Regulation of the Yeast Ace2 Transcription Factor during the Cell Cycle

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Previous studies have revealed many parallels in the cell cycle regulation of the Ace2 and Swi5 transcription factors. Although both proteins begin entry into the nucleus near the start of mitosis, here we show that Ace2 accumulates in the nucleus and binds DNA about 10 min later in the cell cycle than Swi5. We used chimeric fusions to identify the N-terminal region of Ace2 as responsible for the delay, and this same region of Ace2 was required for interaction with Cbk1, a kinase necessary for both transcriptional activation by Ace2 and asymmetric distribution of Ace2. Ace2 and Swi5 also showed differences in prevalence during the cell cycle. Swi5 is apparently degraded soon after nuclear entry, whereas constant Ace2 levels throughout the cell cycle suggest Ace2 is exported from the nucleus. Our work suggests that the precise timing of Ace2 accumulation in the nucleus involves both a nuclear export sequence and a nuclear localization signal, whose activities are regulated by phosphorylation.

Ace2 and Swi5 are yeast transcription factors with a number of similarities. Previous work has shown that ACE2 and SWI5 are both transcribed during the G2 portion of the cell cycle, and the Ace2 and Swi5 proteins accumulate in the nucleus at roughly the same time early in M phase (1–3). Additionally the zinc finger DNA-binding domains of the two proteins are nearly identical, and Ace2 and Swi5 recognize the same DNA sequences in vitro (3, 4). Nonetheless Ace2 and Swi5 activate transcription of different genes in vivo (3, 5). Ace2 activates expression of the CTS1 gene, whereas Swi5 does not. In contrast, Swi5 is required for HO expression, whereas Ace2 is unable to activate HO (3).

The mechanisms controlling the cell cycle regulation of Ace2 and Swi5 have been investigated. The G2-specific transcription of the ACE2 and SWI5 genes is due to binding sites for the Mcm1-Fkh2-Ndd1 complex in both promoters (6–10). A nuclear localization signal (NLS)5 was identified in Swi5, and phosphorylation in the vicinity of the NLS by the Cdc28 cyclin-dependent kinase prevents nuclear accumulation (1). Swi5 is dephosphorylated at anaphase by the Cdc14 phosphatase (11). Importantly mutation of the three phosphorylated serine residues in Swi5 responsible for down-regulation of the NLS results in constitutive nuclear localization of Swi5. The amino acids in Swi5 that are phosphorylated to regulate nuclear localization are conserved in Ace2 (3), and mutation of these residues results in constitutive nuclear localization (12).

Ace2 interacts with the Crm1 nuclear export receptor and contains a nuclear export sequence (NES) (13). A GFP-Ace2 fusion is cytoplasmic, but it is nuclear in the presence of the leptomycin B nuclear export inhibitor (13) or in cells with a mutation in the CRM1 nuclear export protein (14). Importantly a GFP-Swi5 fusion does not respond to leptomycin B. Leucine residues are an essential part of all previously characterized NESs, but mutagenesis of this putative NES region in Ace2 shows that leucines are not required for NES activity, and thus Ace2 has a novel NES (13).

Chimeric fusions between Ace2 and Swi5 have been used to identify functional domains (15). The 83-amino acid DNA-binding domains of Ace2 and Swi5 are highly conserved (83% identity and 94% similarity), and 30–50 residues on either side of the zinc fingers, including the NLSs, are also conserved. The N-terminal regions of Ace2 and Swi5 (578 and 524 amino acids, respectively) are not well conserved with only 18% identity through this region. However, there are three small blocks (20–27 amino acids) of similarity in this large region (see Fig. 1A). We used these small islands of homology as junctions for chimeric fusions in the hope that such fusions would fold properly; all of our fusions are expressed at wild type levels and are able to activate at least a subset of target genes (15). Thus, we defined six regions of each protein where A, B, C, and D are N-terminal to the zinc fingers, E comprises the zinc finger region, and F is the C-terminal region (see Fig. 1A). Studies with these chimeric fusions demonstrated that the zinc fingers do not determine which target genes are activated. Instead region C of Ace2 is required for activation of CTS1, and region D of Swi5 is required for HO expression (15). Moreover region D of Swi5 interacts directly with the Pho2 homeodomain protein and the Gal11(Med15) subunit of the Mediator complex (16, 17).

The abbreviations used are: NLS, nuclear localization signal; NES, nuclear export sequence; GFP, green fluorescent protein; RAM, regulation of Ace2 activity and cellular morphogenesis; ChIP, chromatin immunoprecipitation; GAD, Gal4 activation domain; WT, wild type.
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Interestingly sequences within regions A/B of Swi5 are required for HO expression in a pho2 mutant strain but not in PHO2 cells (18).

Transcriptional activation by Ace2 is severely reduced by mutations in the RAM (regulation of Ace2 activity and cellular morphogenesis) network of genes (19). Mutation of any of these genes, CBK1, MOB2, KIC1, HYM1, and TAO3, results in similar phenotypes, including defects in bud site selection, apical growth of the emerging bud, response to mating pheromone, morphogenesis of mating projections, and cell separation after mitosis (19–22). The cell separation defects of RAM network mutants resemble those of ace2 mutants because expression of Ace2 target genes is sharply reduced in these mutants. Our genetics experiments show that Ace2 overexpression can suppress some defects of RAM network mutations, demonstrating that the RAM genes act upstream of Ace2 (22). For the current study we used strains with the CBK1 kinase deleted; Cbk1 enzymatic activity requires the Mob2 partner (19), and a critical regulatory phosphorylation of Cbk1 requires interaction with Ace2, one of its target proteins (23). The Ace2 protein normally accumulates only in the nucleus of daughters in wild type cells (14, 24). Ace2 is present in both mother and daughter cells in cbk1 or mob2 mutants, and thus Cbk1/Mob2 is required for specific localization of Ace2 in daughter cells.

Transcription factor activity can be regulated by control of subcellular localization, acting on NLS and NES elements (25). NLS elements consist of basic amino acids, and nearby phosphorylation can change the charge and thus inactivate the NLS. Phosphorylation to inactivate an NLS was originally identified in Swi5 (1) and has been seen in a number of transcription factors, including FoxO1, mPer1, Msn2, NF-AT, and Swi6 (25). For other factors, such as Dorsal, SRY, T-antigen, and Xnf7, acetylation or phosphorylation at specific sequence context. Phosphorylation of FoxO1 results in specific sequence context. Phosphorylation of FoxO1 results in conformational changes due to disulfide bond formation between cysteine residues. This can lead to masking of the NLS or NES in Pap1 or Yaf1, respectively, and change nuclear accumulation.

Thus there are multiple mechanisms to regulate nuclear localization, and these respond to signal transduction signals, developmental timing, metabolic signals, or cell cycle timing (25). Importantly there are a number of proteins that show regulation of both their NLS and NES elements, including Crz1, FoxO1, Msn2, NF-AT, Pho4, and PRAK.

In this report we show that the Ace2 and Swi5 factors accumulate in the nucleus and bind DNA at different times. Ace2 accumulated in the nucleus about 10 min later than Swi5, and the two proteins showed marked differences in protein accumulation during the cell cycle. We used protein chimeras between Ace2 and Swi5 to identify regions A/B of Ace2 as important for this cell cycle delay, and these regions were also required for daughter-specific localization and for responsiveness to the Cbk1 kinase. Finally mutating both the NES and eliminating the inhibition of the NLS by phosphorylation resulted in a constitutively nuclear Ace2.

EXPERIMENTAL PROCEDURES

Strains and Media—All yeast strains used are listed in supplemental Table 1 and are isogenic in the W303 background (26). Standard genetic methods were used for strain construction (27). The URA3::lexA-lacZ reporter has been described previously (28). Strains with the integrated chimeras, untagged and Myc-tagged, have been described previously (15), and Ace2(G128E) was Myc-tagged using the same strategy (15). GFP tags were inserted C-terminal to chromosomal SWI5, ACE2, or chimera genes by PCR using plasmid pFA6a: GFP(S65T):KanMX6 (29). Plasmids used are listed in supplemental Table 2. Details of plasmid construction are available upon request. The exact residues present in each chimera fusion are given in supplemental Table 4.

Cells were grown in YPD (yeast extract, peptone, dextrose) medium or in synthetic complete medium with 2% glucose and supplemented with adenine, uracil, and amino acids as appropriate to select for plasmids (27). Strains with the GALp:CDC20 allele (16) were grown in raffinose + galactose medium and synchronized by removing galactose to arrest cells in mitosis, and then galactose was added to release cells from the arrest as described previously (16). To arrest cells with α-factor, log phase cells were grown in YM-1 medium at 25 °C, and α-factor (University of Utah Peptide Synthesis Core Facility) was added to 3 μM; with 90–120 min 95% showed morphology indicating G1 arrest, and samples were taken for Western analysis.

Antibodies—Polyclonal antisera to Ace2 and Swi5 were made by expressing in Escherichia coli a maltose-binding protein fusion to Ace2 residues 1–1467 and a His tag fusion to Swi5 residues 1–709 and injecting into rabbits. Anti-Myc antibody was either 9E11 (Abcam or GeneTex) or 4A6 (Upstate).

Fluorescence Microscopy—Cells were grown in synthetic complete medium supplemented with 0.025% adenine. Cells were synchronized with GALp:CDC20 arrest and release as described above. For the double labeling experiment in Fig. 2D, rhodamine anti-mouse and fluorescein anti-rabbit were used as secondary antibodies along with mouse anti-Myc and rabbit anti-Ace2 antibodies. 4′,6-Diamidino-2-phenylindole staining for nuclear DNA was performed as described previously (30). Fluorescent images were captured with an Olympus BX51 fluorescence/differential interference contrast microscope and a MagnaFire SP S99810 camera.

Other Methods—RNA levels were determined with S1 nuclease protection assays as described previously (17). Chromatin immunoprecipitations were performed as described previously (16) using anti-Myc antibodies and antibody-coated magnetic
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beads (Pan Mouse IgG beads, Dynal Biotech). Chromatin immunoprecipitation (ChIP) assays were analyzed by real time PCR and normalized first to an open reading frame-free chromosome I region (31) as an internal control and then normalized to the total DNA amount from input chromatin for each sample (32). Oligonucleotides used for S1 protection or for ChIP PCRs are listed in supplemental Table 3. Western blots were performed with antibodies to Ace2, Swi5, and Myc using the ECL Western kit (GE Healthcare) or with fluorescent secondary antibodies and the Odyssey Infrared Imaging System (Li-Cor Biosciences). Quantitative measurements of β-galactosidase activity from the strain with an integrated CTS1-lacZ reporter were performed as described previously (33).

RESULTS

Regions A/B of Ace2 Are Required for Cbk1-dependent Activation and Cbk1 Interaction—The Cbk1 gene encodes a protein kinase that interacts with Ace2, and cbk1 mutations change the localization of Ace2 and reduce expression of genes activated by Ace2 (20, 22, 24). To address whether a cbk1 gene disruption affects the amount or the electrophoretic forms of Ace2, we performed Western immunoblots with rabbit polyclonal antisera using extracts from isogenic strains. A cbk1 mutation did not significantly affect the Ace2 protein visible on the Western blot (Fig. 1B). We also examined the Swi5 protein, as Swi5 and Ace2 are related transcription factors with similar patterns of cell cycle regulation, and determined that a cbk1 mutation has little effect on Swi5 protein.

Activation of CTS1 by Ace2 requires the Cbk1 kinase for activation. Ace2 and Swi5 have nearly identical DNA-binding domains, but the N-terminal regions are quite different. Previously, we have described chimeric fusions between Ace2 and Swi5 and how specific portions of the non-conserved N-terminal regions contribute to transcriptional activation of specific target genes (15) (Fig. 1A). We used these chimeric fusions between Ace2 and Swi5 to identify the regions of Ace2 required for regulation of CTS1 expression by the Cbk1 kinase. CTS1 mRNA was measured using an S1 nuclease protection assay and quantitated by Phosphorimager (Fig. 1C). Protein chimeras between Ace2 and Swi5 were able to activate CTS1 expression if they contain region C of Ace2 consistent with our previous results (15). However, not all of the chimeras that can activate CTS1 were dependent on CBK1. Wild type Ace2 (line 1), the Ace2(ABC)-Swi5(DEF) chimera (line 4), and the Ace2(ABCD)-Swi5(EF) chimera (line 5) all showed reduced CTS1 activation in a cbk1 mutant. In contrast, a cbk1 mutation did not substantially affect the ability of the Swi5(AB)-Ace2(CDEF) chimera or the Swi5(AB)-Ace2(C)-Swi5(DEF) three-part chimera to activate CTS1 (lines 6 and 9). Therefore it appears that, although region C is required for CTS1 activation, regions A/B are required to make this activation CBK1-dependent.

We performed experiments to address what region of Ace2 is required for physical interaction with Cbk1. Racki et al. (20) did a two-hybrid screen using a Gal4-DNA-binding domain-Cbk1 fusion as bait, and identified Ace2(220–306) as interacting with Cbk1. This part of Ace2 is within region B, which we have defined as amino acids 201–301 (15). We constructed and tested a LexA-Cbk1 fusion and found it to be a very strong activator of a lacZ reporter with LexA binding sites in the promoter (Fig. 1D) in the absence of an activation domain provided by a two-hybrid partner. These results are not in conflict as Racki et al. (20) used 3-aminotriazole as part of their two-hybrid screen with a HIS3 reporter, and thus higher levels of reporter gene expression were required for a valid two-hybrid signal. Importantly we found that the ability of LexA-Cbk1 to activate the LexA-lacZ reporter is substantially reduced in the absence of Ace2 (Fig. 1D). The Ace2 dependence suggests that LexA-Cbk1 is not itself an activator but that interaction with Ace2 recruits the activation domain present in the Ace2 protein. Mob2 may function as a specificity subunit of the Cbk1-Mob2 kinase (14), and thus it is consistent that LexA-Cbk1 requires Mob2 for interaction with Ace2 (Fig. 1D).

Because the LexA-Cbk1 fusion protein is an Ace2-dependent transcriptional activator, we used the Ace2-Swi5 chimeras to determine what portions of Ace2 are required for strong activation by LexA-Cbk1 (Fig. 1E). Strong activation was seen in the strain with Ace2 (line 1), and this was normalized to 100%. Three of the Ace2-Swi5 chimeras facilitated strong transcriptional activation by LexA-Cbk1 (lines 3, 4, and 5), whereas four chimeras displayed essentially no LexA-Cbk1 activity. Interestingly three of the chimeras showed higher activity (2–6.5-fold) than native Ace2. All of the chimeras are expressed at levels similar to that of native Ace2 (15). The data suggest that the presence of regions C and D of Ace2 limit transcriptional activation in this assay or that regions C and D of Swi5 stimulate transcriptional activation. The three chimeras active in this assay all contain Ace2 regions A/B, suggesting that these regions of Ace2 are sufficient for interaction with LexA-Cbk1.

To further characterize the Ace2-Cbk1 interaction, we constructed two-hybrid plasmids containing various regions of Ace2 fused in frame to the Gal4 activation domain (GAD). An ace2 swi5 mutant strain with an integrated LexA-lacZ reporter was transformed with both the LexA-Cbk1 fusion plasmid and one of the various GAD-Ace2 fusion plasmids. Expression of the lacZ reporter was measured to assess the two-hybrid interaction (Fig. 1F). The results show that neither regions A nor C alone provided two-hybrid activation, whereas region B provided very modest activation in this assay. In contrast, GAD fusions with regions B-C and A-B-C provided strong and very strong activation, respectively. Assuming that the degree of two-hybrid activation reflects the level of interaction between two proteins, this suggests that regions A, B, and C of Ace2 are all required for strong interaction with Cbk1. We considered the possibility that the differences in the stability of the various GAD-Ace2 fusions would influence the two-hybrid activation. We performed Western blots with the GAD-Ace2 fusions, but none of the proteins were visible with either anti-GAD or anti-Ace2 antibodies (data not shown). Based on the results in Fig. 1F showing that Ace2 regions A/B are sufficient for interaction with LexA-Cbk1, we suggest that GAD-Ace2(A-B) may be much less stable than GAD-Ace2(A-B-C). Alternatively the addition of region C, characterized as an activation domain in Swi5-Ace2 chimeras, to the Cbk1-interacting regions A/B in GAD-Ace2(A-B-C) may confer additive transcriptional activation potential with the GAD domain.
Ace2 Accumulates in the Nucleus after Swi5—ChIP experiments were performed to examine the timing of DNA binding by Ace2 and Swi5 within the cell cycle. Strains with either an Ace2-Myc tag or a Swi5-Myc tag were synchronized by a CDC20 arrest and release protocol (16). These cells have the GAL1 promoter integrated in front of the CDC20 cell cycle regulatory gene with arrest and release accomplished by withdrawing and then returning galactose to cells. A high degree of synchrony through the cell cycle after the release was demonstrated by flow cytometry analysis, and cell cycle-regulated expression of CTS1 and HO was demonstrated by S1 protection assays (data not shown).

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A.

B.

C.

D.

E.

F.

GAD vector 49 ± 6
GAD-Ace2(A) 42 ± 9
GAD-Ace2(B) 74 ± 9
GAD-Ace2(C) 48 ± 6
GAD-Ace2(AB) 51 ± 2
GAD-Ace2(BC) 350 ± 50
GAD-Ace2(ABC) 10,600 ± 1050

LacZ Expression
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ChIP assays were performed on synchronized cultures of cells with the Swi5-Myc epitope tag, measuring Swi5-Myc binding to HO and EG12. Although HO is activated only by Swi5, EG12 expression is activated by either Swi5 or Ace2 (22). The experiment in Fig. 2A shows Swi5-Myc binding to these promoters beginning at 15 min and continuing until 40 min after the release. In contrast, ChIP experiments with Ace2-Myc show binding to CTS1 and EG12 beginning at 30 min after the release and persisting through 50 min (Fig. 2B). Thus, there is a major difference in the timing of DNA binding by Ace2 and Swi5 in these synchrony experiments.

The ChIP experiments showing that Swi5 binds to promoters before Ace2 could be explained in two ways. One possibility is that Ace2 accumulates in the nucleus at the same time as Swi5 but is somehow prevented from binding DNA. The alternative is that Ace2 accumulation in the nucleus is actually later than that of Swi5. To differentiate between these two possibilities, we directly examined the kinetics of nuclear localization of Ace2 and Swi5 using strains with either Ace2 or Swi5 tagged with GFP. These cells also contained the integrated GALp:CD20 allele allowing for cell cycle synchronization. As shown in Fig. 2C, Swi5-GFP was present in the nucleus starting 10 min after release from the GALp:CD20 arrest, and some Swi5-GFP was still nuclear after 40 min. In contrast,
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A. Ace2  Swi5

Early

Late

1  2  3  4  5  6  7  8  9

B.

Log Phase

CDC20 Arrest

Early

Late

Myc

Pgk1

C.

| Entry   | Time of Nuclear Entry | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 |
|---------|-----------------------|----|----|----|----|----|----|----|----|----|----|
| 1 Ace2  | Late                  |    |    |    |    |    |    |    |    |    |    |
| 2 Swi5  | Early                 |    |    |    |    |    |    |    |    |    |    |
| 3 Ace2(AB):Swi5(CDEF) | Late    |    |    |    |    |    |    |    |    |    |    |
| 4 Ace2(ABC):Swi5(DEF) | Late    |    |    |    |    |    |    |    |    |    |    |
| 5 Ace2(ABCD):Swi5(EF) | Late    |    |    |    |    |    |    |    |    |    |    |
| 6 Swi5(AB):Ace2(CDEF)  | Early    |    |    |    |    |    |    |    |    |    |    |
| 7 Swi5(ABC):Ace2(DEF)  | Early    |    |    |    |    |    |    |    |    |    |    |
| 8 Swi5(ABCD):Ace2(EF)  | Early    |    |    |    |    |    |    |    |    |    |    |
| 9 Swi5(AB):Ace2(C):Swi5(DEF) | Early |    |    |    |    |    |    |    |    |    |    |

D. Localization

- D only
- M + D
Ace2-GFP did not appear in daughter cell nuclei until 20 min, and Ace2-GFP was still nuclear after 50 min, a time when no Swi5-GFP was visible in the nucleus. These results indicate that Swi5 nuclear accumulation precedes that of Ace2.

To verify these results more precisely, we examined Ace2 and Swi5 localization within single cells. Strain DY6547 contains the integrated GALp:CD20 allele as well as the chromosomal SWI5 gene tagged with a C-terminal Myc tag to follow Swi5 localization with a mouse anti-Myc monoclonal antibody. We generated a rabbit polyclonal anti-Ace2 antibody that was used to follow Ace2 localization, and thus the rabbit anti-Ace2 and the mouse anti-Myc antibodies could be used to detect Ace2 and Swi5 in the same cells by double immunofluorescence staining (Fig. 2D). During the arrest (t = 0), weak Swi5 staining was seen in the nuclei of a few cells. Obvious Swi5 nuclear staining was visible at t = 10 min, and there was much greater Swi5 nuclear staining at t = 20 min. Nuclear Swi5 staining began to diminish at 30 min (data not shown). Importantly the pattern of Ace2 staining was again different with no nuclear staining visible at 10 min and strong staining of daughter cell nuclei from 20 to 40 min. We conclude from these two analyses that Swi5 is localized in the nucleus earlier in the cell cycle than Ace2.

Regions A/B of Ace2 Are Responsible for the Delay—To determine which portions of Ace2 and Swi5 affect the timing of nuclear accumulation we used the chimeric fusions between Ace2 and Swi5. We constructed strains where each of the chimeras is integrated as a single copy in the chromosome and Swi5 tagged with a C-terminal Myc tag to follow Swi5 localization with a mouse anti-Myc monoclonal antibody. We generated a rabbit polyclonal anti-Ace2 antibody that was used to follow Ace2 localization, and thus the rabbit anti-Ace2 and the mouse anti-Myc antibodies could be used to detect Ace2 and Swi5 in the same cells by double immunofluorescence staining (Fig. 2D). During the arrest (t = 0), weak Swi5 staining was seen in the nuclei of a few cells. Obvious Swi5 nuclear staining was visible at t = 10 min, and there was much greater Swi5 nuclear staining at t = 20 min. Nuclear Swi5 staining began to diminish at 30 min (data not shown). Importantly the pattern of Ace2 staining was again different with no nuclear staining visible at 10 min and strong staining of daughter cell nuclei from 20 to 40 min. We conclude from these two analyses that Swi5 is localized in the nucleus earlier in the cell cycle than Ace2.

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Ace2 Protein Is Stable in the Cell Cycle—The timing experiments showed that Ace2 and Swi5 proteins accumulate in the nucleus at slightly different times within the cell cycle. Based on these results, we examined the Ace2 and Swi5 proteins during the cell cycle after a GALp:CD20 arrest and release by Western blotting. As shown in Fig. 4A, the mobility of the Ace2 protein fluctuated during the cell cycle with a major increase in electrophoretic mobility at 20–30 min after release. Interestingly this time corresponds to when Ace2 appears in the nucleus, and the increased mobility may reflect changes in protein phosphorylation. This same increase in electrophoretic mobility was also seen at 100 min in the subsequent cell cycle. Importantly the Ace2 protein remained detectable throughout the cell cycle. We must assume that Ace2 is exported from the nucleus starting at times 50–60 min after release because the protein was detected on the Western blot but was not visible in the nucleus by GFP localization. Like Ace2, Swi5 showed changes in electrophoretic mobility during the cell cycle, presumably due to dephosphorylation, during the first 20 min after release (Fig. 4A, compare lanes 1–3). The timing of this change in mobility is coincident with activation of the Cdc14 phospho-

FIGURE 3. Chimeras identify Ace2 regions required for daughter-specific localization and delayed nuclear accumulation. A, fluorescence microscopy assay of nuclear localization of GFP fusions to integrated Ace2-Swi5 chimeras after cell cycle synchronization of GALp:CD20 strains (DY9311, DY9304, DY10939, DY9378, DY9377, DY9382, DY9379, DY9376, DY10937, and DY9382). Early and “Late” pictures were taken 15 and 20 min after release, respectively, except for the late pictures of Ace2(AB)-Swi5(CDEF) and Ace2(ABC)-Swi5(DEF) that were taken at 30 min. These two strains show nuclear GFP slightly later than Ace2(WT)-GFP and also show speckled cytoplasmic staining at 15 min. Note that all of these proteins are expressed from the native promoter. B, protein levels of the chimeras remain largely constant through the cell cycle. Strains DY9122, DY6546, DY9364, DY9362, DY9361, DY9365, DY9363, DY9360, DY9366, and DY10788 were grown in galactose + raffinose media, and samples were taken (“Log Phase”). Cells were transferred to fresh raffinose media lacking galactose for 4.5 h to arrest cells (“CDC20 Arrest”), galactose was added to release cells, and samples were taken at 15 min after release (Early) and at 20 min after release (Late). As in A, the late samples of Ace2(AB)-Swi5(CDEF) and Ace2(ABC)-Swi5(DEF) were taken at 30 min. Twenty micrograms of protein were loaded onto SDS gels, transferred to nitrocellulose membrane, and probed with anti-Myc antibody and also with anti-Pgk1 antibody as a loading control. C, schematic representation of Ace2-Swi5 chimeras (15). Time of nuclear accumulation for chimeras is shown; representative examples are shown in A. D, quantitative analysis of nuclear localization of chimeras in CBK1 strains showing percentage of cells with equivalent mother and daughter staining (M + D) or daughter-only localization (D only). At least 200 cells were examined for each chimera. Strains DY9311, DY9304, DY7886, DY7882, DY7880, DY7889, DY7884, DY7878, and DY7890 were used.
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A. Time

Ace2

Swi5

| Time | 0 | 10 | 20 | 30 | 40 | 50 | 60 | 70 | 80 | 90 | 100 | 110 | 120 | 130 | 140 | 150 |
|------|---|----|----|----|----|----|----|----|----|----|------|------|------|------|------|------|
| 1    |   |    |    |    |    |    |    |    |    |    |      |      |      |      |      |      |
| 2    |   |    |    |    |    |    |    |    |    |    |      |      |      |      |      |      |
| 3    |   |    |    |    |    |    |    |    |    |    |      |      |      |      |      |      |
| 4    |   |    |    |    |    |    |    |    |    |    |      |      |      |      |      |      |
| 5    |   |    |    |    |    |    |    |    |    |    |      |      |      |      |      |      |
| 6    |   |    |    |    |    |    |    |    |    |    |      |      |      |      |      |      |
| 7    |   |    |    |    |    |    |    |    |    |    |      |      |      |      |      |      |
| 8    |   |    |    |    |    |    |    |    |    |    |      |      |      |      |      |      |
| 9    |   |    |    |    |    |    |    |    |    |    |      |      |      |      |      |      |
| 10   |   |    |    |    |    |    |    |    |    |    |      |      |      |      |      |      |
| 11   |   |    |    |    |    |    |    |    |    |    |      |      |      |      |      |      |
| 12   |   |    |    |    |    |    |    |    |    |    |      |      |      |      |      |      |
| 13   |   |    |    |    |    |    |    |    |    |    |      |      |      |      |      |      |
| 14   |   |    |    |    |    |    |    |    |    |    |      |      |      |      |      |      |
| 15   |   |    |    |    |    |    |    |    |    |    |      |      |      |      |      |      |
| 16   |   |    |    |    |    |    |    |    |    |    |      |      |      |      |      |      |

B. 

C.

The Ace2 protein persists throughout the cell cycle. A, DY6547 cells were synchronized by galactose withdrawal and readdition and at the indicated times after release samples were taken for Western immunoblot analysis with antibodies to Ace2 and Swi5. B, chimeric fusions between Ace2 and Swi5 with C-terminal Myc tags were used to assess what protein regions control degradation in G1. Extracts prepared from either log phase cultures or cells arrested with α-factor (indicated by α) were subjected to immunoblotting using anti-Myc antibody. Strains DY150 (ΔH9251 JOURNAL OF BIOLOGICAL CHEMISTRY -factor arrest was observed with native untagged proteins not present in cells in late G1 phase, for example 40 min after release, whereas Ace2 was present at the same time point (Fig. 4A, lane 5). As an alternative method, α-factor can be used to arrest cells in the G1 phase of the cell cycle to examine the difference in stability between Swi5 and Ace2. When α-factor was used to arrest cells in G1, Swi5-Myc was degraded (Fig. 4B, lane 5), whereas Ace2-Myc remained (Fig. 4B, lane 3). A similar difference in stability of the Ace2 and Swi5 proteins during α-factor arrest was observed with native untagged proteins using polyclonal antisera to probe the immunoblots (data not shown). To address which parts of Ace2 and Swi5 contribute to stability or instability, respectively, we turned to the protein chimeras, which we had tagged previously with the Myc epitope (15). Protein extracts were prepared from logarithmically growing cultures of the seven strains with the various Myc-tagged chimeras, and another seven cultures were arrested in G1 with α-factor before protein extraction. The Western immunoblot in Fig. 4B shows that only two chimeras, Swi5(ABCD)-Ace2(EF) and Swi5(ABC)-Ace2(DEF), displayed the Swi5-like pattern of degradation during α-factor arrest. These results suggest that the Swi5(ABC) region confers instability. Chimeras containing Swi5(AB) but lacking Swi5(C) were stable, suggesting that Swi5(AB) is not sufficient for instability. To test this idea, we created strains expressing Swi5(AB)-Myc and Swi5(ABC)-Myc from the native Swi5 promoter without any C-terminal protein sequences. As shown in Fig. 4C, Swi5(AB)-Myc was stable, whereas Swi5(ABC)-Myc was degraded during α-factor arrest.

Ace2 Nuclear Entry Is Regulated by Both the NLS and the NES—The Swi5 NLS is regulated in the cell cycle. Nearby residues at Ser-522, Ser-646, and Ser-664 are phosphorylated by the Cdc28 cyclin-dependent kinase in G2 phase, apparently inactivating the NLS and keeping the protein cytoplasmic (1). The three residues are dephosphorylated by the Cdc14 phosphatase at anaphase, allowing the NLS to be active and nuclear entry to occur (11). Only mutation of all three phosphorylated residues in Swi5 simultaneously (the S522A,S646A,S664A triple mutant, which we refer to as “AAA”) results in constitutive nuclear entry (1).

The conservation between Swi5 and Ace2 includes the region with the phosphorylated amino acids. O’Conallain et al. (12) mutated the analogous Ace2 residues, Thr-575, Ser-701, and Ser-714, to create Ace2(T575A,S701A,S714A), or Ace2(AAA), and showed that Ace2(AAA)-Myc was constitutively nuclear. In this experiment Ace2(AAA)-Myc was expressed at high levels from the GAL1 promoter, and we repeated this experiment with Ace2(AAA)-GFP expressed from the native chromosomal ACE2 promoter. We found Ace2(AAA)-GFP present only in the nucleus of recently divided cells and only in daughters (Fig. 5A); this is the same pattern as seen for Ace2(WT)-GFP. Thus the kinetics of nuclear accumulation for Ace2(AAA) is different when the protein is overexpressed compared with when it is expressed from the native promoter, and it is possible that overexpression saturates the export machinery.

We next examined nuclear localization of Ace2-GFP with a G128E substitution as this mutation lies within the region of Ace2 defined as an NES, amino acids 122–150. Like wild type, Ace2(G128E)-GFP was in the nucleus only in recently divided cells and only in daughters (Fig. 5A); however, unlike wild type, Ace2(G128E)-GFP was present in both mother and daughter cells as described previously (14, 24). Finally we combined the G128E and AAA mutations, making the G128E, AAA quadruple mutant (G128E,T575A,S701A,S714A). The Ace2(G128E, AAA) protein was present in the nucleus in all cells irrespective of cell cycle position (Fig. 5A).

We repeated this localization experiment using strains with the GALp:CDC20 allele allowing for cell cycle synchronization (Fig. 5B). Wild type Ace2-GFP appeared in the nucleus 20 min
after release from the \( \text{CDC20} \) arrest, and a similar pattern was seen for the \( \text{Ace2(AAA)-GFP} \) and \( \text{Ace2(G128E)-GFP} \) mutants. We did note a small amount of \( \text{Ace2(G128E)-GFP} \) in the nucleus during the arrest and at 10 min after the release that was not seen with wild type \( \text{Ace2-GFP} \). Additionally although the fraction of cells with nuclear staining of \( \text{Ace2(wild type)-GFP} \) was substantially reduced after 40 min, the AAA and G128E mutants still remained prominent in the nucleus in the majority of cells at this time. These results suggest that efficient nuclear export of \( \text{Ace2} \) at this time requires both the Gly-128 region of the NES and the phosphorylation of the NLS that is lost in the AAA mutant. Finally the \( \text{Ace2(G128E, AAA)-GFP} \) protein was present in the nucleus throughout the cell cycle. This suggests that mutating both the NES (G128E) and the residues that inhibit the NLS (AAA) results in a \( \text{Ace2} \) protein that is constitutively nuclear.

**DISCUSSION**

The \( \text{Ace2} \) and \( \text{Swi5} \) cell cycle-regulated transcription factors have essentially identical DNA-binding domains, and previous work identified similarities in their patterns of regulation during the cell cycle. Using ChIP and fluorescence microscopy, we showed that \( \text{Swi5} \) accumulates in the nucleus and binds DNA about 10 min earlier in the cell cycle than \( \text{Ace2} \). Although the \( \text{Swi5} \) protein was degraded from the nucleus during \( G_1 \), \( \text{Ace2} \) levels were constant through the cell cycle, suggesting that \( \text{Ace2} \) down-regulation is achieved by export from the nucleus during \( G_1 \). Two regions of the \( \text{Ace2} \) protein collaborate to determine exactly when during the cell cycle \( \text{Ace2} \) is present in the nucleus: a regulated NES and a regulated NLS.

The \( \text{Ace2} \) NES interacts with the \( \text{Crm1} \) nuclear export receptor (13). GFP-\( \text{Ace2} \) is nuclear when cells are treated with a nuclear export inhibitor, but a GFP-\( \text{Swi5} \) fusion does not respond to the export inhibitor, suggesting that \( \text{Swi5} \) lacks an NES. It has been suggested that \( \text{Cbk1} \) functions to block export of \( \text{Ace2} \) from the nucleus (14). In support of this, the G128E mutation within the NES of \( \text{Ace2} \) partially suppressed the \( \text{Ace2} \) defect in transcriptional activation caused by a \( \text{cbk1} \) mutation (20).6 \( \text{Ace2} \) normally accumulates only in daughter cells, but either an \( \text{Ace2(G128E)} \) mutation or a block of nuclear export by inhibition of \( \text{Crm1} \) activity results in \( \text{Ace2} \) in both mother and daughter cell nuclei (14, 24). Thus, it seems reasonable to propose that the G128E substitution inactivates the \( \text{Ace2} \) NES.

\( \text{Swi5} \) contains a bipartite, basic NLS; deletion of this region eliminates nuclear entry, and this region targets a heterologous protein to the nucleus (1). This region is conserved with \( \text{Ace2} \) as are several consensus \( \text{Cdc28} \) phosphorylation sites near the NLS (12). \( \text{Swi5} \) and \( \text{Ace2} \) are both phosphorylated by the \( \text{Cdc28} \) cyclin-dependent kinase (35, 36), and the \( \text{Cdc14} \) anaphase phosphatase dephosphorylates \( \text{Swi5 in vitro} \) (11) and likely \( \text{Ace2} \) as well. This has led to a model in which phosphorylation during \( G_2 \) acts to inhibit the basic character of the NLS, and the proteins remain cytoplasmic; dephosphorylation by \( \text{Cdc14} \) at anaphase reveals the NLS, and the proteins enter the nucleus. Mutating all three phosphorylated residues in \( \text{Swi5} \) to alanines (the AAA mutant) results in constitutive nuclear localization, and mutation of all three residues is required for this effect (1).

6 M. Sbia and D. J. Stillman, unpublished observations.
Ace2 during the Cell Cycle

The three phosphorylated amino acids in Swi5 are conserved in Ace2, and it was reported that mutation of these three residues to alanines in the Ace2(AAA) mutant results in constitutive nuclear localization (12). In this study Ace2 was expressed at high levels throughout the cell cycle from the strong GAL1 promoter. In contrast, we observed a normal pattern of nuclear localization when Ace2(AAA)-GFP was expressed from the native promoter (Fig. 5). We suggest that the Ace2(AAA) might enter the nucleus prematurely because the AAA substitutions prevent inhibition of the NLS by phosphorylation, but the NES still present in Ace2(AAA) is able to export the protein from the nucleus until the time that the NES is inactivated by Cbk1. The constitutive nuclear localization seen when high levels of Ace2(AAA) were expressed from the GAL1 promoter suggests that the export machinery can be saturated by overexpressed Ace2. Finally combining the G128E mutation in the NES with the AAA mutations that prevent inhibition of the NLS by phosphorylation results in the Ace2(G128E, AAA) protein that was present in the nucleus throughout the cell cycle (Fig. 5). Thus, both the regulated NES and the regulated NLS control the timing of Ace2 accumulation in the nucleus.

We used chimeric fusions between Ace2 and Swi5 to identify the regions of Ace2 with specific activities in vivo. Regions A/B of Ace2 are responsible for determining nuclear accumulation to be later in the cell cycle, whereas regions A/B of Swi5 allow a chimera to accumulate in the nucleus earlier. The daughter-specific localization characteristic of Ace2 also depends on its regions A/B. Importantly the NES is located within this part of Ace2, and this region of Ace2 is not conserved with Swi5. The two-hybrid experiments and assays of the ACE2-dependent activation by a LexA-Cbk1 fusion protein both suggest that regions A/B of Ace2 are required for interaction with Cbk1. Finally the Ace2 property of daughter-specific localization is dependent on Cbk1 (14, 24). Therefore, regions A/B of Ace2 control timing of nuclear accumulation as well as daughter-specific localization and interaction with Cbk1. These results are consistent with the idea that Cbk1 regulates nuclear export and that the presence of Cbk1 exclusively in daughter cells results in Ace2 accumulation in daughter cell nuclei (14). Interestingly we demonstrated previously that region C of Ace2 is sufficient for activating transcription of CTS1 (15), but this region is not required for any localization properties.

The behavior of the Ace2 and Swi5 paralogs also differs during G1 of the cell cycle. It had been reported previously that Swi5 is degraded soon after appearing in the nucleus (37). It was assumed that Ace2 is also degraded after nuclear entry based upon a similar pattern of nuclear localization (3). However, the fact that Ace2 remained detectable on immunoblots throughout the cell cycle suggests that Ace2 is not degraded. Instead we propose that Ace2 is exported from the nucleus later in the cell cycle, consistent with the NES in Ace2 region A (13). The G128E mutation in the NES resulted in Ace2 persisting in the nucleus late into G1 at times when nuclear fluorescence of wild type Ace2-GFP was largely gone. Ace2(AAA)-GFP also persisted in the nucleus later in the cell cycle possibly because it cannot be phosphorylated by the Cdc28/Cnb complex to inactivate the NLS. Ace2 coimmunoprecipitates with the G2 cyclin, and the fraction of cells with Ace2 in the nucleus increases in a clb3 clb4 double mutant, suggesting that the Cdc28/Cnb kinase is involved with excluding Ace2 from the nucleus (35). Thus, there are two mechanisms that contribute to loss of Ace2 from the nucleus after it has activated its target genes, the export region and inhibition of the NLS by phosphorylation.

In contrast to Ace2, Swi5 is degraded not long after entry into the nucleus (37). Analysis of the difference in protein levels of Ace2 and Swi5 using chimeric fusions indicated that regions ABC of Swi5 are required for protein instability. We have a speculative explanation for why regions A, B, and C of Swi5 are required for instability in G1. Swi5 interacts with Pcl2, a specificity subunit for the Pho85 protein kinase, and Pho85/Pcl2 phosphorylates Swi5 in vitro (38). For a number of proteins it has been shown that phosphorylation by Pho85 contributes to protein instability (39). Two-hybrid experiments showed that Swi5 region C was sufficient for interaction with Pcl2 (38). However, there are no Pho85 consensus phosphorylation sites in region C, but the major sites in Swi5 phosphorylated in vitro by Pho85/Pcl2 are in region B (38). Based on these observations, we suggest that region C is required for binding by the Pho85/Pcl2 kinase, which then phosphorylates residues in region B, and this phosphorylation causes Swi5 degradation. Further analysis is needed to understand how differences in post-translational modification, localization, and stability between Ace2 and Swi5 contribute to their divergent transcriptional regulatory roles.

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