. In an asynchronous culture expressing the Ace2-β-galactosidase fusion, several patterns of fluorescent staining can be seen (Fig. 4a). Some cells do not stain with antibody; this may be because Ace2 is not always expressed or may be the result of differences in antibody permeability in individual cells. In some cells, staining is predominantly cytoplasmic. Nuclear staining is seen only in cells with clearly visible mother and daughter nuclei or in cells that are small and unbudded; these cells are in G1 phase. The morphology of a yeast cell is a reliable indicator of position within the cell cycle [Pringle and Hartwell 1981]. A similar pattern of localization was seen using anti-c-myc antibody, and a construct in which a 10-amino-acid epitope derived from the c-Myc protein was inserted before the stop codon of Ace2 [G. Butler and D.J. Thiele, unpubl.]. The differential localization is therefore unlikely to be simply the result of expression of the large Ace2-LacZ fusion protein. Such differential localization is very similar to the cell cycle-regulated localization of Swi5, which is found in the nucleus of cells in late M and G2 phases but is cytoplasmic at all other stages of the cell cycle [Nasmyth et al. 1990].

The localization of Ace2 during the cell cycle was analyzed further by arresting cells carrying the Ace2-β-galactosidase fusion plasmid at specific stages of the cell cycle. Cells were arrested in early M phase by treatment with nocodazole, a microtubule-depolymerizing drug [Jacobs et al. 1988]. Cells arrested with nocodazole display distinct cytoplasmic staining, in a punctate fashion [Fig. 4b]. Punctate cytoplasmic staining was also seen when Swi5 was overproduced [Nasmyth et al. 1990] and may be a result of overproduction from a multicopy plasmid. MATa cells were arrested in G1 using α-factor, and Ace2 is predominantly nuclear localized [Fig. 4c]. It should be noted that during long treatments with α-factor (>3 hr) the β-galactosidase antigen is again found in the cytoplasm, which may result from proteolysis of the Ace2-β-galactosidase fusion, particularly as Ace2 is not expressed during α-factor arrest (see Fig. 2). In any case, these results suggest that the cellular distribution of Ace2 very closely resembles that of Swi5. Both Swi5 and Ace2 are localized to the cytoplasm of cells undergoing DNA synthesis [small buds, S phase] or in early M phase but are localized to the nucleus of cells completing cell division [in late M or early G1].

The regions of Swi5 required for nuclear localization recently have been identified [Moll et al. 1991]. Cell cycle-dependent phosphorylation of three serine residues [amino acids 522, 646, and 664] by the yeast Cdc28 kinase is associated with regulated translocation to the nucleus. A comparison of the protein sequence of Swi5 and Ace2 [Fig. 1] shows that two of these serines (Ser-646 and Ser-664) are absolutely conserved in Ace2, and Ser-522 in Swi5 has been replaced by a threonine in Ace2, which can be phosphorylated in a similar fashion. Sequences surrounding these residues have also been conserved and represent potential phosphorylation sites for Cdc28 kinase [Moreno and Nurse 1990]. Therefore, it is very likely that localization of Ace2 and Swi5 is regulated in a similar fashion. Although the role of these residues in translocation of Ace2 has not been determined, this is presently under investigation.

Differential nuclear localization is emerging as a very important mode of cellular regulation. Certainly transcript...
Figure 4. (See facing page for legend.)
tion factors, such as the glucocorticoid receptor or c-fos, remain cytoplasmic until a specific stimulus is applied (hormone or serum factors), and then enter the nucleus (Picard and Yamamoto 1987; Roux et al. 1990). The yeast Cdc46 protein, which is required for DNA replication, enters the nucleus in a cell cycle-dependent fashion very similar to both Swi5 and Ace2 (Hennessy et al. 1990). The Drosophila Isi1(Ya) protein, required for mitosis, moves between the cytoplasm and the nuclear envelope (an inactive and active state) in a cell cycle-dependent manner (Lin and Wollner 1991). Therefore, it appears that Ace2 is a new member of a class of proteins that show regulated subcellular localization.

ACE2 is a regulator of CTS1

Chitinase is needed to degrade the chitin septum joining mother and daughters. Kuranda and Robbins (1991) have cloned the CTS1 gene, which encodes chitinase, and have shown that disruption of the gene causes a defect in cell separation, which we refer to as clumpiness. We have observed that an ace2 mutation also causes a clumpy phenotype (see below), and we decided to ask whether the CTS1 gene is transcriptionally regulated by ACE2 or SWI5. RNA was prepared from four isogenic strains, and RNA blot hybridization analysis was performed with probes for CTS1 and ACT1 (actin). CTS1 mRNA is absent from the ace2 SWI5 and ace2 swi5 strains (Fig. 5). The ACE2 swi5 strain, however, has CTS1 mRNA levels equivalent to wild type. The results clearly demonstrate that ACE2 is required for CTS1 expression, whereas SWI5 is not. The fact that the ace2 mutation causes a drastic decrease in CTS1 mRNA levels suggests that ACE2 is a major transcriptional activator of CTS1.

CTS1 is cell cycle regulated

The pattern of expression of the CTS1 gene within the cell cycle was determined. One rationale for this experiment is that cell separation, promoted by chitinase, is a landmark of the yeast cell cycle. An additional rationale is that ACE2 and SWI5 are cell cycle regulated in an identical fashion (G2 expression) and that HO, the only gene known to be regulated by SWI5, is also cell cycle regulated (G1 expression). A Northern blot containing RNA samples from cells synchronized by an a-factor arrest-release protocol was hybridized with a CTS1 probe (Fig. 6). CTS1 is expressed during the alpha-factor block, but the CTS1 RNA disappears within 10 min after growth in fresh media and remains off during the first cell cycle. CTS1 RNA appears during the G1 phase of the second cell cycle, disappears during S and G2, and reappears during G1 of the third cell cycle.

CTS1 RNA accumulation clearly is periodic during the cell cycle, with maximal RNA levels seen in G1 phase. As controls, we compared the pattern of CTS1 expression (Fig. 6) with the expression of other genes known to be expressed early in the cell cycle, histone H2B and HO (Hereford et al. 1981; Nasmyth 1983). The periodicity of CTS1 expression is similar to that of HO, in G1. Maximal expression from these two genes occurs slightly before that of H2B, which is expressed in late G1 and S phases of the cell cycle. It has been demonstrated previously that HO mRNA is absent during a-factor arrest (Nasmyth 1983). CTS1 mRNA is present during exposure to mating pheromone, and in this respect CTS1 regulation differs from HO. It appears that the CTS1 gene is transcribed during a-factor arrest, although it is possible that a-factor stabilizes previously synthesized CTS1 mRNA. HO and CTS1 regulation are similar in that mRNA accumulation is substantially reduced during the first cell cycle following release from a-factor arrest (Nasmyth et al. 1987; Breeden and Mikesell 1991). HO and CTS1 differ with respect to cell-type regulation. The HO gene is inactive in diploids (Jensen et al. 1983), whereas the CTS1 gene is expressed in diploids (data not shown).

The SWI4 and SWI6 genes were identified as transcriptional activators of HO and are involved in the cell cycle.

**Figure 4.** Differential nuclear localization of an ACE2-β-galactosidase fusion protein detected by indirect immunofluorescence. (a) Asynchronous culture (strain DTY59) transformed with the ACE2-β-galactosidase fusion plasmid YEpACE2-lacZ. (Left) DAPI-stained DNA; (right) the localization of ACE2-β-galactosidase detected by staining with anti-β-galactosidase primary and FITC-conjugated secondary antibodies. The arrowhead indicates nuclear localization in a G1 cell, the short arrow indicates nuclear localization in a cell just after mitosis, and the longer arrow indicates cytoplasmic localization in a G2 cell prior to nuclear division. (b) Cells (strain DTY59/YEpACE2-lacZ) synchronized in mitosis by growth in media containing nocodazole, and stained as in a. (c) Cells (strain CG378/YEpACE2-lacZ) synchronized in G1 by treatment with the mating pheromone α-factor. This strain shows the same staining pattern as a when grown in the absence of α-factor (data not shown). (d) Cells (DTY59) transformed with the parent vector YEp367, to show degree of nonspecific fluorescence.

**Figure 5.** ACE2 is an activator of the CTS1 gene. A Northern blot was hybridized with a CTS1 probe and an ACT1 probe as an internal control. RNA was prepared from the following isogenic strains: [Lane 1] DY1119 wild type; [lane 2] DY1151 ace2 SWI5; [lane 3] DY1000 ACE2 swi5; [lane 4] DY1149 ace2 swi5.
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Figure 6. CTS1 mRNA levels are cell cycle regulated. Wild-type cells were synchronized by an α-factor block-and-release protocol. RNA samples were prepared from α-factor-blocked cells [lane 1] and at 10-min intervals following release into fresh medium [lanes 2–22]. RNA blot hybridization was performed to assay mRNA levels for CTS1, ACT1, histone H2B, and HO. ACT1 serves as an internal control, H2B RNA levels rise in late G1 and S phases [Hereford et al. 1981], and HO RNA levels rise in G1 phase [Nasmyth 1983]. The same aliquots of cells were used in this experiment and in the experiment shown in Fig. 2.

The ace2 swi5 double mutant is significantly more clumpy than the ace2 SWI5 strain (Table 1). However, no difference in CTS1 RNA levels can be detected between an ace2 swi5 double mutant and an ace2 SWI5 mutant, even when the gel shown in Figure 5 is heavily overexposed (data not shown). This implies that the absence of CTS1 RNA is not the sole cause of the clumpy phenotype. We would therefore suggest that the swi5 mutation contributes to a clumpy phenotype by some means other than control of CTS1 mRNA levels. We speculate that SWI5 is a transcriptional regulator of other, unidentified genes important in cell separation.

A chromosomal HO : lacZ reporter [Breeden and Nasmyth 1987] was used to quantitate HO promoter activity in four isogenic strains differing only at the ACE2 and SWI5 loci. As shown in Table 1, a swi5 mutation reduces HO expression 70-fold. The ace2 single mutation has minimal quantitative effects on HO : lacZ activity, whether the strain is SWI5 or swi5. Therefore, we

regulation of HO [Stern et al. 1984; Breeden and Nasmyth 1987; Breeden and Mikese 1991]. SWI4 and SWI6 are components of a cell cycle-regulated DNA-binding activity and are also transcriptional activators of the G1 cyclins of yeast, CLN1, CLN2, and HSC26 [Andrews and Herskowitz 1989; Nasmyth and Dirick 1991, Ogas et al. 1991]. Because CTS1 is expressed in G1, as are HO and the G1 cyclins, it seemed possible that CTS1 might be regulated by SWI4 and SWI6. RNA was prepared from swi4 and swi6 mutant strains and from an isogenic wild-type strain, and Northern blot analysis demonstrates that the CTS1 transcript is drastically reduced in swi4 and swi6 mutant strains [Fig. 7].

Characterization of ace2 swi5 double mutants

ACE2 and SWI5 encode homologous cell cycle-regulated transcription factors. One might predict that genes encoding cell cycle-regulated transcription factors would be essential, but a strain with a null mutation in either ACE2 or SWI5 is viable. Because ACE2 and SWI5 are homologous, they could provide redundant functions. To answer this question a diploid strain with disrupted alleles of ACE2 and SWI5 was created, this doubly heterozygous diploid strain was then sporulated, and tetrads were dissected. We found that haploid ace2 swi5 double mutant strains are viable. This result does not exclude the possibility that another, unidentified gene provides an essential function and thus permits viability of an ace2 swi5 double mutant.

The ace2 swi5 and the ace2 SWI5 haploid segregants dissected from the ACE2/ace2 SWI5/swi5 diploid strain had an altered colony morphology. The ace2 mutants had surface invaginations and appeared rough at the colony periphery, in contrast to the normal smooth, lustrous colony surface. Microscopic examination revealed a clumpy phenotype [Fig. 8]. The ace2 swi5 double mutant is extremely clumpy, the ace2 single mutant is significantly clumpy, and the swi5 single mutant shows only modest clumpiness. Comparing the extent of the clumpy phenotype of the isogenic strains [Table 1] suggests that the clumpy phenotype is additive in the double mutant. When the clumpy cells were stained with Calcofluor, fluorescence was seen at the junctions between cells [data not shown]. This confirms that the sites of attachment between cells are the chitin septa.

Figure 7. SWI4 and SWI6 are activators of the CTS1 gene. A Northern blot was hybridized with a CTS1 probe and an ACT1 probe as an internal control. RNA was prepared from the following isogenic strains: [Lane 1] DY131 wild type, [lane 2] DY1115 swi4, [lane 3] DY1114 swi6.

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The four strains are isogenic, except for the following mutations: (A) DY1119 wild type; (B) DY1000 ACE2 swi5; (C) DY1151 ace2 SWIS; (D) DY1149 ace2 swi5. These strains were transformed into strain DY1149 [ace2 swi5], which was grown under uracil selection.

**Figure 8. Mutations in ace2 or swi5 lead to a clumpy phenotype.** Logarithmically growing cells were sonicated before phase-contrast micrographs were taken with a Nikon Optiplan microscope. The four strains are isogenic, except for the following mutations: (A) DY1119 wild type; (B) DY1000 ACE2 swi5; (C) DY1151 ace2 SWIS; (D) DY1149 ace2 swi5.

We conclude that ACE2 is not a transcriptional regulator of HO, at least under the conditions assayed.

**Suppression by overproduction of ACE2 or SWIS**

ACE2 and SWIS regulate distinct genes, despite the similarity of Ace2 and Swi5 in their DNA-binding domains, cell cycle-regulated transcription, and regulated cellular localization. Although normal expression from the chromosomal ACE2 gene does not activate HO expression in a swi5 mutant (Table 1), we reasoned that increased levels of Ace2 might activate HO expression in the absence of SWIS. A multicopy plasmid containing ACE2 [YEpACE2] was transformed into an ace2 swi5 mutant strain. The strain contains the lacZ gene driven by the HO promoter, integrated at the HO chromosomal locus. Measurements of lacZ expression [Fig. 9A] demonstrate that ACE2 overexpression can partially complement the swi5 defect. The YEpACE2 plasmid in the ace2 swi5 strain also makes the cells less clumpy than in an ACE2 swi5 strain.

The reciprocal experiment was performed with a multicopy plasmid expressing SWIS transformed into an ace2 swi5 mutant strain [Fig. 9A]. The increase in SWIS expression provided by the YEp plasmid was not sufficient to complement the ace2 defect in CTS1 gene activation. However, the multicopy plasmid containing SWIS [YEpSWIS] did partially suppress the clumpiness of the ace2 swi5 double mutant. Therefore, either very low levels of CTS1 are sufficient for reversion of the clumpy phenotype or the increased expression of an as yet unidentified target gene of SWIS suppresses the clumpy phenotype. To obtain higher levels of Swi5, the ace2 swi5 mutant was transformed with a plasmid that has SWIS function under the control of the inducible GAL1 promoter [Stillman et al. 1988]. When these cells were grown on glucose medium Swi5 was not expressed and no CTS1 transcript was seen. Cells were grown on galactose medium to induce high-level expression of Swi5. Under these conditions CTS1 transcript levels approach the levels observed in wild-type strains [Fig. 9B].

ACE2 and SWIS act as transcriptional activators for the CTS1 and HO target genes, respectively. Each of

| Strain       | HO : lacZ expression (%) | Clumpiness | CTS1 expression |
|--------------|--------------------------|------------|-----------------|
| DY131 SW15 ACE2 | 100.0                    | –          | +++             |
| DY999 swi5 ACE2 | 92.4                     | ++         | +++             |
| DY1148 SW15 ace2 | 1.4                      | –          | –               |
| DY1150 swi5 ace2 | 1.7                      | ++         | –               |

Clumpiness is based on microscopic inspection. HO : lacZ expression is given as a percentage of the wild-type strain. CTS1 expression is from Fig. 5.

**Figure 9. Complementation of ace2 or swi5 defect by overproduction.** (A) Multicopy plasmids YEp24 or YEp24 with SWIS or ACE2 were transformed into strain DY1149 [ace2 swi5] and grown under uracil selection. (B) pGAL : SWIS plasmid was transformed into strain DY1149 [ace2 swi5], which was grown either on SC-Ura [containing 2% glucose] or SC-Ura + Gal [containing 2% galactose] media. HO : lacZ activity is given in quantitative units from ONPG assays [Breeden and Nasmyth 1987]. CTS1 activity is from the Northern blot shown, and clumpiness was determined from microscopic observation.
these zinc finger proteins, when expressed from single copy [either YCp or chromosomal], does not activate transcription of the incorrect target gene. However, overproduction of a DNA-binding protein leads to transcriptional activation of the incorrect target gene. We would suggest that an increase in the Ace2 protein levels in vivo will allow it to interact productively with the HO promoter and act as a transcriptional activator. Similarly, although SWI5 does not normally interact with the CTS1 promoter, these results suggest that it can when expressed at elevated levels. We cannot rule out the possibility that these effects may be indirect.

**Chimeric transcriptional activators**

To address the question of what part of the Ace2 and Swi5 zinc finger proteins are responsible for promoter specificity, protein fusions that exchange domains between Ace2 and Swi5 were constructed. As shown diagrammatically in Figure 10A, Ace2 and Swi5 contain restriction sites KpnI and Clal, respectively, at an equivalent location upstream of the zinc fingers. The restriction sites allowed us to conveniently separate the amino-terminal domain of each protein of the zinc finger domain and to construct protein chimeras by exchanging zinc finger domains. An ace2 swi5 mutant strain was transformed with either a CEN plasmid vector [as a control] or the same plasmid containing either SWI5(Clal-KpnI), ACE2, SWI5 : ACE2[Zn], or ACE2 : SWI5[Zn]. The transformants were scored for HO : lacZ expression, CTS1 mRNA levels, and clumpy phenotype [Fig. 10B].

The two chimeras gave very different results when CTS1 mRNA levels and clumpiness were analyzed. The SWI5 : ACE2[Zn] fusion did not appreciably alter CTS1 expression or clumpiness in the ace2 swi5 mutant strain. In contrast, the ACE2 : SWI5[Zn] chimera significantly stimulated CTS1 expression and reduced the clumpiness to that seen in an ACE2 swi5 strain. We interpret this result to mean that the amino-terminal domain of Ace2 is required for CTS1 promoter activation. This suggests that either zinc finger can function to promote activation of the CTS1 promoter but that promoter specificity resides in the amino-terminal domain.

The results with the HO : lacZ reporter are less clear. Only a slight increase in activity of the HO gene reporter was seen with either chimera. The region of Swi5 needed for HO promoter specificity could be encoded by DNA sequences that span the Clal site used to make the chimeras; therefore, neither chimera is completely functional at HO. Alternatively, it is possible that the SWI5 : ACE2[Zn] fusion protein is nonfunctional or unstable. The fact that the ACE2 : SWI5[Zn] chimera stimulates CTS1 transcription allows us to conclude that this construct produces a protein that is functional but is incapable of activating HO gene transcription.

**Discussion**

The activation of HO by SWI5 presents an interesting pattern of transcriptional regulation. The SWI5 gene is transcribed in the G2 phase of the cell cycle [Nasmyth et al. 1987]. The newly synthesized protein remains cytoplasmic until mitosis, when it moves into the nucleus [Nasmyth et al. 1990]. Swi5 is present in the nucleus in G1, where apparently it rapidly disappears. HO, the only known gene activated by SWI5, is not expressed until later in the cell cycle, in late G1 after START [Nasmyth 1983]. The inactivity of the HO promoter in early G1 is not the result of a lack of competence on the part of Swi5. Nasmyth et al. [1990] have used an HO promoter deletion that is no longer START dependent and have shown that this promoter is activated when Swi5 moves into the nucleus. One of the most interesting facets of this pattern of transcriptional regulation is the asymmet-
ric expression of \textit{HO}. \textit{HO} is only expressed in one of the two cells produced from mitotic division, the mother cell. The mechanism of this mother cell-specific expression is not understood, but several experiments have implicated \textit{SWI5} in mother/daughter control [Nasmyth 1987, Nasmyth et al. 1987].

We have identified a similar pattern of transcriptional regulation of \textit{CTS1} by \textit{ACE2}. \textit{ACE2} is transcribed in G$_2$, and the protein product remains cytoplasmic until late in mitosis. \textit{CTS1}, the gene activated by \textit{ACE2}, is expressed in late G$_1$ phase of the cell cycle. These two regulatory pathways, \textit{SWI5} activation of \textit{HO} and \textit{ACE2} activation of \textit{CTS1}, show a number of common features: The transcription factor gene is expressed in G$_3$, the transcription factor shows cell cycle-dependent cellular localization, and the target gene is expressed late in G$_1$. The \textit{SWI4} and \textit{SWI6} genes are required for expression of both \textit{HO} and \textit{CTS1}, as well as the G$_1$ cyclins of yeast [Breeden and Mikesell 1991; Nasmyth and Dirick 1991; Ogas et al. 1991]. Although these two regulatory pathways reflect the same pattern, they are clearly distinct pathways. There is no cross talk between the \textit{SWI5}/\textit{HO} pathway and the \textit{ACE2}/\textit{CTS1} pathway. This is shown most clearly by the fact that \textit{SWI5} cannot function as an activator of \textit{CTS1} and that \textit{ACE2} cannot activate \textit{HO}. (This is not true when the transcription factors are overproduced.)

The \textit{CTS1} gene encodes chitinase, which is needed for cell separation [Kuranda and Robbins 1991]. The final event in the yeast cell cycle is the formation of the septum, composed of the polysaccharide chitin, which joins the mother cell to the daughter [Byers 1981; Cabib and Roberts 1982]. The chitinase enzyme is needed to degrade the chitin junction between cells and thus allows the daughter cell to begin an independent existence. Because cell separation is a landmark of the yeast cell cycle it is not surprising that \textit{CTS1} expression is cell cycle regulated. Part of the chitinous septum remains with the mother after cell separation, resulting in the structure known as the bud scar. In contrast, the daughter cell is born without a bud scar.

Cell division in budding yeast is inherently asymmetric, and it is possible that the \textit{CTS1} gene, like \textit{HO}, will be asymmetrically expressed in mothers and daughters. Because there are many analogies between the \textit{SWI5}/\textit{HO} and \textit{ACE2}/\textit{CTS1} regulatory pathways, it is reasonable to consider the idea that \textit{CTS1} might be expressed in only one of the two progeny from cell division. The asymmetry of the bud scar is consistent with this notion. Experiments are in progress to address this question.

In addition to common patterns of cell cycle-regulated expression and localization, the \textit{Ace2} and \textit{Swi5} proteins share structural features. Both proteins are roughly the same size—770 amino acids for \textit{Ace2} and 709 amino acids for \textit{Swi5}. Both proteins contain three zinc fingers, with the first two being TFIIIA-like C-X$_2$C-X$_{12}$H-X$_3$C-H zinc fingers, and the third zinc finger a very unusual C-X$_4$C-X$_{12}$H-X$_3$C structure. The zinc finger domains of \textit{Ace2} and \textit{Swi5} are highly conserved, with 83% identical residues and 95% similarity allowing conservative substitutions. Recently, Pavletich and Pabo (1991) solved the structure of a zinc finger–DNA cocrystal, and their data allow us to predict which amino acid residues of the \textit{Ace2} and \textit{Swi5} zinc fingers are likely to be involved in nucleotide sequence recognition. The amino acids predicted to contact DNA in \textit{Ace2} and \textit{Swi5} are identical, and we would therefore predict that \textit{Ace2} and \textit{Swi5} would recognize the same DNA sequence. In support of this, we have obtained evidence that an \textit{Ace2} zinc finger domain purified from \textit{E. coli} binds in vitro to the \textit{Swi5}-binding site at the \textit{HO} promoter [R.M. Brazas and D.J. Stillman, unpubl.]. The multicopy suppression experiments [Fig. 9] provide in vivo support for the idea that \textit{Ace2} and \textit{Swi5} can bind to the same sequences. One experiment showed that \textit{ACE2} overproduction can suppress the \textit{swi5} defect and allow \textit{HO} expression, and the reciprocal experiment showed that \textit{SWI5} overproduction overrides the \textit{ace2} requirement for \textit{CTS1} expression. We have interpreted the results of these experiments as demonstrating that \textit{Ace2} and \textit{Swi5} can bind in vivo to the \textit{HO} and \textit{CTS1} promoters, respectively, although overproduction of a transcription factor could activate gene expression via an indirect mechanism.

If \textit{Ace2} and \textit{Swi5} do recognize the same DNA sequence with equal affinity, how are the \textit{ACE2}/\textit{CTS1} pathway and the \textit{SWI5}/\textit{HO} pathway kept separate? Under normal conditions in which the transcription factors are encoded by single-copy genes (either chromosomal or on CEN plasmids) there is no cross talk between the two pathways, under the conditions tested. We suggest that the zinc finger domains of \textit{Ace2} and \textit{Swi5} are not sufficient to provide promoter specificity. The fact that the \textit{Ace2}/\textit{Swi5} chimera stimulates \textit{CTS1} expression [Fig. 10] suggests that the amino-terminal [non-zinc finger] domain of \textit{Ace2} is needed for \textit{CTS1} activation. It is possible that additional proteins are needed to provide specificity in binding and that these proteins interact with the amino-terminal domains of \textit{Ace2} and \textit{Swi5}. Alternatively, the amino-terminal end of \textit{Ace2} may interact with a coactivator required for \textit{CTS1} expression, with which the \textit{Swi5} amino terminus fails to interact. The divergence in amino acid sequence between the amino-terminal domains of these two proteins is consistent with either of these ideas. We have preliminary evidence for such a factor, which we call \textit{Swi5} stimulatory factor (SSF). \textit{Swi5} binds with low affinity to the \textit{HO}-binding site, and SSF increases the affinity of \textit{Swi5} for specific binding sites [R.M. Brazas and D.J. Stillman, in prep.]. It remains to be seen whether SSF is responsible for the promoter specificity of \textit{Swi5} activating \textit{HO} but not activating \textit{CTS1}, and whether an analogous factor is needed for \textit{Ace2} binding to the \textit{CTS1} promoter.

Materials and methods

\textit{Strains and medium}

Strains of \textit{S. cerevisiae} used are listed in Table 2. A series of isogenic strains were derived from the a/a diploid DY866 [\textit{HO}: \textit{lacZ ade2-1 ade6 can1-100 his3}+ \textit{his4}+ \textit{leu2} trp1 ura3]. The diploid strain was made heterozygous for \textit{ACE2} and
3.65-kb Hpal-Sall fragment from pGBl-1 (Butler and Thiele 1991). The entire promoter region of SWI5 or ACE2 is present in constructs YCpSW15[Clal–Kpnl]. A 3.2-kb Hpal–HindIII fragment of ACE2 from pGB1-1 (Butler and Thiele 1991) was cloned into EcoRV–HindIII-digested Bluescript KS+. This ACE2 fragment was then cloned as a SacI–HindIII fragment into YCplac33, thus creating YCpACE2. YCpSW15: ACE2(Zn) and YCpACE2: SWI5(Zn) were created by exchanging the SacI–Kpnl fragments, which contain the promoter and amino-terminal domain of each gene, between YCpSW15[Clal–Kpnl] and YCpACE2. YEpSW15 was constructed by replacing the Smal–Sphl fragment of YEp24 with the SW15 fragment from pUC19 : SW15. YEpACE2 was constructed by replacing the Smal–Sall fragment of YEp24 with a 3.65-kb Hpal–Sall fragment from pGB1-1 (Butler and Thiele 1991). The entire promoter region of SWI5 or ACE2 is present in constructs YCpSW15[Clal–Kpnl], YCpACE2, YEpSW15: ACE2(Zn), YCpACE2: SWI5(Zn), YEpSW15, and YEpACE2.

YEpACE2–lacZ, containing an in-frame ACE2–β-galactosidase fusion, was constructed in several steps. First, a HindIII site was inserted between the last amino acid and the termination codon of the ACE2 ORF by site-directed mutagenesis (Su and El-Gewely 1988). A 110-bp PvuII–HindIII fragment encompassing this region was then isolated and sequenced to ensure that no other fortuitous changes were introduced. This fragment was then used to reconstitute an ACE2 gene fused in-frame to the β-galactosidase gene in the high-copy-number vector YEp367 (Myers et al. 1986), creating YEpACE2–lacZ. The fusion includes the entire ACE2 ORF and promoter region (to base –517; Butler and Thiele 1991).

**Indirect immunofluorescence**

Ten-milliliter cultures of cells were grown in synthetic complete media minus leucine (SC–Leu) to an OD<sub>600</sub> of 1–1.5, fixed with formaldehyde, and stained with antibodies essentially as described in Pringle et al. [1991]. The primary antibody [mouse monoclonal anti-β-galactosidase, Promega] was applied to 10–20 μg/ml. The secondary antibody, FITC [fluorescein 5-isothiocyanate]-conjugated sheep anti-mouse IgG [Boehringer Mannheim], was used at a final concentration of 12–30 μg/ml. To boost the signal, antibody staining was performed twice. Cells were treated first with the primary antibody for 1.5 hr, then with the secondary antibody for 1.5 hr, and the procedure was then repeated. DAPI (4', 6-diamidino-2-phenylindole dihydrochloride) was included in the mounting medium at a concentration of 45 ng/ml to stain nuclear DNA. Cells were visualized under 100× magnification using a Leitz Orthoplan microscope with an ultraviolet light source and were photographed with Kodak T-Max film at ASA 400. To arrest cells with nocodazole, 10 ml of DTY59 cells carrying YEpACE2–lacZ was grown to an OD<sub>600</sub> of 1–1.5 in SC–Leu, and nocodazole [Sigma] was added to a final concentration of 15 μg/ml and 1% dimethylsulfoxide. Cells were incubated for an additional 2.5 hr, and at that time >90% of the cells were arrested with large buds. To arrest with α-factor, CG378 cells [MATa] transformed with YEpACE2–lacZ were grown in 50 ml of SC–Leu to an OD<sub>600</sub> of 0.3 and washed with sterile water to remove the Bar1 protease. Cells were incubated for an additional 1.5 hr with 5 μg/ml of α-factor [Sigma], until ~70% arrested as small unbudded cells indicative of Δ<sub>C</sub> arrest. Cells arrested by either nocodazole or α-factor were treated and stained as above for indirect immunofluorescence.

**RNA blot hybridization**

Total cellular RNA was prepared as described previously [Nasmush 1983]. Total RNA (1 μg) was subjected to electrophoresis on a 1% agarose-formaldehyde gel, the gel was incubated in 10× SSC [twice, for 10 min each], and the RNA was trans-
ferred to a nylon membrane (Gelman) by a capillary-transfer method (Maniatis et al. 1982). The RNA was then cross-linked to the nylon membranes using a Stratagene UV transilluminator. Hybridizations were carried out in 0.5 M NaPO₄ (pH 7.2), 1 mM EDTA, 7% (wt/vol) SDS, and 1% (wt/vol) bovine serum albumin, at 65°C overnight. Following hybridization, filters were washed once at room temperature in 2× SSC + 1% SDS for 15 min, once in 2× SSC + 0.1% SDS at 65°C for 15 min, and once in 0.5× SSC + 0.1% SDS at 65°C for 15 min. Filters were blotted dry on 3MM (Whatman) filter paper, mounted in plastic bags, and exposed to Dupont Cronex film at -70°C in the presence of an intensifying screen.

Hybridization probes were generated with a Random Primers DNA Labeling System (BRL) and [α-³²P]dATP (3000 Ci/m mole, Amersham) from the following templates: for ACE2, a 736-bp EcoRl-PvuI internal fragment of ACE2, for ACT1, BamHI-linearized pSP65ACT1 (kindly provided by Dave Eide, University of Minnesota, Duluth), for CTS1, a 950-bp fragment corresponding to nucleotides 544-1494 (where the ATG codon is +1); for H2B, an ~950-bp PvuII fragment from plasmid pCC68 (kindly provided by Fred Winston, Harvard Medical School, Cambridge, MA); for HO, a 875-bp HindIII-BamHI fragment containing the 5’ end of the gene; and for SWI5, a 1591-bp Ncol–HindIII internal fragment.

For α-factor synchrony experiments, yeast strain DY150 was grown in YEPD medium to an OD of <0.2, and α-factor (Sigma) was added to a concentration of 10 μg/ml. Microscopic examination demonstrated that >99% of the cells were unbudded immediately prior to release from α-factor arrest, and 10-ml samples were removed at 10-min intervals following release into fresh media. Part of each sample was sonicated and fixed in 3.7% formaldehyde for subsequent microscopic examination to determine the HO gene transcription state.

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