Changes in a SWI4,6–DNA-binding complex occur at the time of HO gene activation in yeast

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The yeast HO gene is transcribed transiently during G1 as cells undergo START. START-specific HO activation requires two proteins, SWI4 and SWI6, which act via a motif (CACGA4) repeated up to 10 times within the URS2 region of the HO promoter. We identified a DNA-binding activity containing SWI4 and SWI6 that recognizes the CACGA4 sequences within URS2. Two forms of SWI4,6–DNA complexes called L and U can be distinguished by their electrophoretic mobility. L complexes can be detected at all stages of the cell cycle, but U complexes are only detected in cells that have undergone START. The formation of U complexes may be the trigger of HO activation. The SWI6 protein is concentrated in the nucleus throughout G1, but at some point in S or G2 significant amounts accumulate in the cytoplasm. This change in cellular location of the SWI6 protein might contribute to the turnoff of HO transcription after cells have undergone START.

[Key Words: Saccharomyces cerevisiae; SWI4,6 complex; HO activation; CACGA4; mating-type switching; START-dependent transcription]

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During balanced growth, every component of a cell must be duplicated during the course of a single cell cycle. For most components, particularly those that exist in large numbers in a cell (e.g., ribosomes), the process of duplication takes place gradually throughout the cell cycle. In contrast, the duplication of rarer structures, such as chromosomes, takes place at a discrete time. A key question in cell cycle control is how the discontinuous events of the cell cycle (e.g., DNA replication, mitosis, and cytokinesis) take place in the correct order.

It is widely assumed, but by no means proven, that cell cycle stage-specific gene expression is an important means of ordering cell cycle events. Though it is not known to what extent stage-specific gene expression determines the order of the cell cycle events, it is known, for example, that many of the genes for enzymes involved in DNA replication are only transcribed as cells approach S phase [Storms et al. 1984; Peterson 1985; Johnston et al. 1987; White 1987].

One way of addressing the role of cell-cycle-regulated gene expression during the cell cycle is to identify the genes for proteins that are involved in cell cycle-specific gene activation and then characterize the phenotypes of mutants in such genes. One would like to know, for example, the immediate consequences on cell cycle progression following the inactivation of such a protein.

Possibly the best example of such a genetic approach concerns the HO gene in the yeast Saccharomyces cerevisiae [Winge and Roberts 1949; Hawthorne 1963]. HO encodes an endonuclease whose pattern of expression determines a well-defined pedigree of mating-type switching [Nasmyth and Shore 1987]. HO transcription is confined to a small window in the cell cycle during late G1 [Nasmyth 1983] and is dependent on the commitment of the cell to S phase, a process known as START, which is dependent on the CDC28 gene product [Hartwell 1974; Pringle and Hartwell 1981]. In addition to cell cycle control, HO is also regulated by the mating-type locus [Jensen et al. 1983] and is only expressed in mother cells [Nasmyth 1983]. The HO promoter is large [up to 1500 bp, Nasmyth 1985a] and complex, containing at least three different types of control element. A simple and highly specific regulatory sequence, the CACGA4 repeat, is sufficient to produce START-dependent transcription when joined to the cytochrome c TATA box [Breden and Nasmyth 1987a]. The URS2 region of the promoter [from -150 to -900 bp] contains 10 copies of the repeat; removal of the repeats causes the promoter to become START independent during G1 [Nasmyth 1985b].

At least six different genes, SWI1–SWI6, are required for efficient HO expression [Haber and Garvik 1977; Stern et al. 1984, Breden and Nasmyth 1987a], however, of these, only SWI4 and SWI6 are required for expression specifically mediated by the CACGA4 repeat [Breden and Nasmyth 1987a; Andrews and Herskowitz 1989a]. It is therefore possible that the cell cycle START dependence of the promoter during G1 may be exclusively a result of changes that occur to SWI4 or SWI6 proteins as cells undergo START. Strains with null alleles of SWI4
SWI4,6-DNA-binding complex and HO activation

(Sockanathan et al. 1991) and SWI6 (Breeden and Nasmyth 1987a) are viable, but double mutants are not, suggesting that these proteins also activate genes that, unlike HO, are essential for cell division. The SWI4 protein has been shown to be a component of a DNA–protein complex formed on CACGA₄ repeats in vitro, suggesting that it may be a sequence-specific DNA-binding protein (Andrews and Herskowitz 1989b).

In this paper we address whether the SWI6 protein also binds to CACGA₄ sequences and whether cell cycle regulation of a SWI4,6-binding activity may be responsible for the START dependence of HO expression. We show that SWI4 and SWI6 are components of specific complexes on several DNA fragments containing CACGA₄ repeats derived from the URS2 region of the HO promoter, and that the state of these complexes changes during passage through G₁ at the time of HO activation.

Results

SWI6 is a nuclear protein during G₁

To characterize the role of SWI6 in HO activation, we have raised antibodies against SWI6 protein produced in Escherichia coli [see Materials and methods]. Our antiserum is highly specific for SWI6, as it detects a 90-kDa protein in a Western blot that is not detectable when extracts are instead prepared from a swi6 deletion strain [see Fig. 1]. To determine whether SWI6 is a nuclear protein, we have analyzed the intracellular distribution of SWI6 using the method of indirect in situ immunofluorescence described by Kilmartin and Adams (1984). In an asynchronous wild-type culture, most cells have nuclear staining exclusively, but some cells also have detectable cytoplasmic staining (Fig. 2a). The bulk of this staining must be due to antibodies recognizing SWI6 protein, as much weaker staining is seen in cells containing a partial deletion of the SWI6 gene [Fig. 2b]. The heterogeneity of SWI6 staining appears to be a result of cell cycle variation in the distribution of SWI6. All unbudded cells have exclusively nuclear staining, whereas those cells that are about to undergo mitosis (i.e., those in which the nucleus is situated in the neck between mother and daughter and is partially extended) have additional cytoplasmic staining. To test this idea, cells were syn-

Figure 1. SWI6 is a 90-kDa protein. Total yeast cell lysates were prepared from cells grown in YEP–raffinose to exponential phase. Galactose (final concentration, 2%) was added to the cultures, and growth was continued for an additional 5–6 hr. Lysates from wild-type cells Y699 (wt), wild-type cells harboring a plasmid containing the SWI6 gene expressed under control of the GAL1–10 promoter (GAL–SWI6) and a strain (Y1748) carrying a SWI6 gene disruption [swi6–] were chromatographed on a 7.5–20.0% gradient SDS-PAGE and immunoblotted with anti-SWI6 antibodies [at 1:1000 dilution]. Numbers at left indicate migration of molecular mass markers (in kDa).
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chronized in early G₁ with the α-factor pheromone [Hartwell 1973] and in mitosis by nocodazole [Jacobs et al. 1988]. Figure 2c shows that all α-factor-arrested cells have nuclear staining, whereas the nocodazole-arrested cells [Fig. 2d] have mainly cytoplasmic staining. The nuclear membrane does not break down during mitosis in yeast, and other proteins [e.g., RAP1] remain in the nucleus at this stage of the cell cycle [Nasmyth et al. 1990].

We have confirmed and extended these results by expressing SWI6 in various temperature-sensitive cell division-cycle [cdc] mutants from the GAL1-10 promoter, which produces at least 10 times more protein per cell than the SWI6 promoter [see Fig. 1] and, therefore, a much stronger immunofluorescence signal. Cells arrested in G₁ as a result of the cdc28-4 mutation have exclusively nuclear staining, cells arrested in G₁ as a result of a cdc4-1 mutation have mainly nuclear staining, and cells arrested in G₂ as a result of a cdc7-1 mutation have both nuclear and cytoplasmic staining, whereas cells arrested in G₂/M phase by cdc13 or in late anaphase as a result of a cdc15 mutation have predominantly cytoplasmic staining [data not shown]. This pattern is similar to that observed for the SWI5 protein [Nasmyth et al. 1990].

The significance of the cell cycle-regulated SWI6 distribution for HO regulation is unclear [see Discussion], but it is important to point out that SWI6 is strongly concentrated in the nucleus at the point during the cell cycle when HO is activated.

**SWI6 forms complexes in vitro on fragments of UR52 that contain CACGA₄ motifs**

SWI6 activates HO via CACGA₄ motifs situated in the UR52 region of the HO promoter between −150 and −900 bp [Breeden and Nasmyth 1987a]. We would therefore expect that if SWI6 were a DNA-binding protein it would bind specifically to this part of the HO promoter. To test this notion, we used an assay [McKay 1981] that measures the avidity of a protein for different DNA fragments by comparing their ability to be immunoprecipitated by specific antibodies. A pBR322 plasmid containing the entire HO gene plus 1800 bp of 5'-flanking sequences [called pH0, Nasmyth 1985a] was digested with RsaI or Sau3AI to produce a mixture of 16 or 40 different fragments, respectively. Figure 3A shows that of the 16 RsaI fragments only 4 (with sizes of 522, 482, 318, and 240 bp) were selectively precipitated with anti-SWI6 antibodies in the presence of SWI6 protein, and that of the 40 Sau3AI fragments only 3 (with sizes 634, 282, and 131 bp) were similarly precipitated. The selective precipitation of this set of fragments was seen neither when the extracts were made from a swi6 deletion strain [Y1354] nor when preimmune serum was used.

The location of the selectively precipitated fragments within the pH0 plasmid is shown in Figure 3C. It is striking that with only one exception all come from the UR52 region of the HO promoter and contain at least one copy of the CACGA₄ motif through which SWI6 is known to act in vivo. The one exception is the 482-bp RsaI fragment, which covers part of the UR51 region of the promoter. It should also be noted that a 43-bp Sau3AI fragment, which contains a single CACGA₄ motif, was not found enriched in SWI6 immunoprecipitates. We suspect that SWI6 may not bind strongly to this fragment because experiments with a gel retardation assay showed that an oligonucleotide containing these sequences also could not compete with another UR52 fragment [sRS2, see below] for the binding of SWI4,6 complexes [data not shown].

Because the SWI4 gene is also required for activation of HO via CACGA₄ motifs, we have investigated whether the SWI4 protein forms complexes with the same set of fragments that are bound by SWI6. In this case, we have used extracts made from a strain expressing a SWI4–DHFR [dihydrofolate reductase] fusion protein and antibodies directed against DHFR. The results are shown in Figure 3B. A similar restricted set of fragments was selectively precipitated with the anti-DHFR antibodies as with the anti-SWI6 antibodies. Control experiments showed that the selective precipitation was not obtained when the SWI4–DHFR fusion protein was neither present in the extract nor when preimmune serum was used. We therefore conclude that SWI6 binds mainly to the same regions of UR52 as SWI4, although there may be minor differences in the ability of SWI4–DHFR and SWI6 to bind to the 482-bp RsaI fragment. The SWI4–DHFR fusion that we have used lacks the carboxy-terminal part of the SWI4 protein and binds to UR52 fragments even in the absence of SWI6 [data not shown, Sockanathan et al. 1991].

Finally, we have addressed the question of whether the specific binding of SWI6 to fragments of UR52 is cell cycle dependent. Instead of using extracts prepared from an asynchronous log-phase culture, we used extracts from cells with an α-mating type that had been uniformly arrested in early G₁ by treatment with α-factor. The same set of fragments was again selectively precipitated [Fig. 3A], suggesting that SWI6 retains the ability to bind to UR52 during a cell cycle stage in which HO is not transcribed.

**Detection of SWI4,6 complexes on CACGA₄ motifs by using a gel retardation assay**

In the McKay assays, described above, we were unable to detect any cell cycle dependence of the sequence-specific DNA binding by SWI4 or SWI6. However, this assay would not be able to distinguish changes in the physical state of protein–DNA complexes unless such changes caused a detectable difference in the strength of binding. To determine whether minor changes in the conformation of protein–DNA complexes take place during the cell cycle, we have also analyzed such complexes using a gel retardation assay. As a probe, we chose a 90-bp oligonucleotide [called small regulatory sequence from UR52–sRS2] containing DNA sequences from the HO promoter between −443 and −358 bp with respect to the translation start site. This region was chosen because it resided within the 240-bp RsaI fragment precipitated...
Figure 3. SWI6 and SWI4-DHFR binding to the HO promoter. pH0 plasmid containing 1.8 kb of the 5'-upstream region of HO [Nasmyth 1985b] plus the HO gene was digested either with RsaI or Sau3AI enzymes and end-labeled. These fragments were first incubated with 40 µg of yeast crude extracts and subsequently with immune (I) or preimmune antiserum (P). Protein A-Sepharose beads were finally added to recover selectively the immunoprecipitated protein–DNA complexes. Those DNA fragments that immunoprecipitated were then analyzed by separation on a 6%, 8.3 M urea-polyacrylamide sequencing gel. The molecular sizes of immunoprecipitated DNA fragments are shown (in bp). (A) To detect SWI6-binding activity, extracts were prepared from a SWI6 + strain (R1355) and a strain carrying a SWI6 gene disruption (swi6-, R1354). The SWI6 + strain was grown in the absence (exponential) or presence of α-factor (α-factor) and incubated with immune and pre-immune SWI6 antisera. (B) In the case of SWI4-DHFR-binding activity, a wild-type strain Y1268 (wt) and a wild-type strain harboring a plasmid containing SWI4--DHFR fusion expressed under control of the GAL1-10 promoter (SWI4-DHFR) were incubated with immune and pre-immune DHFR antisera. (C) The 5'-upstream DNA of HO and the cleavage sites of RsaI and Sau3AI on the URS2 region of the promoter. (Top) The regulatory elements of the HO promoter and the HO gene cloned into a pBR322 plasmid (pHO) [Nasmyth 1985a]. URS1 is required for HO transcription, and URS2 is required for correct cell cycle control. The URS2 region contains 10 copies of a repeat PuNNPyCAGA4, the cis-element, which confers the correct cell cycle regulation [Nasmyth 1985b]. pH0 was digested either with RsaI or Sau3AI, which cleave 16 or 40 different sites, respectively. The URS2 region has four sites for RsaI and six sites for Sau3AI.

Figure 4 shows the pattern of electrophoretic mobility of the radiolabeled sRS2 oligonucleotide in polyacrylamide and agarose gels after incubation with yeast cell extracts. Figure 4A shows that several different types of complexes can be formed on the sRS oligonucleotide; but of these, only two (U for upper and L for lower) are competed by unlabeled sRS2 DNA but not by an unrelated oligonucleotide from the URS1 region of the HO promoter. Because both the U and the L complexes are also competed by an oligonucleotide composed entirely of multiple copies of the CACGA4 motif [Fig. 4A], in the McKay assay and because it contained four copies of the CACGA4 motif. The strength of transcriptional activation by SWI4 and SWI6 seems to be dependent on the number of copies of the CACGA4 motif [Nasmyth 1985b].

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Figure 4. Complexes containing SWI4 and SWI6 form on CACGA₄ sequences from URS2. A 90-bp DNA sequence (sRS2) derived from the URS2 region of the HO promoter was radiolabeled and used as a probe in gel retardation assays with crude yeast cell extracts (40 μg of total protein) as described in Materials and methods. (A) Competition assay. Extract prepared from wild-type strain Y1720 (wt) was assayed with the addition of 100-fold molar excess of oligonucleotide competitors: Unlabeled sRS2 (cold sRS2), an oligonucleotide composed of five copies in tandem of the sequence tcgatcacgAAAA (CACGA₄), and an oligonucleotide containing the SWI5-binding site (non-specific). (B) U and L complexes contain SWI4 and SWI6. Extracts from swi4 mutants (R1071) or swi6 mutants (R1354) do not form U and L complexes on sRS2 in comparison with wild-type strain Y1268. SWI4 and SWI6 are components of both U or L complexes, because U and L are affected in their migration by incubation with specific antibodies (anti-SWI4, 1:200 dilution; anti-SWI6, 1:400 dilution) but not with corresponding preimmune sera. (C) The migration of U and L complexes is retarded by the addition of anti-SWI6 antibodies. This reaction mixture was electrophoresed in a 1.5% agarose gel. The migration of a unique band (wt), presumably comigrating U and L complexes, is retarded by the addition of SWI6-specific antibodies. Lanes 1, 2, and 3 contain decreasing amounts of anti-SWI6 antibodies (1:200, 1:2000, 1:3000 final dilutions) and show complexes with increasing mobility, respectively, as a result of a reduced amount of bound antibodies. (D) L complexes from early G1 cells contain both SWI4 and SWI6. Small G1 cells isolated by centrifugal elutriation form only L complexes whose electrophoretic mobility is affected either by SWI4-specific or SWI6-specific antibodies but not by the corresponding preimmune sera.

and because neither can be detected in extracts from swi4 or swi6 mutants (Fig. 4B), we propose that they are the result of the sequence-specific binding of SWI4 and SWI6 to one of the CACGA₄ motifs within the oligonucleotide.

We have confirmed that SWI6 is a component of both the U and L complexes by showing that both will bind antibodies specific for SWI6 (Fig. 4B-D). The addition of anti-SWI6 antibodies to the binding reaction after mixing the extract and oligonucleotide eliminates both complexes. The antibodies do not prevent the complexes from forming but, rather, produce ternary complexes whose electrophoretic mobility is so reduced that they no longer enter the polyacrylamide gel [Fig. 4B, D]. This notion has been confirmed by running the complexes on an agarose gel [Fig. 4C] where we see only a single complex that is SWI4- and SWI6-dependent (data not shown) and that is retarded by the addition of SWI6-specific antibodies. (We presume that the U and L complexes are not resolved under these conditions and that they comigrate). Andrews and Herskowitz (1989b), using anti-SWI4 antibodies, have shown that a similar gel retardation complex contains SWI4 and is dependent on SWI6. This complex is formed on a fragment of URS2 that contains most of the sRS2 oligonucleotide sequence (CCB DNA sequence between −507 and −394). Using these same anti-SWI4 antibodies, we show that SWI4 is also a component of both U and L complexes (Fig. 4B, D). We will refer to both as SWI4-SWI6 DNA-binding complexes even though we do not yet know whether SWI4 and SWI6 are the sole protein constituents.

U complex formation is cell cycle dependent

Because HO is only activated late in G1 after cells have undergone START, we have addressed the question of whether the formation of either the U or the L complex is cell cycle dependent by comparing extracts from an asynchronous population of cycling cells with those prepared from a uniform population of early G1 (i.e., pre-START) cells. We have used three different means of producing uniform populations of early G1 cells: growth of cdc28-4 mutant cells at the restrictive temperature [Fig. 5A], treatment of MATα cells with the α-factor pheromone [Fig. 5B], and size selection with centrifugal elutriation [Fig. 7A, below]. In each case, we have obtained the same result: U complexes are absent from pre-START G1 cells, whereas L complexes are present. Like those seen in exponentially growing cells, the L complexes seen in early G1 cells obtained by centrifugal elutriation contain both SWI4 and SWI6 proteins (Fig. 4D). Identical results were obtained with G1 cells obtained by α-factor treatment or by shifting cdc28-4 mutants to the restrictive temperature [data not shown].

Formation of the U complex occurs at the onset of HO transcription

The START dependence of the U complex suggests that it might be the transcriptionally active form. To test...
whether the formation of U complex and the activation of HO are correlated in time, we compared the formation of U complex and the activation of HO during the progression of a synchronous culture.

The simplest means of synchronizing the passage of cells through START is to release them from an α-factor-induced G₁ arrest by removal of pheromone from the medium. Unfortunately, the HO gene is not normally expressed during the first cell cycle following such a release [Nasmyth 1983]. The probable reason for this is that HO not only requires SWI4 and SWI6 but also another transcription factor called SWI5, which is known to act via the URS1 region of the HO promoter. SWI5 is itself cell cycle regulated and is not expressed throughout the G₁ period, being activated at some point during S phase [Nasmyth et al. 1987]. It has been proposed that the SWI5 inherited from the previous cell cycle is destroyed during the pheromone treatment and that none can be resynthesized between the release from α-factor and the onset of START [Nasmyth et al. 1987]. We have therefore used a strain [R1793] in our α-factor release experiment that is capable of expressing HO during the first cycle following release because SWI5 is constitutively expressed throughout the cell cycle.

Cells of strain R1793 were released from an α-factor-induced G₁ arrest by harvesting them by filtration and resuspending them in fresh medium lacking the pheromone. At 5- to 10-min intervals following the release, cell samples were harvested to assay DNA binding, to measure RNA levels, and to score cell cycle stage. The progress of these cells through the cell cycle was highly synchronous, with buds (an approximate indication of cells having initiated S phase) forming at 80 min and most cells with elongated spindles initiating anaphase between 130 and 150 min, as scored by indirect immunofluorescence with anti-tubulin antibodies (Fig. 6A). Figure 6B₁ and B₂ shows the pattern of gel retardation with oligonucleotide sRS2, and Figure 6C shows the level of HO RNA as measured by S1 mapping. HO RNAs appear transiently, reaching half-maximal levels between 60 and 65 min and peaking at 75 min. In contrast, the MATα1 transcript remains at a constant level throughout the experiment. As expected, the starting arrested culture lacks the U complex but it begins to appear by 60 min [Fig. 6B₂]. The appearance of U complexes by 60 min is seen more clearly after longer electrophoresis [see Fig. 6B₂]. It seems, therefore, that the appearance of HO RNAs correlates with the appearance of U complexes. Both entities appear in significant amounts by 60 min [for further discussion, see the legend to Fig. 6].

One of the striking features of the pattern of gel retardation as cells progress through the cell cycle is that whereas the appearance of the U complexes parallels HO activation, there is no reciprocal disappearance as the HO promoter is turned off. The ratio of U to L complex seems to increase until it reaches a maximum at 110–130 min, by which time HO is no longer transcribed.

Another notable aspect of this experiment is that there is apparently no loss of the U complex as cells enter G₁ in the second cell cycle following release. Data from three different methods suggest that the U complex is absent in early G₁ cells that have not yet undergone START [see Figs. 5 and 7A]. We would therefore also expect to see a loss of U complexes as cells released from the α-factor block reenter G₁ following mitosis. Our explanation for this apparent contradiction is that cells synchronized by the method of α-factor release are much larger than normal as a result of cell growth while arrested in G₁, and as a consequence they undergo START very soon after completing mitosis; that is, such cells have a very short pre-START G₁ period in the second cell cycle—the only period during which the cells should lack U complexes. A loss of U complexes will therefore be difficult to detect if synchrony is not perfect. The ratio of U to L complex does seem to decrease as cells complete anaphase [see the 150- and 160-min samples in Fig. 6B].

To clarify whether U complexes are absent in G₁ cells and to investigate further whether their appearance correlates with HO activation, we have analyzed the level of HO transcripts and the electrophoretic mobility of SWI4,6-DNA complexes in cells fractionated according to their size with an elutriation rotor [Fig. 7]. An asynchronous culture growing in YEP–galactose (there are too few separated G₁ cells in glucose-grown cultures) was loaded onto an elutriation rotor, and five fractions...
composed of cells with increasing size were collected by gradually raising the flow rate (ideally, we would have liked to isolate enough G₁ cells to make a synchronous culture, but this proved impossible). As in the α-factor release experiment, we see a correlation between the level of U complexes and that of HO transcripts in the five fractions. HO and U complexes are both completely absent in the first fraction; both appear in significant amounts in the third and are maximal in the fourth and fifth fraction. In summary, it is difficult to say conclusively from the α-factor release and elutriation experiments that U complexes appear early enough for them to be the cause of HO activation, but the data are certainly consistent with this hypothesis.

**U complexes in cell cycle mutants**

To confirm the observation that U complexes do not disappear at the time of HO inactivation, we have also assayed the formation of U complex in various temper-
nature-sensitive cdc mutants when their cell cycles are blocked by incubation at the restrictive temperature. Figure 8A shows that the U complex exists in cdc4-, cdc7-, cdc13- and cdc15-blocked cells. cdc4 and cdc7 mutations block cells in G1, but do not prevent HO from being activated. The observation that these mutations also do not prevent the formation of U complex is therefore consistent with its playing a role in HO activation. The observation that U complexes also exist in cdc13 mutants, which arrest in G2/M, and cdc15 mutants, which arrest in late anaphase, suggests that the U complex persists until the very end of the cell cycle. Because HO is expressed neither during the G2 period of wild-type cells nor in cells blocked in G2 or anaphase, we conclude that whereas HO activation may be triggered by the formation of U complex, its subsequent inactivation during S phase is unlikely to be simply the result of the loss of this complex (see Discussion).

In vivo activity of sRS2 during the cell cycle
To investigate whether SW14–SW16 complexes of the type that we have detected in vitro also form in vivo, we have analyzed the in vivo activity of the sRS2 oligonucleotide by fusing it to a form of the CYC1 promoter that lacks an upstream activation sequence (UAS) and is fused to the reporter gene lacZ (see figure legends).

Figure 8B compares the level of lacZ RNAs produced by no UAS (a), by the ribosomal protein gene RP39 UAS (b), and by sRS2 (c). As expected for a sequence that binds SW14 and SW16 in vitro, sRS2 exerts appreciable "upstream activation," producing 40% as much lacZ RNA as RP39. Unlike RP39, the sRS2 activation is completely dependent on SW14 and SW16. We presume, therefore, that the SW14–SW16 complexes that form in vitro also form in vivo and are capable of transcriptional activation.

To compare the cell cycle dependence of this activation with the abundance of U complexes, we have measured lacZ transcripts in cdc28, cdc4, cdc7, cdc13, and cdc15 mutants after stationary-phase cells are incubated in fresh medium at the restrictive temperature for 3 hr. We find that sRS2 is inactive in cells that cannot undergo START (cdc28), in cells arrested in G2 (cdc13), and in cells arrested in a late stage of anaphase (cdc15). In contrast, sRS2 is active in mutants that can undergo START but that arrest before the initiation of DNA replication: cdc4 and, to a lesser extent, cdc7. In the case of cdc28, cdc4, and cdc7, there is a correlation between the ability of cells to form the U complex and sRS2 activity in vivo, but this does not apply to cdc13 and cdc15 mutants in which U complex is formed in vitro but sRS2 is inactive.

Role of CDC28 protein kinase in HO activation
The activation of HO and the formation of the U complex both require an active CDC28 gene product that encodes a protein kinase. It is possible that an activation of the CDC28 protein kinase at some time in G1 triggers HO transcription by site specifically phosphorylating SW14 or SW16. Surana et al. (1991) have found that a form of the CDC28 kinase that binds to Suc1-Sepharose beads (Brizuela et al. 1987) and uses histone H1 as a substrate is active soon after the onset of S phase. To address whether this form of the kinase could be involved in HO activation, we have assayed Suc1-bound kinase activity during the course of the synchronous culture described above. Figure 6D confirms that this form of the kinase is highly cell cycle regulated. Histone H1 phosphorylation is absent in the starting culture of G1-blocked cells and only appears in significant amounts 90–100 min after the release. The kinase activity peaks at 130 min, which is just before the onset of anaphase. It is notable that there is no detectable activity at the time of HO activation and U complex formation. We conclude that this form of the CDC28 kinase is unlikely to be responsible for HO activation, and because the CDC28 gene product is nevertheless required for the onset of HO expression a second form of the kinase must exist that is active earlier in the cell cycle than the form that we have measured. To determine whether phosphorylation might be responsible

Figure 6. The timing of U complex formation following release from α-factor-induced G1 arrest. A synchronous culture of strain R1793 was prepared by releasing cells from a G1 block by treatment with α-factor. Samples of cells were harvested at indicated time points, and extracts were prepared for gel-retardation assays and for RNA and CDC28 histone H1 kinase activity analyses. Synchrony was followed both by scoring the bud formation and by measuring cells with elongated spindles by indirect immunofluorescence with anti-tubulin antibodies. [A] Percentage of cells showing bud formation [○] (roughly coincident with S phase) and elongated spindles [■] (i.e., anaphase) after releasing cells from the α-factor block. [B1] Gel-retardation assay using sRS2 as a probe. [E] Extract from asynchronous cells not treated with α-factor; (blank) the reaction mix to which no extract was added. [B2] Samples 30, 40, 50, 60, 70, 80, and 90 min reanalyzed in a gel-retardation assay identical to B1 but electrophoresed longer. (C) HO RNAs analyzed by S1 protection. MATa1 RNA as an internal control was also mapped. [D] CDC28 histone H1 kinase associated with Suc1 beads. [C] No extract added. The graph shows the amount of radioactivity in the histone H1 band. To compare precisely the kinetics of HO RNA appearance and U complexes would involve comparing, at different time points, the fraction of cells that had initiated START and HO transcription with the fractions of cells containing U complex form. Imperfect synchrony makes it difficult to assess the former. Much of the apparent duration of HO transcription (from 60 to 100 min) is probably a result of asynchrony (i.e., it takes ~30 min for all cells in the culture to pass through their windows of HO activation). Thus, the appearance of half-maximal HO RNA levels at, e.g., 63 min, cannot be taken to mean that 50% of the cells have initiated HO transcription. It is also possible that gel retardation to detect U complex formation is a less sensitive assay than S1 mapping to detect HO RNA. Therefore, it would be more difficult to detect the first molecules of the transcriptionally active complex, although they would be sufficient to initiate transcription of detectable amounts of HO RNA.
Figure 7. Comparison of the levels of U complexes and HO RNA in cells fractionated according to size by centrifugal elutriation. Strain R1793 was exponentially grown in YEP-galactose and at OD_{600} = 2.5, loaded into a Beckman JE-10X centrifugal elutriation rotor. Small G_1 cells were collected initially, followed by subsequent fractions containing increasing numbers of cells with buds. Extracts were prepared for gel-retardation assay and RNAs for mapping HO transcripts by S1 protection. (A) Gel-retardation assay with sRS2 as a probe. (Mix) Cycling cell population; (f1-f5) successive fractions obtained after the initial G_1 population. f2 extract shows a weaker signal because of a low yield in the protein extraction procedure [here, we used 25 μg of total protein]. (B) HO RNAs analyzed by S1 protection. MATa1 RNA as an internal control was also mapped. (C) Percentage of budded cells in each fraction.

for the slower migration of U complexes, we have treated extracts with calf intestinal alkaline phosphatase (CIP) but have found that U and L complexes are equally sensitive [data not shown].

Discussion

In an effort to understand the biochemical basis of START, we and others have been studying the cell cycle regulation of the HO gene [for review, see Herskowitz 1989], which is transiently activated as cells undergo START [Nasmyth and Shore 1987]. HO encodes an endonuclease that initiates mating-type switching in homothallic strains of yeast [Winge and Roberts 1949; Hawthorne 1963]. Its activation is not only dependent on cell cycle stage but also on cell lineage [Nasmyth 1983]. HO is activated at START in only one of the two progeny of a cell division—in the mother cell that has just produced a bud. Two separate parts of the HO promoter and distinct trans-acting gene products are responsible for its lineage and cell cycle specificity [Nasmyth 1985a]. URS2, the region between −150 and −900 bp, contains a repeated motif, CACGA_4, which imparts START-dependent activation dependent on two proteins encoded by the SWI4 and SWI6 genes [Nasmyth 1985b; Breeden and Nasmyth 1987a]. In contrast, URS1, the region between −1000 and −1400 bp, is implicated in mother cell-specific activation that requires a site-specific DNA-binding protein encoded by the SWI5 gene [Stillman et al. 1988]. Cell cycle control of HO is not exclusively the result of URS2. A version of the HO promoter lacking URS2 [called HO::urs2del, Nasmyth et al. 1990] is inactive throughout the S, G2, and M phases of the cell cycle because SWI5 is excluded from the nucleus at these stages. HO::urs2del is only activated when the SWI5 protein suddenly enters the nucleus at the end of mitosis. SWI4 and SWI6 [acting via the CACGA_4 repeat in URS2] somehow restrict the G_1-specific activation of HO exerted by SWI5 to the later part of G_1 during which cells undergo START.

In this study we have addressed two questions: Do SWI4 and SWI6 activate HO by binding to the CACGA_4 motifs within URS2? And, if so, is their binding cell cycle-regulated in a manner consistent with this being the mechanism of START-specific HO activation? We have found that antibodies specific to SWI6 immunoprecipitate a restricted set of fragments from an RsaI or Sau3A restriction digest of a pBR322 plasmid containing the HO gene and promoter [Nasmyth 1985b] when they have been preincubated with extracts from wild-type strains but not from swi6 mutants. With only a single exception, the selectively precipitated fragments come from the URS2 region of the promoter and contain CACGA_4 motifs. Because a similar set of fragments are also selectively precipitated with anti-DHFR antibodies when the extracts are made from cells expressing a SWI4–DHFR fusion protein, we presume that both SWI4 and SWI6 form complexes on the same CACGA_4 motifs. This conclusion is supported by experiments using gel retardation as an assay of DNA binding. Extracts from wild-type cells form complexes on a 90-bp fragment from URS2 [sRS2], which are specifically competed by an oligonucleotide containing multimers of the CACGA_4 motif. Andrews and Herskowitz [1989b] have detected similar complexes on a related DNA sequence and have shown them to contain the SWI4 protein. We have shown that the complexes formed on sRS2 contain both SWI4 and SWI6 proteins as their electrophoretic mobility is retarded further by the binding of SWI4- or SWI6-specific antibodies. However, our experiments do not
address whether these complexes contain any other proteins in addition to SWI4 and SWI6.

Using the gel retardation assay, we have discovered two forms of SWI4–SWI6–DNA complexes that have different electrophoretic mobilities. The faster migrating L form can be detected in cell extracts from all stages of the cell cycle, whereas the slower U form is absent in all forms of G1 cells that have not yet undergone START. When cells treated with α-factor are released from their G1 arrest, the U form appears with the same kinetics as HO RNA. This raises the possibility that the START-dependent activation of HO transcription may be the result of a change in the physical state of SWI4–SWI6 complexes brought about by the activity of the CDC28 protein kinase.

What activates HO transcription at START?

Because START-specific activation of HO via multiple copies of a CACGA4 motif within URS2 requires SWI6 (Breeden and Nasmyth 1987a), we have analyzed whether there are any changes in the state of the SWI6 protein as cells progress through the cell cycle, which might shed light on the mechanism of HO activation. Our first discovery, using the technique of in situ indirect immunofluorescence (Kilmartin and Adams 1984), is that the location of SWI6 changes during the cell cycle. SWI6 is concentrated in the nucleus during G1 and S phases but becomes distributed throughout the cytoplasm some time during G2 and only reaccumulates in the nucleus as cells complete mitosis. This implies that there may be a major change in the state of the SWI6 protein as cells enter G1. Although the change in SWI6 location may be necessary for HO activation, it cannot be the actual trigger, because HO is not activated until later in G1 when cells undergo START. Our observation raises the possibility that SWI6 might have a function [unrelated to HO activation] during G1 prior to START.

Our second discovery concerning the state of SWI6 at different stages of the cell cycle may be more relevant to the triggering of HO activation. There are two types of complex formed between SWI4, SWI6, and CACGA4–
containing oligonucleotides: U and L forms. The slower migrating U complexes are cell cycle regulated and appear at the time of HO activation. We propose that the formation of U-type complexes on the HO promoter may be the trigger that activates HO. This proposal is consistent with our observation that U complexes were never detected in cells prevented from undergoing START (e.g., cdc28 mutants, cells treated with α-factor, and size-selected G₁ cells) but are always formed in cells that have undergone START even though their subsequent cell cycle progression is arrested (e.g., cdc4 mutants that cannot initiate DNA replication but nevertheless express HO). CDC28 is the only CDC gene required for START (for which there are mutations with tight phenotypes) and is also the only one required for U complex formation.

The timing of U complex formation is consistent with its involvement in the activation of HO. HO RNA reaches half-maximal levels 65 min after α-factor release, by which time U complexes have also just formed (60 min). Because the appearance of U complexes does not precisely coincide with the first appearance of HO RNA (55 min), it could be argued that these complexes may instead be involved in repression rather than activation of HO transcription. This seems inconsistent with genetic data showing that SWI4 and SWI6 are activators of HO transcription. Our inability to detect the formation of U complexes at the precise time of the appearance of HO RNA may simply be the result of a difference in the sensitivity of the two assays.

What causes the formation of U complexes?

Because a very similar type of complex, the slightly faster migrating L complex, exists in cells before they undergo START, it is likely but not proven that U complexes arise from L complexes. In this case, understanding the physical difference between U and L complexes may be crucial to understanding what triggers HO transcription. We have shown that both types of complex contain SWI4 and SWI6. We cannot yet exclude the possibility that the complexes contain other proteins in addition to SWI4 and SWI6. We think it likely that the complexes observed by Andrews and Herskowitz (1989b) on a similar but larger DNA fragment are equivalent to those described in this paper. Because we are only able to resolve U from L complexes under particular electrophoresis conditions, it is possible that the difference between the U and L complexes may be a subtle one, such as a conformational change induced by the modification of one of the constituent proteins, rather than the addition of a new protein.

Because the formation of U complexes is dependent on the CDC28 gene function, it is possible that the transition from the L to the U complex is caused by one of the proteins in the complex being phosphorylated by CDC28 protein kinase at the time of START. CDC28 is required not only for exit from G₁ but also for mitosis, and it is a strong possibility that it is active in one form or another throughout the S, G₂, and M phases of the mitotic cycle, that is, throughout the entire period during which we can detect U complexes. We have measured the level throughout the cell cycle of a CDC28-dependent histone H1 kinase that binds to the Schizosaccharomyces pombe Suc1 protein [Brizuela et al. 1987] but have found that this form of the kinase does not become active until well after the activation of HO and the formation of U complexes. This implies that another form of the CDC28 kinase (which either does not bind to Suc1 or fails to recognize histone H1 as a substrate) must be responsible for HO activation. Such a kinase may be formed by association of CDC28 with G₁-specific cyclins (Wittenberg et al. 1990).

What inactivates HO transcription?

Cell-cycle-regulated promoters must not only be activated but also repressed at specific stages during the cell cycle. The hypothesis that the cell cycle-dependent formation of U complexes on URS2 triggers HO activation cannot explain why the HO promoter is inactivated soon after START because U complexes persist even until the end of mitosis, that is, long after HO has been repressed. If our hypothesis concerning HO activation is correct, we must conclude that HO is repressed by a mechanism that is not simply a reversal of its activation. One possibility is suggested by the behavior of another HO activator, SWI5. The location of the SWI5 protein is strongly cell cycle regulated [Nasmyth et al. 1990]. The protein synthesized during S or G₂ accumulates in the cytoplasm and does not enter the nucleus until cells complete mitosis and enter G₁. It is possible, therefore, that an alteration of SWI5 following START might be sufficient to inactivate HO in early S phase in the absence of any loss of active U complexes at this time.

We have shown that the very same oligonucleotide sequence (sRS2) that will form U complexes throughout G₁ and mitosis cannot activate transcription when cells are arrested at these stages of the cell cycle, even though its activation is independent of SWI5. Therefore, there must be another mechanism for HO inactivation that involves SWI4 and SWI6. One possibility is that regulation of intracellular localization of SWI6 may be involved. SWI6, like SWI5, accumulates in the cytoplasm in cells blocked in G₂ or mitosis and there may be insufficient protein present in the nucleus for HO to remain active. Thus, the SWI4,6 U complexes that we detect in vitro may be of nuclear origin when isolated from G₁ cells but of cytoplasmic origin when isolated from G₂ cells. If this is so, U complexes may only exist in the nucleus for a short period of time following START while SWI6 protein is still concentrated in the nucleus. Another mechanism for HO repression is suggested by our observation that cell cycle mutants that fail to initiate DNA replication, for example, cdc4 and cdc7, also fail to inactivate CACG₃A₄-dependent transcription. It is possible that DNA replication itself is necessary for the removal of active U complexes from the HO promoter. DNA replication per se could be a mechanism for the
repression of transcription of certain cell cycle-regulated genes.

Materials and methods

Yeast strains

Y1268: MATa, leu2, trpl, ura3, prb1-1122, pep4-3, prcl-407, gal2 (Shore et al. 1987)

Y699: MATa, ade2-1, trpl-1, can1-100, leu2-3 112, his3-11, 15, GAL, psi+ 

Y1052: MATa, ura3, swi4::LEU2

Y1748: MATa, ade2-1, can1-100, leu2-3 112, his3-11, 15, GAL, psi+, swi6::TRP1

R1720: MATa, ade2-1, trpl-1, can1-100, leu2-3 112, his3-11, 15, GAL, psi+, pep4::URA3 

R1355: Y1268 derivative, bar1::LEU2

Y1719a: MATa, cdc28-4, can1-100, ura3

Y1719b: MATa, cdc28-4, can1-100, pep4::URA3

Y1728a: MATa, hmlα, HMRAa, cdc15, HOXbo129-102, ura3

Y1728b: MATa, hmlα, HMRAa, cdc15, HOXbo129-102, pep4::URA3

Y1731a: MATa, cdc4-1, ura3

Y1731b: MATa, cdc4-1, pep4::URA3

Y1733a: MATa, cdc7-1, ura3

Y1733b: MATa, cdc7-1, pep4::URA3

Y1735a: MATa, cdc13, ura3

Y1735b: MATa, cdc13, pep4::URA3

R1354: Y1268 derivative, swi6::TRP1

R1071: MATa, HOa, HMRAa, swi4-100, ho/SUP4-O, HIS, leu2, trpl, ade2-1, can1-100, pep4::URA3

R1793: MATa, ade2-1, bar1::LEU2, can1-100, RP39::SWI5pro, ho, pep4::URA3

Preparation of yeast extracts

Stationary-phase cells grown in YEPD were used to inoculate (1:30 dilution) a fresh culture of the same medium and grown to an OD600 of 1.2–1.5. Cells were harvested, washed with cold distilled water, and broken by vortexing with 0.5-mm glass beads for 3 min. The breakage buffer contained 0.2 M Tris-HCl (pH 8.0), 10 mM MgCl2, 1 mM dithiothreitol (DTT), 10% glycerol, 1 mM PMSF, 0.5 mM PCMK, 25 μM TLCK, 2 μg/ml of pepstatin A, 15 mM p-nitrophenylphosphate, 0.1 mM Na-meta-vanadate, 60 mM 2-glycerophosphate, and 50 mM NaF. Cell debris and glass beads were cleared from the suspension after a quick spin in the microcentrifuge. After another spin for 20 min, the crude extract was aliquoted, frozen immediately in liquid N₂, and stored at –70°C.

Probe for gel-retardation assay and specific competitors

To detect specific formation of DNA-protein complexes on URS2 sequences, the oligonucleotides were synthesized by using an automated Applied Biosystems 380B DNA synthesizer. Oligonucleotides synthesized were srs2 [from −443 to −358 bp of the 5′-upstream regulatory HO DNA], a 90-bp sequence containing the motifs CAGCAATA, TACGAAAT, CACGAAA, and CCAGAAA; an 80-bp sequence containing 5X TGATCCAGAAA [CAGCAATA] and a 53-bp sequence containing the SWI6-binding site (Stillman et al. 1988). Oligonucleotides were used as competitors or probe. As probe, the radiolabeling was performed with Klenow enzyme and [α-32P]CTP.

Purification of SWI6 protein and raising of antibodies

SWI6 was cloned previously in a 5.7-kb HindIII–HindIII fragment (Breeden and Nasmyth 1987b). Oligonucleotide-directed mutagenesis in an M13 system (Zoller and Smith 1983) was used to generate an NdeI site at the initiation codon of the SWI6 reading frame. A 2.5-kb fragment was derived from the construct, starting from the new NdeI site to the BglII site that lies ~200 bp downstream of the terminating codon. This was cloned in the T7–RNA polymerase-inducible p RK171a vector (McLeod et al. 1987) at the NdeI and BamHI sites and designated as T7–SWI6. The intact SWI6 protein was expressed and purified in the following manner: Competent BL21 (DE3) cells were transformed with T7–SWI6, one of the resulting ampicillin-resistant colonies was used to grow a 3-liter culture in 2X TY medium with ampicillin to an OD600 of 0.6–0.8. At this point, IPTG was added to the culture to a final concentration of 1 mM, inducing the expression of SWI6. Induction was allowed for 3 hr before cells were spun down, washed, and resuspended in 20 ml of lysis buffer consisting of 200 mM KCl, 20 mM Tris (pH 7.5), 10 mM MgCl2, 8 mg/ml of lysozyme, 1 mM DTT, and 1 mM PMSF. The resuspended cells were allowed to lyse on ice for 1 hr. After sonication, the lysis mixture was centrifuged at 45,000 rpm for 1 hr. SWI6 is present in the supernatant (soluble fraction) at >1% of the total soluble protein as determined by 7.5–20% gradient SDS-PAGE with Coomasie blue staining. The soluble fraction was dialyzed against 20 mM Tris (pH 7.5) and 1 mM DTT, and saturated ammonium sulfate was added to a concentration of 25%. The mixture was incubated on ice for 2 hr and centrifuged as described previously. The supernatant was collected, and saturated ammonium sulfate was added to a final concentration of 40%. Most of the SWI6 protein precipitates at this point and represents ~50% of the total protein precipitated. After centrifugation, the pellet was resuspended in 2 ml of 20 mM Tris (pH 7.5) and 1 mM DTT. After dialysis against the same buffer, the protein was loaded on a Fast Flow Q column and eluted against a NaCl gradient. SWI6 elutes at an estimated 75% homogeneity with a salt concentration of ~0.25 M. The eluate was again dialyzed against 20 mM Tris (pH 7.5) and 1 mM DTT and loaded onto a heparin–Sepharose column. With the same NaCl gradient, SWI6 elutes at ~0.2 M salt and has a homogeneity of ~95%. After dialysis, as described previously, the eluate was used as antigen to raise antibodies against SWI6. Three rabbits were injected with 400 μg of SWI6 with Freund’s complete adjuvant as the initial immunization; 6 weeks later, a booster of 200 μg of protein with Freund’s incomplete adjuvant was used. Fourteen days later, test bleeds were taken, and all rabbits were found to have high titers of SWI6 antibodies.

Immunoprecipitation assay

To identify binding sites for the SWI6 protein on the HO gene promoter, an indirect immunoprecipitation assay was employed (McKay 1981). For each binding reaction 1 μl of polyclonal antisera or a nonspecific antisera was incubated with 20 μl of protein A–Sepharose beads (Sigma) in 380 μl of lpp500 [500 mM NaCl, 10 mM Tris-HCl (pH 8.0), 0.1% NP-40, and 0.1% Na2CO3] on a rotating wheel at 4°C for 90 min. The beads were collected by centrifugation and washed twice with 300 μl of lpp500 and twice with 400 μl of lpp400 [lpp400 is identical to lpp500, except that it contains only 50 mM NaCl]. Binding reactions were set up as described below (gel-retardation assay) by using per reaction 75 ng of pHO DNA (Nasmyth 1985b) digested to completion with either Rsal or SoolAI and end-labeled using by T4 polynucleotide kinase and [γ-32P]ATP; the reactions were incubated on ice for 30 min before addition to
the beads. Incubation was continued for an additional 10 min, and the beads were then washed four times with 400 μl of Ippso (at 4°C) and resuspended in 400 μl of HM [300 mM NaCl, 50 mM Tris-HCl (pH 7.4), 5 mM EDTA, 1.5% SDS, 1.5 mg/ml of proteinase K]. Digestion was carried out at 37°C for 30 min, and the supernatant was extracted with phenol–chloroform–isoamyl alcohol [25:25:1]. The DNA was recovered from the aqueous phase by ethanol precipitation following addition of 10 μg of carrier RNA, dissolved in 5 μl of loading buffer (80% formamide, 10 mM NaOH, 1 mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol blue), denatured by boiling, and loaded onto a 6% acrylamide, 8.3 μm urea, 1 x TBE (pH 8.3) gel.

Note that the experiments involving SWI4 antibodies in gel-retardation assays were performed during the period of preparation of this manuscript. For this reason, we did not use them in the initial MacKay assays.

**Gel-retardation assay**

Band-shift assays were performed in 20-μl reactions in buffer containing 20 mM Tris-HCl (pH 7.5), 3 mM MgCl₂, 50 mM NaCl, 1 mM DTT, 50 μg/ml of BSA, 0.1 mM spermine, 5% glycerol, and 2–3 μg of poly[d(I-C)]. Forty micrograms of total protein from yeast extracts was diluted in the buffer described above. After adding ~0.03 pmole of labeled probe, the reactions were incubated at room temperature for 15–20 min and loaded onto a 4% polyacrylamide gel (20:1 cross-linking) or 1.5% agarose gel in 0.5 x TBE buffer. In competition experiments, the competitors were added to 100-fold molar excess (no difference was observed when competitors were added before, at the same time, or after the probe). When antibodies were used, 1 μl of diluted antiserum was added to the reaction mixture, and after 5 min of further incubation at room temperature the reaction mixture was loaded onto the gel. Polyacrylamide gels were prerun for 30 min at 130 V at 28–30°C, and after loading the samples the same running conditions were used until the bromphenol blue had just passed the bottom of the gel (~1.5 hr). An alternative and possibly better means of resolving U and L complexes is to run longer gels with higher voltage so that the temperature in the gel reaches 28–30°C. The prerun in this case is important for at least 1 hr to equilibrate the temperature. Agarose gels were run at 4°C for 1.5 hr. After fixing (10% methanol, 10% acetic acid) and drying, the gels were exposed to autoradiography at ~80°C.

**Other procedures**

S1 mapping analysis was performed as described by Nasmyth (1985a). Measurement of CDC28 H1-kinase activity was as in Surana et al. (1991).

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