Direct induction of G₁-specific transcripts following reactivation of the Cdc28 kinase in the absence of de novo protein synthesis

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In *Saccharomyces cerevisiae*, the genes encoding the *HO* endonuclease, G₁-specific cyclins *CLN1* and *CLN2*, as well as most proteins involved in DNA synthesis, are periodically transcribed with maximal levels reached in late G₁. For *HO* and the DNA replication genes, cell cycle stage-specific expression has been shown to be dependent on the Cdc28 kinase and passage through START. Here, we show that cells released from *cdc28^{ts}* arrest in the presence of cycloheximide show wild-type levels of induction for *HO*, *CLN1*, and *CDC9* (DNA ligase). Induction is gradual with a significant lag not seen in untreated cells where transcript levels fluctuate coordinately with the cell cycle. This lag may be due, at least in part, to association of the Cdc28 peptide with G₁ cyclins to form an active kinase complex because overexpression of *CLN2* prior to release in cycloheximide increases the rate of induction for *CDC9* and *HO*. Consistent with this, release from pheromone arrest (where *CLN1* and *CLN2* are not expressed) in cycloheximide shows no induction at all. Transcriptonal activation of *CDC9* is likely to be mediated through a conserved promoter element also present in genes for other DNA synthesis enzymes similarly cell cycle regulated. The element contains an intact *MluI* restriction enzyme recognition site (consensus ~5'-A/TPuACGCGTNA/T-3'). Insertion of a 20-bp fragment from the *CDC9* promoter (containing a *MluI* element) upstream of *LacZ* confers both periodic expression and transcriptional induction in cycloheximide following release from *cdc28^{ts}* arrest. High levels of induction depended on both the *MluI* element and *CDC28*. These results suggest that the activity of *trans*-acting factors that operate through the *MluI* element may be governed by phosphorylation by the Cdc28 kinase.

[Key Words: Cell cycle; *Saccharomyces cerevisiae*; *CDC28*; transcription]

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In the budding yeast *Saccharomyces cerevisiae*, entry into the mitotic cell cycle is regulated at a point within the G₁ interval, operationally defined as START (for review, see Pringle and Hartwell 1981). At this control point, cells assess their external environment and subsequently either proceed into S-phase or exit the cell cycle (by mating, sporulating, or entering into stationary phase). Passage through START requires a functional *CDC28* gene, which encodes a 34-kD cell cycle-specific protein kinase (Reed et al. 1985; Wittenberg and Reed 1988) that also regulates entry into mitosis (Piggott et al. 1982; Reed and Wittenberg 1990). In *Schizosaccharomyces pombe*, the homologous Cdc2 protein similarly regulates START as well as the G₂/M transition (Nurse and Bissett 1981). It is now known that both proteins are members of an evolutionarily conserved family of protein kinases that constitute the catalytic subunit of the

universal mitotic regulator, maturation promoting factor, or MPF (for review, see Murray and Kirschner 1989).

Although *CDC28* function is essential for completing START and initiating successive rounds of cell division, the precise role it plays in this transition is undetermined. There are no reports to date positively identifying any in vivo substrates for the G₁ form of the Cdc28 kinase complex. A 40-kD protein has been found in extracts of G₁ cells that coimmunoprecipitates with the *CDC28* gene product and serves as a substrate for the Cdc28 kinase in vitro (Mendenhall et al. 1987). However, its function is not known. To understand the mechanisms of START, it is necessary to define the in vivo substrates of *CDC28* and how they influence cellular changes necessary for the onset of S-phase.

In *S. cerevisiae*, there are several classes of genes that exhibit START (*CDC28*)-dependent, cell cycle-regulated expression, reaching peak transcript levels in late G₁. For example, many of the genes encoding the enzymes necessary for DNA synthesis or dNTP precursor production,

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including DNA polymerase α [*POL1* (Johnston et al. 1987)], DNA polymerase δ (*CDC2*), and proliferating cell nuclear antigen or PCNA [*POL30* (Bauer and Burgers 1990)], the subunits of DNA primase [*PRI1* (Plevani et al. 1987) and *PRI2* (Foiani et al. 1989)], DNA ligase (*CDC9* [Peterson et al. 1985]), ribonucleotide reductase [*RNR1* (Elledge and Davis 1990)], thymidylate kinase [*CDC8* (White et al. 1987)], and thymidylate synthase [*CDC21* (Storms et al. 1984)] are periodically expressed in late G_1 . In addition, the *CDC6* gene, which has an unknown function but is apparently required for S-phase entry, is similarly cell cycle regulated (Zhou et al. 1989, 1990).

Analysis of the promoter regions of these genes has revealed the presence of a conserved sequence, 5'-A/TPuACGCGTNA/T-3', which contains the recognition site (or 5/6 match) of the *MluI* restriction enzyme. The site is sometimes repeated twice, ~100–200 bp upstream of the initiation codon. Cell cycle-specific transcription of *CDC21* and *POL1* has been shown to be mediated through the *MluI* sequences [called the *MluI* cell cycle box, or MCB (Gordon and Campbell 1991; McIntosh et al. 1991)]. Furthermore, a synthetic trimer of the ACGCGT hexanucleotide can confer on a reporter gene some features of the regulated expression of this class of genes (Lowndes et al. 1991). A binding factor specific for the MCB has been purified (Verma et al. 1991), which also binds to several other promoters containing the *MluI* motif. Therefore, it appears that regulation of this set of genes may be coordinated by a common factor.

In addition to the DNA synthesis genes, transcription of *HO*, which encodes a site-specific endonuclease that initiates mating-type switching, also occurs post-START in late G_1 (Nasmyth 1985). Periodic expression of *HO* is conferred by a discrete promoter element [CACGAAAA (called the cell cycle box or CCB)], which is repeated 10 times in the upstream regulatory region (Nasmyth 1985). When tandemly repeated, this sequence promotes transcription of a reporter gene with the correct periodicity and START dependency (Breedon and Nasmyth 1987a). Genetic and biochemical data indicate that both the Swi4 and Swi6 proteins act through the CCB element to mediate cell cycle-specific transcription of *HO* (Breedon and Nasmyth 1987a; Andrews and Herskowitz 1989a,b). Swi4 and Swi6 are therefore prime candidates for direct transcriptional activators responsible for G_1 -specific transcription of *HO*.

Recently, CCB-like sequence repeats have been found in the upstream promoter region of the G_1 cyclin genes *CLN1* and *CLN2* (Nasmyth and Dirick 1991; Ogas et al. 1991; C. Wittenberg, unpubl.), whose expression is also limited to G_1 . G_1 -specific transcription of *CLN1* and *CLN2* has been shown to have at least two modes of regulation: a cell size-dependent, *CDC28*-independent mode, as well as one that is dependent on *CDC28* activity (Cross and Tinkelenberg 1991; Dirick and Nasmyth 1991; D. Lew et al. 1992). The latter form of regulation is responsible for generating a positive feedback loop whereby Cln accumulation is autoamplified. That is, as Cln proteins accumulate and activate the Cdc28 kinase, *CLN1* and *CLN2* transcription is stimulated further and

more Clns accumulate. By analogy with *HO*, *CDC28*-dependent transcription of *CLN1* and *CLN2* may be mediated by *SWI4* and/or *SWI6* by CCB-like sequences (Nasmyth and Dirick 1991; Ogas et al. 1991).

The regulatory complexes that mediate the G_1 -specific transcription of the above-mentioned genes must all somehow sense cell cycle position, especially regarding passage through START. Until now, however, it was not clear exactly how far downstream of the Cdc28 signal the activation of these genes lay. In this paper we demonstrate that cells released from a *cdc28^{ts}* arrest in the absence of de novo protein synthesis show wild-type levels of induction for the *HO*, *CLN1*, and *CDC9* (DNA ligase) transcripts. Thus, all the components necessary for the START (*CDC28*)-dependent transcriptional activation of these genes are present at the *CDC28* execution point. Under these conditions, transcript levels peak after a significant lag and remain high. The observed lag can be reduced by overexpression of *CLN2*, suggesting that it is partly the result of association of the Cdc28 polypeptide with G_1 cyclins to form an active kinase complex. Insertion of a 20-bp fragment from the *CDC9* promoter (containing the *MluI* motif) upstream of a reporter gene confers both periodic expression and transcriptional induction in the absence of protein synthesis following release from *cdc28^{ts}* arrest. The most economical interpretation of these results is that the activity of transcription factors that mediate the G_1 activation of these genes is governed by phosphorylation by the Cdc28 kinase.

Results

Coordinate expression of three classes of G_1 -specific genes

The premise for identifying transcripts that can be induced in the absence of protein synthesis following release from a *cdc28^{ts}* block is that these genes must be regulated by factors whose activity is somehow intimately related to activation of the Cdc28 kinase at START. It is likely, moreover, that such transcription factors serve as in vivo substrates for Cdc28 and are thus activated (or deactivated) by this phosphorylation. Identification of such genes and ultimately the respective *trans*-acting factors will therefore help us to better understand the molecular aspects of passage through START and the role of Cdc28.

The success of identifying transcriptional induction under these conditions, however, is predicated on the ability of cells to recover from a *cdc28^{ts}* block and proceed synchronously through the cell cycle. Figure 1 shows the results from such a cell cycle synchrony experiment. Cells bearing a *cdc28-13* allele were arrested at START by incubation at the restrictive temperature followed by shift to the permissive temperature as described in Materials and methods. At the indicated times following the temperature shift, RNA was isolated and the cells were scored morphologically to determine their

Figure 1. D13au (*cdc28-13*) cells were arrested at START by incubation at 37°C for 3 hr and shifted to 23°C. The cells were scored morphologically, and RNA was isolated at the indicated times following the shift. Transcript levels were quantitated from densitometric scans of Northern blots (bottom) normalized to the *CDC36* transcript. For *HO*, peak transcript levels are relative to the 15-min time point. The budding profile for the culture is shown in the top panel.

position in the cell cycle. Both the budding profile and the correct periodic appearance of late G_1 , *CDC28*-dependent transcripts indicated good cell cycle synchrony through at least the first cell cycle following release from *cdc28^{ts}* arrest. The three transcripts measured [*CDC9* (DNA ligase), *HO*, *CLN1*] always showed peak expression levels (relative to *CDC36* mRNA) 30–45 min following shift to the permissive temperature and prior to the onset of budding (which is coincident with initiation of S-phase). Thus, the timing of cell cycle traverse under these conditions is similar to that achieved by synchro-

nization with mating pheromone. Good cell cycle synchrony was only achieved, however, using the *cdc28-13* allele, as *cdc28-4* cells recovered very poorly upon shift to the permissive temperature.

It is interesting to note that the three transcripts shown in Figure 1 display similar kinetics of accumulation, with all reaching peak levels at the same time following the temperature shift. Because *CDC9* is representative of a class of periodically expressed genes encoding proteins involved in DNA synthesis (see introductory section), it seems likely that this set of genes in addition to *CLN1*, *CLN2* (not shown), and *HO* are all simultaneously transcribed shortly following activation of the Cdc28 kinase at START. It should be re-emphasized that although *CLN1* and *CLN2* encode G_1 cyclins that can activate the Cdc28 kinase, presumably by binding the $p34^{CDC28}$ catalytic subunit, part of their transcriptional regulation is dependent on Cdc28 activation, thus creating a positive feedback loop whereby *CLN1* and *CLN2* expression is autoamplified. It appears as if the kinetics of activation of this positive feedback loop are similar to that of induction of *CDC9* and *HO* transcription.

Direct dependence on the Cdc28 kinase

To determine whether protein synthesis was required for transcriptional induction following reactivation of Cdc28, a similar experiment was performed except that cycloheximide was added to the culture 10 min prior to shift to the permissive temperature (see Materials and methods). Under the conditions employed here, total cellular protein synthesis was inhibited 96–97%, with the residual largely the result of mitochondrial protein synthesis (Schatz and Saltzgeber 1969). The cells do not form buds nor do they grow or initiate DNA replication (as determined by light scattering and fluorescence) even at prolonged incubation at the permissive temperature. As shown in the Northern analysis in Figure 2, the *CDC9*, *HO*, and *CLN1* transcripts were all induced in the absence of protein synthesis following Cdc28 kinase activation. This induction is not an artifact of increased message stabilization caused by cycloheximide because no increase in transcript levels was seen at the restrictive temperature for Cdc28. In fact, the *CLN1* message was rapidly lost under these conditions.

Although peak transcript levels in cycloheximide-treated cells were roughly similar to those in untreated cells (cf. Figs. 1 and 2), there was a lag not seen in untreated cells. Typically, transcripts accumulated for ~2 hr (vs. 30–45 min in cycling cells), at which point the levels reached a plateau and remained high for at least 4 hr following the shift to the permissive temperature (see Fig. 5, below). Therefore, it appears that cells in this state are “frozen” in the cell cycle in late G_1 . Even though it may seem from the quantitative densitometric analysis (Fig. 2, top) that the *CLN1* message reached its maximal level prior to that of *CDC9*, these data were from different experiments. Within any one experiment, the timing of induction in cycloheximide-treated cells was the same for each of the three transcripts shown.

Figure 2. D13au [*cdc28-13*] cells were grown and arrested as in Fig. 1. Cycloheximide was then added to 10 μ g/ml, and the cells were incubated further for 10 min at 37°C. The culture was then split and half was shifted to 23°C while the other half was maintained at 37°C. RNA was isolated at the indicated times. *CDC9* and *CLN1* mRNA levels were quantitated (top panel) from densitometric scans of Northern blots (bottom panels) and normalized to *CDC36* transcript levels.

We conclude from these experiments that all of the regulatory factors necessary for the Cdc28-dependent activation of *CDC9* (and probably other DNA synthesis genes similarly regulated), the periodically transcribed G_1 cyclins (*CLN1* and *CLN2*), and *HO* are present at the *CDC28* execution point. As stated above, the activity of these factors must be closely related to Cdc28 kinase activation and may be a direct result of phosphorylation by Cdc28. In this regard, it is interesting that periodic transcription of the DNA replication genes seems to be

driven by a different promoter element (and possibly by different transcription factors) than that of *CLN1*, *CLN2*, and *HO* (see introductory section).

G₁ cyclins are stable in cycloheximide

The delayed and gradual accumulation of transcripts seen in cycloheximide-treated cultures seems curious because activation of Cdc28 presumably requires association with a G_1 cyclin. In addition to the inherent instability of Cln protein (15-min half-life; Wittenberg et al. 1990), Cln1 and Cln2 biosynthesis by a Cdc28-dependent positive feedback loop is prevented in this experiment by the addition of cycloheximide. Thus, at the *cdc28^{ts}* arrest point, one might predict only low levels of Cln protein that would turn over rapidly following cycloheximide addition. As shown in Figure 2, however, the signal for transcriptional induction is steady and persists for 2 hr following shift to the permissive temperature. It is possible that although only a low level of Cdc28 kinase may be transiently activated following shift to the permissive temperature and subsequently inactivated by Cln proteolysis, the targets are stably phosphorylated and, thus, the signal persists after the kinase has been inactivated. Alternatively, it is possible that under these conditions, Cln protein (and, therefore, kinase) is stabilized and leads to chronic, low-level kinase activity in cycloheximide-treated cells at the permissive temperature.

To address this question, protein extracts were made from cells treated with cycloheximide as in Figure 2 and analyzed for the presence of Cln2 protein by immunoblotting. The results (Fig. 3) demonstrated that unlike Cln2 in cycling cells, Cln2 in cycloheximide-treated cells is stable for at least 4 hr. During the time course there appears to be an accumulation of post-translationally modified forms of the Cln2 protein. Such forms of Cln2 have been observed previously (Wittenberg et al.

Figure 3. D13au [*cdc28-13*] cells were grown and arrested as in Fig. 1. Cycloheximide (CHX) was then added to 10 μ g/ml and the cells were incubated further for 10 min at 37°C. The culture was then shifted to 23°C and maintained at that temperature for 4 hr. Samples were taken just prior to addition of CHX (lane 1), just prior to shift to the permissive temperature (lane 2), and at the indicated times following temperature shift (lanes 3–5). Protein was isolated from each sample and analyzed by immunoblotting with Cln2 antiserum. Cln2-specific bands are indicated by arrows; asterisks (*) indicate post-translationally modified forms of Cln2 (see text).

1990) and are partially the result of phosphorylation (C. Wittenberg, pers. comm.). It is interesting, therefore, that these forms accumulate following release from a *CDC28* block in the absence of protein synthesis.

In any event, given the stability of Cln2, it is likely that a low level of Cdc28 kinase activity is chronically active for the duration of this experiment. Consistent with this, a constant, low level of histone H1 kinase activity was detected in anti-Cdc28 immunoprecipitates from cells similarly treated with cycloheximide through at least 2 hr following shift to the permissive temperature (data not shown).

G₁ cyclins are rate limiting for transcriptional induction

Given the low level of Cln2 protein present in cycloheximide-treated cultures, it is likely that the levels of Cln protein become rate limiting for binding to and activating Cdc28 and triggering the transcriptional response. To test this possibility directly, the Cln2 protein was overexpressed from the inducible *GAL1* promoter prior to shift to the permissive temperature in the presence of cycloheximide. NMY1 (*cdc28-13 GAL1^P::CLN2*) cells

were grown in YEP-sucrose and arrested at 37°C for 4 hr. The culture was split, and galactose was added to one-half. Both cultures were incubated at 37°C for an additional 30 min, at which point cycloheximide was added to each and the cultures were shifted to the permissive temperature. A longer incubation at the restrictive temperature was required for uniform arrest as a result of the significantly longer cell cycle of these cells in sucrose-based media.

As shown in Figure 4A, overexpression of *CLN2* markedly increased the rate of *CDC9* transcript induction. This effect is not simply the result of galactose addition because cells lacking the inducible *CLN2* allele showed no difference in *CDC9* induction in a similar experiment with or without galactose (not shown). In the culture without addition of galactose, the induction appeared weak relative to similar experiments with glucose-based media (cf. Fig. 4A with Figs. 2 and 5B). This may be directly the result of media differences or it may simply reflect a lengthening of the cell cycle in YEP-sucrose and the need for longer incubations to reach peak transcription levels. In any event, it is clear that overproduction of Cln2 increases the initial rate of *CDC9* transcriptional induction. Similar results were obtained with *HO* (data

Figure 4. (A) NMY1 (*cdc28-13 GAL1^P::CLN2*) cells were grown in YEP-sucrose and shifted to 37°C for 4 hr. The culture was split, and galactose (final conc. 2%) was added to one-half. Both cultures were incubated further for 30 min at 37°C. Cycloheximide was then added (10 µg/ml) to each culture, and after 10 min, both cultures were shifted to 23°C. RNA was then isolated at the indicated times, and *CDC9* mRNA levels were quantitated as in the previous figures. (B) GCY11 (*bar1::LEU2*) cells were arrested by the addition of α -factor to 60 ng/ml for 3 hr. The culture was then split, and cycloheximide (CHX) was added to one half. After 10 min, pheromone was washed out and the cells were resuspended in fresh media. RNA was isolated at the indicated times, and the *CDC9* mRNA level was quantitated as before. (Top) The budding profile of the control culture following α -factor release.

not shown). Therefore, it appears that G_1 cyclins are rate limiting for Cdc28 activation under these conditions; and by increasing the rate of kinase activation in the absence of protein synthesis, one can increase the rate of Cdc28-dependent transcriptional induction.

Even though the level of Cln protein is apparently rate limiting at the *CDC28* execution point, these cells still contain the mRNA of all three functionally redundant G_1 cyclin genes, *CLN1*, *CLN2*, and *CLN3* (Fig. 1; Wittenberg et al. 1990). Unlike that of *CLN1* and *CLN2*, the *CLN3* transcript does not vary as a function of the cell cycle but remains constitutively expressed (Nash et al. 1988; Wittenberg et al. 1990). The levels of *CLN1* and *CLN2* transcripts, however, are low in the absence of the Cdc28-dependent positive feedback loop. Phormone-arrested cells, on the other hand, contain detectable mRNA (and presumably protein) levels only of *CLN3* (Wittenberg et al. 1990). Thus, release from phormone arrest in the presence of cycloheximide should display an even more striking lag of transcriptional induction than release from *cdc28^{ts}* arrest. As shown in Figure 4B, the *CDC9* transcript is not induced at all following release from α -factor arrest under these conditions. In control (–CHX) cells, however, the message exhibited normal cell cycle periodicity. A similar result has been reported for *RNR1*, a gene encoding a subunit of ribonucleotide reductase coordinately regulated with *CDC9* (Elledge and Davis 1990). Given that cells deleted for both *CLN1* and *CLN2* are still viable (Richardson et al. 1989), the absence of any detectable transcript induction (and presumably kinase activation) in this experiment may indicate either that, unlike the message, the Cln3 protein is no longer present after prolonged exposure to α -factor or that it must somehow be activated following release from phormone arrest in a way that requires protein synthesis.

A target of Cdc28-dependent activation is the MCB

As stated in the introductory section, a number of genes encoding proteins involved in DNA synthesis are coordinately regulated and all contain a conserved promoter element (the MCB; Verma et al. 1991), which contains at least an intact *MluI* restriction enzyme site or a 5/6 match. Nine base pairs surrounding one *MluI* site in the *CDC21* promoter (McIntosh et al. 1991) and 19 bp encompassing an *MluI* site from the *POL1* promoter (Gordon and Campbell 1991) confer correct periodic expression on heterologous genes. Similarly, a synthetic trimer of the ACGCGT hexamer can direct periodic expression of a reporter gene (Lowndes et al. 1991). With this synthetic upstream activating sequence (UAS), however, transcript levels were high in phormone-arrested cells and fell through the first cell cycle following release only to fluctuate properly in the second round of cell division. Thus, all of the regulatory information cannot reside solely in *MluI* hexanucleotide.

It has been shown previously that a 60-bp fragment from the *CDC9* promoter (containing the *MluI* site) can direct β -galactosidase (*lacZ*) transcription in the correct

cell cycle-dependent fashion following release from α -factor arrest (Lowndes et al. 1991). To further define the MCB and to ask whether this promoter element itself can confer inducibility on a heterologous gene as in the previous experiments, a *MluI::lacZ* expression plasmid was constructed (Fig. 5A). The region from –137 to –117 (relative to the initiation codon) in the *CDC9* promoter was inserted upstream of *lacZ* in the UAS-deficient plasmid pCZ Δ (Buchman and Kornberg 1990). This region includes an intact *MluI* site, a 5/6 match nearby (a feature of some promoters in this class of genes), and 3 bases on either side of these elements.

The resulting plasmid, pMlu20, was transformed into *cdc28-13* cells, and *lacZ* expression was monitored following release from a *cdc28^{ts}* block in the absence or presence of cycloheximide. It is clear from the Northern analysis (Fig. 5B) that the *lacZ* transcript behaves exactly as does *CDC9* both in cycling and cycloheximide-treated cells. The only visible difference is the existence of a significant basal level of constitutive, Cdc28-independent synthesis of *CDC9*, not seen in *lacZ*, which is probably driven independently of the MCB. Transcriptional induction therefore appears greater for *lacZ* relative to the zero time point. That this induction is dependent on Cdc28 activation is shown in Figure 6. NMY2 (*cdc28-13* pMlu20) cells released from *cdc28^{ts}*-arrest in the presence of cycloheximide at the permissive temperature show significantly greater induction of *lacZ* than those kept at the restrictive temperature, similar to the *CDC9* message in the same cells. However, there appears to be a slight induction of *lacZ* at the restrictive temperature. This is likely the result of stabilization by cycloheximide of a low background level of transcription independent of the *MluI* element. The parent plasmid pCZ Δ also exhibited a very low level of *lacZ* transcription that was not periodic but was weakly induced (similar to the 37°C *lacZ* samples in Fig. 6) at both the restrictive and permissive temperatures in a similar experiment (not shown). We conclude from these experiments that all of the information necessary to direct the G_1 periodicity of *CDC9* and induction in the absence of protein synthesis following release from *cdc28^{ts}* arrest is contained within these 20 bp from the *CDC9* promoter, which encompass the MCB. Thus, transcription factors that operate through the MCB or proteins regulating them are likely substrates of the G_1 form of the Cdc28 kinase.

Discussion

In this paper we have shown that cells released from *cdc28^{ts}* arrest in the absence of de novo protein synthesis are capable of transcriptionally activating the *HO*, *CLN1*, and *CDC9* genes. Furthermore, we have defined a small region from the *CDC9* promoter containing the MCB that is responsible for mediating the *CDC28*-dependent G_1 periodicity of *CDC9* as well as induction in cycloheximide following release from the *cdc28^{ts}* block. Thus, whatever factors operate through the MCB are present at the *CDC28* execution point and are potential substrates for the Cdc28 kinase. By analogy to *CDC9*,

Figure 5. (A) Complementary oligonucleotides were constructed such that the region from -137 to -117 (from the initiation codon) in the *CDC9* promoter was directionally cloned into the polylinker of the UAS-deficient expression vector pCZΔ (gift of R. Kornberg). This region contains a conserved *MluI* restriction enzyme site (black box) that is present in the upstream region of many periodically expressed genes encoding proteins involved in DNA synthesis. A nearby 5/6 match to a *MluI* site (also a feature of some genes in this class) is outlined. The resulting plasmid is pMlu20. (B) NMY2 (*cdc28-13* pMlu20) cells were treated and analyzed essentially as in Fig. 1 for the $-CHX$ samples and as in Fig. 2 [at 23°C] for the $+CHX$ samples. In the $-CHX$ culture, *lacZ* transcript levels are relative to the 15-min time point owing to the very low level at 0 min.

transcription factors that operate through the CCB of *HO*, *CLN1*, and *CLN2* (the Swi4 and/or Swi6 proteins) are also present at this point and are also potential substrates.

Although little is known concerning MCB-binding factors, much is known about SWI4 and SWI6. Genetic and biochemical data indicate that both the Swi4 and Swi6 proteins act through the CCB of *HO* and CCB-like sequences of *CLN1* and *CLN2* to promote *CDC28*-dependent G_1 -specific expression of these genes (Breedon and Nasmyth 1987a; Andrews and Herskowitz 1989a,b; Nasmyth and Dirick 1991; Ogas et al. 1991). Both proteins bind to these regulatory sequences as part of a multisubunit transcription complex (Andrews and Herskowitz 1989b; Nasmyth and Dirick 1991; Ogas et al. 1991).

As stated earlier, G_1 -specific transcription of *CLN1* and *CLN2* is thought to have two modes of regulation: a size-dependent, *CDC28*-independent mode, and one that is dependent on *CDC28* activity (Cross and Tinklenberg 1991; Dirick and Nasmyth 1991; D. Lew et al.

1992). The latter form of regulation is thought to lead to a positive feedback loop whereby Cln accumulation activates the Cdc28 kinase, which further stimulates *CLN1* and *CLN2* transcription leading to more Cln accumulation, and so on. Just as for *HO*, Swi4 and Swi6 probably mediate the Cdc28-dependent signal necessary for autoamplification of *CLN1* and *CLN2* transcripts.

Although Swi4 and Swi6 have not been shown to be *in vivo* substrates of the G_1 form of the Cdc28 kinase, both proteins contain consensus Cdc28 phosphorylation sites within regions that are highly conserved in a wide variety of proteins from diverse species (Breedon and Nasmyth 1987b; Andrews and Herskowitz 1989b, 1990). Perhaps the most notable of these is the *S. pombe cdc10* gene, which is also involved in START progression in fission yeast and may have a similar function. Furthermore, Swi4 is apparently a phosphoprotein (Andrews and Herskowitz 1990). This, combined with the results presented here, make it very likely that START-dependent activation of *HO* and *CLN1* and *CLN2* is brought about

Figure 6. NMY2 cells were released from *cdc28^{ts}* arrest in the presence of cycloheximide as in the legend to Fig. 2. *CDC9* and *lacZ* RNA levels were quantitated for both the 23°C culture and the 37°C cultures exactly as described previously.

by direct phosphorylation of the Swi4 and/or Swi6 proteins by Cdc28.

A protein that specifically binds the MCB from the *POL1* promoter has recently been purified (Verma et al. 1991). This protein has an apparent molecular mass of 17 kD and can also specifically bind the MCB from several other DNA synthesis genes. It remains to be determined, however, whether this protein actually functions in the periodic regulation of these genes (either alone or in a complex like Swi4/6) or can serve as a substrate for Cdc28.

It seems likely that transcriptional induction in the absence of protein synthesis following release from *cdc28^{ts}* arrest is indicative of direct phosphorylation of transcriptional regulators by Cdc28. Although it is possible that intermediate steps exist, we think it unlikely for several reasons. First, although transcript levels of *HO* (Nasmyth 1983), *POL1* (Johnston et al. 1987), *CDC8*, *CDC9*, and *CDC21* (White et al. 1987) are low or absent in *cdc28^{ts}*-arrested cells, they are fully activated in *cdc4^{ts}*-arrested cells. The *CDC4* execution point is immediately following that of *CDC28* in late G₁ (Pringle and Hartwell 1981). Second, as shown in Figure 4A, the level of Cln protein at the *CDC28* block is rate limiting for transcriptional induction in the absence of further protein synthesis. With *CLN2* overexpression, the rate of induction is increased; thus, there is a direct relationship between Cdc28 kinase activation and transcriptional induction. Finally, it has been proposed that the Cdc28-dependent positive feedback loop whereby Cln synthesis is autoamplified serves to create a decisive burst of Cln accumulation that is necessary to fully activate the Cdc28 kinase and commit the cell to a new round of cell division. It seems very unlikely, therefore, for this regulatory loop to include intermediate steps other than direct phosphorylation of Swi4 and/or Swi6. Given the fact that the rate of transcriptional induction of *CLN1* in cy-

cloheximide is the same as that of *HO* or *CDC9*, we believe that all of these are likely to represent direct interactions between Cdc28 and the respective transcription factors. It should be pointed out that although phosphorylation by the Cdc28 kinase may activate *trans*-acting factors, it is equally plausible that this phosphorylation inactivates negative regulators of transcription.

The question then arises as to how these regulatory molecules might be affected by phosphorylation. Lowndes et al. (1991) have shown that a DNA-binding activity specific for a fragment containing a synthetic trimer of the ACGCGT sequence fluctuates coordinately with *lacZ* transcription driven by the same sequence as a UAS element. The *MluI* trimer, however, does not respond correctly to α -factor arrest (i.e., *lacZ* transcript levels are high in pheromone-arrested cells) nor does it peak in the first G₁ phase following α -factor removal. Correct periodicity is seen, though, in the subsequent cell division cycles. We have performed gel mobility-shift assays using a probe from the polylinker region of plasmid pMlu20, which contains the MCB element from *CDC9*. Although sequence-specific binding activity was detected in cell extracts, we have never observed fluctuation of this activity as a function of cell cycle traverse using a variety of synchrony methods (N.J. Marini and S.I. Reed, unpubl.). Similarly, the binding of Swi4/6 to the CCB sequences apparently does not quantitatively vary with the cell cycle (Andrews and Herskowitz 1989a; Nasmyth and Dirick 1991) although, under certain conditions, a slower migrating species appears near the time of *HO* activation (presumably the result of phosphorylation; Nasmyth and Dirick 1991).

If, then, these transcription factors are constitutively bound to their respective promoter elements and subsequently activated by phosphorylation while DNA associated, this scenario would fit a familiar theme in yeast. In *S. cerevisiae*, the heat shock transcription factor (HSF) is apparently bound to the heat shock element even at low temperature and shows heat-induced phosphorylation, which is thought to trigger heat shock gene transcription (Sorger and Pelham 1988). Likewise, there is good evidence that the *STE12* protein, which mediates transcriptional induction of the pheromone-responsive genes, is bound to the pheromone response element (PRE) in the absence of pheromone and activated by phosphorylation upon exposure to pheromone (even in the absence of protein synthesis; Song et al. 1991). Alternatively, it is possible that phosphorylation may influence nuclear localization. The yeast Swi5 protein (also a *trans*-activator of *HO* responsible for mother cell-specific expression) enters the nucleus in early G₁, and this event is probably mediated by cell cycle-regulated phosphorylation by the Cdc28 kinase (Moll et al. 1991). In this latter type of situation, it is possible that whole-cell extracts will not exhibit cell cycle-specific DNA-binding activity by a gel mobility-shift assay, similar to what we have seen for MCB-binding activity.

It is interesting to note that the MCB element may also play a role in regulating the DNA damage inducibility of this class of genes. In addition to being cell cycle

regulated, *POL1* (Johnston et al. 1987), *RNR1* (Elledge and Davis 1990), and *CDC9* (Barker et al. 1985; Peterson et al. 1985) transcripts are also induced in response to a variety of agents that damage DNA. In the case of *RNR2*, damage inducibility is conferred by a damage response element (DRE), which has been localized to a small promoter segment containing a 5/6 match to the *MluI* site (Elledge and Davis 1989). Similarly, an intact *MluI* site has been shown to be essential for damage inducibility of *RAD54* (Cole and Mortimer 1989). *RAD54* is also weakly periodic (about threefold) through the cell cycle and is expressed coordinately with *CDC9* (N.J. Marini and S.I. Reed, unpub.). Further experiments are necessary, however, to determine the role of the MCB in DNA damage induction.

To explain the results presented here, we propose that one role for the Cdc28 kinase at START is to phosphorylate, and thereby activate, transcription factors involved in regulating the periodic expression of *HO*, *CLN1* and *CLN2*, and several genes encoding proteins involved in DNA replication. Because these genes are cell cycle regulated by strikingly different promoter elements, it is possible that different factors (and, therefore, different substrates) are involved. Alternatively, it is also possible that the Swi4 or Swi6 proteins, which have been shown to be part of multimeric transcription complexes on the CCB, may also be a part or somehow control the activity of complexes that bind the MCB.

Materials and methods

Yeast strains and plasmids

All yeast strains used in this study were derivatives of BF264-15D, *MAT α ade1 his2 leu2-3,112 trp1-1a* (Reed et al. 1985). The genotypes of the strains used were 15 Dau, *MAT α ura3 Δ NS* (Cole et al. 1990); D13au 15 Dau *cdc28-13*; GCY11 15 Dau *bar1::LEU2*; NMY1, D13au *leu2::GAL1^P::CLN2 LEU2*; NMY2, D13au (pMlu20). All yeast transformations were carried out by the alkali cation method described by Ito et al. (1983).

Plasmid YIpG2[*CLN2*] (containing the *CLN2* allele under transcriptional control of the *GAL1* promoter) was created from YCpG2[*CLN2*] (Wittenberg et al. 1990) by removal of the 4.7-kb *SalI*-*BamHI* fragment encompassing the *URA3*, *CEN4*, and *ARS1* loci. To create strain NMY1, *GAL1^P::CLN2* was integrated at the *leu2* locus of D13au by transformation with YIpG2[*CLN2*] linearized at the *BstEII* site within the *LEU2* allele remaining on the plasmid. Integration of this plasmid at the *leu2* locus was confirmed by Southern analysis of genomic DNA.

Plasmid pMlu20 was created by directionally inserting the following complementary oligonucleotides into the polylinker region of pCZA (Buchman and Kornberg 1990) doubly digested with *EcoRI* and *XhoI* (see Fig. 4A).

5'-AATTGTGAACGCGAAAACGCGTGAAC
CAATTGCGCTTTTGCGCACTTGAGCT-5'

These oligonucleotides contain the region from -137 to -117 (relative to the initiation codon) in the *CDC9* promoter (Barker et al. 1985). pCZA is a UAS-deficient expression plasmid containing the yeast *CYC1* promoter fused to LacZ protein-coding sequences.

Growth conditions and cell cycle synchrony

Yeast cultures were grown on YEPD (1% yeast extract, 2% Bacto-peptone, 0.005% adenine, 0.005% uracil, 2% glucose). When plasmid selection was required, cells were grown in minimal medium supplemented with appropriate amino acids and/or bases (Sherman et al. 1982). Galactose induction experiments were performed by growing cells in YEP, supplemented with sucrose (2%), and adding galactose to 2%.

To obtain synchronously growing cultures following release from *cdc28^{ts}* arrest, cells bearing a *cdc28-13* allele were grown to mid-log phase, incubated at 37°C for 3 hr (unless otherwise noted), and shifted to 23°C. Synchronization of yeast cultures by mating pheromone treatment was described by Wittenberg et al. (1990). Where indicated, de novo protein synthesis was inhibited prior to Cdc28 reactivation by adding cycloheximide (final concentration, 10 μ g/ml) 10 min prior to shift to the permissive temperature or α -factor washout. This concentration of cycloheximide was determined to inhibit 96–97% of total cellular protein synthesis in both asynchronous and *CDC28*-arrested cultures as measured by TCA-precipitable incorporation in cells labeled with [³⁵S]methionine essentially as described (Wittenberg and Reed 1988; Yaffe 1991). The residual level of protein synthesis is probably largely the result of mitochondrial protein synthesis (Schatz and Saltzgeber 1969).

RNA preparation and northern analysis

Total RNA was isolated from $\sim 10^8$ cells essentially as described previously (Elder et al. 1983) and electrophoretically separated on 1% agarose gels containing 2.2 M formaldehyde (Maniatis et al. 1989). RNA was transferred to Biotrans (ICN) nylon membrane, and hybridization and washes were performed as described by Reed et al. (1982).

Radiolabeled probes were prepared using a random primer DNA labeling kit (Boehringer Mannheim) according to the manufacturer's instructions. The DNA fragments used as probes were as follows: *CDC9* and *CDC36*, the 2.7-kb *SacI* fragment from YEp13[*CDC9.2*] which contains regions of both genes (Peterson et al. 1985); *HO*, the 875bp *HindIII*-*BamHI* fragment from YCp50[*HO*] (Russell et al. 1986); *CLN1*, the 1.6-kb *BamHI* fragment encompassing the open reading frame (Hadwiger et al. 1989); and *lacZ*, the 3.0-kb *PstI* fragment from pHO-lac-cl2 (Russell et al. 1986). An Ultrascan XL laser densitometer (LKB) was used to scan autoradiographs to quantitate transcript levels relative to *CDC36* mRNA, whose level is invariant through the cell cycle (Peterson et al. 1985). Filters were stripped and re-probed as described (Maniatis et al. 1989).

Cln2 immunoblotting

Protein extracts were prepared from $\sim 10^8$ cells by vortexing with glass beads (0.5 mm) in 100 μ l of 300 mM NaCl, 50 mM Tris-HCl (pH 7.2), 10 mM Na₂P₂O₇, 50 mM NaF, 0.5 mM Na₃VO₄, and 1 μ g/ml of each aprotinin, leupeptin, and pepstatin. Lysates were spun in a microcentrifuge for 15 min, and 0.75 A₂₈₀ units of supernatant was subjected to 4%/10% discontinuous SDS-PAGE followed by immunoblotting using anti-Cln2 serum (prepared and characterized as in Wittenberg et al. 1990) and alkaline phosphatase-conjugated goat anti-rabbit antiserum (Promega Biotec). Chemiluminescent signal was detected using LumiPhos (Boehringer Mannheim) according to the manufacturer's instructions.

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Direct induction of G1-specific transcripts following reactivation of the Cdc28 kinase in the absence of de novo protein synthesis.

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