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# Regulated nuclear localisation of the yeast transcription factor Ace2p controls expression of chitinase (*CTS1*) in *Saccharomyces cerevisiae*

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**Abstract** The yeast transcription factor Ace2p regulates expression of the chitinase gene *CTS1* in a cell cycle-dependent manner. Nuclear localisation of Ace2p is restricted to late M and early G1 phases of the mitotic cell cycle. We show here that this nuclear localisation is directly associated with regulation of *CTS1* expression. Using a version of Ace2p tagged with a c-myc epitope, we show that the protein is excluded from the nucleus of cells during most phases of the mitotic cell cycle. A mutant derivative in which one threonine and two serine residues, which are candidate phosphorylation sites, were replaced by alanine (to mimic constitutive dephosphorylation) is localised in the nucleus throughout the cell cycle. The mechanism of localisation of Ace2p therefore involves regulation of its phosphorylation state, and closely resembles that used by the homologous transcription factor Swi5p. The wild-type Ace2 protein associates with Cdc28p in vivo, suggesting this may be the kinase that mediates the phosphorylation event. The stability of the protein is greatly reduced in a mutant that is constitutively localised to the nucleus, but is restored in a deletion derivative which remains in the cytoplasm. Ace2p is therefore controlled throughout the cell cycle at three levels: transcription, nuclear localisation, and proteolysis.

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## Introduction

Endochitinase activity in the yeast *Saccharomyces cerevisiae* is encoded by a single chitinase gene, *CTS1*, with two allelic variants (Kuranda and Robbins 1987). Chitinase is responsible for breaking down the chitin septum between mother and daughter cells after cell division. The enzyme is a secretory protein, and is stored in vesicles in the periplasmic space (Elango et al. 1981). The secretory vesicles fuse to the plasma membrane and release the enzyme, some of which apparently binds to the septum via chitin-binding domains. Chitin represents only 1% of the components of the cell wall, and is localised specifically in the septum. Deficiencies in chitinase production therefore lead to defects in cell separation (Kuranda and Robbins 1991; Dohrmann et al. 1992).

Expression of chitinase is controlled by the transcription factor Ace2p, first identified as a regulator of the metallothionein gene *CUP1* (Butler and Thiele 1991; Dohrmann et al. 1992). In strains carrying a deletion of *ace2*, expression of *CTS1* is greatly reduced. Ace2p is very similar, particularly in the C-terminal region that includes the DNA binding domains, to the yeast transcription factor Swi5p, which regulates expression of the gene for the HO endonuclease (Nasmyth et al. 1987; Butler and Thiele 1991). Both proteins recognise the same DNA sequence (Dohrmann et al. 1996). *ACE2* and *SWI5* are regulated throughout the mitotic cell cycle in a similar manner – both are expressed in G2, and the proteins remain in the cytoplasm until late M phase (Nasmyth et al. 1990; Dohrmann et al. 1992). Their target genes (*CTS1* and *HO*, respectively) are expressed in G1.

Despite these similarities, the proteins have very different functions. Swi5p does not normally function as a regulator of *CTS1* expression, though overexpression of *SWI5* can partially compensate for the chitinase

deficiency phenotype of an *ace2* deletion mutant (Dohrmann et al. 1992). As well as regulating the expression of the HO endonuclease (Nasmyth et al. 1987), Swi5p is also required for maximal expression of *SIC1*, *EGT2*, *ASH1*, *CDC6*, *RME1, PCL2* and *PCL9* (Piatti et al. 1995; Toone et al. 1995; Bobola et al. 1996; Kovacech et al. 1996; Toyn et al. 1997; Aerne et al. 1998). Overexpression of *ACE2* can lead to expression of HO in a *swi5*-deleted strain, and *ACE2* plays a role in regulation of some of the other genes. Ace2p and Cts1p (but not Swi5p) are also involved in pseudohyphal growth (King and Butler 1998). There is also a difference in timing between Ace2p- and Swi5p-specific regulation, because in strains deleted for *swi5*, Ace2p-driven expression of both *SIC1* and *EGT2* occurs later in G1 than in the wild type (Kovacech et al. 1996; Toyn et al. 1997).

Regulation of the nuclear localisation of Swi5p has been well characterized. Phosphorylation of three serine residues by the cell cycle kinase Cdc28p causes the protein to remain in the cytoplasm, and dephosphorylation is associated with translocation to the nucleus (Nasmyth et al. 1990; Moll et al. 1991). We show here that entry of Ace2p into the nucleus is regulated in a similar manner, and that constitutive nuclear localisation results in reduced stability of Ace2p, and increased expression of *CTS1*. The stability of a deletion derivative of the protein that remains in the cytoplasm is not affected, however.

## Materials and methods

### Strains and media

The yeast strains used were DTY59 (*MATα*, *his6, leu2-3, -112*, *ura3-52*, *ace1Δ-225*, *CUP1$^{R-3}$*; Butler and Thiele 1991), LKY6 (*MATα*, *his6*, *leu2*::*CTS1-lacZ*, *ura3-52, ace1Δ-225*, *ace2*::*hisG*, *CUP1$^{R-3}$*) (King and Butler 1998), W303/CDC28-HA (*MATα*, *leu2*, *ura3*, *CDC28-HA, his3*) (gift from N. Lowndes, ICRF), CG378 (*MAT***a**, *ade5, canR, leu2-3, -112*, *trp1-289*, *ura3*) (Craig Giroux) and PSY580 (*MAT***a**, *ura3-52, leu2Δ1*, *trp1Δ63*) (Winston et al. 1995). In W303/CDC28-HA the endogenous *CDC28* gene has been replaced with an HA-tagged version (Sorger and Murray 1994). All yeast strains were grown in rich (YEPD) medium or synthetic complete (SC) medium lacking nutrients as specified (Sherman et al. 1986) at 30° C or 25° C.

### Plasmids

To tag Ace2p with an epitope marker, a *NotI* site was introduced immediately upstream of the termination codon by site-directed mutagenesis, using the pALTER mutagenesis kit (Promega) and the oligonucleotide 5′-GAAACTGATGCTGCGGCCGCCTCT GACGAACA-3′. The myc tag sequence was isolated from pUC119 encoding three tandem copies of the c-myc 9E10 epitope (gift from S. Kron and D. Kornitzer) by PCR, using the oligonucleotides 5′-GGGGGCGGCCGCTCCTCTAGAGGTGAACAAAAGT-3′ and 5′-GGGGGCGGCCGCCTATCCGTTCAAGTCTTCT-3′, which place *NotI* sites at either end of the epitope in the same reading frame as the site in *ACE2*. The inserts were verified by sequencing. A *Hin*dIII site was introduced immediately upstream of the *ACE2* start codon to allow the generation of a *GAL1-ACE2* fusion, using the oligonucleotide 5′-TAAAGAAAGCTTTAGG-CCTAAAAACGG-3′. The *GAL1-10* promoter was isolated from pBM272 using *Hin*dIII and *Eco*RI, and cloned into the centromeric vector pRS316 (Sikorski and Heiter 1989) at the same sites. The tagged *ACE2* gene was introduced at the *Hin*dIII site in this plasmid, and is expressed from the *GAL1* promoter. Plasmid pGAL-ACE2myc3 expresses a version of the wild-type Ace2p marked with three copies of the myc tag, under the control of the *GAL1* promoter. Plasmid pGAL-AAAmyc3 is a similar construct, but contains one threonine to alanine, and two serine to alanine substitutions as described below. Plasmid pGAL-HA contains a triple HA tag inserted at the introduced *Not*I site. Plasmid pMM2 contains the entire *ACE2* ORF, together with promoter and terminator sequences, isolated from YEpACEr on a *Hpa*I-*Hin*dIII fragment (Butler and Thiele 1991) and cloned into pRS316. pMM2-TRIP is a similar construct, containing the three alanine substitutions.

The wild-type *ACE2* sequence contains two *Cla*I sites, at codons 62 and 414. To generate an N-terminal truncation, a PCR reaction was performed using the oligonucleotides TRUI (5′-TACTATAACATCGATGAC-3′), which correspond to the region surrounding the first *Cla*I site, and TRU2 (5′-CCCCATCGATT-TTCCCTTATATGGTCAA-3′), which introduces a *Cla*I site at codon 181. The PCR product was used to replace the *Cla*I fragment in the wild-type *ACE2* gene in pGAL-AAAmyc3, resulting in a product that lacks amino acids 181–414. The plasmid is called pGAL-ΔAAAmyc3.

### Directed mutagenesis of threonine/serine codons

Threonine 575 and serines 701 and 714 of Ace2p were replaced by alanines, using the site-directed mutagenesis procedure of Kunkel (1985), and the oligonucleotides 5′-AACAAGAACTTACAG-CGCCCAA-3′, 5′-CATGACACAGCTCCCGTAAAAG-3′, and 5′-CCTAGGACTGCGCCAATG-3′. The mutagenesis procedure was carried out on a 770-bp *Eco*RI-*Hin*dIII fragment, which was later sequenced completely and used to replace the same fragment in the wild-type *ACE2* gene.

### Immunoblot analysis and co-immunoprecipitation

Cells were grown to an $OD_{600}$ of approximately 0.8 in synthetic medium containing raffinose as sole carbon source, induced by the addition of 2% galactose, and allowed to grow for up to 3 h. CG378 cells were arrested with α-factor at 1 μg/ml for 3 h where indicated (see below). Lysates were prepared from up to 100 ml of cells. Cells were pelleted and resuspended in 100 μl of lysis buffer (50 mM TRIS-HCl pH 7.5, 150 mM NaCl, 1 mM $MgCl_2$, 5 mM EDTA, 10 mM NaF, 50 mM *β*-glycerophosphate, 1 mM DTT, 0.5 mM PMSF, 25 μg/ml chymostatin, leupeptin and antipain, 5 μg/ml pepstatin) per 10 ml of cells, and lysed with glass beads. The crude extract was clarified by centrifugation at 13,000 rpm at 4° C for 30 min, and the protein concentration was determined using a protein assay (Bio-Rad). For detection of myc-tagged Ace2p, 100 μg of total protein was fractionated on a 7.0% SDS-polyacrylamide gel, and transferred to nitrocellulose membranes using a semi-dry blotter (C.B.S.). The anti-c-myc monoclonal antibody 9E10 (1 mg/ml) was obtained from Noel Lowndes (ICRF) or purchased from Boehringer Mannheim, and used at a dilution of 1/1000 in 0.5 × blocking solution (Boehringer Mannheim). HRP-linked anti-mouse IgG (Sigma) was used at a dilution of 1/700 and detected by luminol-based chemiluminesence (Boehringer Mannheim; Pierce). Anti-HA monoclonal antibodies (12CA5 at 0.4 mg/ml, Boehringer Mannheim) were used at a dilution of 1/1000 to detect HA-tagged Cdc28 and Ace2 proteins. To verify protein concentrations, membranes were stripped following the manufacturer's instructions, and tubulin levels were measured with the monoclonal antibody YOL1/34 (0.5 mg/ml, Harlan Sera-Labs), at a dilution of 1/500, and HRP-conjugated anti-rat IgG (Sigma). For immunoprecipitation, 250 μg of clarified extract was incubated with 4 μg of 9E10 antibody at 4° C for 1 h. 25 μl of Protein G

beads (Sigma) was added and the incubation continued for a further hour at 4° C. The immunoprecipitated material was washed five times with lysis buffer, resuspended in SDS sample buffer and fractionated by electrophoresis on a 10% SDS polyacyrlamide gel. HA-tagged Cdc28p and c-myc-tagged Ace2p were detected as above.

### Immunofluorescence

The method used was adapted from that of Adams and Pringle (1994). Cells were grown overnight in selective synthetic medium, and expression of the *GAL1* promoter was induced by growth in YP containing 2% galactose for 3 h. CG378 cells were arrested with alpha factor (1 μg/ml) for 45 min. The cells from 5 ml of culture were harvested and fixed in 1 ml 0.1 M potassium phosphate (pH 6.5) containing 4% formaldehyde. Cells were incubated at room temperature for 2 h, washed and resuspended in 250 μl of 0.1 M potassium phosphate pH 6.5, 1.2 M sorbitol. Cell walls were digested for times varying up to 15 min with 38 μg of lyticase (in 50% glycerol) and mercaptoethanol (1.25 μl). The cells were washed and resuspended in 100 μl of 0.1 M potassium phosphate pH 6.5, 1.2 M sorbitol. Then 15 μl of cell suspension was applied to polylysine-coated slides (Sigma) for 3 min, and plunged into methanol at −20° C for 6 min and acetone at −20° C for 30 s. Non-specific antibody binding was blocked by incubation for 30 min in 1% BSA in phosphate-buffered saline pH 7.5. The primary anti-c-myc monoclonal antibody 9E10 was applied at a concentration of 5 μg/ml in blocking solution and a fluoroisothiocyanate (FITC)-conjugated anti-mouse IgG secondary antibody was applied at a concentration of 10 μg/ml in blocking solution (Jackson Immunoresearch Laboratories). The cells were incubated for a minimum of 1 h with 9E10 and 45 min with anti-mouse IgG, and washed with blocking solution between antibody treatments. The cells were also stained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma) at a concentration of 0.5 μg/ml in phosphate-buffered saline pH 7.5, to visualise cell nuclei. The mounting solution used contained 90% glycerol and 10% phosphate-buffered saline pH 7.5. To reduce photobleaching, 1,4-diazabicyclo-[2.2.2]octane (DABCO; Sigma) was added to the mounting solution at a concentration of 2.5%. Cells were visualized under 100× magnification using an Olympus BX60 microscope with a UV light source, and were photographed with Ilford HP400 film at an ISO of 1600.

## Results

### Localisation of Ace2p

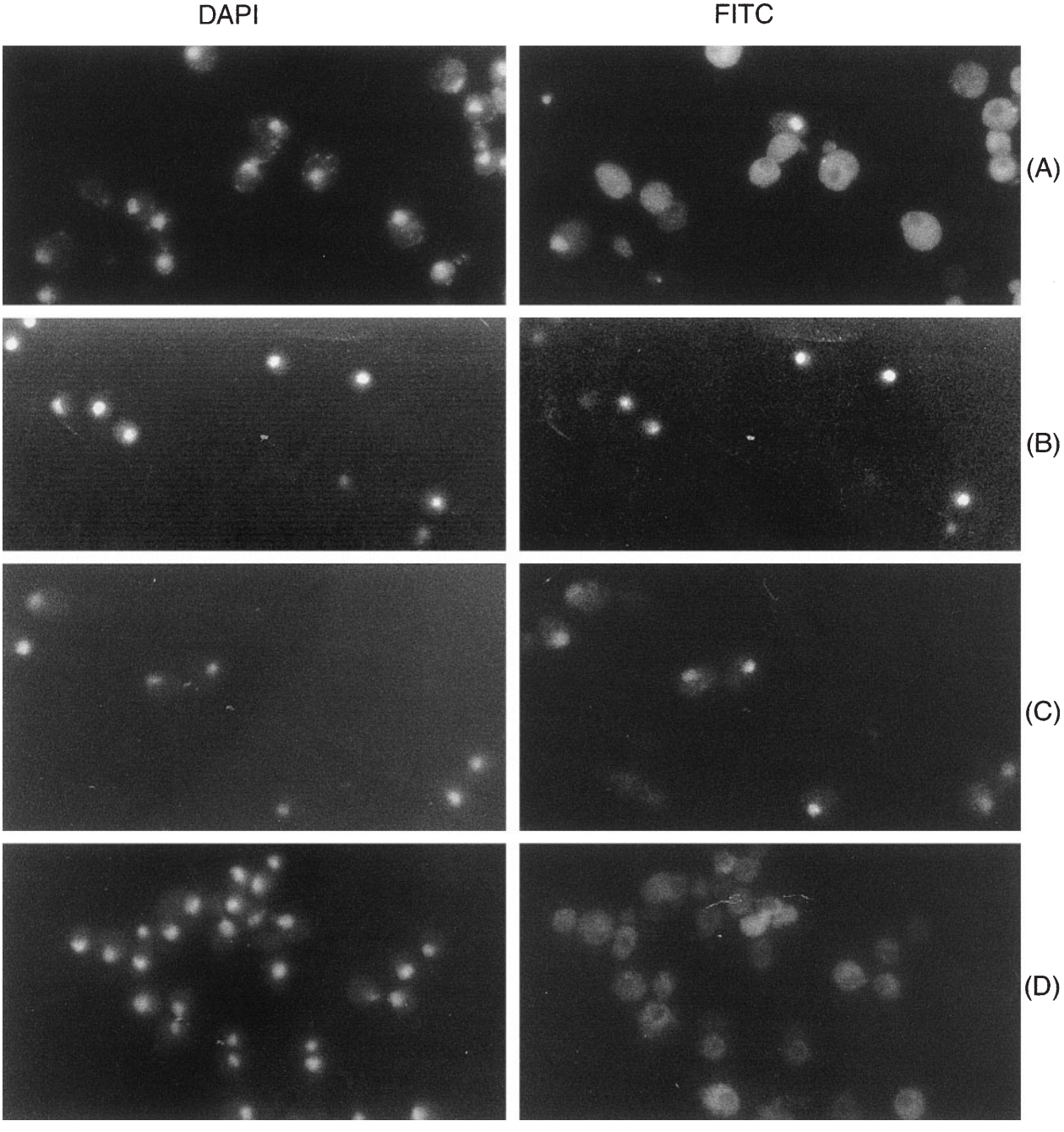
We have previously demonstrated, using an ACE2-*β*-galactosidase fusion, that the location of the Ace2 protein varies during the cell cycle (Dohrmann et al. 1992). In G2 or early M phase cells, Ace2p is found in the cytoplasm, and it translocates to the nucleus in late M phase, remaining there into G1. The similarities in sequence between Ace2p and Swi5p suggested that their localisation might be regulated in similar fashion (Dohrmann et al. 1992). Cell cycle-specific phosphorylation of Swi5p at three serine residues by the kinase Cdc28 causes retention in the cytoplasm; translocation to the nucleus in late M phase is associated with dephosphorylation (Nasmyth et al. 1990; Moll et al. 1991). Two of these serines are conserved in Ace2 (at positions 701 and 714), and the third is replaced by a threonine (at position 575), which is also part of a Cdc28p recognition site (Fig. 1). To investigate the role of these residues in the


**Fig. 1** Comparison of the Ace2p and Swi5p proteins. The two proteins were aligned with the aid of the Gap program (GCG Wisconsin package); identical amino acids are indicated by *vertical lines*, and similar amino acids by *colons* or *periods*. The conserved potential Cdc28p recognition sites are *boxed*. One site overlaps part of the nuclear localization signal in Swi5p (Tebb et al. 1993) that is also conserved in Ace2p (indicated by the *stippled boxes*). The region deleted to generate the truncated ΔAAAp protein is indicated by the *arrows*

localisation of Ace2p, a mutant protein was constructed in which the threonine and the serines were replaced by alanines, to mimic constitutive dephosphorylation (Ace2-AAAp). *β*-Galactosidase fusions proved to be markedly unstable (data not shown), so the wild-type and mutant proteins were tagged with a triple c-myc epitope (Bobola et al. 1996). Expression was driven from the *GAL1* promoter to prevent any effects due to cell cycle-dependent regulation of expression of the constructs. The fusions are biologically active, as they complement the chitinase deficiency phenotype of an

*ace2* deletion mutant (data not shown). The tagged proteins were detected by immunofluorescence.

The subcellular localisation of Ace2p varies with cell cycle phase, even when the gene is ectopically expressed from the *GAL1* promoter (Fig. 2A). In cells in G1 phase (characterised by round unbudded cells with a single nucleus), Ace2p is localised to the nucleus, and no cytoplasmic staining is evident (Fig. 2A). This is more clearly seen in Fig. 2B, where cells have been arrested in G1 with alpha-factor. In most other cells the cytoplasm, but not the nucleus, is heavily stained (Fig. 2A). Ace2p is therefore excluded from the nucleus in these cells, even when expressed from an ectopic promoter, suggesting an active mechanism is required for nuclear entry. In the mutant protein containing the triple alanine substitutions, staining is predominantly nuclear (Fig. 2C). Although not all cells express the protein, when present it is found in the nucleus at all stages of the cell cycle; the cytoplasmic localisation and stage-dependent exclusion from the nucleus described for the wild-type protein is never observed. Substitution of alanine at these positions is therefore associated with increased nuclear localisation of Ace2p, as observed with the corresponding derivative of Swi5p (Nasmyth et al. 1990).



## Stability of Ace2p

Both wild-type and triple-alanine mutant myc fusion proteins were expressed from the *GAL1* promoter, and detected by Western analysis (Fig. 3A). The wild-type Ace2 protein (Fig. 3A, lane 2) was detected at much higher levels than the triple alanine mutant version (lane 3), although screening with an anti-tubulin antibody demonstrates that approximately equal amounts of total protein were loaded in both lanes. This suggests the mutant protein is much less stable than the wild type, and this reduced stability is correlated with its cellular location. It is likely that the change in stability is a direct result of entry into the nucleus rather than an effect of the alanine substitutions on protein structure, as the wild-type Ace2p is also unstable when cells are arrested with α-factor (Fig. 3B). In these cells, Ace2p is also localised in the nucleus (Fig. 2B; Dohrmann et al. 1992). The protein used in this experiment was tagged with a triple HA-epitope, which allows detection at very low levels.

Swi5p is also an unstable protein, and the instability is associated with a region towards the N-terminal end of the protein (Tebb et al. 1993). Ace2p and Swi5p are not very similar in this region, but do share a sequence which may form an α-helical structure (Fig. 1). To determine the role of the N-terminal region in regulating the stability of Ace2p, we generated a protein derivative that contained the three alanine substitutions, but was also deleted for amino acids 181–414 (ΔAAAp). As shown in Fig. 3C, the levels of protein detected are much greater than for the equivalent alanine mutant, which is almost undetectable (Fig. 3C; compare lane 4 with lane 2). The protein levels are also approximately 25% higher than that of wild-type Ace2p. The size of the protein is reduced by 26 kDa, as a result of the deletion. Rather surprisingly however, this mutant derivative is not constitutively localised in the nucleus, but rather is excluded from the nucleus (Fig. 2D). It is therefore apparent that nuclear localisation requires N-terminal sequences, and that stability is directly associated with cellular localisation.

**Fig. 2A–D** A mutant version of the Ace2p (Ace2-AAAp) is constitutively localised to the nucleus. Yeast cultures were transformed with plasmids expressing Ace2p, Ace2-AAAp or a deletion variant (ΔAAAp) from the *GAL1* promoter. All three proteins are tagged with the c-myc epitope, and detected with anti-c-myc (9E10) and FITC-conjugated secondary antibodies. The panels on the *right* (FITC) show detection of the Ace2 proteins, and those on the *left* (DAPI) show the location of the nucleus containing DAPI-stained DNA. In **A**, Ace2p is clearly excluded from the nucleus in most cycling cells, and is found in nucleus only in round unbudded (G1) cells. Panel **B** shows the localisation of Ace2p in α-factor arrested cells. Panel **C** shows Ace2-AAAp, which is predominantly nuclear when present, and is never excluded from the nucleus. Panel **D** shows the localisation of a version of Ace2-AAAp (ΔAAAp), which is deleted for 234 amino acids in the N terminal region. This derivative is always excluded from the nucleus. The host strains used were DTY59 (**A**), CG378 (**B**) and PSY580 (**C** and **D**). The host strain chosen has no effect on the patterns seen

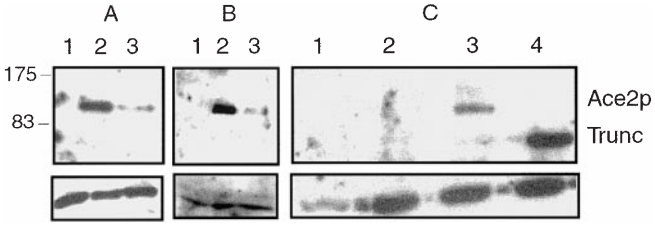



**Fig. 3A–C** Nuclear localisation of Ace2p is associated with decreased stability. **A** A 100-μg aliquot of total protein from cells expressing c-myc tagged Ace2p (lane 2) or the nuclear derivative Ace2-AAAp (Lane 3) from the *GAL1* promoter was fractionated on a 7% SDS gel, and detected using anti-c-myc primary and HRP-linked secondary antibodies. Panel **B** shows 60 μg of total protein from cycling cells (lane 2) or α-factor arrested cells (lane 3) expressing HA-tagged Ace2p. Panel **C** shows the detection of c-myc tagged Ace2-AAAp (lane 2), Ace2p (lane 3) and ΔAAAp (lane 4) in 100-μg aliquots of protein. The wild type Ace2p and truncated (Trunc) proteins are indicated. The *upper* sections show detection of Ace2p, and the *lower* profiles detection of tubulin. Lane 1 is a negative control, and is underloaded in panel **C**

## Ace2p is associated with Cdc28p in vivo

Because approximately 2% of yeast genes encode protein kinases (Hunter and Plowman 1997) it is important to determine which kinase is responsible for phosphorylation of Ace2p in vivo. As Swi5p is phosphorylated by Cdc28p (Moll et al. 1991) we investigated potential interactions of Ace2p with this kinase. A co-immunoprecipitation experiment was carried out using an Ace2 protein tagged with a triple c-myc epitope, and a yeast strain in which the endogenous Cdc28 had been replaced by Cdc28 tagged with an HA epitope (Sorger and Murray 1994). The wild-type and mutant Ace2 proteins were immunoprecipitated using the anti c-myc antibody (9E10). Subsequent screening of the immunoprecipitate with anti-HA antibody (12CA5) revealed the presence of a protein of the same size as Cdc28-HA, which co-immunoprecipitated with Ace2p (Fig. 4, lane 3). The protein was also detected, though in lower amounts, when Ace2-AAAp was immunoprecipitated (Fig. 4, lane 2). This may be due to the reduced stability of the triple alanine construct, or may reflect a biologically relevant difference. The fact that the wild-type Ace2p and Cdc28p co-immunoprecipitate however suggests the two proteins interact in vivo, and the resulting phosphorylation event is associated with retention of Ace2p in the cytoplasm.

## Regulation of *CTS1*

To determine the effect of nuclear localisation of Ace2p on the expression of chitinase, a *CTS1-lacZ* fusion was integrated in the genome of a yeast strain carrying an *ace2* disruption. Expression of chitinase could therefore be monitored by measuring β-galactosidase levels. The wild-type or triple alanine mutant form of Ace2p was supplied on a single-copy plasmid, under the control of

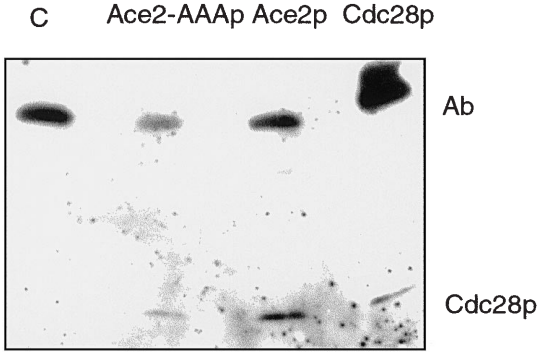



**Fig. 4** Ace2p and Cdc28p form a complex. Ace2-AAAp (lane 2) and Ace2p (Lane 3) were immunoprecipitated from W303 cells expressing Cdc28-HA using anti-c-myc antibody (9E10) and Protein G beads. The immunoprecipitate was then screened with anti-HA (12CA5) antibody to detect Cdc28-HA. Lane 4 shows direct precipitation of Cdc28-HA using the 12CA5 antibody, and lane 1 is a mock precipitation using untagged Ace2p. The heavy chains of the primary antibody (Ab) are indicated

the endogenous promoter. As reported previously, expression of *CTS1* is greatly reduced in the absence of the Ace2 protein (Dohrmann et al. 1992, 1996). In the presence of a wild-type Ace2p, expression of *CTS1-lacZ* is reduced in cells arrested in early M phase by treatment with nocodazole, as compared to cycling cells (Fig. 5). When expression is driven from the triple alanine mutant however, *CTS1-lacZ* levels are higher than wild type in cycling cells, and remain high in nocodazole-arrested cells. High-level expression of *CTS1* therefore correlates with increased amounts of Ace2p in the nucleus.

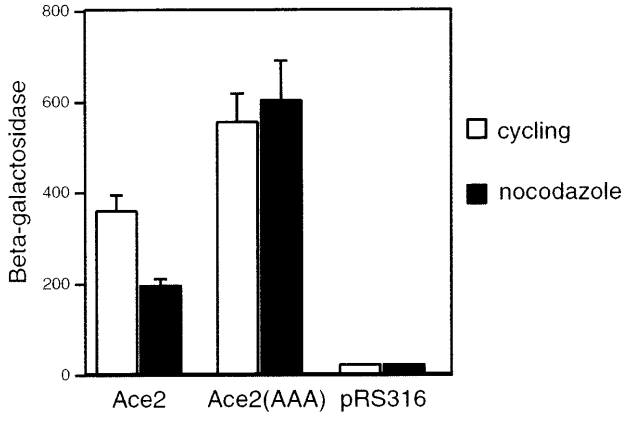



**Fig. 5** Constitutive nuclear localisation of Ace2p results in constitutive expression of *CTS1*. Yeast strain LKY6 carrying an *ace2* deletion and an integrated *CTS1-lacZ* fusion was transformed with the single-copy plasmid pRS316 [pRS316], the same plasmid carrying a wild-type *ACE2* gene [Ace2], or the triple alanine mutant [Ace2(AAA)]. The *ACE2* derivatives were expressed from the endogenous promoter. $\beta$-Galactosidase levels were measured in cycling cells (*empty bars*), and in cells arrested in early M phase by treatment with nocodazole (*filled bars*). The values are averages from at least three independent transformants, and the standard errors are indicated

## Discussion

Our results suggest that cell cycle-regulated entry of Ace2p into the nucleus is associated with dephosphorylation of one threonine and two serine residues, and the mechanism closely resembles that previously described for Swi5p. The protein is associated with Cdc28 kinase in vivo, suggesting that this may be the kinase responsible for the phosphorylation event. Alteration of phosphorylation levels is a common mechanism for regulating transport of diverse proteins to the nucleus (Jans et al. 1995; Vandromme et al. 1996). In many cases phosphorylation is associated with transport into the nucleus (Mosialos et al. 1991; Rihs et al. 1991; Vancurova et al. 1995), and phosphorylation of the SV40 large T antigen increases the binding affinity of the nuclear localisation signal for the nuclear import machinery (Hübner et al. 1997; Xiao et al. 1998). However, phosphorylation by a cell cycle-dependent kinase (CDK) has been shown to correlate with retention in the cytoplasm (Moll et al. 1991; Sidorova et al. 1995; O'Neill et al. 1996), and dephosphorylation of the NF-AT family of transcription factors by calcineurin is associated with nuclear localisation (Beals et al. 1997; Scott et al. 1997). Our results suggest that the dephosphorylation event is a positive signal for nuclear transport, as the wild-type Ace2 protein is actively excluded from the nucleus in most cycling cells, even when the protein is overexpressed from the *GAL1* promoter (Fig. 2A). This exclusion was not previously described for Swi5p. A mutant version of Ace2p, containing three alanine substitutions, is constitutively localised to the nucleus (Fig. 2C).

Although Swi5p is a major activator of expression of *HO*, constitutive nuclear entry of Swi5p does not result in constitutive expression of *HO*. The transcription factor Swi6p also regulates *HO* expression, and exhibits phosphorylation-dependent cell cycle-regulated nuclear localisation (Sidorova et al. 1995). Constitutive nuclear localisation of Swi6p also does not affect cell cycle regulation of *HO* (Sidorova et al. 1995). We demonstrate here that a mutant form of Ace2p which is localised to the nucleus throughout the cell cycle (Fig. 2C) causes an associated increase in expression of *CTS1* (Fig. 5). Expression of *CTS1* directed by the Ace2p triple alanine mutant remains high in cells arrested in early M phase (Fig. 5). The localisation of Ace2p therefore plays an important role in regulating expression of its target gene. Ace2p belongs to a growing number of transcription factors whose localisation is controlled, and this is an important element in the regulation of their activity (Jans 1995; Vandromme et al. 1996). It is likely that retention of transcription factors in the cytoplasm, owing to phosphorylation by CDKs, is a major mechanism of transcriptional regulation in eukaryotes, as the Swi5 protein is localised in a similar manner when expressed in mammalian cells (Jans et al. 1995).

The stability of Ace2p varies with cell cycle phase, and dramatically decreases when the protein is localised

in the nucleus (Fig. 3). Swi5 protein is also extremely unstable when localised in the nucleus (Tebb et al. 1993). Cell cycle-regulated proteolysis of specific proteins has been implicated in driving cell cycle progression (King et al. 1997). Many cyclins contain PEST sequences (Rechsteiner and Rogers 1996), which target them to a ubiquitin-dependent conjugation pathway leading to degradation by the 26S proteasome (Hilt and Wolf 1996). Proteolysis at the metaphase-anaphase transition is directed through a different ubiquitin-dependent pathway, represented by the Anaphase-Promoting Complex (King et al. 1997; Zachariae et al. 1997). The degradation signal is a 9-amino acid motif called the destruction box (D box). Swi5p contains a PEST sequence that is detected using the program PESTFIND (Rechsteiner and Rogers 1996), but this motif is not in the region determined to be responsible for instability (Tebb et al. 1993). The Ace2 protein sequence does not contain any regions with close matches to either PEST sequences or D-boxes. The region of Swi5p that confers instability has been localised to a region between amino acids 182 and 326 (Tebb et al. 1993). Comparison of this sequence with Ace2p reveals the presence of two conserved regions, a glutamine-rich region which may form an amphipathic α-helix between residues 186 and 234 of Ace2p, and a second region between amino acids 290 and 322 (Fig. 1). Both of these regions were deleted in the stable derivative described here. It is therefore possible that degradation of Ace2p and Swi5p is regulated through one or both of these regions.

Ace2p and Swi5p are very similar, both in sequence and in function. Both proteins bind to the same DNA sequence in vitro (Dohrmann et al. 1996) and we have now demonstrated that regulation of nuclear localisation of both is similar. However, nuclear localisation of Ace2p requires sequences in the N-terminal segment of the protein, which has not been reported for Swi5p (Fig. 2D). The two transcription factors also activate expression of different target genes: Swi5p, but not Ace2p, regulates expression of the genes for the endonuclease HO and the cyclin-like proteins Pcl2 and Pcl9 (Aerne et al. 1998), and Ace2p alone regulates expression of *CTS1* (Dohrmann et al. 1992). Disruption of *ACE2*, but not *SWI5*, allows production of pseudohyphae in some genetic backgrounds (King and Butler 1998). Both proteins are required for maximal expression of *RME1* (which encodes a negative regulator of meiosis) (Toone et al. 1995), *ASH1* (required for mother cell-specific expression of *HO*; Bobola et al. 1996), the cyclin kinase inhibitor Sic1 (Knapp et al. 1996; Toyn et al. 1997) and the early G1 transcript *EGT2* (Kovacech et al. 1996). Interestingly, expression of the Ace2p-driven *CTS1* gene occurs later in G1 than that of the Swi5p-driven *SIC1* (Toyn et al. 1997). In a *swi5*-deleted strain, Ace2p-driven expression of *SIC1* is later then Swi5p-regulated expression, and coincides with expression of *CTS1*. *SIC1* is also expressed during telophase, while *CTS1* is not. Expression of *EGT2*, which is primarily controlled by Swi5p, is also delayed in a *swi5* deletion background (Kovacech et al. 1996). These differences may be due to a difference in the timing of nuclear localisation of Ace2p and Swi5p which is not detectable in our experiments, or may be due to different interactions between Swi5p and Ace2p with ancillary proteins. Swi5p binding to the *HO* promoter is enhanced by the Pho2p homeodomain factor (Brazas and Stillman 1993; Brazas et al. 1995), but Ace2p does not interact with Pho2p (Dohrmann et al. 1996). It also appears that some factors prevent either Ace2p or Swi5p binding to the wrong promoters. A series of mutations in the *CTS1* promoter (NCE mutations) allow Swi5-driven expression of *CTS1* in the absence of Ace2 (Dohrmann et al. 1996). Mutations in *SIN5* allow Ace2p-driven expression of *HO*, suggesting Sin5p may normally prevent Ace2p binding to the *HO* promoter (Stillman et al. 1994; Dohrmann et al. 1996). It will be interesting to determine the range of genes regulated by the two transcription factors and their degree of overlap, and experiments are underway to investigate the role of the timing of nuclear entry and proteolysis on the functions of these two proteins.

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