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.

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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 cocrystal [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 the *CTS1* gene. The *CTS1* 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 that *CTS1* and *HO* display a similar pattern of cell cycle-regulated transcription. The structural similarity of the two transcrip-

tional 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 blot 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* and *CLB2*, with the *ACE2/SWI5* match, and significant homology was seen. It should be noted that the two 9/12 matches identified for the *SCB2* gene are actually in tandem. This high degree of similarity seen in

Figure 2. *ACE2* 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 *ACE2*, *ACT1*, histone *H2B*, and *SWI5*. *ACT1* serves as an internal control, *H2B* RNA levels rise in late G_1 and S phases (Hereford et al. 1981), and *SWI5* RNA levels rise in G_2 phase (Nasmyth et al. 1987).

promoters of G_2 -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 G_1 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 G_1 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 G_1 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 G_1).

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. Certain transcrip-

<i>SWI5</i>	607 before ATG	T T C T T T C A T T A A
<i>ACE2</i>	191 before ATG	T T C T T T C A T T A A
<i>CLB1/SCB1</i>	349 before ATG	T T C T T T T A T G A A
<i>CLB2</i>	297 before ATG	T T C T T T C T T T T T
<i>CLB2</i>	285 before ATG	T T C T T T C T T T T T

Figure 3. Promoter sequences in G_2 -expressed genes. The promoter sequences of *SWI5* and *ACE2* were compared, and a 12-nucleotide match was observed. This 12-nucleotide consensus was then used to search the promoters of the G_2 cyclin genes *CLB1/SCB1* and *CLB2*. The two *SCB2* matches are in tandem.

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* fsl(Ya) protein, required for mitosis, moves between the cytoplasm and the nuclear envelope (and an inactive and active state) in a cell cycle-dependent manner (Lin and Wolfner 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 (G_2 expression) and that *HO*, the only gene known to be regulated by *SWI5*, is also cell cycle regulated (G_1 expression). A Northern blot containing RNA samples from cells synchronized by an α -factor arrest-and-release protocol was hybridized with a *CTS1* probe (Fig. 6). *CTS1* is expressed during the α -factor

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*.

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 G_1 phase of the second cell cycle, disappears during S and G_2 , and reappears during G_1 of the third cell cycle.

CTS1 RNA accumulation clearly is periodic during the cell cycle, with maximal RNA levels seen in G_1 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 G_1 . Maximal expression from these two genes occurs slightly before that of *H2B*, which is expressed in late G_1 and S phases of the cell cycle. It has been demonstrated previously that *HO* mRNA is absent during α -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 α -factor arrest, although it is possible that α -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 α -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 YEp*ACE2-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 G_1 cell, the short arrow indicates nuclear localization in a cell just after mitosis, and the longer arrow indicates cytoplasmic fluorescence in a G_2 cell prior to nuclear division. (b) Cells (strain DTY59/YEp*ACE2-lacZ*) synchronized in mitosis by growth in media containing nocodazole, and stained as in a. (c) Cells (strain CG378/YEp*ACE2-lacZ*) synchronized in G_1 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 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 G_1 and S phases (Hereford et al. 1981), and *HO* RNA levels rise in G_1 phase (Nasmyth 1983). The same aliquots of cells were used in this experiment and in the experiment shown in Fig. 2.

regulation of *HO* (Stern et al. 1984; Breeden and Nasmyth 1987; Breeden and Mikesell 1991). *SWI4* and *SWI6* are components of a cell cycle-regulated DNA-binding activity and are also transcriptional activators of the G_1 cyclins of yeast, *CLN1*, *CLN2*, and *HSC26* (Andrews and Herskowitz 1989; Nasmyth and Dirick 1991; Ogas et al. 1991). Because *CTS1* is expressed in G_1 , as are *HO* and the G_1 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.

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 : lacZ* activity 70-fold. The *ace2* single mutation has minimal quantitative effects on *HO : lacZ* activity, whether the strain is *SWI5* or *swi5*. Therefore, we

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*.

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 Optiplot microscope. The four strains are isogenic, except for the following mutations: (A) DY1119 wild type; (B) DY1000 *ACE2 swi5*; (C) DY1151 *ace2 SWI5*; (D) DY1149 *ace2 swi5*.

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

Suppression by overproduction of *ACE2* or *SWI5*

ACE2 and *SWI5* 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 *SWI5*. A multicopy plasmid containing *ACE2* (YEp-*ACE2*) was transformed into an *ace2 swi5* mutant strain. The strain contains the *lacZ* gene driven by the *HO* pro-

Table 1. *SWI5* and *ACE2* genotype differentially affect *HO* expression, clumpiness, and *CTS1* expression

Strain	<i>HO : lacZ</i> expression (%)	Clumpiness	<i>CTS1</i> expression
DY131 <i>SWI5 ACE2</i>	100.0	—	+++
DY999 <i>swi5 ACE2</i>	1.4	++	+++
DY1148 <i>SWI5 ace2</i>	92.4	++++	—
DY1150 <i>swi5 ace2</i>	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.

motor, integrated at the *HO* chromosomal locus. Measurements of *lacZ* expression (Fig. 9A) demonstrate that *ACE2* overexpression can partially complement the *swi5* defect. The YEp-*ACE2* 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 *SWI5* transformed into an *ace2 swi5* mutant strain (Fig. 9A). The increase in *SWI5* expression provided by the YEp plasmid was not sufficient to complement the *ace2* defect in *CTS1* gene activation. However, the multicopy plasmid containing *SWI5* (YEp-*SWI5*) 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 *SWI5* suppresses the clumpy phenotype. To obtain higher levels of Swi5, the *ace2 swi5* mutant was transformed with a plasmid that has *SWI5* 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 *SWI5* act as transcriptional activators for the *CTS1* and *HO* target genes, respectively. Each of

Figure 9. Complementation of *ace2* or *swi5* defect by overproduction. (A) Multicopy plasmids YEp24 or YEp24 with *SWI5* or *ACE2* were transformed into strain DY1149 (*ace2 swi5*) and grown under uracil selection. (B) *pGAL : SWI5* 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 (Breen and Nasymth 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 from 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

Figure 10. *CTS1* activation by *ACE2:SWI5(Zn)* chimera. (A) Diagram of YCpSWI5(*Clal-KpnI*), YCpACE2, YCpSWI5 : *ACE2* (Zn), and YCpACE2 : *SWI5*(Zn) genes. The genes are aligned at the zinc finger domains. The *Clal* site of Swi5 was converted to a *KpnI* site, generating *SWI5(Clal-KpnI)*. The *KpnI* sites of *SWI5(Clal-KpnI)* and *ACE2* were used to exchange domains and create the chimeras. Swi5 is 709 amino acids in length, with the zinc finger encompassing amino acids 550–637. The *Clal* site encodes amino acids 538–539. Ace2 is 770 amino acids in length, with the zinc finger encompassing amino acids 603–690. The *KpnI* site encodes amino acids 589–590. The *Clal* site of *SWI5* and the *KpnI* site of *ACE2* are at equivalent positions 11 and 13 amino acids, respectively, upstream of the zinc finger. (B) Strain DY1149 (*ace2 swi5*) was transformed with the indicated single-copy plasmid and grown under uracil selection. *HO:lacZ* activity is given in quantitative units from ONPG assays (Breen and Nasmyth 1987), *CTS1* activity is from the Northern blot shown in C, and clumpiness was determined from microscopic observation. (C) Northern blot hybridized with *CTS1* and *ACT1* probes.

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 *HO*. *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 *SWI5* in mother/daughter control (Nasmyth 1987; Nasmyth et al. 1987).

We have identified a similar pattern of transcriptional regulation of *CTS1* by *ACE2*. *ACE2* is transcribed in G_2 , and the protein product remains cytoplasmic until late in mitosis. *CTS1*, the gene activated by *ACE2*, is expressed in late G_1 phase of the cell cycle. These two regulatory pathways, *SWI5* activation of *HO* and *ACE2* activation of *CTS1*, show a number of common features: The transcription factor gene is expressed in G_2 , the transcription factor shows cell cycle-dependent cellular localization, and the target gene is expressed late in G_1 . The *SWI4* and *SWI6* genes are required for expression of both *HO* and *CTS1*, as well as the G_1 cyclins of yeast (Breedon 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 *SWI5/HO* pathway and the *ACE2/CTS1* pathway. This is shown most clearly by the fact that *SWI5* cannot function as an activator of *CTS1* and that *ACE2* cannot activate *HO*. (This is not true when the transcription factors are overexpressed; see below.)

The *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 *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 *CTS1* gene, like *HO*, will be asymmetrically expressed in mothers and daughters. Because there are many analogies between the *SWI5/HO* and *ACE2/CTS1* regulatory pathways, it is reasonable to consider the idea that *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 Ace2 and Swi5 proteins share structural features. Both proteins are roughly the same size—770 amino acids for Ace2 and 709 amino acids for Swi5. Both proteins contain three zinc fingers, with the first two being TFIIIA-like C-X₄-C-X₁₂-H-X₃-H zinc fingers, and the third zinc finger a very unusual C-X₁-C-X₁₂-H-X₅-C structure. The zinc finger domains of Ace2 and 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 Ace2 and Swi5 zinc fingers are likely to be involved in nucleotide sequence recognition. The amino acids predicted to contact DNA in Ace2 and Swi5 are identical, and we would therefore predict that Ace2 and Swi5 would recognize the same DNA sequence. In support of this, we have obtained evidence that an Ace2 zinc finger domain purified from *E. coli* binds in vitro to the Swi5-binding site at the *HO* promoter (R.M. Brazas and D.J. Stillman, unpubl.). The multicopy suppression experiments (Fig. 9) provide in vivo support for the idea that Ace2 and Swi5 can bind to the same sequences. One experiment showed that *ACE2* overproduction can suppress the *swi5* defect and allow *HO* expression, and the reciprocal experiment showed that *SWI5* overproduction overcomes the *ace2* requirement for *CTS1* expression. We have interpreted the results of these experiments as demonstrating that Ace2 and Swi5 can bind in vivo to the *HO* and *CTS1* promoters, respectively, although overproduction of a transcription factor could activate gene expression via an indirect mechanism.

If Ace2 and Swi5 do recognize the same DNA sequence with equal affinity, how are the *ACE2/CTS1* pathway and the *SWI5/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 Ace2 and Swi5 are not sufficient to provide promoter specificity. The fact that the Ace2:Swi5(Zn) chimera stimulates *CTS1* expression (Fig. 10) suggests that the amino-terminal (non-zinc finger) domain of Ace2 is needed for *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 Ace2 and Swi5. Alternatively, the amino-terminal end of Ace2 may interact with a coactivator required for *CTS1* expression, with which the 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 Swi5 stimulatory factor (SSF). Swi5 binds with low affinity to the *HO*-binding site, and SSF increases the affinity of 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 Swi5 activating *HO* but not activating *CTS1*, and whether an analogous factor is needed for Ace2 binding to the *CTS1* promoter.

Materials and methods

Strains and medium

Strains of *S. cerevisiae* used are listed in Table 2. A series of isogenic strains were derived from the a/α diploid DY866 (*HO*:*lacZ ade2-1 ade6 can1-100 his3/+ his4/+ leu2 trp1 ura3*). The diploid strain was made heterozygous for *ACE2* and

Table 2. List of *S. cerevisiae* strains

CG378	MATa <i>ade5 can1 KIL-O leu2-3,112 trp1-289 ura3-52</i>
DTY59	MATα <i>ace1Δ225 CUP1R-3 his6 leu2-3,112 ura3-52</i>
DY131 ^a	MATa HO : <i>lacZ ade2-1 ade6 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3</i>
DY150	MATa <i>ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-52</i>
DY999 ^a	MATa HO : <i>lacZ swi5Δ : TRP1 ade2-1 ade6 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3</i>
DY1000 ^a	MATα HO : <i>lacZ swi5 : TRP1 ade2-1 ade6 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3</i>
DY1114 ^a	MATa HO : <i>lacZ swi6Δ : TRP1 ade2-1 ade6 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3</i>
DY1115 ^a	MATa HO : <i>lacZ swi4Δ : LEU2 ade2-1 ade6 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3</i>
DY1119 ^a	MATα HO : <i>lacZ ade2-1 ade6 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3</i>
DY1148 ^a	MATa HO : <i>lacZ swi5Δ : TRP1 ace2Δ : HIS3 ade2-1 ade6 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3</i>
DY1149 ^a	MATα HO : <i>lacZ swi5Δ : TRP1 ace2Δ : HIS3 ade2-1 ade6 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3</i>
DY1150 ^a	MATa HO : <i>lacZ ace2Δ : HIS3 ade2-1 ade6 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3</i>
DY1151 ^a	MATα HO : <i>lacZ ace2Δ : HIS3 ade2-1 ade6 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3</i>

^aIsogenic strains.

SWI5 by gene replacement (Rothstein 1991) with *ace2Δ : HIS3* and *swi5Δ : TRP1* null alleles. Haploid segregants differing only at the *MAT*, *ACE2*, and *SWI5* loci were identified after tetrad dissection of the heterozygous diploid. The *ace2Δ : HIS3* allele was created by the γ gene disruption method of Sikorski and Heiter (1989). *ACE2* sequences from the *Bgl*III site to the *Kpn*I site (nucleotides +212 to +1764, where the ATG is +1) have been deleted. The *swi5Δ : TRP1* allele deletes DNA between the *Bgl*III and *Nsi*I sites, removing sequences extending from -875 to +1825. Strains DY1114 (BY600) and DY1115 (BY606) were provided by Linda Breeden (Hutchison Cancer Research Center, Seattle, WA) and are isogenic to the strains derived from DY866. Strain DTY59 has been described previously (Butler and Thiele 1991). Strain CG378 was obtained from Dr. Craig Giroux (Wayne State University, Detroit, MI). Cells were grown either in rich YEPD medium or in synthetic complete media lacking either leucine (SC-Leu) or uracil (SC-Ura) for plasmid selection (Sherman 1991). Standard genetic methods were used for strain construction and tetrad analysis (Rothstein 1991; Sherman and Hicks 1991). Yeast cells were transformed by the method of Ito et al. (1983).

Plasmid constructions

A *SWI5* fragment extending from -1209 to +2660 was cloned into pUC19 to create pUC19 : *SWI5*. The *Kpn*I site in pUC19 : *SWI5* was destroyed by digestion with *Asp*718 and filling in with Klenow, and the *Clal* site of *SWI5* was then converted to a *Kpn*I site using a *Clal*/*Kpn*I adaptor (CGGTAC), thus creating pUC19 : *SWI5*(*Clal*-*Kpn*I). This adds 2 amino acids to Swi5 but maintains the reading frame. *Sac*I and *Sph*I sites in the pUC19 polylinker were used to clone the *SWI5*(*Clal*-*Kpn*I) fragment into YCplac33 (Gietz and Sugino 1988), thus creating

YCpSWI5(*Clal*-*Kpn*I). A 3.2-kb *Hpa*I-*Hind*III fragment of *ACE2* from pGB1-1 (Butler and Thiele 1991) was cloned into *Eco*RV-*Hind*III-digested Bluescript KS+. This *ACE2* fragment was then cloned as a *Sac*I-*Hind*III fragment into YCplac33, thus creating YCpACE2. YCpSWI5 : *ACE2*(Zn) and YCpACE2 : *SWI5*(Zn) were created by exchanging the *Sac*I-*Kpn*I fragments, which contain the promoter and amino-terminal domain of each gene, between YCpSWI5(*Clal*-*Kpn*I) and YCpACE2. YEpSWI5 was constructed by replacing the *Sma*I-*Sph*I fragment of YEp24 with the *SWI5* fragment from pUC19 : *SWI5*. YEpACE2 was constructed by replacing the *Sma*I-*Sall* fragment of YEp24 with a 3.65-kb *Hpa*I-*Sall* fragment from pGB1-1 (Butler and Thiele 1991). The entire promoter region of *SWI5* or *ACE2* is present in constructs YCpSWI5(*Clal*-*Kpn*I), YCpACE2, YCpSWI5 : *ACE2*(Zn), YCpACE2 : *SWI5*(Zn), YEpSWI5, and YEpACE2.

YEpACE2-*lacZ*, containing an in-frame *ACE2*- β -galactosidase fusion, was constructed in several steps. First, a *Hind*III 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 *Pvu*II-*Hind*III 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 OD₆₀₀ 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 at 10–20 μ g/ml. The secondary antibody, FITC (fluorescein 5-isothiocyanate)-conjugated sheep anti-mouse IgG (Boehringer Mannheim), was used at a final concentration of 12–30 μ g/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 $\times$ 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 OD₆₀₀ of 1–1.5 in SC-Leu, and nocodazole (Sigma) was added to a final concentration of 15 μ g/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 OD₆₀₀ of 0.3 and washed with sterile water to remove the Bar1 protease. Cells were incubated for an additional 1.5 hr with 5 μ g/ml of α -factor (Sigma), until ~70% arrested as small unbudded cells indicative of G₁ 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 (Nasmyth 1983). Total RNA (1 μ g) was subjected to electrophoresis on a 1% agarose-formaldehyde gel, the gel was incubated in 10 $\times$ 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 + 0.1% 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/mmol, Amersham) from the following templates: for *ACE2*, a 736-bp *EcoRI*-*PvuII* internal fragment of *ACE2*; for *ACT1*, *Bam*HI-linearized pSP65*ACT1* (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 *Hind*III-*Bam*HI fragment containing the 5' end of the gene; and for *SWI5*, a 1591-bp *Nco*I-*Hind*III 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 μ g/ml. Microscopic examination demonstrated that >99% of the cells were unbudded after 195 min, which is indicative of G₁ phase arrest. The cells were filtered on a 0.22- μ m nitrocellulose filter and inoculated into fresh, prewarmed media. One 10-ml aliquot was removed 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 evaluate cell cycle synchrony, and the remainder was used to prepare RNA.

β -Galactosidase determinations

Quantitative determinations of β -galactosidase activity were performed as described previously (Breen and Nasmyth 1987). Each value reported is the average of determinations of at least three independent transformants.

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

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