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NDD1, a High-Dosage Suppressor of *cdc28-1N*, Is Essential for Expression of a Subset of Late-S-Phase-Specific Genes in *Saccharomyces cerevisiae*

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cdc28-1N **mutants progress through the G_1 and S phases normally at the restrictive temperature but fail to undergo nuclear division. We have isolated a gene, *NDD1*, which at a high dosage suppresses the nuclear-division defect of *cdc28-1N*. *NDD1* (nuclear division defective) is an essential gene. Its expression during the cell cycle is tightly regulated such that *NDD1* RNA is most abundant during the S phase. Cells lacking the *NDD1* gene arrest with an elongated bud, a short mitotic spindle, 2N DNA content, and an undivided nucleus, suggesting that its function is required for some aspect of nuclear division. We show that overexpression of Ndd1 results in the upregulation of both *CLB1* and *CLB2* transcription, suggesting that the suppression of *cdc28-1N* by *NDD1* may be due to an accumulation of these cyclins. Overproduction of Ndd1 also enhances the expression of *SWI5*, whose transcription, like that of *CLB1* and *CLB2*, is activated in the late S phase. Ndd1 is essential for the expression of *CLB1*, *CLB2*, and *SWI5*, since none of these genes are transcribed in its absence. Both *CLB2* expression and its upregulation by *NDD1* are mediated by a 240-bp promoter sequence that contains four *MCM1*-binding sites. However, Ndd1 does not appear to be a component of any of the protein complexes assembled on this DNA fragment, as indicated by gel mobility shift assays. Instead, overexpression of *NDD1* prevents the formation of one of the complexes whose appearance correlates with the termination of *CLB2* expression in G_1. The inability of *GAL1* promoter-driven *CLB2* to suppress the lethality of *NDD1* null mutant suggests that, in addition to *CLB1* and *CLB2*, *NDD1* may also be required for the transcription of other genes whose functions are necessary for G_2/M transition.**

An orderly progression through the cell division cycle requires that the relevant cellular events occur in a strict temporal sequence. In the budding yeast *Saccharomyces cerevisiae*, as in many other organisms, this is achieved by a combination of transcriptional and posttranslational regulations of various effectors. One of the best-studied examples of such posttranslational controls is the way in which the activity of the key protein kinase Cdc28 is regulated. Phosphorylation of specific tyrosine and threonine residues (19, 34, 35, 49), the association with a variety of cyclins (14, 43, 48, 53), and their proteolytic destruction at various stages of the cell cycle (2, 3, 10, 18) all contribute to sharply define not only the timing of Cdc28 activation-inactivation but also, presumably, its substrate specificity (35). Similarly, an anaphase-promoting complex whose activity is crucial for chromosome segregation appears to be regulated at the posttranslational level (24, 54, 55).

Although posttranslational modifications of the mitotic regulators are perhaps the most effective way for the cell to quickly respond to the changing cellular context, these responses may be further sharpened by controls at the transcriptional level. Indeed, it has been estimated by DNA microarray hybridization that about 800 genes of the budding yeast *S. cerevisiae* are transcribed only at specific stages of the cell cycle (50). Many of these periodically transcribed genes play crucial roles in cell cycle progression. For instance, the transcripts for the G_1 cyclins *CLN1* and *CLN2* appear in late G_1 phase approximately when the cells traverse START. The transcription of these genes is regulated by a sequence motif called SCB (Swi4/Swi6 cell cycle box) within their promoter (22). This motif is also found in the promoters of the genes encoding *HO* endonuclease and the cyclin-like protein Hcs26 (6, 11), which are also expressed in late G_1. A specific heteromeric transcription factor complex (SCF) containing Swi4 and Swi6 proteins drives the cell cycle stage-specific expression by binding to the SCB element (37, 38). The S-phase cyclin genes *CLB5* and *CLB6* and DNA synthesis genes such as *POL1*, *TMP1*, and *CDC9* are also expressed in late G_1 but their expression is driven by another promoter element called MCB (Mlu1 cell cycle box) which binds a transcription factor complex (MBF) containing Mbp1 and Swi6 (23, 28). At the onset of the S phase, histones and, somewhat later, *CLB3* and *CLB4* cyclin genes are transcribed (15).

The transcription of another set of genes is activated near the completion of the S phase. This includes *FAR1*, a protein required for pheromone-induced G_1 arrest, the transcription factors *SWI5* and *ACE2*, mitotic cyclin genes *CLB1* and *CLB2*, and *CDC5*, a gene encoding a Polo-like protein kinase. It is not clear whether the periodic expression of these genes is regulated by a common promoter element and transcription factors. An analysis of *SWI5* promoter revealed a 55-bp element that is sufficient for the periodic expression of *SWI5* (29). It has been proposed that Mcm1, a DNA-binding protein important for the determination of mating-type specificity, forms a complex with Sff (*SWI5* transcription factor) and binds to this 55-bp promoter element (29). The gene encoding Sff has not yet been identified. In subsequent studies, it was shown that Mcm1 is required for the expression of *CLB1*, *CLB2*, *SWI5*, *CDC5*, and *ACE2* (1, 30). This is consistent with the fact that the promoter regions of *CLB1*, *CLB2*, and *SWI5* contain consensus Mcm1

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TABLE 1. Yeast strains

Strain	Relevant genotype
W303	*MAT***a** *ade2-1 trp1-1 can1-100 leu2-3 his3-11 ura3*
US100	*MAT***a** *cdc28-4 ade2-1 trp1-1 can1-100 leu2-3 his3-11 ura3*
US102	*MAT***a** *cdc28-1N ade2-1 trp1-1 can1-100 leu2-3 his3-11 ura3*
US239	*MAT***a**/α *ade2-1 trp1-1 can1-100 leu2-3 his3-11 ura3*
US241	*MAT***a**/α *NDD1/ndd1Δ::LEU2 ade2-1 trp1-1 can1-100 leu2-3 his3-11 ura3*
US262	*MAT***a** *ndd1Δ::LEU2* pGAL1-NDD1 *ade2-1 trp1-1 can1-100 leu2-3 his3-11 ura3*
US356	*MAT***a** *ade2-1 trp1-1 can1-100 leu2-3 his3-11 ura3 bar1Δ::hisG*
US449	*MAT***a** *gal4 gal80 his3 ade2-101 trp1-901 leu2-3 ura3-52 URA3::GAL1-lacZ LYS2::GAL1-HIS3*, Y190
US737	*MAT***a** *ade2-1 trp1-1 can1-100 leu2-3 his3-11 ura3 bar1Δ::hisG* pGAL1-NDD1
US1005	*MAT***a** *prc1-407 prb1-1122 pep4-3 leu2 trp1 ura3-52 gal2* (BJ2168)
US1013	*MAT***a** *prc1-407 prb1-1122 pep4-3 leu2 trp1 ura3-52 gal2* pGAL1-HA_3-NDD1
US1052	*MAT***a** *mcm1 ade2-1 leu2-3 his3-11*
US1139	*MAT***a** *ndd1Δ:LEU2 pGAL1-HA_3-NDD1 ade2-1 trp1-1 can1-100 leu2-3 his3-11 ura3 pep4Δ::URA3*
US1354	*MAT***a** *cdc15 ade2-1 trp1-1 can1-100 leu2-3 his3-11 ura3* pGAL1-HA_3-NDD

binding sites. Interestingly, Mcm1 occupies these sites throughout the cell cycle, implying that the timing of expression is perhaps determined either by specific modifications of the bound factors or by other associated factors (1). It has been recently reported that a novel promoter element ECB (early cell cycle box), which renders the expression of *SWI4*, *CDC6*, *CDC47*, and *CDC46* M/G_1 specific, also binds Mcm1 (32). Since Mcm1 is also involved in the regulation of other cellular processes that are not directly linked to the cell cycle progression (26, 31, 33, 51), it seems unlikely that Mcm1 is the critical factor in determining the periodicity of gene expression.

As Cdc28 is the key protein kinase required at various stages for cell cycle progression, most *CDC28* mutants exhibit defects in progression through both START and G_2/M transition (39, 41). However, *cdc28-1N* is a unique mutant in that, although normal with respect to its START functions, it fails to undergo nuclear division at the restrictive temperature and, consequently, arrests with a large elongated bud, a short mitotic spindle, 2N DNA content, high histone H1 kinase activity, and an undivided nucleus (39, 53). Although the molecular nature of its mitotic defect is still unknown, the *cdc28-1N* mutant has served well for the identification of a number of novel genes. *CLB1*, *CLB2*, and *CLB4* were first isolated as allele-specific, high-dosage suppressors of the *cdc28-1N* mutation (53). Similarly, *CIM3* and *CIM5* genes encoding the 26S proteosome subunits were identified as mutations that exhibit synthetic lethal behavior in combination with *cdc28-1N* (17). We report here the isolation of a novel allele-specific, high-dosage suppressor of *cdc28-1N*, termed *NDD1*, and examine its role in cell cycle progression. We describe its basic characterization and show that *NDD1* is an essential component, as is *MCM1*, of the mechanism that activates the expression of a set of late-S-phase-specific genes.

MATERIALS AND METHODS

Yeast strains and growth media. Yeast strains (Table 1) used in this study were derivatives of the standard wild-type strain W303. Strain BJ2168 (US1005) was used in the mobility shift DNA-binding assay. Yeast cells were routinely grown in yeast extract-peptone medium containing adenine (50 mg/liter) supplemented with either glucose or galactose. Raffinose was also added to the galactose medium. All media were prepared as described by Rose et al. (44).

To obtain cultures synchronized in G_1, *bar1*Δ cells (US356) were treated with α-factor (0.8 μg/ml) for 3 h. In experiments where a synchronous release from late telophase was required, *cdc15* cells were grown to log phase at 24°C before they were filtered and resuspended in prewarmed growth medium at 37°C. After 3 h, cells were allowed to resume cell cycle progression at 24°C.

Isolation of *NDD1*. *cdc28-1N* cells were transformed with a library of yeast genomic fragments cloned into the *Bam*HI site of the YEp13 (2μm) vector (36), allowed to recover at 24°C for 24 h, and then shifted to 37°C for 3 days. The plasmids were retrieved from various transformants and were categorized into seven distinct groups based on the patterns of fragments generated by restriction enzyme digestion. One group of plasmids harbored a 2.7-kb *Pvu*II genomic fragment containing the *NDD1* gene.

DNA manipulations. All DNA manipulations were performed as described by Sambrook et al. (46). The 2.7-kb *Pvu*II fragment containing the entire *NDD1* gene was blunt-end cloned into the *Sma*I-*Bgl*II site of pIC19H vector to yield pUS529. To construct the *NDD1* gene under the control of *GAL1* promoter, an *Nco*I site was created immediately upstream of the ATG start codon by PCR. The *Bam*HI-*Bgl*II fragment from pUS529 was then replaced by the *Bam*HI-*Bgl*II-digested 0.97-kb PCR product. The complete *NDD1* gene was then excised with *Nco*I-*Hin*dIII and blunt-end ligated to the *GAL1* promoter in a *URA3*- or *TRP1*-selectable CEN vector.

Gene disruptions were performed by using the one-step gene replacement method of Rothstein (45) with a 2.7-kb *Pvu*II fragment in which the 450-bp *Eco*RV fragment within the open reading frame (ORF) was replaced by a 1.6-kb *Xho*I-*Sal*I fragment containing the *LEU2* gene. The disruption of the *NDD1* gene was confirmed by Southern blot analysis. To tag *NDD1* with three tandem copies of the hemagglutinin (HA) epitope (GRIFYPYDVPDYAGYPYDVPDYAGSYPYDVPDYAAQC), a *Not*I site was introduced just after the ATG start codon by PCR. The triple HA tag (as a 111-bp *Not*I fragment) was cloned into the newly created *Not*I site. The resulting HA_3-tagged *NDD1* was excised as an *Nco*I-*Sma*I fragment and blunt-end ligated to the *GAL1* promoter in a *TRP1*-selectable CEN vector to yield pGAL1-HA_3-NDD1.

To test the upstream activation sequence (UAS) activity, *CLB2* promoter sequences were cloned upstream of a *CYC1* TATA box fused to a *ubiY lacZ* reporter, which expresses β-galactosidase activity with a half-life of 10 min instead of more than 20 h (8). The 340-bp *CLB2* UAS (extending from −863 to −523 bp from ATG) was generated by PCR and cloned into the *Xho*I-*Bgl*II site of pDL1498 plasmid containing the *ubiY lacZ* reporter (29) as a *Sal*I-*Bgl*II fragment (sites introduced by PCR). The 240-bp *CLB2* UAS (−863 to −627 bp from ATG) was made by dropping out a 110-bp *Xho*I-*Bgl*II fragment from the 340-bp *CLB2* UAS reporter construct and religating the plasmid. The 55-bp *CLB2* UAS (−698 to −643 bp from ATG) was synthesized by mutually priming two oligonucleotides (5′-TACAGAATTCTCGAGAATATAGCGACCGAATC AGGAAAAGGTCAACAACGA-3′ and 5′-ACTGAATTCAGATCTCATCCA TATCGCGAACTTCGTTGTTGACCTTTTCC-3′) as described in Ausubel et al. (7) and cloned into pDL1498 as a *Xho*I-*Bgl*II fragment (sites introduced by PCR). The recombinant reporter constructs were excised by *Sma*I-*Nco*I digestion and blunt-end cloned into the *Eco*RI site of a *URA3*-based integrative plasmid and subsequently integrated at the *URA3* locus by homologous recombination. All integration events were checked by Southern blot analysis, and β-galactosidase activities were measured in at least three independent isolates from each integrative transformation. Activities were expressed as Miller units.

To clone the full-length *NDD1* in frame with the *GAL4* DNA-binding domain of pGBT9 (9), *NDD1* was excised from pUS529 as a *Nco*I-*Xho*I fragment and blunt-end ligated into the *Sma*I site of pGBT9. For *GAL4-NDD1* deletion mutant analysis, all constructs were made similarly except that different restriction enzymes were used to excise the various *NDD1* fragments from pUS529. The isolated fragments were subsequently cloned in frame into pGBT9 to yield the various *GAL4-NDD1* deletion fusion constructs (see Table 2). *NDD1* lacking the polyglutamine domain was constructed by PCR with the following sets of primers: 5′-TTGATTGGATCCATGGACAGAGATATAAGC-3′ and 5′-TCTGCT GATGCTGCAGTAATATAC-3′ to obtain the sequence 5′ of the polyglutamine domain and 5′-TTCTTCTGTTCTGCAGTTCGGCAAC-3′ and 5′-TATTGTT AGATCTTAGCGGCGTTCT-3′ to obtain the sequence 3′ of the polyglutamine domain. The two PCR products were digested with *Nco*I-*Pst*I and *Pst*I-*Bgl*II and triple ligated into an *Nco*I-*Bgl*II-cut pUS529. This resulted in a *NDD1*$^{-\Delta Q}$ construct which lacks amino acids 93 to 143. This construct was subsequently excised as an *Nco*I-*Xho*I fragment and blunt-end ligated in frame with the *GAL4* DNA-binding domain in pGBT9.

Random spore analysis. To determine the terminal phenotype of cells deficient in *NDD1*, a diploid strain heterozygous for *ndd1Δ::LEU2* (US241) was sporulated in liquid medium. Spores purified by centrifugation (7) were inoculated into medium lacking leucine. Samples were withdrawn 6 h after germination and were used for in situ immunofluorescence and photomicroscopy.

Gel mobility shift assay. Yeast protein extracts were prepared from protease-deficient (*pep4*$^-$) strains essentially as described by Company et al. (12). Double-stranded DNA probes were prepared by using the Klenow enzyme to fill in the protruding 5′ end of a restriction site in the presence of radioactive [α-^{32}P]dATP. The labelled probes were purified from the unincorporated nucleotides by using

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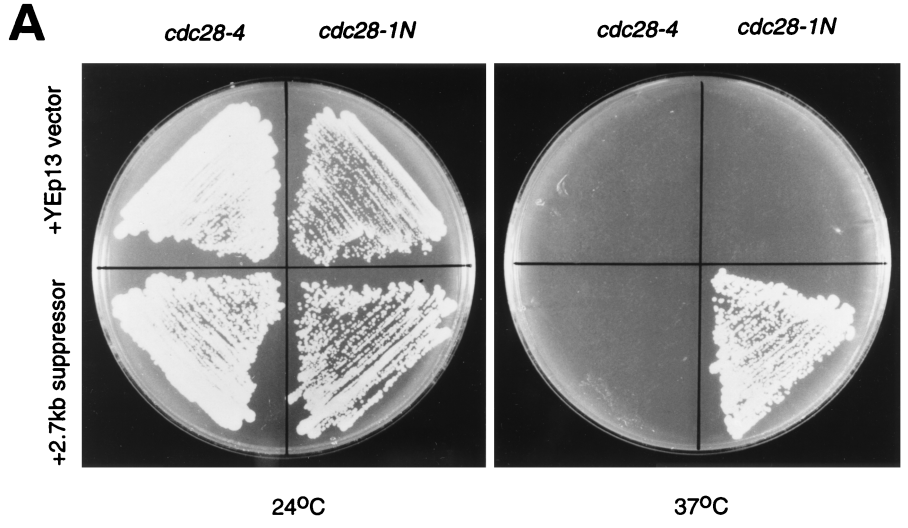

FIG. 1. (A) *NDD1* is an allele-specific suppressor of the *cdc28-1N* mutation. *cdc28-4* and *cdc28-1N* cells were transformed with YEp13 (2μm) or the vector containing *NDD1* as the 2.7-kb *Pvu*II fragment. The transformants were plated on leucine-deficient plates and incubated at 24 or 37°C for 48 h. (B) Nucleotide sequence and the predicted amino acid sequence of *NDD1*. The polyglutamine stretch is shown in boldface, and the three well-conserved *cdc2*/*CDC28* histone H1 phosphorylation sites are underlined.

NucTrap probe purification columns (Stratagene). DNA binding reactions were carried out in 1.5-ml Eppendorf tubes. Generally, 10,000 to 20,000 cpm (Cerenkov) of probe was used for each reaction. Each reaction contained 4 μl of 5× BS buffer (100 mM Tris-HCl [pH 7.5], 250 mM NaCl, 15 mM $MgCl_2$, 5 mM dithiothreitol, 25 mM spermidine, 250 μg of bovine serum albumin per ml, 100 mM EDTA), 2 μl of 50% glycerol, 2.5 μl of a 1-mg/ml concentration of poly(dI-dC), radiolabelled probe, water, and 20 μg of protein extract to give a final volume of 20 μl. Protein extracts were always added last, and the reactions were incubated at 24°C for 15 min followed by a further 15 min of incubation at 4°C. Conditions for competition experiments were exactly the same except that cold competitor DNAs were added to the reaction mixture before the addition of radiolabelled probe. For antibody supershift assays, the appropriate antibodies were added 6 min after the addition of the protein extracts and processed as described above. The gel used for resolving the protein-DNA complexes was 4% (20:1) acrylamide-bisacrylamide gel in 0.5× Tris-borate-EDTA (TBE). The gels were prerun at 4°C for 2 h at 200 V (~20 mA) in 0.5× TBE. After incubation, the reaction mixtures were loaded onto the gel and run at 4°C for 3 h at 200 V (~8 mA). The gels were dried and autoradiographed with an intensifying screen at −70°C.

Other techniques. The lithium acetate method was used for all yeast transformations. Total RNA was isolated as described by Cross and Tinkelenberg (13), and Northern (RNA) blot analyses were performed as described by Price et al. (40). The method of Kilmartin and Adams (21) was used for immunofluorescence and photomicroscopy. DNA distribution analysis by flow cytometry was performed as described by Lim et al. (27).

RESULTS

***NDD1* is an allele-specific suppressor of the *cdc28-1N* mutation.** Most *CDC28* mutants (i.e., *cdc28-1*, *cdc28-4*, and *cdc28-13*) are predominantly defective in traversing START and therefore arrest in late G_1 phase as unbudded cells with 1N DNA content (20, 42). Some of them also exhibit a defect in progression through mitosis (39, 41). The *cdc28-1N* mutant is unique in that it progresses through START and S phase normally but fails to undergo nuclear division at the nonpermissive temperature, despite the high Clb2-associated histone H1 kinase activity (39, 53). We isolated *NDD1* in a genetic screen designed to identify genes whose overexpression can suppress the mitotic defect of *cdc28-1N* mutant. To determine whether *NDD1* overexpression causes suppression of other *cdc28* mutations, a high-copy-number (2μm) vector carrying *NDD1* was introduced into both *cdc28-4* and *cdc28-1N* mutants, and the transformants were tested for growth at 37°C. While *NDD1* allowed growth of *cdc28-1N* cells at 37°C, it failed to suppress the *cdc28-4* mutation (Fig. 1A). Overexpression of *NDD1* also failed to suppress the *cdc28-4* mutation when tested at 31 and 35°C (data not included). Hence, *NDD1* is an allele-specific suppressor of the *cdc28-1N* mutation. This behavior is identical to that of the mitotic cyclin genes *CLB1* and *CLB2*, which were isolated in the same screening (53). This suggests that the role of *NDD1* is related to the mitotic function of *CDC28*.

NDD1 gene, located on chromosome XV, contains a 1.66-kb ORF (*Saccharomyces* Genome Database ORF sequence YOR372c) that encodes a protein of 554 amino acids (Fig. 1B) with no significant overall homology to any known protein in the databases. The protein contains an unbroken stretch of 15 glutamine residues in its N terminus. The glutamine stretches are found in a number of proteins with diverse functions, including some transcription activators, but the precise role this motif serves in these proteins is not known. At least in the case of Ndd1, the glutamine tract does not seem to play a crucial role for Ndd1 function, since Ndd1 lacking this stretch can complement *NDD1*-null mutant and is also able to suppress *cdc28-1N* mutation (see below). Ndd1 also contains three putative Cdc28 phosphorylation sites in the middle region, but their relevance to Ndd1 function is not clear. As there are no other features that match any of the known motifs, the amino acid sequence of Ndd1 currently provides no clues to its possible function.

***NDD1* is an essential gene whose function is required during mitosis.** To determine whether *NDD1* is necessary for cell viability, the ORF in one of the two copies of *NDD1* in a wild-type diploid was replaced by the *LEU2* gene (Fig. 2A). The resulting heterozygous mutant (US241) was allowed to sporulate, and the tetrads were dissected on rich medium. Only two spores in each tetrad survived and gave rise to normal colonies that failed to grow when replica plated on leucine-deficient medium. By inference, the absence of viable Leu^+ segregants in these dissections suggests that all segregants that were Leu^+ and therefore lacked intact *NDD1* ORF were nonviable. Microscopic examination of these segregants revealed that the spores had germinated but that most had arrested as large-budded cells, while others had undergone one or two

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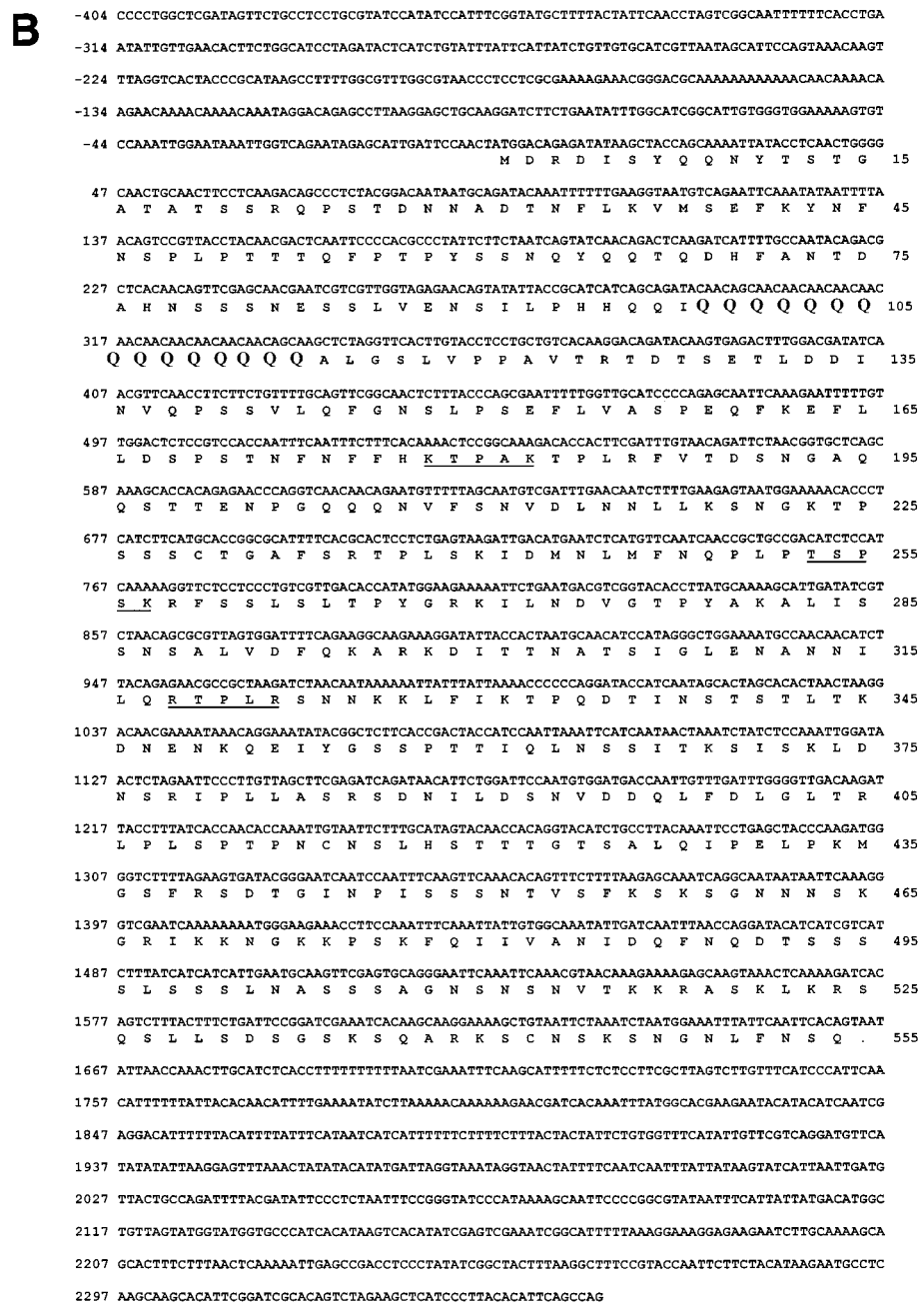

FIG. 1—*Continued.*

divisions before cessation of growth. This suggests that *NDD1* is essential for vegetative growth.

To characterize the cell cycle arrest phenotype of *NDD1*-deficient cells, diploid cells heterozygous for *NDD1* disruption (US241) were grown in YPD medium and then transferred to sporulation medium. After 20 h, spores were purified and inoculated into medium lacking leucine to allow germination and growth of only those spores in which the *NDD1* ORF had been replaced by the *LEU2* gene. Immunofluorescence microscopy revealed that *NDD1*-deficient cells arrested with a large, elongated bud, an undivided nucleus, and a short mitotic spindle (Fig. 2B). In a parallel experiment, *NDD1*-deficient haploid cells kept alive by a *CEN* vector carrying *GAL-NDD1* (US262) was first grown in galactose and then transferred to glucose medium. At the end of 6 h, the cells had arrested, with a phenotype identical to that observed in the spore outgrowth experiment. Fluorescence-activated cell sorter analysis showed that these cells had arrested with 2N DNA content (Fig. 2B). Thus, *NDD1*-deficient cells are capable of bud emergence, DNA replication, and spindle formation, but they fail to proceed to anaphase. This suggests that *NDD1* is essential for some aspect of nuclear division.

Expression and localization of Ndd1 during the cell cycle. Since *NDD1* appeared to be necessary for the nuclear division process, we asked whether its expression is restricted to this stage of the cell cycle. Exponentially growing wild-type cells

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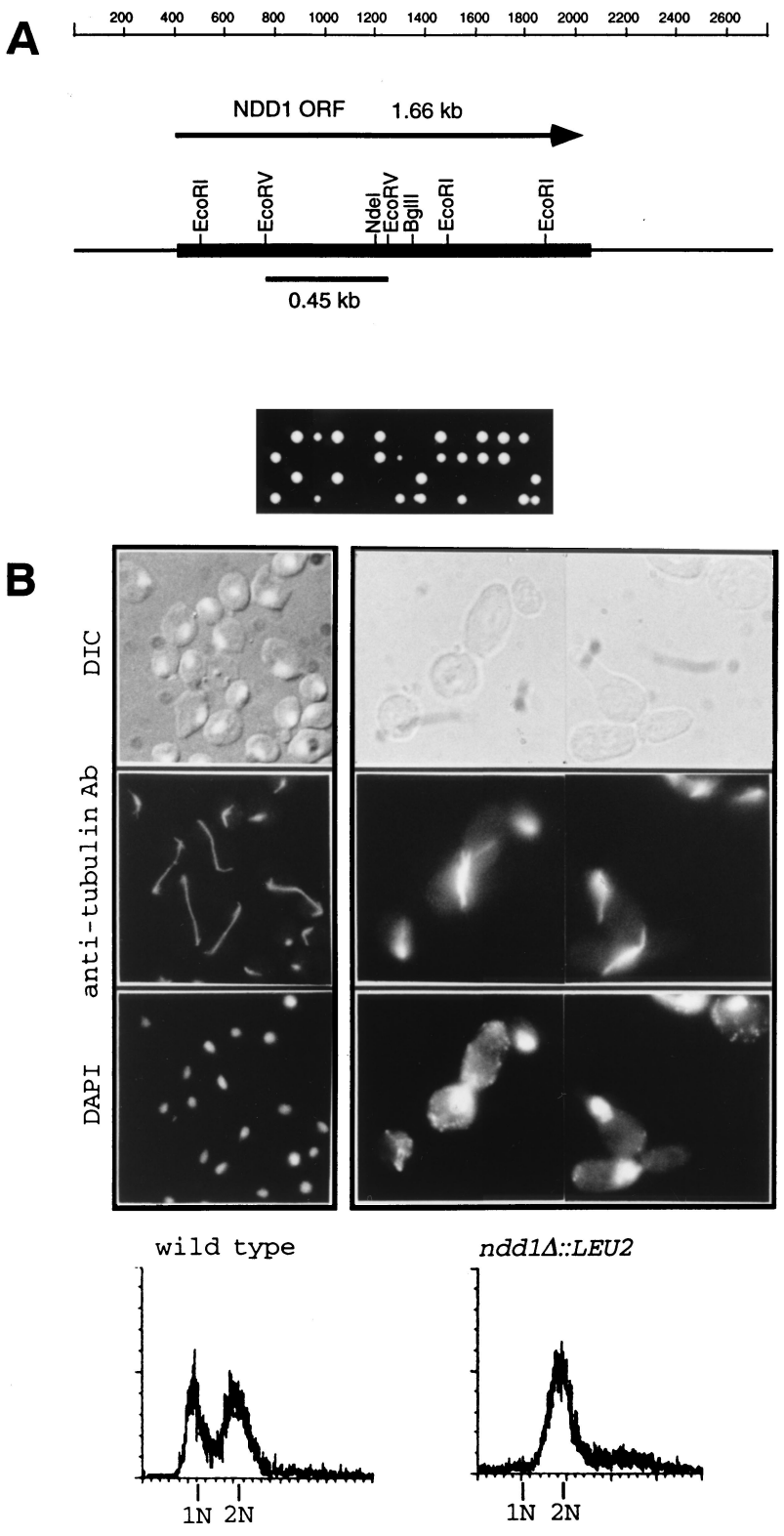

FIG. 2. *NDD1* is an essential gene. (A) Partial restriction map of the 2.7-kb *Pvu*II fragment containing *NDD1*. The 0.45-kb *Eco*RV fragment in the ORF was replaced by the *LEU2* gene to generate the disruption mutant *ndd1*Δ*::LEU2*. The scale bar is in base pairs. Diploid cells heterozygous for *ndd1*Δ*::LEU2* disruption were sporulated and dissected on rich medium. Tetrads show a 2:2 segregation pattern for viability. All survivors were Leu$^-$. (B) Cell cycle arrest phenotype of *ndd1*Δ. Spores from a wild-type diploid strain or an *ndd1*Δ*::LEU2* heterozygous diploid strain were purified and germinated in either complex medium or medium lacking leucine, respectively. Samples were withdrawn 6 h after germination and used for differential-interference-contrast (DIC) microscopy, staining with anti-tubulin antibodies, and DAPI (4′,6-diamidino-2-phenylindole) staining. The lower panel shows DNA distribution profiles of asynchronously growing wild-type and Ndd1-depleted cells.

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were synchronized in G_1 by α-factor treatment for 3 h at 25°C and then allowed to resume cell cycle progression in α-factor-free medium. Total RNA was isolated, and *NDD1*-specific transcript was detected by Northern blot analysis. The *NDD1* RNA, first detectable above the basal level at 30 min after the release from G_1 arrest, peaks at 50 to 60 min and then declines ca. 30 min before the majority of cells reach anaphase (Fig. 3A). The transcript appears again in the next cycle when the proportion of cells with anaphase spindle has reached its minimum. The window of *NDD1* expression lies between the peaks of *CLN1* and *CLB2* transcription (Fig. 3A), a period that corresponds to the S phase. It is not yet clear as to what restricts *NDD1* transcription to the S phase. So far we have not found any known *cis*-regulatory elements within the 2-kb region upstream of the ORF except one loosely conserved SCB (Swi4/Swi6 cell cycle box) in reverse orientation.

To determine the cellular location of Ndd1 protein, *NDD1*-ORF was fused in frame with the gene encoding β-galactosidase and put under the control of *GAL1* promoter on a *CEN* plasmid. This fusion construct was fully functional, since a *NDD1* null mutant harboring this plasmid grew normally on galactose but remained nonviable in glucose medium. Wild-type cells carrying the fusion construct were grown in galactose medium for 3 h and then fixed with formaldehyde. Immunofluorescence staining with anti-β-galactosidase antibodies showed brightly stained nuclei (Fig. 3B). No nuclear staining was observed in cells expressing only β-galactosidase from *GAL1* promoter (not shown). This implies that Ndd1 is a nuclear protein (Fig. 3B). The protein was always seen in the nucleus, irrespective of both the bud size and the state of nuclear division, suggesting that nuclear localization of Ndd1 is perhaps not cell cycle stage dependent.

Overexpression of Ndd1 enhances the expression of *CLB1*, *CLB2*, and *SWI5* genes. It is known that elevated levels of the B-type cyclins Clb1, Clb2, and Clb4 can suppress the nuclear division defect of the *cdc28-1N* mutant (53). We reasoned that any gene whose overexpression can cause the accumulation of these cyclins, either by upregulation of transcription or by increasing their stabilization, would also be identified by our genetic screen. Therefore, we tested *NDD1* for its ability to enhance the transcription of *CLB1* and *CLB2*. Wild-type cells carrying *GAL-NDD1* on a *CEN* plasmid (US737) were grown in raffinose medium (in which *GAL1* promoter is not active) for 3 h and then one-half of the culture was induced to express Ndd1 by the addition of galactose. Total RNA was prepared at various time points and analyzed for the presence of *CLB1*, *CLB2*, *CLB3*, *SWI5*, *CDC20*, and *NDD1* transcripts. Of these, the *CLB1*, *CLB2*, *SWI5*, and *CDC20* genes are transcribed in late S/G_2 phase, whereas *CLB3* is expressed slightly earlier (22). The transcription of *CLB1*, *CLB2*, and *SWI5* continues throughout mitosis and is switched off as the cells exit from mitosis (29, 53). *URA3* served as an internal control for equal RNA loading since it is transcribed constitutively during the cell cycle. The overexpression of *NDD1* did not cause any change in the level of the *CLB3*, *CDC20*, and *URA3* transcripts. However, there was a three- to fivefold increase in the steady-state levels of *CLB1* and *CLB2* RNA (Fig. 4A). The *SWI5* expression was also noticeably enhanced (Fig. 4A). *CDC5* and *ACE2* are two other genes that are expressed in late-S/G_2 phase, but their expression is not affected by the overexpression of *NDD1* (data not shown). Thus, *NDD1* modulates expression of only a subset of the late-S/G_2 genes. These data also suggest that the ability of *NDD1* to suppress the *cdc28-1N* mutation is perhaps linked to its capacity to augment the expression of the mitotic cyclins *CLB1* and *CLB2*.

The increase in the steady-state level of *CLB1* and *CLB2* RNAs may be because *NDD1* overexpression abolishes the cell cycle regulation of these cyclins so that they are constitutively expressed in response to excess Ndd1. To test this, *cdc15* cells carrying *GAL-NDD1* on a *CEN* vector (US1354) were grown in raffinose medium at 25°C and then synchronized in telophase by incubation at 37°C for 2 h. Cells were induced to produce *NDD1* for the next 2 h by the addition of galactose before they were allowed to resume cell cycle progression at 25°C in galactose medium. In a control experiment, *cdc15* cells carrying a vector without *GAL-NDD1* were subjected to an identical experimental regimen. When released from telophase arrest, *cdc15* cells traverse the cell cycle in a highly synchronous manner (52). Total RNA was prepared at various time points and was analyzed for the presence of *CLB2* transcript by Northern blotting. As expected, *CLB2* expression in cells without *GAL-NDD1* showed characteristic undulation such that it is switched off 30 min after the release from telophase and is turned on again when the cells are well into the next cycle (Fig. 4B). The overexpression of *NDD1* from *GAL1* promoter does not change this pattern; instead it causes a three- to fivefold increase in the overall expression of *CLB2* (Fig. 4B). Thus, excess *NDD1* amplifies the *CLB2* expression but does not abrogate its cell cycle regulation.

***NDD1* is essential for *CLB1*, *CLB2*, and *SWI5* expression.** Since Ndd1 can modulate the expression of *CLB1*, *CLB2*, and *SWI5*, we asked whether *NDD1* is essential for the transcription of these genes. Since our efforts to isolate a "tight" temperature-sensitive allele of *NDD1* were not successful, we used for these experiments an *ndd1*Δ strain kept alive by a *CEN* plasmid carrying *GAL-NDD1* (US262). When transferred to glucose medium, this strain exhibits an arrest phenotype within 4 to 5 h. The cells were first grown in galactose until they reached log phase and were then shifted to glucose medium to switch off the *NDD1* expression. Total RNA was isolated from samples withdrawn at various time intervals and analyzed for the presence of *CLB1*, *CLB2*, *SWI5*, *CLB3*, *CDC20*, *NDD1*, and *URA3* RNAs by Northern blotting. The cells began to exhibit their characteristic terminal phenotype after 5 h and thereafter remained arrested with 2N content DNA, a short mitotic spindle, and an undivided nucleus. While the *CLB3*, *CDC20*, and *URA3* transcripts were present throughout the course of the experiment, the *CLB1*, *CLB2*, and *SWI5* RNAs became undetectable within 4 h of shifting to glucose medium and remained so for the remainder of the time course (Fig. 5A). As expected, *NDD1* was not transcribed during this period. These results suggest that *NDD1* is essential for the transcriptional activation of *CLB1*, *CLB2*, and *SWI5* genes but not for the expression of *CLB3* or *CDC20*.

Clb1 and Clb2 are B-type cyclins that associate with the protein kinase Cdc28 and govern its mitotic activity (16, 53). Cells lacking either *CLB1* or *CLB2* are viable but the *clb1*Δ *clb2*Δ double mutant is unable to proceed through mitosis and arrest with a short spindle, an undivided nucleus, and 2N DNA content (53). This phenotype is very similar to that of the *ndd1*Δ mutant. Since *NDD1* is required for *CLB1* and *CLB2* transcription, it is possible that the nonviability of *ndd1*Δ mutant may be entirely due to a lack of these mitotic cyclins. To test this, we performed a plasmid shuffle experiment in which a *TRP1*-selectable *CEN* plasmid carrying either *GAL-CLB2* or *GAL-NDD1* was introduced into an *ndd1*Δ mutant kept alive by the native-promoter-driven *NDD1* on a *URA3*-selectable vector. The transformants were plated on galactose medium containing 5-fluoro-orotic acid (5-FOA) to select for clones that had lost the *URA3* vector bearing *NDD1* but that expressed either *GAL-NDD1* or *GAL-CLB2*. While the cells containing *GAL-NDD1* grew readily on 5-FOA plates, the

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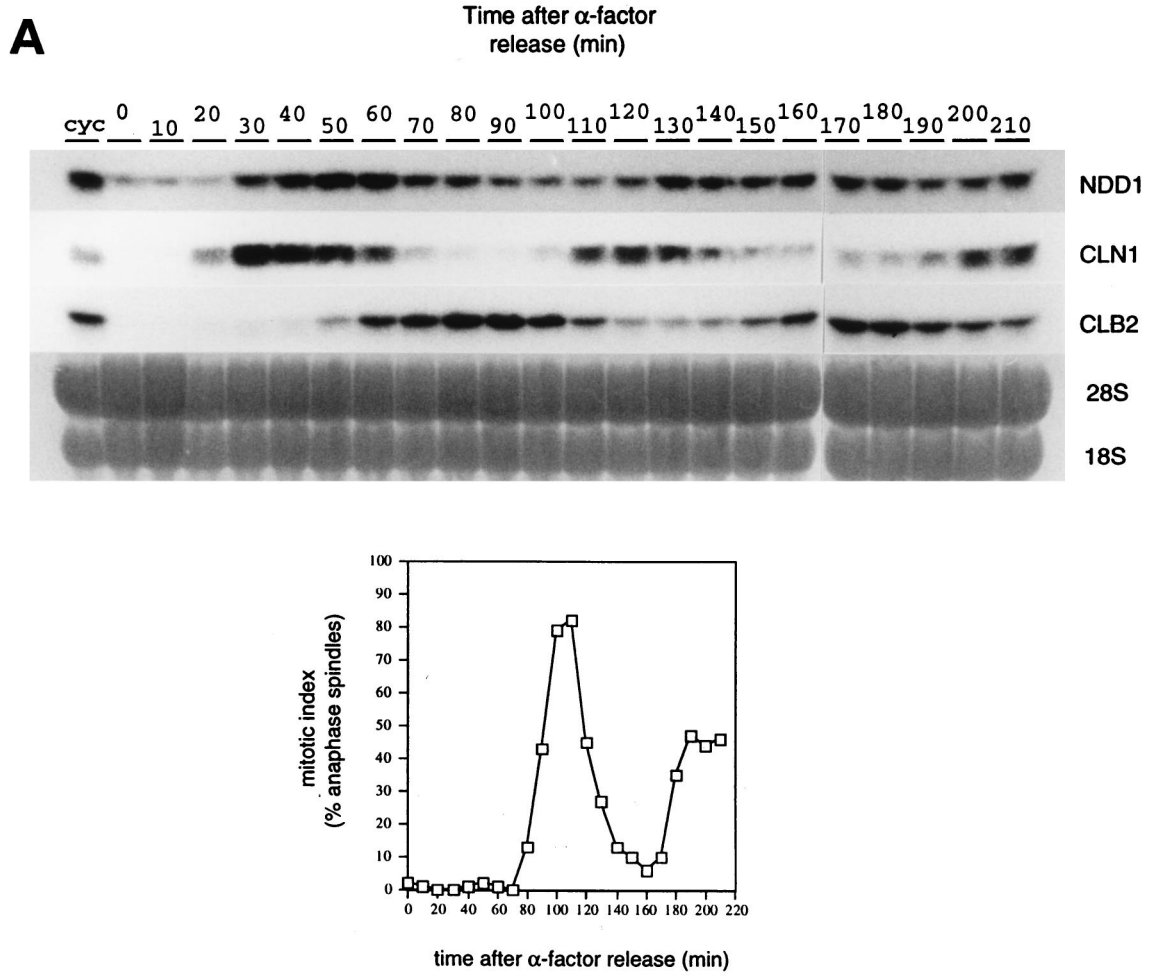

FIG. 3. (A) *NDD1* gene expression during the cell cycle. Wild-type cells lacking *BAR1* gene were arrested in G_1 by α-factor treatment and then released into growth medium without α-factor. Samples were withdrawn at 10-min intervals, and total RNA was analyzed by Northern blotting with an internal *Eco*RI fragment of *NDD1* as a probe. *CLN1* and *CLB2* RNA transcripts are also shown for comparison. The amount of total RNA loaded (30 μg) was visualized by methylene blue staining of the rRNAs. The graph at the bottom shows the percentage of cells with anaphase spindles after release. (B) Nuclear localization of the Ndd1-lacZp. Wild-type cells carrying pGAL1-NDD1-lacZ were grown in glucose or galactose medium and then used for both indirect immunofluorescence staining with anti-β-galactosidase antibodies (upper panels) and nuclear staining with DAPI (lower panels).

strain harboring *GAL-CLB2* did not give rise to any colonies (Fig. 5B), suggesting that Clb2 overexpression alone cannot compensate for the absence of Ndd1. Hence, the lethality caused by *NDD1* deficiency is not solely due to the lack of mitotic cyclins. This implies that, in addition to *CLB1* and *CLB2*, *NDD1* may also be required for the expression of other genes whose function is necessary for the G_2/M transition.

Transcriptional activation by *NDD1*. Since our results raise the possibility that Ndd1 may be a transcription activator, we tested its ability to activate transcription in a heterologous context by using a yeast one-hybrid assay. The *NDD1* coding region, fused in frame with the DNA-binding domain of the transcription factor *GAL4*, was put under the control of the *ADH* promoter and was transformed into a tester strain that carried the *lacZ* and *HIS3* genes as reporters, both driven by three copies of a 17-mer *Gal4*-binding sequence. The *Gal4-Ndd1* fusion was able to activate transcription of the reporter constructs, resulting in both the appearance of His^+ colonies and the expression of β-galactosidase (data not shown).

It has been shown previously that *MCM1* participates in the mechanism that coordinates the expression of a group of genes (including *CLB1*, *CLB2*, and *SWI5*) whose transcription is activated during the late-S/G_2 phase of the cell cycle (1). In light of our observation that *NDD1* is required for the transcription of a subset of these genes and that it can activate transcription in the yeast one-hybrid assay, it is possible that *NDD1* interacts functionally with *MCM1*. Indeed, a multicopy vector (2μm) carrying native-promoter-driven *NDD1*, although not able to support growth of the *mcm1* mutant cells at 37°C, does allow them to grow at 35°C (1a). Thus, excess *NDD1* is able to suppress, to some extent, the lethality caused by the lack of Mcm1 function.

Normal periodic transcription of *CLB2* requires a 240-bp promoter sequence. Maher et al. (30) have described a 55-bp UAS within the *CLB2* promoter which contains one consensus Mcm1 binding site [DCCY(A/T)(A/T)(T/A)NN(G/A)G; D ≠ C, Y = T or C] (25) and is both necessary and sufficient for the cell-cycle-regulated expression of *CLB2*. This 55-bp UAS is similar to a 55-bp element in the *SWI5* promoter in both its sequence and its ability to bind a Mcm1-containing ternary complex (30).

In addition to the one Mcm1 consensus element (MCE) and an Sff response element (SFRE) in the 55-bp UAS, there are four other MCEs in the *CLB2* promoter. The relative positions

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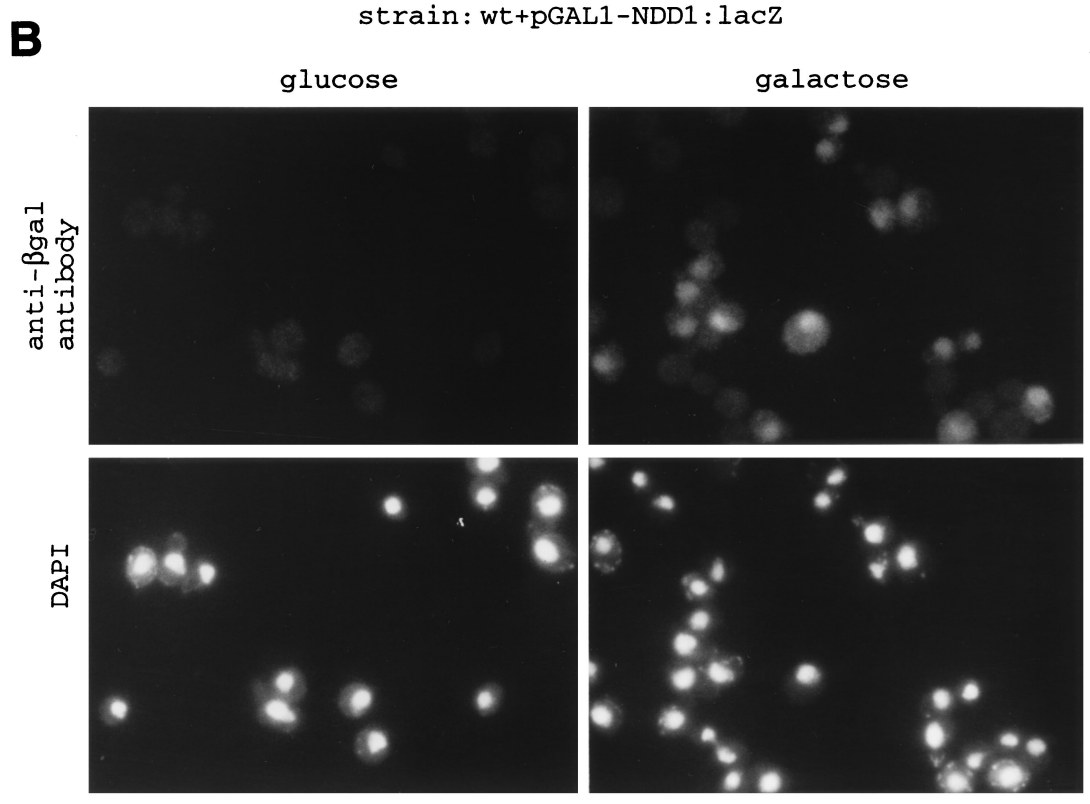

FIG. 3—*Continued.*

of these elements are shown in Fig. 6A. Some of our initial experiments had led us to suspect that the previously documented 55-bp UAS (30) may not be sufficient for the normal cell-cycle-regulated expression of *CLB2*. We reasoned that MCEs contained within the sequences flanking the 55-bp UAS might also be required. We therefore compared the 55-, 240-, and 340-bp promoter fragments, which contained one, four, and five MCEs, respectively, for their ability to regulate transcription of a reporter gene. The promoter fragments were placed immediately upstream of the *CYC1* TATA box linked to a reporter gene *ubiY lacZ* (29), and these constructs were subsequently integrated in chromosome I at the *URA3* locus in wild-type cells. Constructs without any UAS ("dead") or with the 55-bp UAS of *SWI5* gene were used as negative and positive controls, respectively. To determine the pattern of *lacZ* expression at various stages of the cell cycle, the growth of wild-type cells carrying these constructs was arrested by treatment with α-factor (late G_1), hydroxyurea (early S phase), or nocodazole (pre-nuclear division). Total RNA was prepared and analyzed by Northern blotting for the presence of both *lacZ* and the endogenous *CLB2* transcripts. The 240-bp, 340-bp, and *SWI5* UAS fragments were all able to drive the *lacZ* expression in cycling, hydroxyurea (HU)- and nocodazole (NOC)-arrested cells but were transcriptionally silent in α-factor-treated cells (Fig. 6B). This expression pattern closely matches with that of the endogenous *CLB2* RNA, except that the level of *lacZ* transcription from the recombinant constructs is somewhat lower. The 55-bp fragment of *CLB2* promoter, however, failed to activate *lacZ* transcription in all instances (Fig. 6B). Identical results were obtained when the reporter constructs were tested on 2μm vectors (results not shown).

To confirm that the 240-bp fragment can regulate *lacZ* expression during the progression through the cell cycle, cells containing an integrated copy of the 240-bp-driven *ubiY lacZ* were first synchronized in G_1 by α-factor treatment and then allowed to traverse the cell cycle in α-factor-free medium. Total RNA was isolated from samples collected at various times and analyzed for the presence of the *lacZ* and endogenous *CLB2* RNAs by Northern blotting. As in the case of cells arrested at various stages of the cell cycle, the pattern of *lacZ* expression in synchronously cycling cells is indistinguishable from that of endogenous *CLB2* gene (Fig. 6C), except that the level of *lacZ* expression is lower in the second cycle. These results suggest that the 240-bp promoter fragment, but not the 55-bp element, is sufficient for the cell-cycle-regulated expression of *CLB2*.

The 240-bp fragment mediates potentiation of *CLB2* expression by *NDD1* overexpression. Since the 240-bp sequence is sufficient to impose cell cycle regulation on *CLB2* transcription, we asked whether the enhancement of *CLB2* expression by excess Ndd1 is effected through this promoter fragment. Therefore, we compared the efficacy of the 55-, 240-, and 340-bp fragments to elicit the upregulation of *CLB2* transcription. A *CEN* vector carrying *GAL-NDD1* was introduced into wild-type strains containing *ubiY lacZ* reporters driven by the various promoter fragments (described in the preceding section) at the *URA3* locus. The cells were first grown in raffinose medium and then induced to express *NDD1* for 2 h by the addition of galactose. The total RNA was analyzed for the presence of *lacZ* and the endogenous *CLB2* transcripts. Whereas no *lacZ* RNA was detected when the reporter construct was under control of the 55-bp fragment, both the 240- and the 340-bp-fragment-driven reporters were transcribed to a readily detectable level (Fig. 7). The transcription from both

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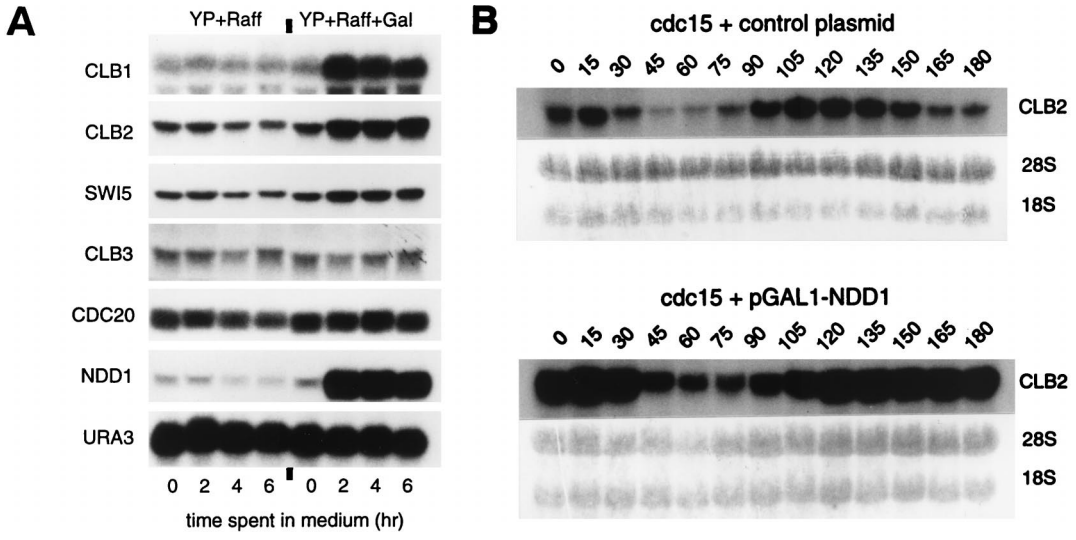

FIG. 4. (A) Overexpression of *NDD1* results in enhanced expression of *CLB1*, *CLB2*, and *SWI5*. A wild-type strain containing pGAL1-NDD1 was grown to mid-log phase in raffinose medium. While one-half of the culture continued to grow in raffinose medium (left lanes), the other half was induced by the addition of galactose to express Ndd1 (right lanes). Total RNA was prepared from samples withdrawn at 2-h intervals and analyzed by Northern blotting for *CLB1*, *CLB2*, and *SWI5* transcripts. *CLB3* and *CDC20* RNA transcripts are also shown for comparison. (B) *NDD1* overexpression does not abrogate the cell-cycle-regulated transcription of *CLB2*. *cdc15* cells carrying either a control plasmid (upper panel) or a plasmid harboring pGAL1-NDD1 (lower panel) were synchronized in telophase by growth in raffinose medium at 37°C for 2 h. *NDD1* expression was induced for 2 h by the addition of galactose before the cells were released into galactose medium at 25°C. Samples were withdrawn at 15-min intervals and then analyzed by Northern blotting with *CLB2*-specific probe. The amount of total RNA loaded (30 μg) was visualized by methylene blue staining of the rRNAs.

the 240- and the 340-bp constructs was upregulated in response to the overexpression of *NDD1* (Fig. 7). All three strains transcribed the endogenous *CLB2* gene and, as expected, the expression was enhanced when Ndd1 was overexpressed. Hence, Ndd1 exerts its effect on *CLB2* transcription via the 240-bp promoter sequence.

Ndd1 prevents formation of protein complexes on the 240-bp sequence, whose absence correlated with the expression of Clb2. Our results thus far strongly suggest that the 240-bp promoter fragment mediates both the cell-cycle-regulated expression of *CLB2* and its potentiation by excess Ndd1. To determine whether Ndd1 is recruited to the protein complexes assembled on this *cis*-regulatory sequence, a radioactively labelled 240-bp fragment and the protein extract from cells expressing native-promoter-driven, fully functional HA_3-*NDD1* were analyzed in a gel mobility shift assay. The assay

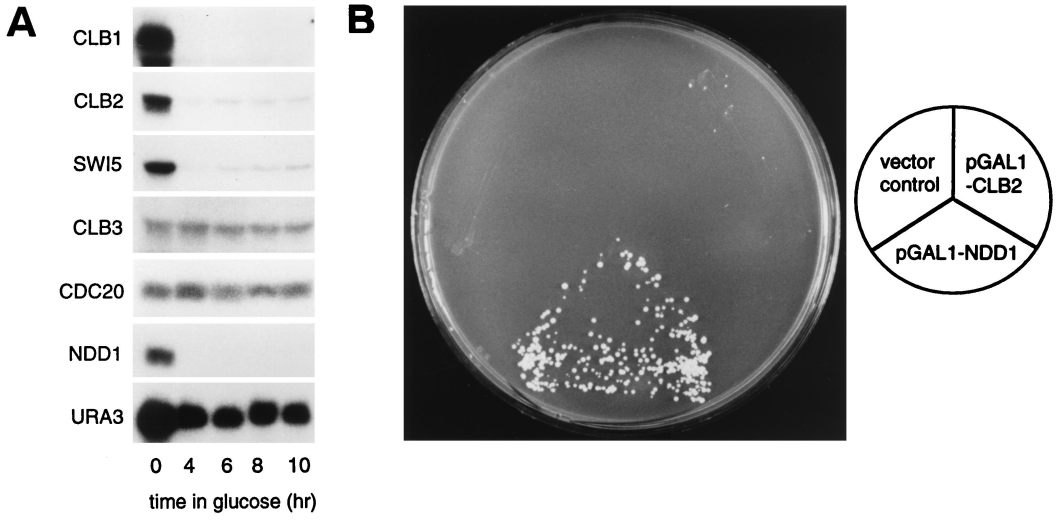

FIG. 5. (A) *NDD1* is required for the normal expression of *CLB1*, *CLB2*, and *SWI5*. *ndd1Δ::LEU2* cells carrying pGAL1-NDD1 were grown to log phase in galactose medium. After 3 h, *NDD1* expression from the *GAL1* promoter was terminated by transferring the cells into glucose medium. RNA samples were taken after 4, 6, 8, and 10 h of incubation in glucose and then analyzed by Northern blotting with *CLB1*-, *CLB2*-, and *SWI5*-specific probes. *CLB3* and *CDC20* RNA transcripts are also shown for comparison. (B) Overexpression of *CLB2* does not suppress the *ndd1Δ::LEU2* null mutation. *ndd1Δ::LEU2* cells, whose growth is dependent on wild-type *NDD1* on a *URA3* plasmid, were transformed with either *TRP1*-based control vector or *TRP1* vector carrying either *GAL1-CLB2* or *GAL1-NDD1*. Transformants were streaked onto galactose medium containing 5-FOA to counter-select for the *URA3*-based plasmid and to induce *NDD1* expression from the *GAL1* promoter. After incubation at 24°C for 3 days, only the cells carrying pGAL1-NDD1 survived.

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yielded four specific complexes (designated I, II, III, and IV) (Fig. 8A, left panel, lane 2), all of which disappeared when challenged with a molar excess of nonradioactive probe (Fig. 8A, left panel, lane 6). However, when a molar excess of nonradioactive 55-bp fragment was used to challenge the binding, complex IV (and to some extent complexes I and II) was abrogated. This suggests that this particular complex is assembled on the core 55-bp region of the 240-bp fragment (Fig. 8A, right panel, lane 4). Complexes I, II, and IV contained Mcm1 because inclusion of anti-Mcm1 antibodies in the binding reactions abolished their formation (Fig. 8A, left panel, lane 5). Mcm1 is perhaps also present in complex III since anti-Mcm1 antibodies diminish, though do not abolish, its formation. However, anti-HA antibodies failed to supershift any of the complexes, indicating that Ndd1 is not present in these complexes (Fig. 8A, left panel, lanes 3 and 4). The failure of anti-HA antibodies to elicit any supershift in our experiments is not due to their general ineffectiveness in gel retardation assays, since the same antibodies (12CA5) have been shown to cause supershifts in a variety of different contexts (37a).

If Ndd1 does not appear to physically associate with the protein assemblage on the 240-bp promoter sequence, then how does it modulate the expression of *CLB2* and the other target genes? To address this question, we first compared the pattern of protein complexes formed on the 240-bp fragment during the stages when *CLB2* promoter is either active or inactive. *pep4*$^-$ cells (US1005) were arrested in late G_1 phase, early S phase, or in mitosis by treatment with α-factor, HU, or NOC, respectively. Cell extracts from these cultures were used in a gel mobility shift assay with the 240-bp sequence as a probe, and total RNA was analyzed for the presence of the *CLB2* transcript. As expected, the cycling extract yielded four specific protein complexes (I, II, III, and IV), identical to the ones described in the preceding paragraph (Fig. 8B, lane 2). While the same four bands were detected in extracts from HU-arrested cells, complexes I, II, and III were either absent or diminished in NOC extracts (Fig. 8B, lanes 4 and 5). Interestingly, a slow-migrating complex (N) was detected in α-factor extract, which was conspicuously absent from early-S-phase or mitotic extracts (Fig. 8B, lane 3). Anti-Mcm1 antibodies abolish its formation, suggesting that this complex also contains Mcm1 (Fig. 8B, lane 7). As expected, *CLB2* was not expressed in G_1 (α-factor arrest), but it was efficiently transcribed in both cycling and HU- or NOC-arrested cells (Fig. 8B, bottom panel). Hence, the appearance of the slow-migrating N complex in α-factor extracts correlates with the absence of *CLB2* RNA. Similarly, the high-level expression of *CLB2* in NOC-arrested extracts is concomitant with a dramatic reduction in the abundance of complex III.

The *CLB2* transcription is activated in late S/G_2 phase; it continues through most of the M phase and is then switched off when the cells exit mitosis to enter the G_1 phase of a new cycle (15, 53). Since Ndd1 is required for the expression of *CLB2*, we wondered whether the turning off of *CLB2* transcription in G_1 is, at least in part, caused by the absence of the Ndd1 protein. This inquiry seemed pertinent in light of our observation that *CLB2* transcription shuts off less efficiently in cells expressing *GAL-NDD1* (Fig. 4B and our unpublished observations). We therefore determined the relative stability of Ndd1 protein during the cell cycle. *cdc15* mutant cells carrying *GAL-HA$_3$-NDD1* on a *CEN* plasmid (US1354) were first synchronized in late telophase by incubation at 37°C for 2 h in raffinose medium. They were induced to express HA_3-Ndd1 for the next 2 h by the addition of galactose and then allowed to resume cell cycle progression at 25°C in galactose medium. Constitutive expression of Ndd1 from *GAL1* promoter in this experiment allowed us to determine not only the timing of its degradation but also the timing of its stabilization. Cell extracts were prepared from samples withdrawn at various time points after the release and were analyzed for the presence of both *HA$_3$-Ndd1* and Clb2 proteins by Western blotting. The abundance of Ndd1 protein begins to decline 30 min after the release from telophase arrest, as the cells begin to disassemble the mitotic spindles (Fig. 8C). Ndd1 remains unstable until cells enter a new cycle at 75 min, as indicated by the absence of anaphase spindles; thereafter, the protein continues to accumulate until it becomes unstable again at 180 min, when the second cycle of spindle disassembly begins. The timing of Ndd1 degradation after the release from telophase arrest matched closely with that of endogenous Clb2, but Clb2 accumulation showed an apparent delay of 40 min (Fig. 8C). This delay is expected in this experiment since the endogenous *CLB2* gene, unlike *GAL1*-driven *NDD1*, is under the control of its native promoter and is transcribed significantly later than is Ndd1. Thus, the disappearance of Ndd1 protein in G_1 correlates well with the silencing of *CLB2* transcription.

The rapid degradation of Ndd1 upon exit from mitosis also correlates with the appearance of the slow-migrating DNA-protein complex (N) (Fig. 8B). Therefore, we asked if the expression of Ndd1 in G_1 would abrogate the formation of this protein complex. *pep4*$^-$ cells carrying three copies of *GAL-NDD1* integrated at the *URA3* locus were first synchronized in G_1 by α-factor treatment in raffinose medium for 2 h. One-half of the culture was then induced to express *NDD1* by the addition of galactose, while the other half received glucose to repress the *GAL1* promoter. After 2 h, cell extracts were prepared and were used in combination with the 240-bp promoter fragment in a gel mobility shift assay. The G_1 arrest induced by α-factor was maintained through the entire course of the experiment. While both the slow-migrating N complex and complex III were present in the glucose-grown cells, their abundance was dramatically reduced in cells expressing Ndd1 from *GAL1* promoter (Fig. 8D, lanes 3 and 4), suggesting that Ndd1 may prevent the formation of these protein complexes on the *CLB2* promoter. As before (Fig. 8B), the N complex was competed out by both the anti-Mcm1 antibodies and a molar excess of the nonradioactive probe (Fig. 8D, lanes 5 and 6). The overexpression of Ndd1 also diminishes complexes I, II, and III, suggesting that presence of Ndd1 in G_1 also affects their assembly.

Together, these data suggest a possible mechanism by which *NDD1* may participate in the regulation of *CLB2* expression. According to this scheme, complexes such as N and III may act as repressors of *CLB2* transcription, since their appearance is inversely correlated to the expression of *CLB2*. That Ndd1 can cause abrogation of both complexes N and III (Fig. 8D) implies that Ndd1 may modulate *CLB2* transcription by preventing the formation of these repressor complexes, thereby allowing the activation of transcription. Thus, the rapid degradation of Ndd1 in G_1, which would allow (or enhance) the assembly of various repressor complexes, may constitute an important step in the silencing of *CLB2* expression upon exit from mitosis. Although we describe this scheme with a particular focus on complexes N and III, complexes I and II could also be repressor aggregates of various protein compositions since, like N complex, they are present in protein extracts from α-factor-arrested cells and diminish in abundance in NOC extracts.

A deletion analysis of Ndd1. Based on the requirement of Ndd1 for *CLB1*, *CLB2* and *SWI5* expression and its ability to activate transcription in a yeast one-hybrid assay, we had raised the possibility that Ndd1 may act as an activator of transcription. Since transcription activation in a heterologous context

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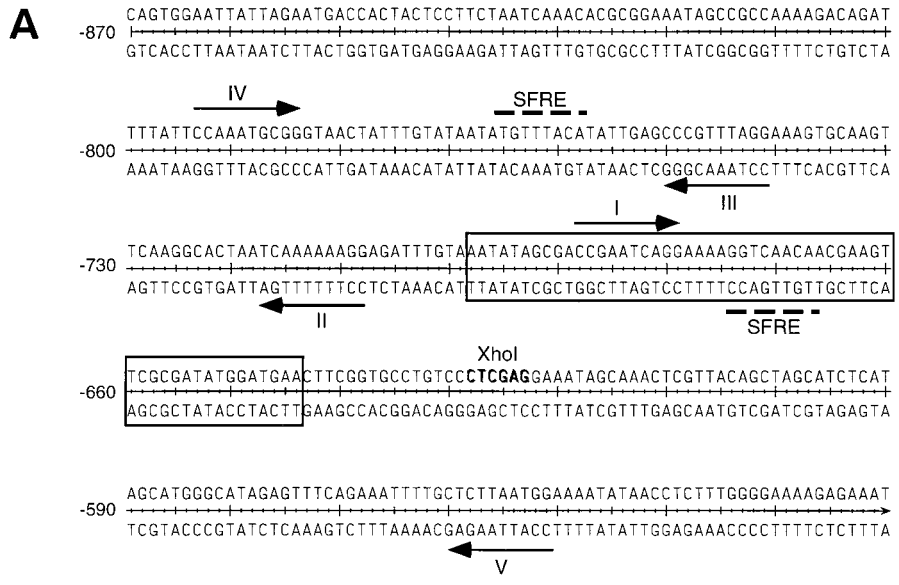

FIG. 6. (A) The 340-bp region of the *CLB2* promoter. The 5′ flanking region of *CLB2* gene from −870 to −520 bp is shown. The boxed region indicates the presumptive 55-bp core promoter sequence. Mcm1 binding sites (MCE I, II, III, IV, and V) are indicated by arrows. Sff binding sites (SFRE) are indicated by dashed lines. The endogenous *Xho*I site is also shown. (B) Cell-cycle-regulated *CLB2* transcription requires 240-bp (−863 to −627) *CLB2* UAS. To test the UAS activity at different stages of the cell cycle, cells carrying a single, integrated copy of the various UAS-*ubiY lacZ* reporter fusions were arrested in α-factor (αF), HU, or NOC. RNA samples were taken and analyzed by Northern blotting for *lacZ* and *CLB2* transcripts. The amount of total RNA loaded (30 μg) was visualized by methylene blue staining of the rRNAs. The designations are as follows: dead, *ubiY lacZ* reporter without UAS sequences; 55 bp, *CLB2* UAS$^{[-698\ to\ -643]}$-*ubiY lacZ*; 240 bp, *CLB2* UAS$^{[-863\ to\ -627]}$-*ubiY lacZ*; 340 bp, *CLB2* UAS$^{[-863\ to\ -523]}$-*ubiY lacZ*; and SWI5, 55-bp *SWI5* UAS-*ubiY lacZ*. (C) Pattern of gene expression from the 240-bp *CLB2* UAS and the endogenous *CLB2* promoter. Cells carrying one integrated copy of 240-bp *CLB2* UAS-*ubiY lacZ* reporter gene were first arrested in G_1 with α-factor treatment (1 μg/ml) at 24°C and then allowed to resume cell cycle progression in medium without α-factor. RNA samples were collected at 10-min intervals and analyzed by Northern blotting for *lacZ* and endogenous *CLB2* RNA expression. The amount of total RNA loaded (30 μg) was visualized by methylene blue staining of the rRNAs. The degree of synchrony was measured in terms of the proportion of cells with anaphase spindles (lower panel).

such as a one-hybrid assay can be artifactual, we asked whether the biological activity of Ndd1 (such as the suppression of *cdc28-1N* and *mcm1* mutations) is related to its ability to activate transcription in a one-hybrid assay. Therefore, we made various constructs in which the DNA-binding domain of the *GAL4* transcription factor was fused in frame with different parts of the *NDD1* ORF and then tested these constructs for both their biological activity and their ability to activate transcription. Such a deletion analysis was also expected to identify the regions important for Ndd1 function.

The deletion constructs (Table 2) were introduced into a yeast strain carrying the *lacZ* reporter under the control of *GAL4* UAS (US449), and the extent of transcriptional activation was estimated by quantitative measurement of the β-galactosidase activity. To determine the biological activity, the hybrid fusions were also introduced into the *ndd1*Δ, *cdc28-1N*, and *mcm1* mutants. As evident from Table 2, the full-length *NDD1* fused to the *GAL4* DNA-binding domain is able to both activate transcription and suppress the growth defects of *ndd1*Δ, *cdc28-1N*, and *mcm1* mutants (row 2). A comparison of various deletion mutants shows that the elimination of the C-terminal portion (residues 232 to 554; Table 2, rows 3, 4, and 5) results in a loss of the biological activity of Ndd1 but it does not diminish its ability to activate transcription. Hence, the N-terminal part of the protein is sufficient for transcriptional activation but not for the biological function. Although the C-terminal half is required for the Ndd1 function, by itself it is both biologically and transcriptionally inactive (row 9). Interestingly, the polyglutamine stretch in the N-terminal half, the most conspicuous feature of the protein, is dispensable since its deletion does not affect Ndd1 function, although it does elicit a higher level of transcription activation (row 12). These results imply that the ability of Ndd1 to activate transcription in a one-hybrid assay may be unrelated to its biological function. However, it is possible that Ndd1 serves as a transcription activator in other cellular contexts.

DISCUSSION

The activities of many of the cell cycle effectors are regulated by protein modification, as well as by the controls operating at the level of transcription. Such multitiered regulation is presumably necessary in order to sharpen the timing of various cellular events during the progression through the division cycle. The *cis*-acting DNA sequences and the factors that activate the transcription of genes at START or at the onset of the S phase have been described in some detail (23, 28, 37, 38). However, the transcriptional regulation of the late S/G_2-phase-specific genes is poorly understood. So far, Mcm1 and another putative transcription factor, Sff, have been shown to participate in the control of gene expression at this stage of the cell cycle (1, 29). However, since Mcm1 is also required for the regulation of a number of other genes whose transcription is not periodic (31, 33, 51), it is unlikely that Mcm1 itself is the major target for cell cycle regulation. This notion is consistent with the finding that Mcm1 occupies both *SWI5* and *CLB2* promoters at all times, although their expression is cyclic (1).

We have identified and characterized a new gene, *NDD1*, that plays an essential role in the expression of the late-S-phase genes *CLB1*, *CLB2*, and *SWI5*, such that none of these genes are transcribed in its absence. Thus, it is a new player in the regulation of gene expression during late S phase. Ndd1 is not only essential for the transcription of *CLB1* and *CLB2*, but its excess enhances the level of their expression. This observation may provide an explanation for the ability of Ndd1 to suppress the *cdc28-1N* mutation. The requirement of *NDD1* for *CLB1* and *CLB2* expression may argue that the lethality of the *ndd1*Δ mutant is solely due to a lack of these mitotic cyclins. However,

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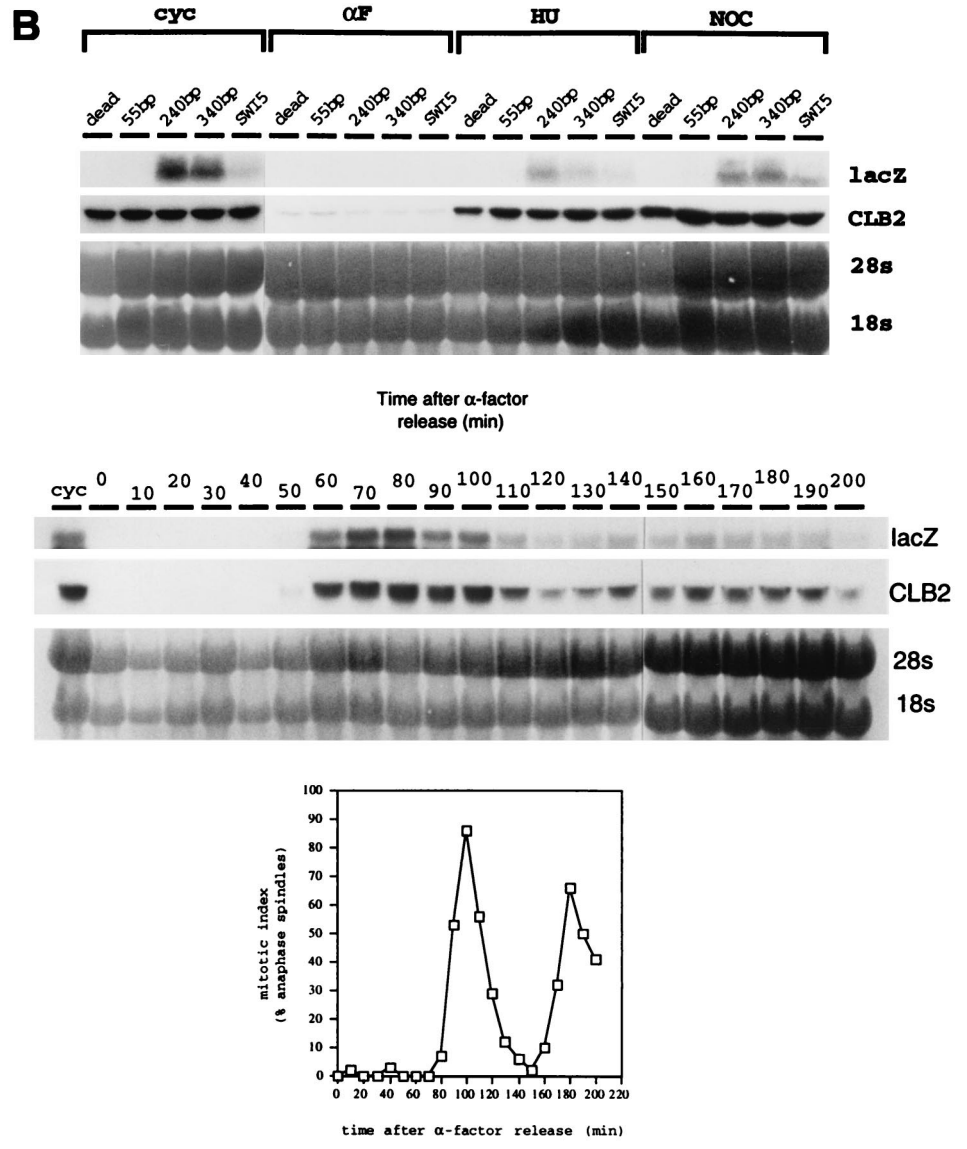

FIG. 6—*Continued.*

overexpression of *CLB2* from the *GAL1* promoter fails to suppress the nuclear-division defect of *NDD1*-deficient cells (Fig. 5B), implying that *NDD1* is necessary for the expression of additional genes that participate in the process of nuclear division. *NDD1* was also independently identified as a gene whose overexpression could suppress a crippling mutation in the *SWI5* UAS, which abolishes *SWI5* transcription (29a).

NDD1 is expressed in a cell-cycle-stage-dependent manner such that its expression peaks in S phase just prior to the expression of its target gene *CLB2* (Fig. 3A). This may implicate *NDD1* as a critical factor in the timing of *CLB2* expression. Our observation that the constitutive expression of Ndd1 does not alter the cell-cycle-regulated pattern of *CLB2* transcription (Fig. 4B) suggests that *NDD1*, though necessary for the activation of a subset of late-S-phase genes, is not a crucial determinant in the temporal control of their transcription. However, since the constitutive expression of Ndd1 leads to inefficient silencing of *CLB2* transcription in late telophase (unpublished observations), the turning off of *CLB2* transcription in late telophase may involve regulation of the Ndd1 protein. It is conceivable that the inactivation of the Ndd1

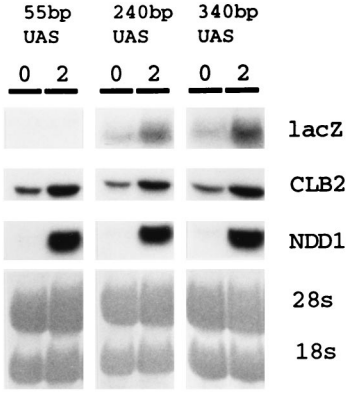

FIG. 7. Enhancement of *CLB2* transcription by excess Ndd1 requires the 240-bp *CLB2* UAS. Strains containing the 55-, 240-, or 340-bp *CLB2* UAS-*ubiY lacZ* reporter were transformed with *pGAL1-NDD1* and grown in raffinose medium to mid-log phase. Galactose was then added to induce overexpression of *NDD1* from the *GAL1* promoter. RNA samples were withdrawn after galactose induction (at 0 and 2 h, respectively) and analyzed by Northern blotting with *lacZ*- and *CLB2*-specific probes. The amount of total RNA loaded (30 μg) was visualized by methylene blue staining of the rRNAs.

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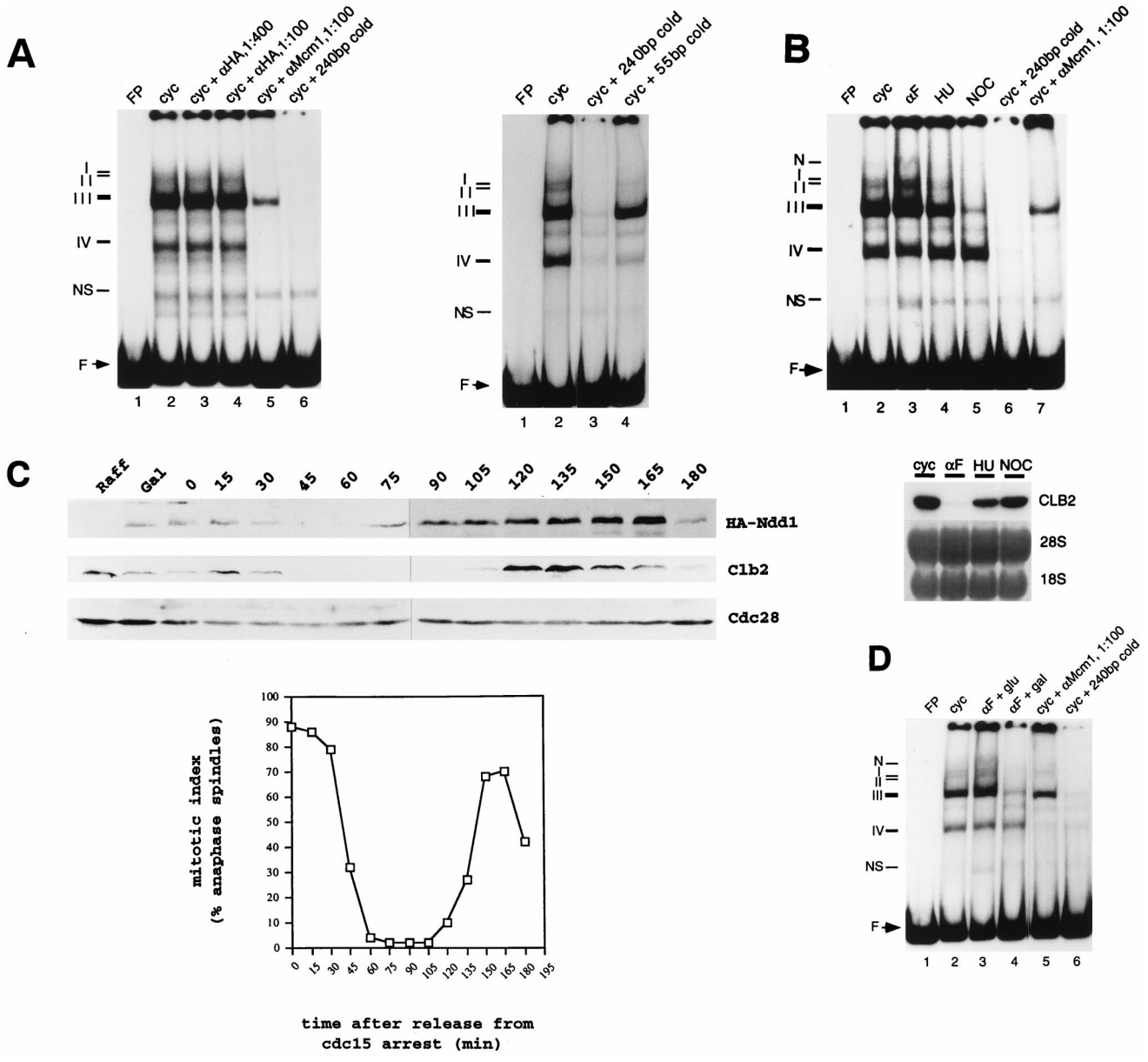

FIG. 8. (A) DNA-protein complexes formed on 240-bp *CLB2* UAS do not contain Ndd1. Radioactively labelled double-stranded 240-bp *CLB2* UAS probe was used for gel retardation assays. The probe was mixed with 20 μg of protein extracts prepared from *pep4*$^-$ cells carrying native-promoter-driven *HA*$_3$*-NDD1*. The designations are as follows: I, II, III, and IV, specific DNA-protein complexes I, II, III, IV; NS, nonspecific band; F, free probe. Antibody supershift reactions were performed with HA- or Mcm1-specific antibodies (left panel, lanes 3, 4, and 5). For competition, excess nonradioactive 240- or 55-bp competitor probe was added to the reaction prior to the addition of radiolabelled probe (left panel, lane 6; right panel, lanes 3 and 4). (B) Appearance of N complex on 240-bp *CLB2* UAS coincides with the absence of *CLB2* expression. Exponentially growing (cyc) *pep4*$^-$ cells were synchronized either in G_1 phase, S phase, or mitosis by α-factor (αF), HU, or NOC treatments, respectively. Protein extracts made from these cultures were incubated with radiolabelled 240-bp *CLB2* UAS probe (upper panel). Designations for the major, specific DNA-protein complexes are as in panel A. The antibody supershift reaction was performed with Mcm1-specific antibodies (lane 7). RNA samples from the cultures were analyzed by Northern blotting with *CLB2*-specific probe. The amount of total RNA loaded (30 μg) was visualized by methylene blue staining of the rRNAs. (C) HA-Ndd1 is unstable in G_1. *cdc15* cells carrying pGAL1-HA$_3$-NDD1 were first synchronized in telophase by growth at 37°C and then released into 25°C to resume cell cycle progression as described in the text. Samples were withdrawn at 15-min intervals, and the stability of HA-Ndd1 was monitored by Western blotting with anti-HA antibodies (12CA5). Clb2 protein was detected with anti-Clb2 antibodies. The amount of Cdc28 protein, whose level remains constant throughout the cell cycle, shows equal loading of samples in all lanes. (D) Conditional overexpression of Ndd1 in G_1 prevents formation of N complex. *pep4*$^-$ cells carrying three integrated copies of *GAL1-NDD1* were arrested in G_1 by α-factor treatment in raffinose medium at 24°C. After 2 h, glucose was added to one-half of the culture, while the other half was induced for 2 h to express Ndd1 by the addition of galactose. Protein extracts made from these cells were used for gel retardation assay by employing radiolabelled 240-bp *CLB2* UAS as a probe. Antibody supershift was performed with Mcm1-specific antibodies (lane 5). For competition, excess nonradioactive 240-bp competitor probe was added to the reaction prior to the addition of radiolabelled probe (lane 6).

protein might be one of the steps required to switch off *CLB2* expression at the end of mitosis. The instability of Ndd1 due to rapid degradation during mitotic exit is consistent with this notion (Fig. 8C). The mechanism, which renders Ndd1 unstable in G_1, remains unclear. We found four destruction-box-like sequences in the middle region of the protein, but their removal did not affect its stability (unpublished results). Another important factor in silencing *CLB2* expression may be the

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TABLE 2. Deletion analysis of Ndd1[a]

Row	Gal4-Ndd1 deletion mutants	β-Galactosidase activity (Miller units)	Ability to:		
			Complement *ndd1Δ*	Suppress *cdc28-1N*	Suppress *mcm1*
1	control Gal4	0	–	–	–
2	1-554 Gal4 full length	25	+	+	+
3	1-321 Gal4	86	–	–	–
4	1-266 Gal4	59	–	–	–
5	1-231 Gal4	33	–	–	–
6	1-216 Gal4	0	–	–	–
7	1-133 Gal4	0	–	–	–
8	1-100 Gal4	0	–	–	–
9	267-554 Gal4	0	–	–	–
10	40-321 Gal4	41	–	–	–
11	134-284 Gal4	0	–	–	–
12	ΔQ1-554 Gal4	76	+	+	+
13	ΔQ1-321 Gal4	79	–	–	–
14	ΔQ1-266 Gal4	88	–	–	–

[a] Various sequences of *NDD1* were fused in frame to the *GAL4* DNA-binding domain (residues 1 to 147). These fusions were transformed into a strain containing the *lacZ* reporter gene whose expression is driven by sequences bearing Gal4-binding sites. The Gal4 DNA-binding domain alone (Gal4) was used as a control. Numbers represent amino acid residues of Ndd1 fused to the Gal4 DNA-binding domain. Polyglutamine domain deletions are denoted by ΔQ. Results for β-galactosidase activities are the average of three independent assays. The ability to complement *ndd1* null mutant was tested by growth on glucose after transforming the fusion constructs into *ndd1Δ::LEU2* pGAL1-NDD1. For the suppression assays, the fusion constructs were introduced into *cdc28-1N* and *mcm1* mutants, and the transformants were plated at the nonpermissive temperatures of 37 and 35°C, respectively.

Cdc28-Clb kinase itself. It has been previously shown that Cdc28-Clb kinase complex stimulates *CLB2* expression via a positive feedback loop (4). Consequently, inactivation of the kinase by abrupt proteolysis of Clb proteins during the M/G_1 transition could lead to a rapid decline in *CLB2* expression.

The genetic interaction with *MCM1* and its ability to enhance *CLB1*, *CLB2*, and *SWI5* transcription lead us to suspect that *NDD1* may influence gene expression by modulating their promoter activity. During the course of our studies, we discovered that the previously reported 55-bp UAS (30) in the *CLB2* promoter, which contains a pair of regulatory sites comprised of one MCE and one SFRE, is unable to drive the expression of a reporter gene in our assay system (see Materials and Methods). In an identical assay, the 55-bp UAS of *SWI5* gene not only elicited transcription but also showed the expected pattern of expression during the cell cycle. This suggests that the inactivity of the 55-bp *CLB2* UAS in our experiments is not due to a faulty assay system. By further investigations, we have identified a 240-bp fragment that is sufficient for both the expression and the cell cycle regulation of *CLB2*. The 240-bp sequence contains within it the 55-bp presumptive UAS and another pair of MCE and SFRE flanked by two additional MCE (Fig. 6A) (reference 1 and this study). This configuration is also capable of mediating the transcriptional enhancement caused by the overexpression of Ndd1; the 55-bp UAS alone, on the other hand, remains unresponsive (Fig. 7). The *CLB1* promoter also harbors two pairs of MCE and SFRE but their spatial arrangement is not identical to that in the *CLB2* promoter (1). It is intriguing that while one set of MCE and SFRE can appropriately regulate *SWI5* expression, it is not sufficient for the activation of *CLB2* transcription. Perhaps this is due to the differences in both the regulatory sequences themselves and in the promoter sequence within which these elements are embedded.

The 240-bp promoter fragment promotes the assembly of four prominent protein complexes. While the exact composition of these complexes remains unknown, they all appear to contain Mcm1 (Fig. 8A). Extracts prepared from cells traversing synchronously through the cell cycle are capable of assembling, albeit to various extents, all four complexes on this *CLB2* promoter fragment (data not shown). Interestingly, Ndd1 is not recruited to any of these protein complexes, as is suggested by the gel mobility shift assay (Fig. 8A), despite its requirement for *CLB1*, *CLB2*, and *SWI5* transcription and its ability to enhance gene expression. In immunoprecipitation experiments, Ndd1 neither associates with Mcm1 nor with any of the major components of the general transcription machinery such as yeast *TBP*, *TAF145*, or *TAF90* (unpublished data). These observations suggest that Ndd1 cooperates indirectly with the transcriptional apparatus, possibly through intermediary proteins, to regulate the expression of late-S-phase genes. How then, does Ndd1 exert its effect on gene expression? One possibility is that Ndd1 is an effector of Cdc28-Clb kinase activity and that it affects *CLB2* transcription by modifying the efficacy of the positive-feedback loop. However, this seems unlikely, because the Ndd1-depleted cells during NOC-induced arrest contain significant levels of the mitotic kinase activity but yet do not transcribe *CLB2* (unpublished results).

Alternatively, Ndd1 may prevent a repressor from binding to the *CLB2* promoter, thus allowing the activation of transcrip-

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tion. The finding that Mcm1, an activator of transcription, occupies the *CLB2* promoter throughout the cell cycle (1) strengthens the possibility that a repressor may be, in part, responsible for the termination of *CLB2* transcription in G_1. In the context of Ndd1 function, this repressor model is consistent with some of our findings: (i) a new protein complex N is detected on the 240-bp promoter fragment during G_1 when *CLB2* transcription is abruptly switched off; (ii) this complex is undetectable in HU- or NOC-arrested cells where *CLB2* is actively transcribed; (iii) overexpression of Ndd1 in G_1 abolishes the formation of the N complex; and (iv) in wild-type cells, Ndd1 is rapidly degraded in G_1, concomitant with the silencing of *CLB2* expression. Complex III behaves like complex N in that its abundance is enhanced during G_1, dramatically reduced during NOC-induced arrest when *CLB2* is maximally transcribed, and negatively influenced by the overexpression of Ndd1. Thus, complexes N and III could be repressor assemblages capable of terminating *CLB2* expression. Although the presence of Ndd1 abolishes the formation of the presumptive repressor complexes, its overexpression does not elicit *CLB2* expression in G_1. This implies that a lack of Ndd1 protein may not be the sole reason for the termination of *CLB2* transcription; a progressive weakening of the positive-feedback loop upon proteolysis of Clb proteins may also be critical. The nature of the hypothetical repressor is so far unknown. To identify such a repressor, we have embarked upon a genetic screen to isolate mutations that will lead to inappropriate *CLB2* expression in G_1.

With the identification of *NDD1*, we have added a new element in the regulation of gene expression in late S phase. However, further investigations will be required to uncover the mechanism that ensures the correct timing of the onset and termination of the expression of these genes.

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