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EGT2 Gene Transcription Is Induced Predominantly by Swi5 in Early G_1

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In a screen for cell cycle-regulated genes in the yeast *Saccharomyces cerevisiae*, we have identified a gene, *EGT2*, which is involved in cell separation in the G_1 stage of the cell cycle. Transcription of *EGT2* is tightly regulated in a cell cycle-dependent manner. Transcriptional levels peak at the boundary of mitosis and early G_1. The transcription factors responsible for *EGT2* expression in early G_1 are Swi5 and, to a lesser extent, Ace2. Swi5 is involved in the transcriptional activation of the *HO* gene during late G_1 and early S phase, and Ace2 induces *CTS1* transcription during early and late G_1. We show that Swi5 activates *EGT2* transcription as soon as it enters the nucleus at the end of mitosis in a concentration-dependent manner. Since Swi5 is unstable in the nucleus, its level drops rapidly, causing termination of *EGT2* transcription before cells are committed to the next cell cycle. However, Swi5 is still able to activate transcription of *HO* in late G_1 in conjunction with additional activators such as Swi4 and Swi6.

One of the most crucial phases in the cell cycle of a yeast cell is G_1. At this stage, a cell has the potential to undergo different developmental fates. Under normal nutritional conditions, a cell enters S phase and continues to divide. Under certain starvation conditions, however, a haploid cell arrests in G_0 (21, 52), whereas diploid cells enter the meiotic division cycles (27) or undergo pseudohyphal growth (18). Also, pheromones signal haploid cells to arrest at a point in late G_1 called Start and to prepare for mating in order to continue growth as a diploid organism.

If nutritional conditions allow a yeast cell to divide, it passes Start and is irreversibly committed to a new round of cell cycle progression. While the regulation of this transition has been extensively studied (for a review, see reference 30), little is known about the events that take place in early G_1, the stage at which the cell has just finished mitosis and has not yet accumulated sufficient levels of active S-phase-promoting kinase. At this stage, cell separation takes place resulting in a larger mother cell and a smaller daughter cell. To separate a daughter cell from its mother, specific enzymatic processes which lead to partial degradation of cell wall components at the mother-daughter cell junction are activated. One gene involved in this process is *CTS1*, which encodes chitinase (25). This enzyme partially removes chitin, which is deposited in the cell wall between mother and daughter cell during bud formation and septation. The removal of chitin is a prerequisite for cell separation. Mutant cells which lack chitinase grow as large cell clusters (25). In addition to chitin, other cell wall components such as glucans and mannoproteins have to be removed to complete yeast cell division (22).

CTS1 is transiently expressed. It is activated at its execution point in early G_1 by the transcription factor Ace2 (9, 14). Transient expression has been attributed to a number of genes. It has been estimated that up to 250 genes are expressed in *Saccharomyces cerevisiae* in a cell cycle dependent manner (38). It is now well-established that transcriptional activation of particular genes contributes to the precise completion of key cell cycle events such as DNA replication and mitosis. The G_1 cyclin genes, *CLN1* and *CLN2*, are expressed only in G_1 (13, 32, 55) where they induce budding and activate transcription of *CLB5* and *CLB6* genes, which themselves are necessary for S-phase entry and spindle formation (16, 47). The G_2 cyclins, Clb1 to Clb4, are synthesized in late S phase and during G_2 phase (17, 41, 50). Furthermore, it has been shown that the transcriptional activation of the G_2 cyclin gene *CLB2* in late S phase not only leads to activation of mitotic kinase but also is required for the transcriptional repression of the G_1 cyclin genes, *CLN1* and *CLN2* (2). This change of gene expression ensures that G_1 kinase cannot be formed in a cell cycle stage where it may interfere with normal cell cycle progression.

Recently, we developed a screen for cell cycle-regulated genes in *S. cerevisiae* with the aim of detecting genes whose expression is rate limiting for ordered cell cycle progression (38). This screen was based on a Northern blot analysis of temporally staged RNAs that were probed with individual DNA fragments of single phage from Maynard Olson's ordered λ phage library (26, 36, 38) and described the transcriptional behavior of approximately 3,000 genes (39). To investigate the regulation of cell growth in early G_1, we scanned our data for genes expressed during this stage with the aim of testing them for a possible function in early cell cycle events. This scan turned up two genes, *EGT1* (38) and *EGT2*. While a function for *EGT1* is not yet known, analysis of *EGT2* suggests a role in the correct timing of cell separation after cytokinesis. Its transcription is activated at the boundary of mitosis and early G_1 and inactivated again before cells pass Start. This identifies *EGT2* as a cell cycle-regulated gene that is exclusively expressed at this early cell cycle stage. Our work detects the transcription factor Swi5 as the main activator of *EGT2*. Swi5 has previously been found as one of the activators of the *HO* gene, whose expression takes place in late G_1 at a point in the cell cycle at which *EGT2* is not transcribed (7, 33, 34). We present two models which explain how cell cycle-specific transcription may be induced by Swi5 at two different cell cycle stages.

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TABLE 1. *S. cerevisiae* strains used in this study

Strain[a]	Genotype	Source or reference
Y32	*MAT***a**	W303
Y33	*MAT*α	W303
Y111	*MAT***a**/*MAT*α	W303
Y121	*MAT***a** *egt2*::*hisG-URA3-hisG*	This work
Y168	*MAT***a** *cln1*::*hisG cln2*::*del cln3*::*LEU* YCp *GAL-CLN1*(*URA3*)	12
Y203	*MAT***a**/*MAT*α *egt2*::*HisG*/*egt2*::*HisG*	This work
Y213	*MAT***a** *egt2*::*hisG*	This work
Y214	*MAT***a** *cdc28-4*	50
Y272	*MAT***a** *swi5*::*LEU2*	This work
Y286	*MAT***a** *swi5*(*S522A S646A S664A*)	28
Y287	*MAT*α *ace2*::*hisG-URA3-hisG*	This work
Y288	*MAT***a**/*MAT*α *ace2*::*hisG-URA3-hisG*/*ACE2 swi5*::*LEU2*/*SWI5*	This work
Y479	*MAT***a**/*MAT*α *ace2*::*hisG-URA3-hisG*/*ace2*::*hisG*	This work
Y838	*MAT***a** *hml***a** *HMR***a** *HO*::*URS2del ade2-1 can1-100 trp1-1 leu2-3 ura3 cdc15-2*	38
Y1170	*MAT***a** *gal2 bar1*::*URA3*	38

[a] Except for Y838 and Y1170, which have been described elsewhere, all strains were isogenic derivatives of the Y32 strain, whose full genotype is *MAT***a** *ho ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 GAL psi*$^+$. The original *cdc28-4* mutant was extensively backcrossed and thus made isogenic with Y32.

MATERIALS AND METHODS

Yeast strains and media. The yeast strains used in this study are listed in Table 1. Unless otherwise stated, cells were grown at 30°C in YEP (1% yeast extract, 2% Bacto Peptone, 50 mg of adenine per liter) supplemented with 2% glucose (YEPD), with 2% raffinose and 2% galactose (YEPraf+gal), or with 2% raffinose (YEPraf). Synthetic medium lacking uracil was prepared as described previously (43).

Plasmid constructions and genetic manipulations. DNA manipulations were performed by standard techniques (45). The DNA fragments of phage c2425 (according to Maynard Olson's classification) that hybridized with *EGT2* RNA have been sequenced as described previously (8). The detected open reading frame encoding *EGT2* was disrupted by the one-step gene disruption technique (44). The 1,540-bp *Eco*RV-*Spe*I fragment of the coding region was then replaced with a 3.8-kb *hisG-URA3-hisG* fragment (1). The diploid strain Y111 was transformed with the linearized *egt2* deletion construct by the alkali cation method as described previously (19) and selected on agar plates without uracil. Southern blots verified the correct replacement of the *EGT2* gene with the deletion construct. Haploid URA$^+$ clones (Y121 is one of them) were obtained by sporulation and dissection. The *ura3 egt2* mutant (Y213) was obtained from Y121 by selecting against *URA3* on minimal medium plates containing 5-fluoroorotic acid (Sigma) (3).

To restore the wild-type phenotype to the *egt2* deletion, a 6.5-kb fragment (*Hin*dIII-*Eco*RI) encompassing the entire *EGT2* gene together with its 5′ and 3′ flanking regions was cloned into the pFL38 vector (4) and transformed into the deletion mutant (Y213).

Overexpression of *EGT2* was accomplished with the *GAL1* promoter to drive the gene. The N-terminal part of the *EGT2* coding region was replaced with a PCR fragment containing an *Eco*RI site upstream of the putative start codon, and the resulting 4.34-kb *Eco*RI fragment was cloned into the *Eco*RI site of the YCp50 vector under control of the *GAL1-10* promoter fragment (described in reference 8). To express *SWI5* and *SWI5-AAA* from the *GAL1-10* promoter, the *cdc28-4* strain (Y214) was transformed with the centromeric plasmid YCp *GAL-SWI5* and YCp *GAL-SWI5-AAA* (28, 33).

To disrupt the *ACE2* gene (kindly provided by Dennis J. Thiele), the internal 2.12-kb *Nru*I-*Sma*I fragment was replaced with the 3.8-kb *hisG-URA3-hisG* fragment. The resulting construct was linearized and transformed into Y33. A positive clone (Y287) was verified by Southern blot hybridization and showed the clumpy phenotype characteristic of cells lacking the *ACE2* gene product (9).

swi5 ace2 double mutants were obtained by crossing strains Y272 and Y287. The resulting diploid strain, Y288, was sporulated, and tetrads were dissected. Double-mutant strains were selected for their ability to grow on media lacking both leucine and uracil.

Cell biological and biochemical techniques. Several methods were used to synchronize yeast cultures. Either cells were arrested with pheromone α-factor in G_1 and released from the block by resuspending the cells in medium lacking α-factor or cells containing the *cdc15-2* thermosensitive allele were arrested at the restrictive temperature and synchronously released at the permissive temperature (38). To arrest strain Y272 (*BAR1*$^+$), 100 μg of α-factor per ml was added. A third way to synchronize cells involved reversible deprivation of the G_1 cyclin function. Cells of strain Y168 were grown to early exponential phase in YEPraf+gal and arrested in YEPraf. After 2.5 h of incubation, all cells arrested uniformly in G_1 and were released from the block by the addition of galactose. Mitotic arrest with nocodazole was performed as described previously (38).

To express *SWI5* ectopicaly in late G_1, stationary *cdc28-4* cells (Y214) transformed with a centromeric plasmid carrying either wild-type *SWI5* or *SWI5-AAA* under the *GAL1* promoter were incubated in fresh YEPraf at 37°C for 1 h and then supplied with galactose. After 1 h of induction, glucose was added to repress *SWI5* and *SWI5-AAA* expression again.

To visualize chitin, cells were grown to early logarithmic phase, sonicated, and fixed in 3.7% formaldehyde–0.1 M potassium phosphate (pH 6.4) for 1 h at room temperature. They were then washed three times in 0.1 M potassium phosphate buffer (pH 6.4) and stained with Calcofluor White M2R New (Sigma) as described previously (40). Stained cells were mounted onto polylysine-coated slides and examined with a Leitz model DMRX fluorescence microscope.

For flow cytometric DNA quantitation of synchronous cultures of Y32 and Y213, the α-factor-arrested cells were released into prewarmed fresh YEPD. Samples were taken every 10 min and prepared for flow cytometry as described in reference 16.

RNA was isolated, and Northern blot analysis was performed as described previously (10, 38) with 10 μg of total RNA per lane. Probes for hybridization were prepared as described previously (38).

The β-1,3-glucanase assay was performed as described in reference 48. Briefly, cells were grown to an A_{600} of 0.9, chilled on ice, mildly sonicated, washed, and resuspended in 1 ml of assay buffer (50 mM potassium phosphate [pH 7.5]). Recombinant β-1,3-glucanase (ICN) was added to a final concentration of 1 U/μl (48) and incubated for 20 min at 30°C in a shaking water bath. In some cases either 2-mercaptoethanol (10 mM) or proteinase K (50 μg/ml; Merck) was added. Cell clusters were monitored by microscopic observation.

RESULTS

***EGT2* codes for a protein with a novel structure.** With the aim of studying early cell cycle events in the yeast *S. cerevisiae*, we searched our data collection, which we have put together in a screen of Maynard Olson's ordered λ phage library (38), for genes that are expressed in early G_1. We identified a DNA fragment of phage c2425 containing a novel gene which appeared to be strongly activated in early G_1 (data not shown). We call this gene *EGT2* (early G_1 transcript 2). It is positioned on the end of the left arm of chromosome XIV.

We sequenced the region that contains *EGT2* and detected an open reading frame of 3,123 bp encoding a protein with a length of 1,041 amino acids (Fig. 1). Its DNA sequence has recently been released as part of a cosmid sequence which is available to the public under GenBank accession no. Z46259. There, *EGT2* is represented by the gene *NO320*.

The most remarkable structural feature of Egt2 protein was an array of nine 35-amino-acid repeats of a novel type in its C-terminal part (Fig. 1, underlined sequences). While repeats 2, 4, 5, and 6 are particularly conserved, the others are rather distantly related. Repeats 7 and 8 are almost identical. The central parts of all repeats contain predominantly neutral and negatively charged residues. Their C-terminal regions, however, carry small clusters of hydrophobic amino acids. Searches of the available databases did not reveal any homology to known motifs.

Egt2 also contains a putative signal sequence with a length of 18 to 20 amino acids at its N terminus (Fig. 1, dotted line). The sequence shares general features with other known leader peptides (53, 54). This finding suggests that the protein is transported via the secretory pathway to its final position where it may function. Furthermore, a 13-amino-acid hydrophobic tail starting with Ile-1029 at its C terminus (Fig. 1, sequences underlined with circles) suggests that Egt2 may be attached to a membrane.

Deletion of *EGT2* delays cell separation. To identify a function for Egt2, we deleted one copy of *EGT2* in the diploid strain Y111. Sporulation and dissection of tetrads yielded four viable segregants. Microscopic observation of *egt2* cells of an early-log-phase culture after sonication revealed the presence of clusters of three or four cells (Fig. 2A). In addition, fluo-

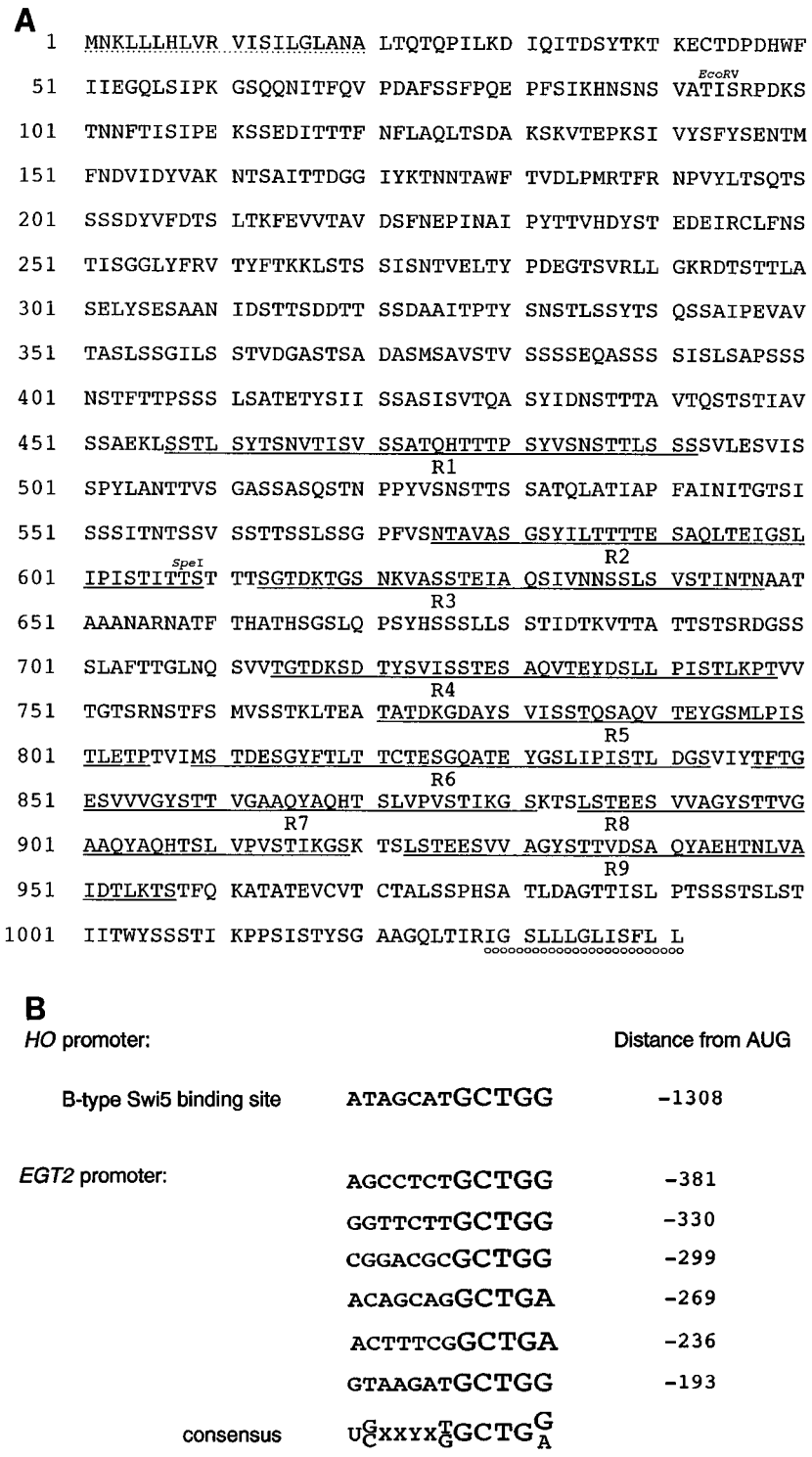

FIG. 1. (A) Sequence of the Egt2 protein. A putative leader peptide at the amino-terminal end of the Egt2 protein is marked with a dotted line. The nine 35-amino-acid repeats (R1 to R9) are underlined with solid lines while the stretch of hydrophobic amino acids at the very carboxy terminus is indicated by open circles. The *Eco*RV-*Spe*I fragment has been deleted in the disruption construct. (B) Putative Swi5 binding sites in the *EGT2* promoter. The sequences of the six putative binding sites are shown, and a consensus site is derived, where U represents a purine, Y indicates a pyrimidine, and X is any base. The sequences are compared with the B-type Swi5 binding motif of the *HO* promoter, which is in opposite orientation to the *EGT2* sequences.

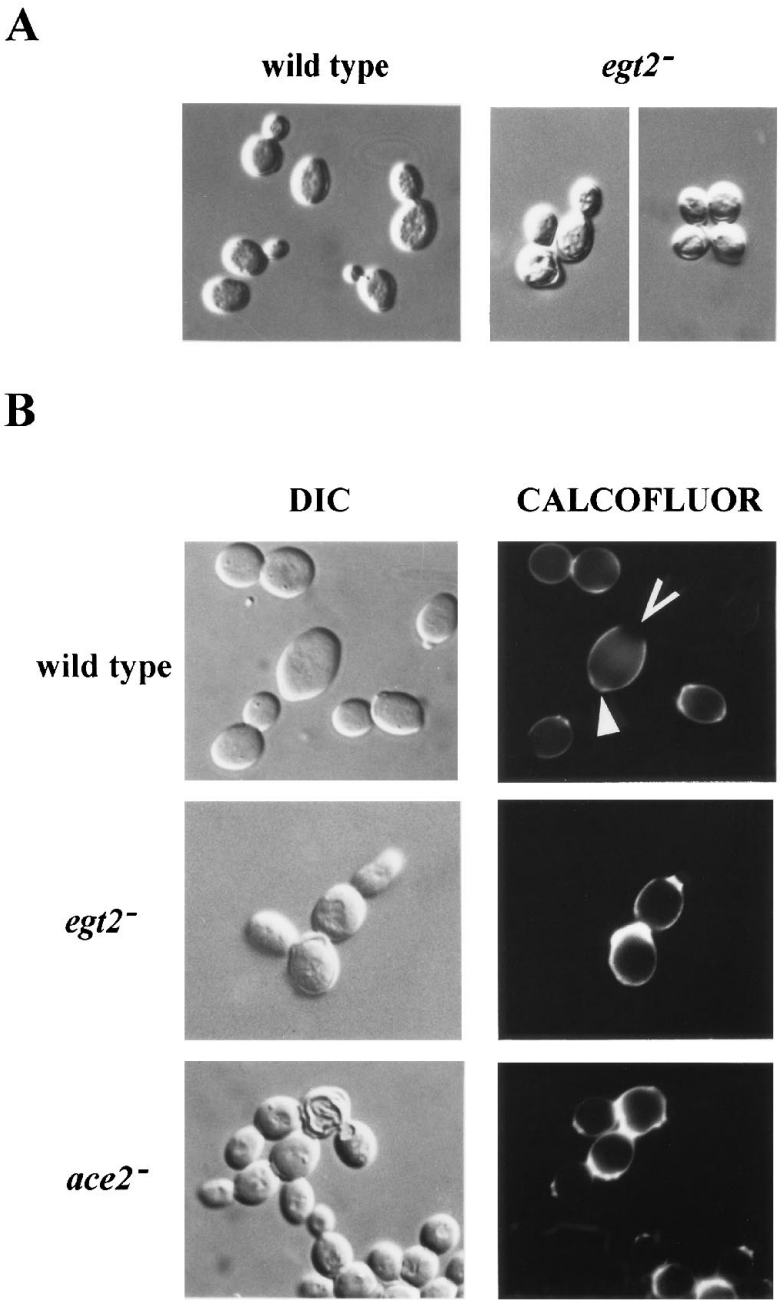

FIG. 2. *egt2* cells form clusters of up to four members. (A) Haploid wild-type (Y32) and *egt2* (Y213) cells from exponentially growing cultures (optical density at 600 nm of 0.9) were chilled on ice, mildly sonicated, and mounted in glycerol on polylysine-coated slides. (B) Egt2 deficiency does not affect chitin degradation during cell separation. Diploid wild-type (Y111), *egt2* (Y203), and *ace2* (Y479) cells were grown to early log phase, chilled on ice, mildly sonicated, and stained with Calcofluor White to visualize chitin. On the surface of wild-type cells, chitin shows a typical pattern of distribution: brightly stained bud scars (normal chitin distribution; arrowhead) and dark birth scars (no chitin; caret). For clustered *egt2* cells, a similar pattern is discernible although cells have not yet separated. In contrast, the neck region of *ace2* cells does not show any sign of chitin degradation and displays uniform calcofluor staining on both mother and daughter sides. DIC, differential interference contrast.

rescence-activated cell sort analysis (FACS) of haploid cycling *egt2* cells showed that the number of cells with a 1N DNA content is reduced approximately threefold compared with wild type cells (Fig. 3). To show that lack of *EGT2* function is responsible for this mutant phenotype, we expressed the *EGT2* gene ectopically in *egt2* cells. This expression entirely restored the *EGT2* phenotype of the *egt2* cells (data not shown). The behavior of mutant cells suggests that *EGT2* is involved in cell separation. Since we never observed clusters of more than four cells, we assumed that *egt2* cells do separate but at a later stage of the cell cycle than wild-type cells.

To test this, we carried out a FACS analysis of temporal staged cells to examine division behavior of cells that lack functional Egt2. We arrested a wild-type strain (Y32) and an isogenic strain with a deletion of *EGT2* (Y213) with pheromone α-factor in G_1. After 2.5 h of arrest we observed that

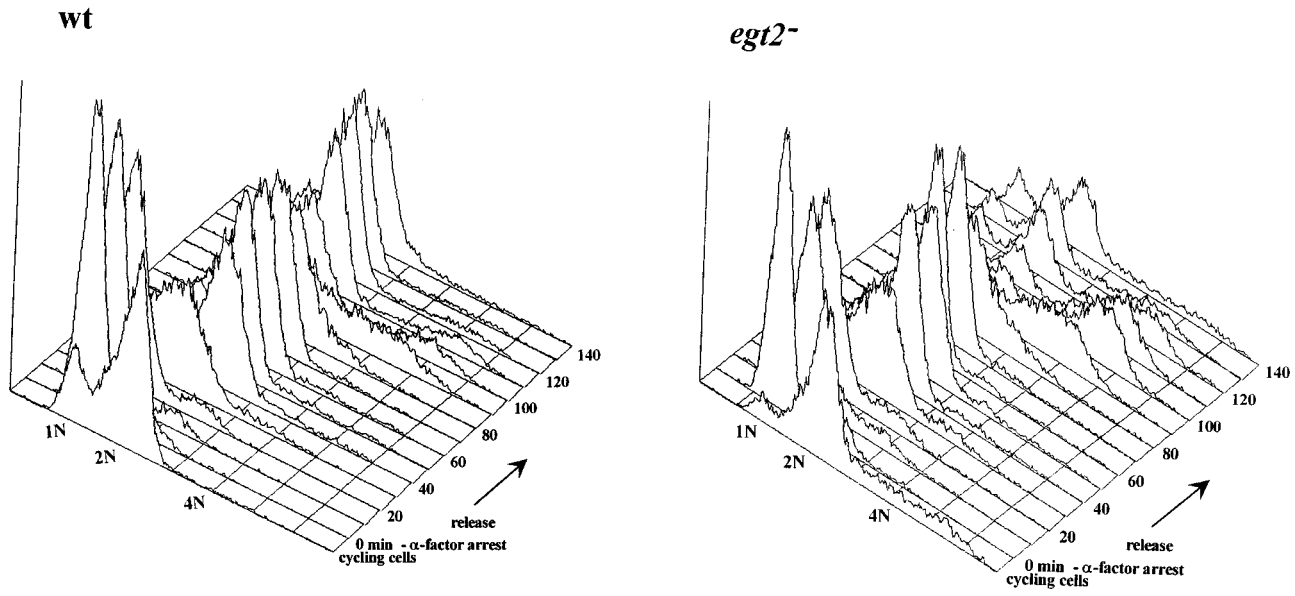

FIG. 3. *egt2* cells prevalently fail to separate in G_1 but do so later in the cell cycle as shown by FACS analysis of a synchronously growing haploid *egt2* (Y213) cell culture. The first lane shows an analysis of exponentially growing cells. The time point 0 min represents cells arrested by α-factor for 2.5 h. The subsequent time points indicate the time of release (in minutes) from the G_1 block. In the second cell cycle after release, the majority of *egt2* cells replicate their DNA before cell separation occurs, which is indicated by a predominant 4N population (90 to 130 min). Contrary to the mutant, only a minority of wild-type cells (Y32) replicate their DNA before separation. This behavior, however, can be attributed to the long pheromone arrest.

most cells of the mutant strain separated to form single cells as the wild-type strain did (Fig. 3). The release from the α-factor block shows that the 1N peak, which accounts for cells in G_1, decreases quickly to generate a 2N peak, representing cells in G_2 after 30 min from the release in both cases. At 80 min after release they enter the second cell cycle and start to replicate their DNA again. Here, the mutant strain shows a predominant 4N peak, representing the four-cell clusters, which lasts up to 140 min. At this time point the four-cell clusters start to separate into two dumbbell-shaped cells, which are in the G_2 stage of the second cell cycle after release since their DNA content is doubled. The small 4N peak appearing 80 min after release in the case of wild-type cells can be attributed solely to the long α-factor arrest. This arrest leads to enlarged G_1 cells that in this strain have an accelerated initiation of replication in the second cycle after release, which results in the lack of reappearance of a G_1 peak in the FACS analysis. This outcome suggests that the *EGT2* gene product is involved in the precise timing of cell separation after nuclear division has occurred. It does not affect DNA replication since this would lead to an altered growth rate and cell viability.

Delayed separation of mutant cells might be caused by incomplete cytokinesis. This would hint a possible role of *EGT2* either in the last stage of mitosis or in septum formation between mother and daughter cells. To examine whether this is the case, we treated cycling cells with Glusulase to remove the cell wall. If mother and daughter cells have not yet assembled membranes at the bud neck, we would expect to see either clustered spheroplasts or, in case of severe damage, dead cells and ghosts. However, we observed the disappearance of cell clusters without any visible breakage of spheroplasts. Furthermore, using plating assays, we found that these spheroplasts are still viable (data not shown). In a third experiment we broke cells partially open by vortexing them with glass beads. Breakage of individual cells in clusters should result in dead neighbors if cytokinesis has not yet been completed. Phase-contrast microscopic examination, however, showed that this was not the case. From this result, we concluded that the delay of cell disjunction is not caused by retarded mitotic events but that cytokinesis has already been completed. These findings rather suggest that a retarded cell wall degradation between mother and daughter cell leads to the delayed separation.

***Egt2* is involved in mother-daughter cell disjunction.** The cell wall of *S. cerevisiae* has a bilayered structure in which the outer layer is formed by a cross-linked network of mannoprotein and the inner one is formed by the polysaccharides glucan and chitin (for a review, see reference 22). Chitin is predominantly deposited in the neck region between a mother cell and its growing bud (22, 49). In early G_1, during separation, it is partially digested by a chitinase encoded by *CTS1* (25). At the mother cell this process gives rise to a bud scar; at the daughter cell it removes the chitin moiety at the birth scar (40). To test whether retarded degradation of chitin is the cause of delayed separation of *egt2* cells, we used Calcofluor White to compare chitin deposits at the mother-daughter cell junction in diploid wild-type, *egt2*, and *ace2* cells. As seen in Fig. 2B, the wild-type cells show normal chitin distribution with large deposits at the bud scar and no chitin at all at the birth scar. *egt2* cells show a similar chitin pattern. Although mother and daughter cells are still connected, it is evident that chitin has been removed from the wall of the daughter's part of the junction while the mother cell wall shows chitin deposits. A completely different picture is obtained for *ace2* mutants. These mutant cells cannot synthesize chitinase, since *ACE2* encoding the transcription factor that activates *CTS1* has been deleted (14). They still contain a chitin ring around the neck between mother and daughter cells. These data show that Egt2 deficiency does not hinder chitin degradation, but that it is involved in degradation of other cell wall components, such as mannoproteins and glucans. While treatment of *egt2* cells with recombinant β-1,3-glucanase or proteinase K did not separate the clusters, the double digestion led to their complete disassembly and, as in the case of wild-type cells, to formation of spheroplasts (data not shown). Since it is known that the glucan layer is respon-

sible for the mechanical strength of the cell wall but not the mannoproteins (22), we suggest that *EGT2* encodes either an enzyme which is involved in the metabolism of glucans in the neck region between mother and daughter cells or a regulatory protein controlling this metabolic event.

***EGT2* is expressed during a short period in early G_1.** *EGT2* transcription is activated in early G_1 when its gene product is required for cell separation. Since this requirement suggests that its transcriptional activation is essential for completion of a defined step in cell division, we decided to study the molecular events which initiate *EGT2* transcription. To pursue the time of its transcriptional activation, we carried out a Northern blot analysis of temporal staged RNAs of three differently synchronized cell cultures. In the first case, total RNAs were prepared from samples of a culture which was synchronized by release from α-factor arrest. Northern blots were prepared and hybridized with an *EGT2* gene probe. As shown in Fig. 4A, *EGT2* is not expressed in the arrest. Its mRNA first appears in G_1 of the second cell cycle after release from the arrest 10 min before *HO* and *RNR1* (15) expression rises. This timing is different from that of the chitinase gene *CTS1*, which is expressed at the α-factor block. In the second cycle, however, *CTS1* appears to be transcribed at the same time as *EGT2*, probably because the cell culture was gradually losing its synchrony during later cell cycle stages.

To exclude the possibility that G_1 arrest by pheromone treatment suppresses *EGT2* transcription in the first cycle after release from the block, we chose a different G_1 arrest, using strain Y168, which is deficient in all three *CLN* genes (*cln1 cln2 cln3*) but kept alive by *CLN1* expressed from the galactose-inducible *GAL1* promoter (12). These cells arrest in medium lacking galactose at around the same point in G_1 as cells treated with pheromone. We arrested them in raffinose-containing medium for 2.5 h and synchronously released them by adding galactose. In this experiment *EGT2* transcript levels decrease rapidly during the raffinose block (Fig. 4B). At the time when all cells are arrested in G_1, *EGT2* mRNA is not detectable. After the addition of galactose, the cells enter S phase and the *RNR1* transcript becomes visible, while the *EGT2* transcript appears in the second cell cycle after release, as shown for the release from the α-factor block.

These data show that *EGT2* is not expressed during G_1 arrest caused by α factor or a deficiency of the Cln function or during later stages of the first cell cycle after release from the block. Since expression is turned on in the following cell cycle 10 to 20 min before *RNR1* transcript levels appear (Fig. 4A and B), we asked whether *EGT2* is transcribed when cells exit mitosis but before they reach Start. To investigate this, we arrested cells of strain K838, which contains a thermosensitive allele of *CDC15*, at the restrictive temperature of 37°C for 3 h in mitosis and released the cells at the permissive temperature of 25°C. Figure 4C shows that *EGT2* is expressed in the first cell cycle at a point where *SWI5* transcription is already repressed and *RNR1* is not yet induced. *EGT2* transcript levels even reach their maximum before *CTS1* when cells have not yet changed their mitotic morphology since they still contain long mitotic spindles (Fig. 4C, graph).

The results of these three experiments show that the expression of *EGT2* is confined to a very small window of the cell cycle, namely between the end of mitosis and early G_1. Since our data suggest that Egt2 is involved in the correct timing of cell separation, its expression takes place at exactly that stage of the cell cycle when it is needed for its proper function.

Swi5 and Ace2 activate *EGT2* in early G_1. The *HO* gene encodes a double-strand nuclease that is involved in mating-type interconversion. Its expression takes place in late G_1 just before cells enter S phase (29) and is controlled by two upstream regulating sequences, URS1 and URS2. While URS2 is bound by heterodimeric complexes composed of Swi4 and Swi6, the activator Swi5 binds at URS1 (7, 29, 33). Deletion of URS2 results in a gene solely driven by Swi5 that leads to premature expression of *HO* at the end of mitosis and the beginning of G_1 (31) (Fig. 4C). This finding encouraged us to investigate whether *EGT2* is activated by the same transcription factor, Swi5, as the *HO* gene.

We deleted *SWI5* in Y32 and examined *EGT2* expression in cells in early logarithmic growth phase. Compared with wild-type cells, *swi5* cells show *EGT2* transcript levels reduced by more than 50% (Fig. 5). This result demonstrates that Swi5 is involved in *EGT2* transcription and that there might be another factor which activates *EGT2*.

It has recently been shown that *CTS1* is activated in early G_1 by the transcription factor Ace2 (14). This transcription factor is related to Swi5 (9). Overexpression of *ACE2* partially complements an *SWI5* defect and induces *HO* transcription, while Swi5 overproduction leads to partial *CTS1* expression in an *ace2* strain (14). This result led us to ask whether Ace2 is also involved in *EGT2* expression. When we deleted just *ACE2*, we did not see a difference in *EGT2* expression between wild-type and mutant cells; deletion of both *SWI5* and *ACE2* led to a complete abolition of *EGT2* expression (Fig. 5). This result shows that Swi5 and Ace2 activate *EGT2* transcription and that Ace2 at least partially complements Swi5 in its absence. It also suggests that Swi5 possesses the dominant function in activation since deletion of *ACE2* does not lead to a reduced *EGT2* transcript level. However, in an *swi5* deletion mutant, Ace2 itself is able to activate *EGT2* transcription in a cell cycle-dependent manner in G_1. We show this in a Northern blot of RNA samples of a synchronized *swi5* cell culture (Y272) which was hybridized with an *EGT2* gene fragment. The results shown in Fig. 4D indicate that, in this strain, *EGT2* is expressed later than in wild-type cells (Fig. 4A) or in $SWI5^+$ *ace2* cells (data not shown). *EGT2* transcript levels peak at around the same time in the second cell cycle as *RNR1* transcript levels.

Recently, the Swi5 binding sites of the *HO* promoter have been determined (51). A search for similar sequences in the *EGT2* promoter identified a 210-bp region with an array of six DNA sequences which resemble the B-type Swi5 binding site of the *HO* promoter. Their comparison showed almost 100% identity of the 3′ part of the motif while the 5′ half does not appear to be well conserved (Fig. 1B). Whether these sequences are responsible for cell cycle-dependent activation of *EGT2* has to be determined.

Swi5 protein accumulation in the nucleus is required for *EGT2* transcription. When *SWI5* is transcribed during G_2 and early mitosis, its translation product accumulates in the cytoplasm (31, 33) (Fig. 4C). It is prevented from entering the nucleus because of phosphorylation of three serine residues presumably by M-phase-promoting kinase. As soon as this kinase is inactivated at the end of mitosis, the serine residues are dephosphorylated and Swi5 enters the nucleus (28), where it induces *EGT2* transcription.

If this translocation step of Swi5 into the nucleus is responsible for activation of *EGT2* in early G_1, then a mutant Swi5 protein that is able to enter the nucleus at other stages of the cell cycle should activate *EGT2* transcription as soon as it is made. Such a mutation (*SWI5-AAA*) replaces the three serine residues whose phosphorylation during M phase retains the protein in the cytoplasm with alanines (28). To test whether this altered transcription factor is now able to stimulate *EGT2* transcription in mitosis, we arrested cells of Y286 expressing the mutant *SWI5-AAA* gene from its own promoter for 2.5 h

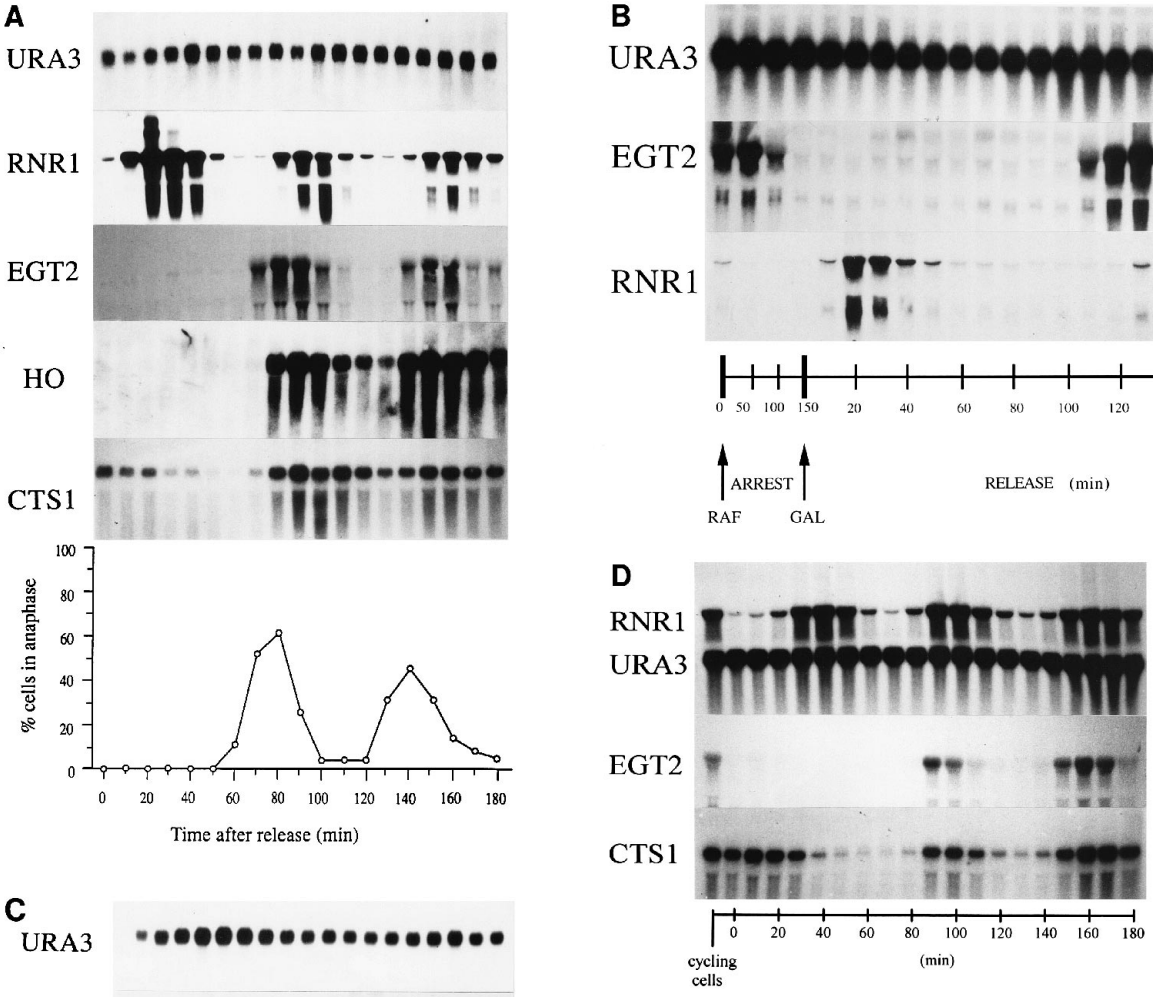

FIG. 4. *EGT2* expression is confirmed to a narrow window in G_1. Its cell cycle-specific transcription has been analyzed in different Northern blot experiments. *RNR1*, *CTS1*, *HO*, and *SWI5* are used as stage-specific markers; *URA3* served as a loading control. A missing signal in the first lane in panel C represents transcriptional repression of the *URA3* signal during the *cdc15* arrest. The graphs below the Northern blot hybridizations indicate the percentage of cells in anaphase determined by scoring for the presence of anaphase spindles following in situ immunofluorescence with rat anti-tubulin monoclonal antibodies (38). (A) *EGT2* is not expressed in the first cell cycle after release from an α-factor block. Haploid cells (H1170) of mating type **a** were arrested with α-factor in G_1. After release into fresh medium, samples were taken every 10 min, and RNA was isolated. The *EGT2* gene is not expressed in the first cell cycle, but in the second one its mRNA appears 10 min before the *HO* RNA (70 versus 80 min). The *RNR1* gene is expressed in the late G_1/early S phase, while *CTS1* is transcribed during G_1. (B) *EGT2* is not expressed in cells arrested in G_1 by depletion of G_1 cyclin function. *cln1 cln2 cln3 Gal-CLN1* cells (Y168) were grown to early log phase in YEPraff+gal and then transferred into YEPraf to arrest them in G_1 (time point 0). After 150 min of arrest, cells were released by the addition of galactose. Total RNA was prepared from samples taken every 10 min. The Northern blot hybridization shows that the *EGT2* transcript disappears during the arrest and appears again in the second cell cycle before the *RNR1* transcript. (C) *EGT2* is expressed only for a short time immediately after mitosis. Cells carrying the *cdc15* temperature-sensitive allele (K838) were arrested in late mitosis at 37°C and synchronously released by a temperature shift to 25°C. Samples for RNA preparation were taken every 15 min. The peak level of *EGT2* transcription precedes those of *RNR1* and *CTS1* by at least 15 min and quickly disappears. *SWI5* is expressed during late S phase, G_2, and mitosis. *HO::URS2del* is a mutated version of the *HO* gene which has a deletion of the Swi4/Swi6 binding site and thus is transcriptionally dependent solely on Swi5. (D) In *swi5* mutants (Y272) *EGT2* is activated only by Ace2 and expressed in a cell cycle-specific manner similar to that of wild-type cells (see panel A). Cells were arrested with α-factor in G_1 and synchronously released into the next cell cycles. Samples were taken every 10 min after release, and RNA was isolated. *EGT2* is expressed in cells kept in early logarithmic growth phase but not in the first cell cycle after release. In the second cell cycle its RNA levels peak at around the same time as *RNR1* transcript levels do.

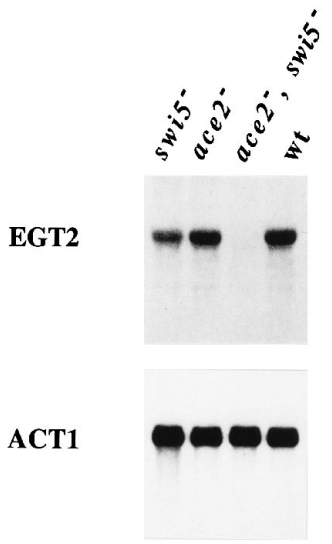

FIG. 5. Swi5 and Ace2 activate *EGT2*. Northern blots of total RNAs isolated from cycling wild-type (wt), *swi5*, *ace2*, and *swi5 ace2* double-mutant cells (derived from segregants of a single tetrade of sporulated Y288) show that a deletion of *SWI5* reduces *EGT2* expression by more than 50% as quantified with a Molecular Dynamics PhosphorImager. A deletion of the homologous *ACE2* gene, however, did not have any influence on *EGT2* expression. Only the double deletion extinguishes *EGT2* expression totally. This finding demonstrates that Swi5 is the main activator of *EGT2*, even though Ace2 is able to complement an Swi5 defect partially. An *ACT1* probe served as the loading control.

with nocodazole in anaphase and tested their *EGT2* expression by Northern blots. Figure 6 shows that mutant Swi5 does activate *EGT2* expression in the arrested cells, even though the level does not appear to be as high as that in cycling cells. Furthermore, cells arrested for 3.5 h exhibit an even stronger *EGT2* transcript signal. This result suggests that Swi5 levels accumulating in the nucleus determine the intensity of expression.

As soon as Swi5 enters the nucleus in early G_1, it becomes unstable and is rapidly degraded (31). This drop in concentration may cause the immediate inactivation of *EGT2* transcription before cells pass Start. If this is true, ectopic overexpression of *SWI5* in late G_1 should lead to accumulation of newly synthesized Swi5 in the nucleus followed by a reactivation of *EGT2* transcription. Furthermore, at this cell cycle stage not only mutant Swi5-AAA but also wild-type Swi5 will be able to enter the nucleus (31). To test this hypothesis we transformed cells of strain Y214 which contain a *cdc28-4*-thermosensitive allele with a centromeric plasmid harboring either a wild-type *SWI5* or *SWI5-AAA* gene, each driven by the *GAL1* promoter. Stationary cells were resuspended in fresh medium containing raffinose, incubated at 37°C for approximately 1 h, and then induced with galactose to activate *SWI5* and *SWI5-AAA* transcription. After 60 min of induction we supplemented the cultures with glucose to repress *SWI5* RNA synthesis. As can be seen in Fig. 7, induction of *SWI5* as well as *SWI5-AAA* transcription leads to *EGT2* expression, which ceases again as soon as *SWI5* RNA levels decrease. Furthermore, we observe that *EGT2* mRNA levels appear to be increased when *SWI5* RNA levels accumulate. From these experiments we conclude that cell cycle-specific *EGT2* expression is dependent on the accumulation of Swi5 protein in the nucleus in early G_1 and that expression levels drop as soon as Swi5 levels decline.

DISCUSSION

Most genes in the yeast *S. cerevisiae* are expressed in a cell cycle-independent manner (38). They are thought to supply cells with components that are necessary for growth. However, cells have to react to environmental changes and respond to signals which are either received from neighboring cells or generated inside the cell in order to regulate the discontinuous steps of cell growth. A cell can do this by posttranscriptional and posttranslational modification of already existing proteins, for example, by phosphorylation. A further possibility for a cell is to express genes transiently, during periods when the gene products are required. We are interested in identifying genes in *S. cerevisiae* whose transcriptional activation is dependent on specific cell cycle stages and whose gene products carry out important functions in cell division control. Our search for such genes turned up *EGT2*, a novel gene which is expressed at the boundary between mitosis and G_1.

Function of Egt2. In a fast-growing culture, *egt2* deletion mutant cells generate clusters of up to four connected cells. Most daughter cells do not separate in G_1 after they have completed mitosis and cytokinesis. But, at the end of a second round of division, the first mother cell separates from its first daughter, resulting in two dumbbell-shaped cells. This mutant phenotype appears to be leaky, since single G_1 cells can also be observed. Compared with wild type cells, however, their appearance is reduced approximately threefold. Our data suggest that Egt2 protein is necessary for degradation of the cell wall at the neck region between the mother and daughter cells in early G_1.

The yeast cell wall consists mainly of mannoproteins and structural polysaccharides such as glucan and chitin. The cross-linked network of mannoprotein forms the outer surface of the wall that communicates with the environment, while the inner layer of glucan and chitin is responsible for mechanical strength (for a review, see reference 22). Chitin is also preferentially deposited at the bud site prior to bud emergence where it forms a ring-like structure (22, 49). After septum formation, the chitin ring is partially digested by chitinase, which is required for separation of a daughter cell from its mother (25). Cells that do not produce functional chitinase do not separate but form large clusters. Egt2 does not seem to be required for degradation of chitin during cell separation, because Calcofluor White staining showed that the chitin at the neck re-

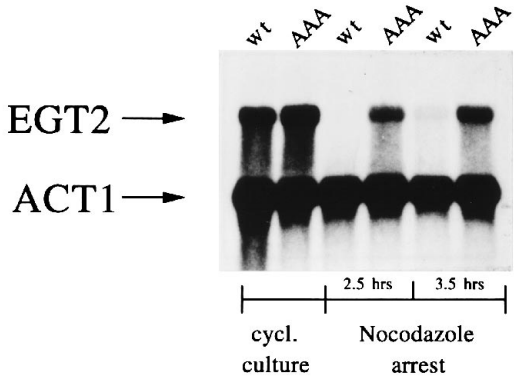

FIG. 6. A mutant Swi5 protein exhibiting constitutive nuclear entry is able to activate *EGT2* gene expression in mitosis in a dose-dependent manner. Both wild-type SWI5 (wt; Y32) and SWI5-AAA mutant (AAA; Y286) cells were grown to early log phase (cycl. culture samples) and arrested in mitosis by the addition of nocodazole for 2.5 and 3.5 h. *EGT2* expression was examined by Northern blot hybridization. An *ACT1* probe served as an internal control.

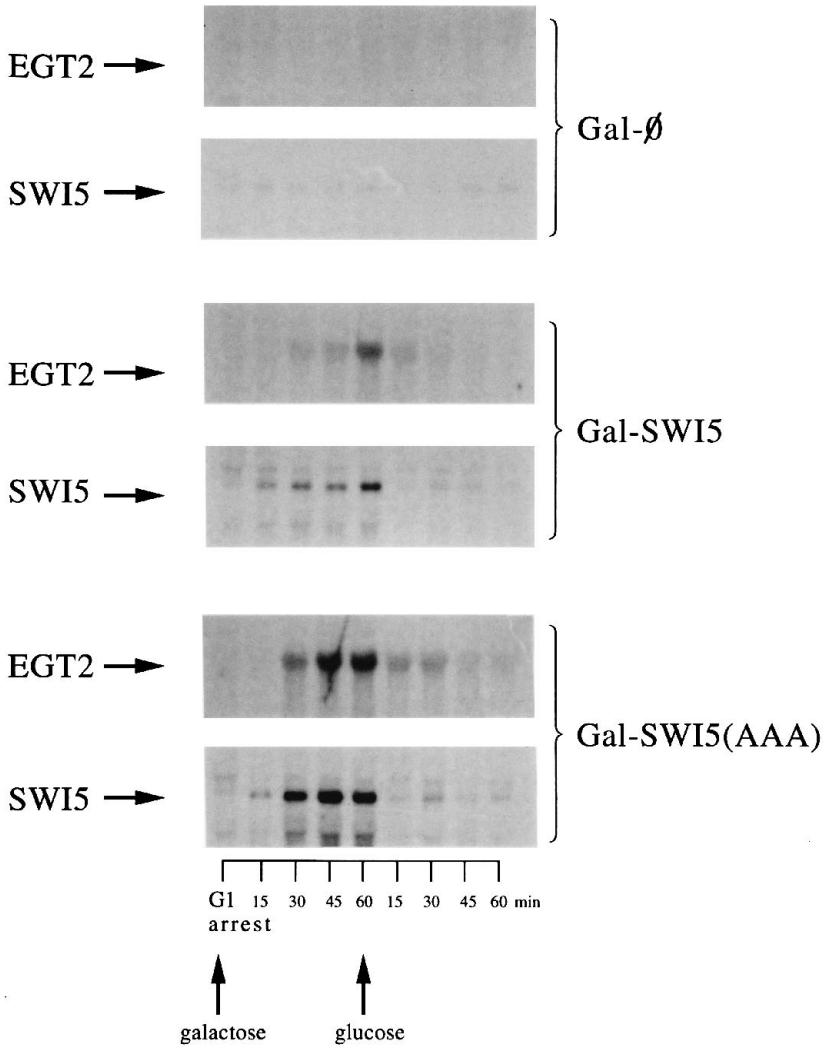

FIG. 7. Ectopic expression of wild-type SWI5 and mutant *SWI5-AAA* genes reactivates *EGT2* expression in late G_1. Cells carrying a *cdc28-4* temperature-sensitive allele and a wild-type or a mutant *SWI5-AAA* gene under *GAL1* promoter control were arrested at the nonpermissive temperature in G_1. The addition of galactose induced *SWI5* transcription, which, in turn, resulted in activation of *EGT2*. After 1 h of induction, *SWI5* expression was repressed by the addition of glucose, which led to the immediate inactivation of *EGT2* transcription. Cells which did not express *SWI5* ectopically (GAL-Ø) were unable to activate *EGT2* at the *cdc28-4* arrest.

gion in clustered *egt2* cells is digested in a manner similar to that of wild-type cells (Fig. 2B). Treatment of cells with only proteinase K or β-mercaptoethanol, which is thought to digest or modify the outer mannoprotein layer, respectively, in conjunction with recombinant β-1,3-glucanase, broke the clusters apart. This result indicates either that Egt2 protein is a glucan-degrading enzyme or that it activates certain glucan-metabolizing enzymes.

In *egt2* cell cultures we observe only clusters of four cells or fewer, since cells are able to separate at later cell cycle stages. These clusters even disappear when cells are arrested in G_1 by α-factor. This suggests that yeast cells possess additional mechanisms for cell separation. The cell wall is a dynamic structure that changes its composition while the cell proceeds through the cell cycle or reacts to stress conditions such as pheromone arrest and starvation. It is known that many enzymes involved in cell wall metabolism are released into the periplasmic space or trapped in the cell wall to support these changes (11, 22, 53). One of these enzymes may be able to cleave cell wall components in the absence of Egt2, and this cleavage then leads to separation of mother and daughter cells later in the cell cycle.

***EGT2* expression is tightly regulated.** *EGT2* is expressed in a cell cycle-dependent manner. Its transcripts accumulate during a very narrow window in early G_1 immediately after mitosis, and the RNA levels peak before cells reach late G_1. We have demonstrated that *EGT2* is activated by Swi5 and Ace2. Its transient expression pattern is due to transcriptional and not posttranscriptional controls since RNAs expressed from the *GAL1* promoter do not fluctuate during the cell cycle (24). Furthermore, ectopic expression of *SWI5* in cells arrested at Start leads to immediate transcriptional activation of *EGT2*, suggesting a direct dependency of gene activity on the presence of Swi5.

Compared with wild-type cells, *swi5* mutants show reduced *EGT2* expression levels while *ace2* mutants do not. In addition, they seem to express *EGT2* later in the cell cycle than do wild-type or *ace2* cells. From this, we concluded that Swi5 is the dominant activator of *EGT2* and that Ace2 does not seem to be required for full gene induction; but it can complement Swi5 function partially if Swi5 is absent. As previously shown, Swi5 and Ace2 also have overlapping functions in regulating *HO* and *CTS1* when they are overexpressed (14). However, it

is not yet known whether Ace2 binds the same sequences in the *HO* promoter which were shown to be bound by Swi5 (51). To understand Swi5 and Ace2 function better, it will be important to determine whether these factors bind the six motifs that we identified in the *EGT2* promoter.

EGT2 driven by the *GAL1* promoter in a cell cycle-independent manner does not seem to hinder normal cell growth. If its transient expression does not seem to be required, why does a yeast cell regulate *EGT2* so tightly? It is known that constitutive expression of most cell cycle-regulated genes seems to have little effect on cell growth (20, 23). However, global constitutive expression of transiently transcribed genes may waste energy pools and may lead to severe growth defects or even to cell death because of disruption of normal cell cycle control. The Egt2 protein is involved in certain enzymatic processes that degrade cell wall composition during early G_1. Expression of *EGT2* in all cell cycle stages may therefore impair survival. The effect of its deregulated expression, however, might not be discernible under laboratory conditions.

Swi5 activates *EGT2* in a concentration-dependent manner. The transcription factor Swi5 is synthesized during G_2 and early mitosis (34). As soon as it is made, it is phosphorylated at three serine residues presumably by the Clb/Cdc28 mitotic kinase. This prevents Swi5 from entering the nucleus, and it accumulates in the cytoplasm (28). When the kinase is inactivated at the end of mitosis, the serine residues are dephosphorylated, and Swi5 enters the nucleus, leading to a transient burst in its nuclear concentration (31). There it binds to specific sites in the *EGT2* promoter and activates *EGT2* transcription in early G_1 phase. The Swi5 protein, however, appears to be very unstable in the nucleus and is rapidly degraded (31). We believe that this decrease of the Swi5 moiety destabilizes its binding to the *EGT2* promoter and leads to inactivation of transcription before the cell reaches the pheromone arrest point in G_1. This model is supported by our observation that *EGT2* can be expressed in mitosis if it is driven by a mutant Swi5 transcription factor (Fig. 6). Furthermore, when we express Swi5 from a galactose-inducible promoter during the *cdc28-4* arrest in late G_1, we observe accumulating levels of the *EGT2* transcript depending on the time *SWI5* is expressed (Fig. 7). As soon as *SWI5* RNA levels drop, *EGT2* expression declines as well. Thus, *EGT2* is activated by Swi5 in a concentration-dependent manner.

In contrast, *HO* is activated by Swi5 in late G_1 after Start. This finding indicates that nuclear entry of Swi5 is not sufficient to stimulate *HO* expression. Transcription of *HO* also requires the Swi4/Swi6 transcription factor complex which is activated during late G_1. While *EGT2* expression seems to require high level of Swi5 in the nucleus, which occurs only transiently, *HO* is activated when nuclear Swi5 pools are low. This result suggests that Swi5 may undergo different fates depending on the promoter at which it binds: it binds at the EGT2 promoter only transiently when highly accumulated in the nucleus but may remain stably bound at the *HO* promoter even when Swi5 levels are low (5, 6).

Another possibility to explain Swi5 function is a "hit-and-run" model (42, 51). In this case Swi5 would bind at both promoters when it enters the nucleus, prime them for transcription, and leave them in an active state until gene transcription is fully induced. This sequence leads to immediate expression of *EGT2* while initiation of *HO* transcription needs additional activation by Swi4/Swi6, which occurs in late G_1. This model is supported by the transcriptional behavior of the *HO*::*URS2del* gene construct. Here, the Swi4/Swi6 binding sites are deleted, and transcriptional activation is dependent only on Swi5, which activates *HO*::*URS2del* even earlier than *EGT2*, possibly because of higher affinity to the URS1 sequence (Fig. 4C). As soon as expression reaches peak levels, the *HO*::*URS2del* gene is inactivated and no transcripts are detectable when cells enter late G_1.

This work demonstrates that Swi5 is an early G_1-specific transcription factor. Recently, it has been shown that *CDC6* transcription is activated at the boundary of mitosis and early G_1 at least partially by Swi5 (37). It will be interesting to see whether more genes which are activated by Swi5 in early G_1 will be identified in the near future. A further possible candidate may be *SIC1*, which encodes a negative regulator of S phase entry by blocking Clb5/Cdc28 kinase (35, 46). It is transcribed throughout the cell cycle but activated to higher levels around the same time that *EGT2* is expressed.

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