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Regulation of *CDC9*, the *Saccharomyces cerevisiae* Gene That Encodes DNA Ligase

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We have cloned *CDC9*, the structural gene for *Saccharomyces cerevisiae* DNA ligase, and investigated its transcriptional regulation both as a function of cell cycle stage and after UV irradiation. The steady-state level of DNA ligase mRNA increases at least fourfold in late G1, after the completion of start but before S phase. This high level of *CDC9* mRNA then decays with an apparent half-life of ca. 20 min and remains at a low basal level throughout the rest of the cell cycle. The accumulation of *CDC9* mRNA in late G1 is dependent upon the completion of start but not the *CDC7* and *CDC8* functions. Exposure of cells to UV light elicits an eightfold increase in DNA ligase mRNA levels.

The yeast *Saccharomyces cerevisiae* offers the opportunity to combine sophisticated genetic and biochemical analyses to the study of eucaryotic cell division. In attempting to elucidate the molecular events of the cell division cycle, researchers have asked whether yeast genes are expressed periodically during the cell cycle. Initial studies on the activities of various enzymes in cultures of synchronously dividing cells did show significant periodic fluctuations in activity (21). However, subsequent work which examined synthesis of over 200 major yeast proteins found no evidence for periodic synthesis; instead, the proteins analyzed showed exponential synthesis during the cell cycle (7). It was suggested that the previously observed activity modulations were the result not of changes in enzyme synthesis but rather of periodic changes in regulatory molecules affecting enzyme activity (8). The only exceptions noted were the histones; these proteins did show a cell cycle periodicity in synthesis, being synthesized maximally in early S phase (7). Expression of the yeast histone genes is now known to be cell cycle regulated by both transcriptional and posttranscriptional controls (10, 13). To our knowledge, the histone genes and the homothallism gene *HO* (24) are the only identified yeast genes known to be subject to mitotic cell cycle regulation at the level of synthesis of gene products. However, a recent examination of ca. 900 *S. cerevisiae* proteins revealed eight other proteins of unknown identity which appeared to be synthesized periodically in the cell cycle (19).

Of the many yeast *cdc* (cell division cycle) mutants isolated, *cdc9* is one of the few in which the biochemical defect has been identified. Johnston and Nasmyth demonstrated that *cdc9* mutants are defective in DNA ligase activity (17). While this manuscript was in preparation, we became aware that *CDC9* was recently cloned and shown to encode a DNA ligase by virtue of its ability to complement the *Schizosaccharomyces pombe cdc17* mutation, which lies in the DNA ligase structural gene of that organism (1, 25). We report here the independent molecular cloning and transcriptional characterization of *CDC9*.

DNA ligase functions in at least three processes in vivo: DNA replication, repair, and recombination. Since DNA replication is periodic and levels of DNA repair and recombination are known to be variable in response to a number of cellular conditions, the regulation of *CDC9* in cells preparing for each of these processes is potentially interesting. Accordingly, we used our cloned *CDC9* DNA sequences as hybridization probes to answer various questions concerning possible transcriptional regulation of the DNA ligase structural gene.

Since the need of a cell for DNA ligase probably peaks once per cell cycle during S phase, *CDC9* is an excellent subject for investigating the question of cell cycle regulation. A priori, it seems economically favorable that DNA ligase would be regulated at the level of transcription of the structural gene, *CDC9*. However, the results of the cell cycle studies discussed above argue against cell cycle transcriptional regulation but instead would support only posttranslational regulatory mechanisms. We tested these alternate hypotheses directly, and we present evidence that the steady-state level of DNA ligase mRNA is in fact cell cycle regulated.

The regulation of *CDC9* also is pertinent to studies of DNA repair processes. Cells about to undertake DNA repair may utilize preexisting enzymes, or they may synthesize the required enzymes de novo. To address this issue, we asked whether the steady-state levels of *CDC9* mRNA were altered by irradiation of cells. Our finding that the levels of *CDC9* mRNA do increase significantly after UV irradiation suggests that the DNA ligase needed for repair is synthesized de novo.

MATERIALS AND METHODS

Strains, media, plasmids and cloning procedures. *Escherichia coli* HB101 (*leuB pro thi thr lacY1* Str^r *hsdR hsdM recA*) and JA300 (*thr leuB6 thi thyA trpC1117 hsr$_k$ hsm$_k$* Str^r) were used as appropriate for growing plasmids. The *S. cerevisiae* yeast strains used and their genotypes are given in Table 1. Strain 381G (*bar1-1*) was provided by D. Jenness. Strain A364A and its derivatives 13052 and 4008 were obtained from Lee Hartwell and grown in MV medium (10).

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

Strain	Genotype
EH2-7B	*MAT***a** *cdc902 leu2-3 leu2-112 met (8,15) trp1-289 ura3-52*
LP2724-3C	*MAT*α *cdc36-16 leu2-3 leu2-112 trp1-289 ura3-52*
381G(*bar1-1*)	*Mat***a** *bar1-1 cry1 ade2^0 his4^a lys2^0 trp1^a tyr1^0 SUP4-3*(TS)
A364A	*MAT***a** *ade1 ura1 his7 lys2 tyr1*
4008	*MAT***a** *cdc7-4 ade1 ura1 his7 lys2 tyr1*
13052	*MAT***a** *cdc8-3 ade1 ura1 his7 lys2 tyr1*
X2180-1B	*MAT*α *SUC2 gal2 mal CUP1*
B-635	*MAT***a** *cyc1-115 his1-1 lys2-1 trp2 RAD$^+$ CDC$^+$*

Yeast and bacterial culture media and transformations were as described by Tschumper and Carbon (42). YEPD/A medium consists of 1% yeast extract, 2% peptone, 2% glucose, and 30 μg of adenine per ml. Plasmids YRp7 (40), YEp13 (4), and YEp20 (2) have been described previously. Restriction fragments from plasmids YRp7(*CDC36*.1) (3), YEp24 (2), and YEp13(*CDC9*.2) (described here) were purified from polyacrylamide gels for use as hybridization probes. Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs or Bethesda Research Laboratories and used according to the specifications of the manufacturer.

Isolation and construction of *CDC9* plasmids. *S. cerevisiae* EH2-7B (Table 1) carrying the alleles *cdc9-2 leu2-3 leu2-112* was transformed with plasmid DNA from the YEp13 yeast DNA library made by Nasmyth and Reed (26). Plasmid DNA was isolated from Leu$^+$ Cdc$^+$ transformants and used to transform *E. coli* for amplification and analysis. In this way we obtained 12 plasmids containing unique *S. cerevisiae* DNA inserts which complemented the *cdc9-2* mutation. Partial restriction analysis of these 12 plasmids revealed restriction site homologies among all of them. Plasmid YEp13(*CDC9*.1), which contained the smallest insert of 7 kilobase pairs (kbp), was chosen for further study. A 4.6-kbp *Bam*HI-*Bgl*II fragment present within the yeast DNA insert of YEp13(*CDC9*.1) was ligated into the *Bam*HI site of YEp13 to generate plasmid YEp13(*CDC9*.2), which complemented the *cdc9-2* mutation. The construction of YEp13(*CDC9*.3) is described in the text. pRC2(*CDC36*.2) has been described previously (3). YEp20(*CDC9*.1) was constructed by insertion of the 2.3-kbp *Pst*I fragment of YEp13(*CDC9*.2) into the *Pst*I site of YEp20.

Synchronization by α factor treatment. *S. cerevisiae* 381G(*bar1-1*) (Table 1) was grown to a density of 0.5×10^7 to 1×10^7 cells per ml in YEPD/A medium at room temperature. Under these conditions the generation time is ca. 190 min. α factor (Sigma Chemical Co.) was added to 300 ng/ml, and the culture was incubated with shaking for ca. 6 h, at which time nearly all of the cells were large G1-arrested shmoos. The α factor-containing medium was then removed by filtration through a disk (Millipore Corp.) Cells collected on the disk were washed by filtering ca. 20 ml of YEPD/A medium without α factor through them. The washed cells were resuspended at the previous density in fresh YEPD/A medium without α factor to initiate a synchronous cell division. The time of α factor removal was taken as the beginning of the 20-ml YEPD/A medium filtration wash. For determining cell number and percent budded cells, samples were diluted 1:1 with ice-cold fixing solution (0.9% NaCl, 2% formaldehyde) and held on ice for examination within 12 h. The samples were then sonicated briefly and counted on a hemacytometer. Cultures of A364A strains were synchronized by incubation with α factor prepared from *MAT*α cell culture supernatants as described previously (10).

Synchronization by sedimentation selection. *S. cerevisiae* X2180-1B (Table 1) was grown to ca. 2×10^7 cells per ml in YEPD/A medium at room temperature. The generation time of this strain under these conditions is ca. 120 min. Cells (5×10^9) were collected by centrifugation, resuspended in culture supernatant to a total volume of 9 ml, and sonicated briefly. About 2.2 ml of the cell suspension was layered onto each of four preformed 70-ml 10 to 40% sorbitol gradients in YEPD/A medium in centrifuge tubes (2.7 by 14 cm). The gradients were centrifuged in swing-out buckets at a relative centrifugal force of $180 \times g$ for 5 min and again at a relative centrifugal force (RCF) of $400 \times g$ for 8 min. Visual inspection of the gradients showed that the thickest cell layer had moved about 3/4 of the way down the tubes (see reference 22). The upper layers containing the smallest G1 cells were withdrawn, and the cells were collected by centrifugation, whereupon they were resuspended in fresh YEPD/A medium under growth conditions. The entire selection operation was performed at room temperature and took ca. 60 min.

Preparation of RNA, RNA blotting, hybridizations, and autoradiography. For some experiments (see Fig. 2 and 5), total RNA was prepared as described previously (32) except that for each time point ca. 5×10^7 cells were lysed by vortexing with glass beads and phenol in a 1.5-ml Eppendorf tube for six 30-s intervals. RNA was prepared from UV-irradiated cells in the following manner. Strain B-635 (RAD$^+$; Table 1) was grown in YEPD medium to a density of ca. 5×10^7 cells per ml. Cells were pelleted by centrifugation, washed once with water, and resuspended at the same density in sterile water. Twenty-milliliter samples were irradiated with constant stirring in petri dishes (150 by 20 mm) at 1.00 J/m^2 as described previously (18). Irradiated cells were pelleted by centrifugation, resuspended in ca. 3/4 volume of YEPD medium, and incubated in the dark at 30°C with constant shaking. At various times after UV irradiation, 14-ml samples were removed and RNA was prepared. All operations were performed in yellow light until the addition of glass beads and phenol to avoid photoreactivation. Samples also were withdrawn before and after UV irradiation for viability determinations.

For some experiments (see Fig. 2 and 5), RNA electrophoresis and transfer were carried out essentially as described by Thomas (41), except that agarose gels were run in 40 mM morpholinepropanesulfonic acid buffer (pH 7) rather than in 10 mM sodium phosphate. The RNA blots (Fig. 3 and 4) are the same as those shown in Hereford et al. (10; Fig. 3 and 4, respectively) after washing and hybridization to *CDC9* and H2A probes. For UV-irradiation experiments, RNA was electrophoresed through formaldehyde gels and transferred to Gene Screen by the protocol provided by New England Nuclear Corp. Blots were hybridized to nick-translated DNA restriction fragment probes (33) in the presence of 10% dextran sulfate (Pharmacia Fine Chemicals) as has been described previously (32). For cell cycle experiments, exposure to film and densitometric quantitation have been described previously (3). For UV-irradiation experiments, densitometric scans were carried out with an LKB soft laser scanning densitometer, and peaks were integrated with a Tektronix 4956 digitizer.

RESULTS

Isolation and identification of *CDC9*. *S. cerevisiae* DNA sequences capable of complementing *cdc9* mutations were

obtained by transformational complementation of an *S. cerevisiae cdc9* strain (see above). A 4.6-kbp *Bam*HI-*Bgl*II *cdc9*-complementing yeast DNA fragment was subcloned into vector YEp13 to make plasmid YEp13(*CDC9*.2). A partial restriction map of the 4.6-kbp *Bam*HI-*Bgl*II insert is shown in Fig. 1. It was found that plasmid YEp13(*CDC9*.2) also complements the *cdc36-16* mutation. Since *cdc9* and *cdc36* are 1.2 map units apart on the left arm of chromosome IV (35), we conclude that the 4.6-kbp *Bam*HI-*Bgl*II fragment originates from the chromosomal *CDC9-CDC36* locus and contains both of these genes.

As part of an independent study of *S. cerevisiae* start genes, *CDC36* had been cloned and localized on a 4.6-kbp *Bam*HI-*Bgl*II yeast DNA fragment also originating from the Nasmyth and Reed library (3). A comparison of the restriction maps of the two independently isolated fragments revealed that they were identical. This second fragment had been isolated by virtue of its ability to complement the *cdc36-16* mutation and was shown to derive from the genomic *CDC36* locus by its ability to direct plasmid integration at that site, presumably by homologous recombination (3). Transcripts arising from this sequence were identified by RNA blot analysis and R-loop mapping, and transcriptional orientation of the coding regions was established by using bacteriophage M13 vectors to prepare strand-specific probes, followed by hybridization to yeast RNA blots (3) (Fig. 1). It can be seen that three transcripts are identified: *TSL36* (transcribed sequence to the left of *CDC36*), *CDC36*, and *TSR36*. Subcloning analysis showed the central 615-base-pair transcribed sequence to represent *CDC36* (3). Since *cdc36* and *cdc9* are not allelic (unpublished data), the *CDC9* transcript must be either *TSL36* or *TSR36*.

To determine which gene is *CDC9*, we constructed the subclone plasmids shown in Fig. 1. YEp13(*CDC9*.3) was constructed by removal from YEp13(*CDC9*.2) of an *Sph*I fragment extending from the *Sph*I site near the middle of *TSR36* to the *Sph*I site in the vector. Thus, YEp13(*CDC9*.3) has an extensive deletion of *TSR36* but has *TSL36* and *CDC36* intact (Fig. 1); this plasmid complements both the *cdc36-16* and *cdc9-2* mutations. Additionally, pRC2 (*CDC36*.2) complements the *cdc36-16* mutation (3) but not the *cdc9-1* mutation, whereas YEp20(*CDC9*.1) complements neither *cdc9* nor *cdc36* mutations. These results, taken together with genetic evidence that *CDC9* and *CDC36* are not allelic (unpublished data), indicate that *CDC9* is in fact *TSL36*. Additionally, the size of the *TSL36* transcript is ca. 2,500 bases and so can potentially encode a polypeptide of up to 100,000 daltons in size, which is consistent with most reported sizes of eucaryotic DNA ligases (37). DNA blot hybridization analysis (38) indicates that *CDC9* is a unique yeast genomic sequence (data not shown).

Periodic modulation in steady-state levels of *CDC9* mRNA. To test the possibility of cell cycle regulation of *CDC9*, we examined the intracellular steady-state levels of *CDC9* mRNA in a population of synchronous cells as they progressed through the cell cycle. The synchronous culture was prepared by treating haploid 381G(*bar1-1*) *MAT***a** cells (Table 1; 39) with the mating pheromone α factor, which causes cells to arrest cell division at start, a point late in the G1 segment of the cell cycle. The *bar1-1* mutation renders cells ca. 20-fold more sensitive to α factor than wild-type, because it is thought that cells carrying the *bar1-1* mutation lack an activity normally present in wild-type cells which degrades α factor (5, 20). To ensure that all cells were arrested at start, they were held in α factor for 6 h, whereupon the pheromone was removed by filtration and the cells were washed and resuspended in fresh medium to initiate a synchronous round of division. At various times throughout the experiment, samples of cells were removed from the main culture for (i) counting and visual examination of cell morphology, which serves as an indicator of cell cycle stage, and (ii) preparation of total RNA. Equal amounts of the RNA preparations were subsequently denatured with glyoxal, electrophoresed on agarose gels, and blotted to nitrocellulose (41). These RNA blots then were hybridized with radiolabeled probes to determine the relative intracellular steady-state levels of *CDC9* and control gene mRNAs throughout the course of the α factor-induced arrest and the subsequent synchronous cell division cycle. The total RNA preparations are composed predominantly of rRNA, and equal amounts of each preparation (as determined by A_{260}) were loaded in each sample lane. As such, the amount of mRNA detected by hybridization is normalized to the concentration of rRNA, which should remain constant, and so the intensities of hybridization observed reflect the relative intracellular steady-state levels of each mRNA species at the various times of the experiment.

There is a graph in Fig. 2A which shows the cell concentrations and the percent budded cells as determined at the indicated times of the experiment. α factor was added to the asynchronous culture at 0.5 h and removed at 6.5 h. The cell cycle-arresting effect of α factor is apparent from the graphs, where it can be seen that after 2.5 h of exposure to α factor the cell number plateaued and the percent budded cells decreased to ca. 5%. Visual examination showed that greater than 90% of the cells were arrested as unbudded cells which continued to grow to form the misshappen cells known as shmoos. Shortly after removal of the α factor, the cells underwent a synchronous round of budding, reaching greater than 90% budded cells at 8.5 h (Fig. 2A). The drop in percent budded cells at 9 and 9.5 h, coincident with an increase in cell number, indicates cytokinesis in progress. The percent budded cells drops to less than 70% at 9.5 h but does not approach 0 because the newly divided cells immediately initiated a second round of budding. The unbudded period between the two budded cycles is very short, since during the extensive α factor block most cells had grown to such a size that they would pass quickly through G1 to S phase (36).

Figure 2B shows the autoradiographs of the RNA blots prepared in this experiment. The sequence of lanes corre-

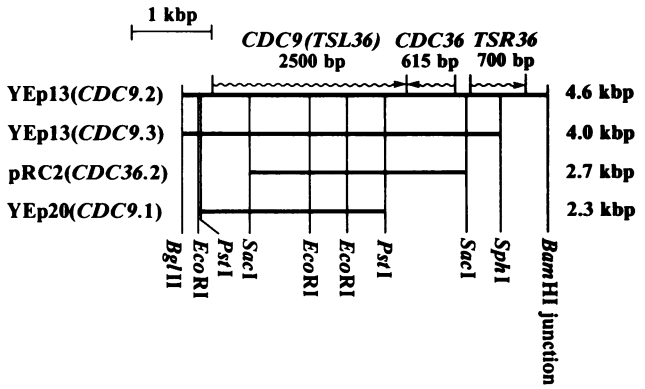

FIG. 1. Plasmid inserts containing *CDC9* sequences. The isolation of *CDC9* sequences by transformational complementation and the construction of derivative plasmids is described in the text. The transcribed regions of the uppermost 4.6-kbp *Bam*HI-*Bgl*II insert were mapped and oriented as described previously (3).

sponds to RNA prepared from cells samples at the times shown directly above each lane in panel A. The blots were hybridized with two radiolabeled DNA probes: the 2.7-kbp *Sac*I fragment containing *CDC9* and *CDC36* sequences (Fig. 1) and a 1.1-kbp *Hin*dIII fragment of YEp24 containing *URA3* sequences (2). The level of *URA3* mRNA is not expected to vary in these experiments, and so serves as a control for equality of RNA amounts loaded in each lane. Inspection of the autoradiograph shows that the level of *CDC9* mRNA changes significantly, whereas the levels of *URA3* and *CDC36* mRNA appear roughly constant throughout the experiment. The lower regions of lanes 3 through 9 (Fig. 2B) appear faint due to a blotting artifact. The changes in *CDC9* mRNA levels were quantitated by densitometric scanning of the autoradiograph (Fig. 2A). It is apparent that the level of *CDC9* mRNA is about twofold lower in cells arrested at start by α factor than in the asynchronous population. The α factor was removed at 6.5 h; the RNA sample prepared shortly afterwards, at 7 h, shows a fourfold increase in the *CDC9* mRNA level. This point occurs shortly before entry into S phase as indicated by the onset of budding (15, 46). The *CDC9* mRNA level remains high at the following 7.5-h point and then drops to a low level at the 8- and 8.5-h points, as the cells complete S phase and traverse G2. The *CDC9* mRNA level increases again at 9 h, when cells enter the late G1 phase of the following cell cycle. By comparison to the peaks of percent budded cells, it can be seen that the peaks in *CDC9* mRNA level are separated by one cell cycle and occur before the onset of budding of each cycle. Hence, *CDC9* mRNA increases to a high level just after start and before S phase, whereas it is at a low level throughout the rest of the cell cycle. Additionally, the level of *CDC9* mRNA decreases 10-fold between the 7- and 8-h samples (Fig. 2A). This indicates that the *CDC9* message has a half-life of ca. 20 min under these conditions. This is a maximum estimate of the stability of the message since it does not take into account the possibility of ongoing *CDC9* transcription and imperfect synchrony. The data on *CDC9* mRNA accumulation (Fig. 2) are shown in tabular form in experiment 1 of Table 2. This α factor synchronization experiment was repeated, and these data are presented in experiment 2 of Table 2. As can be seen by comparison of the results of the two independent experiments, essentially the same pattern of *CDC9* fluctuation was observed. As a control, a cell culture was treated exactly as in the previous experiments except that the α factor was removed 5 min after addition. RNA blot analysis showed no significant variation in *CDC9* mRNA levels (data not shown). This control shows that the changes in *CDC9* mRNA level described above are due to the α factor-induced synchronization of the cells and are not due to some stress response elicited by the washing steps or the α factor per se. Additionally, *CDC9* mRNA levels were examined in A364A cells (BAR^+; Table 1) synchronized with α factor obtained from *MAT*α cell culture supernatant (10). Periodicity in *CDC9* mRNA levels again was observed, and it was seen to parallel the periodicity of histone mRNA accumulation (data not shown). We conclude that the intracellular steady-state levels of *CDC9* mRNA undergo significant modulation as cells progress through the cell cycle.

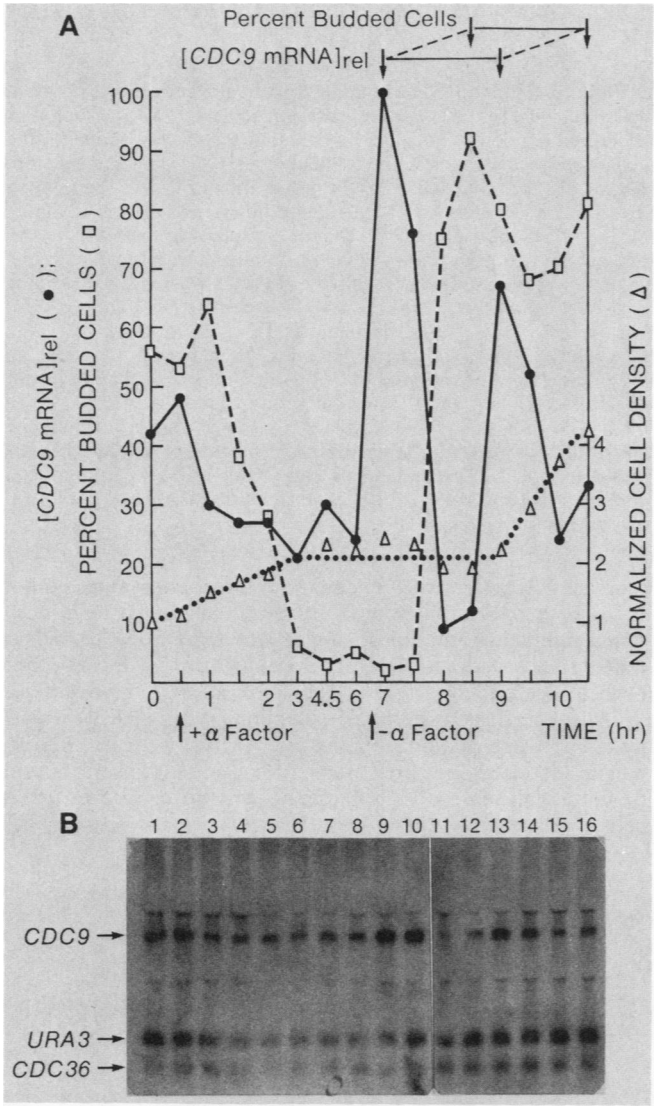

FIG. 2. Regulation of *CDC9* mRNA levels in α factor-synchronized cells. (A) Relative levels of *CDC9* mRNA (●), percent budded cells (□), and normalized cell density (△) are plotted against time. α factor was added to a log-phase culture of *S. cerevisiae* cells at 0.5 h and removed at 6.5 h. Samples of the culture were withdrawn at the indicated times, cell number and percent budded cells were determined by counting on a hemacytometer, and RNA was extracted for Northern blot analysis of *CDC9* mRNA levels. The cell density at time 0 was 5.1×10^6 cells per ml; subsequent points are normalized to that value. The relative levels of *CDC9* mRNA were determined by densitometric scanning of the autoradiograph in panel B and are expressed as a percentage of the maximum at 7 h. The parallelogram at the top of the figure illustrates that the peaks in *CDC9* mRNA level occur before the onset of budding and are separated by one cell cycle. Note that the time scale is not linear during the period of exposure to α factor; rather, the data points are equally spaced in line with the corresponding lanes in panel B. Percent budded cells was determined at 11 and 11.5 h and equalled 68 and 56%, respectively. (B) Autoradiographs of RNA blots displaying RNA prepared from cells sampled at the times shown directly above each lane in panel A. The blots were probed with the radiolabeled 2.7-kbp *Sac*I DNA fragment, which has homology to both *CDC9* and *CDC36* mRNAs (Fig. 1), and the 1.1-kbp *Hin*dIII DNA fragment of YEp24, which hybridizes to *URA3* mRNA (2). The faint bands above and below the *CDC9* bands are rRNA-associated artifacts often seen in these total RNA preparations. The lower regions of lanes 3 through 9 appear faint due to a blotting artifact.

***CDC9* mRNA levels in *cdc7* and *cdc8* cells.** We asked whether the accumulation of *CDC9* mRNA at the G1-S phase boundary is dependent upon the completion of cell cycle events occurring in G1 and S phase. The temperature-sensitive cell division cycle mutation *cdc7* arrests cells in late G1, after the point of α factor arrest (12). *cdc7*(Ts) cells were synchronized with α factor at the permissive temperature, whereupon the α factor was removed and the cells were resuspended in media prewarmed to the restrictive temperature of 36°C. The culture was incubated at 36°C, and steady-state levels of *CDC9* mRNA were determined at intervals by RNA blotting as described previously (10). The results (Fig. 3) show that *CDC9* mRNA begins to accumulate at 30 min after removal of α factor, reaches a maximum at 50 min, and thereafter maintains a significant, though decreasing, level. Although the final *CDC9* mRNA level is 50% of the maximum seen at 50 min, it is still significantly greater than the basal levels seen at 0, 10, and 20 min. These data are qualitatively similar to that of H2A mRNA (Fig. 3) and H2B mRNA as previously quantitated (10). This result indicates that, like the histones, accumulation of *CDC9* mRNA is not dependent upon completion of the *cdc7*-sensitive step.

We similarly determined the relative steady-state levels of *CDC9* mRNA in cells synchronized by α factor and then released to the *cdc8* block point. At the restrictive temperature, cells carrying the *cdc8*(Ts) mutation execute the *CDC7* step and initiate DNA synthesis but arrest in early S phase due to a DNA chain elongation defect (9). The accumulation of *CDC9* mRNA in these cells is shown in Fig. 4. It can be seen that *CDC9* mRNA begins accumulating at ca. 30 min after removal of α factor, reaches a maximum at 40 min, and then rapidly decreases towards basal levels. With the exception of some fluctuations between 60 and 90 min, the relative levels of *CDC9* mRNA parallel those of the previously quantitated H2B mRNA (10) and H2A mRNA (Fig. 4). We will show later that the level of *CDC9* mRNA increases after treatment of cells with DNA-damaging agents. It is conceivable that temperature-arrested *cdc8* cells contain a significant amount of single-stranded and nicked DNA due to the cessation of DNA chain elongation, whereas other replicative functions (e.g., topoisomerases) remain active. We suggest, therefore, that the fluctuations in *CDC9* mRNA levels between 60 and 90 min (Fig. 4) may be attributed to partial increases in *CDC9* mRNA level in response to a real or perceived state of DNA damage in these *cdc8*-arrested cells.

TABLE 2. Accumulation of *CDC9* mRNA in cells synchronized by α factor[a]

Expt 1		Expt 2	
Time (h)	(*CDC9* mRNA)$_{rel}$	Time (h)	(*CDC9* mRNA)$_{rel}$
0	42	0	16
0.5	48	0.8	9
1	30	1.8	7
1.5	27	2.8	5
2	27	3.8	7
3	21	4.8	3
4.5	30	5.8	7
6	24	6.9	26
7	100	7.3	66
7.5	76	7.8	40
8	9	8.3	10
8.5	12	8.8	20
9	67	9.3	100
9.5	52	9.8	96
10	24	10.3	43
10.5	33	10.8	20

[a] Log-phase cells were synchronized by a 6-hour exposure to α factor, and the relative levels of *CDC9* mRNA were determined at the indicated times as discussed in the text and the legend to Fig. 2. The data for experiment 1 are plotted in Fig. 2. α factor was added at 0.5 h and removed at 6.5 h. Experiment 2 is an independent repeat to experiment 1. α factor was added at 0.1 and removed at 6.2 h. (*CDC9* mRNA)$_{rel}$ are the relative levels of *CDC9* mRNA expressed as a percentage of the maximum level reached in each experiment. The values of (*CDC9 mRNA*)$_{rel}$ were determined by densitometric scanning of autoradiographs of RNA blots, as described in the text and the legend to Fig. 2.

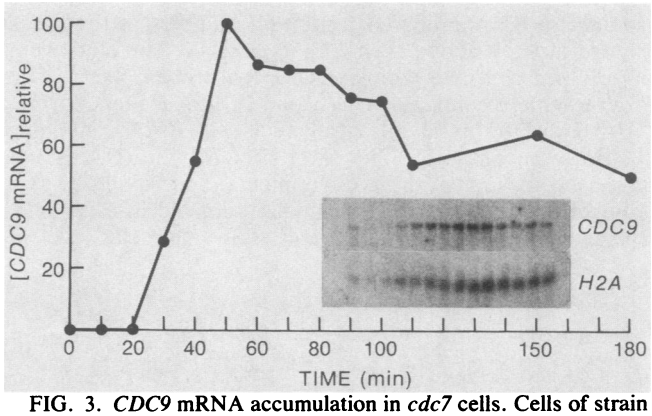

FIG. 3. *CDC9* mRNA accumulation in *cdc7* cells. Cells of strain 4008 (*cdc7-4*; Table 1) were synchronized in G1 with α factor as described previously (10, 13). At time 0, α factor was removed, the cells were resuspended in fresh medium at 36°C, and samples were removed at the indicated times for preparation of RNA. The relative levels of *CDC9* mRNA were quantitated by densitometric scanning of the RNA blot shown in the inset and are expressed as a percentage of the maximum level at 50 min. The RNA blot is the same one shown previously (10; Fig. 3) after washing and probing with the radiolabeled 2.7-kbp *Sac*I fragment (*CDC9* and *CDC36*; Fig. 1) and TRT-3 *Hin*dIII fragment E′ (H2A mRNA; 13). The *CDC9* mRNA bands at times 0, 10, and 20 min are faintly visible in the original autoradiograph but below the limits of detection of our densitometer. *CDC36* mRNA comigrates with H2A mRNA; since *CDC36* is not cell cycle regulated, it is a minor, unchanging constituent of the band labeled H2A. The rate of DNA synthesis was determined by pulse labeling and shown to be greatly reduced compared with wild-type cells, confirming that these *cdc7* cells are arrested before S phase (10).

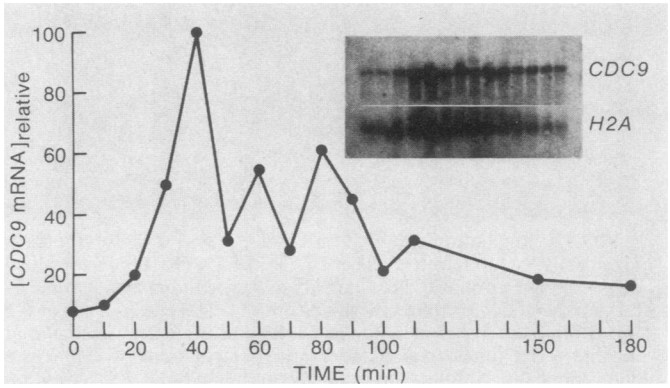

FIG. 4. *CDC9* mRNA accumulation in *cdc8* cells. The details of this experiment are the same as in Fig. 3, except that cells of strain 13052 (*cdc8-3*; Table 1) were used. α factor was removed at time 0, and the cells were resuspended in fresh medium at 36°C. The RNA blot is the same as used previously (10; Fig. 4). The rate of DNA synthesis in these *cdc8* cells was greatly reduced compared with that in wild-type cells (10).

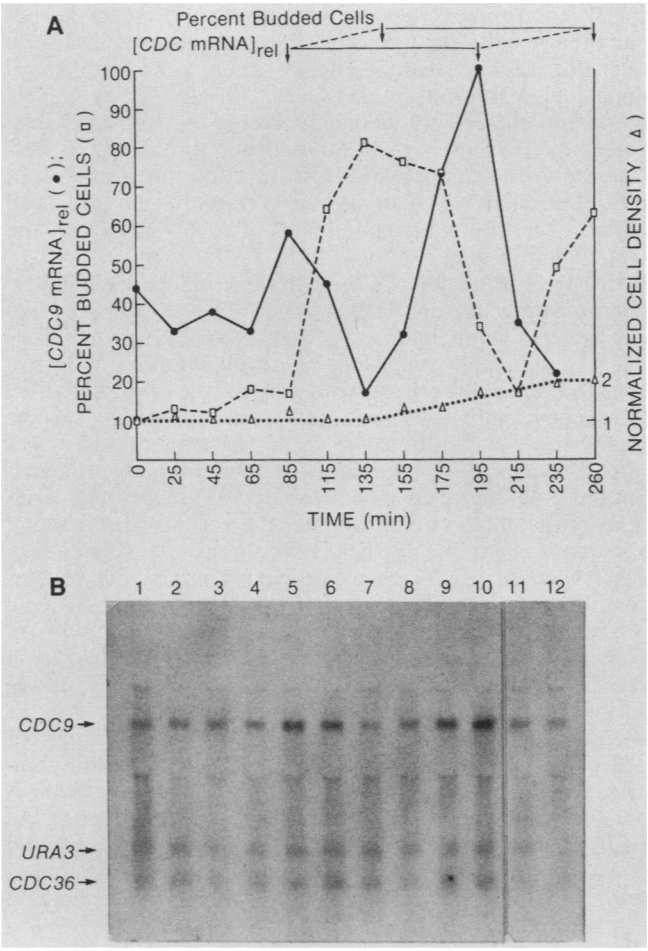

FIG. 5. Regulation of *CDC9* mRNA levels in size-selected synchronous cells. (A) Legend is the same as that for Fig. 2, except that log-phase cells (X2180-1B; Table 1) were sedimented through a 10 to 40% sorbitol gradient to separate cells on the basis of size and density. The fractions containing the smallest, newly abscised cells were isolated and resuspended in fresh medium to initiate a synchronous round of division. The cell density at time 0 was 1.1×10^7 cells per ml. The relative levels of *CDC9* mRNA were determined by densitometric scanning of the blots in panel B and are expressed as a percentage of the maximum level at 195 min. Note that the time scale is not linear throughout; rather, the data points are equally spaced in line with the corresponding lanes in panel B. (B) Autoradiographs of RNA blots made with RNA prepared from cells sampled at the times shown directly above each lane in panel A. The blots were hybridized with the same probes used in Fig. 3 to detect *CDC9*, *CDC36*, and *URA3* mRNAs.

Modulation of *CDC9* mRNA levels in cells synchronized by size selection. We were concerned that the apparent cell cycle regulation of *CDC9* seen in α factor-synchronized cultures may represent an artifact associated with α factor-induced arrest. To address this concern, we prepared synchronous cultures by a method which could not produce any α factor-induced artifacts. Log-phase X2180-1B cells (Table 1) were sedimented through a 10 to 40% sorbitol gradient to separate cells on the basis of size and density (22). The fractions containing the smallest, newly abscised daughter cells were isolated and resuspended in fresh medium to initiate a synchronous round of division. Cell samples were periodically withdrawn for visual examination, counting, and preparation of total RNA as in previous experiments.

Figure 5A shows the cell concentrations and percent budded cells determined in the experiment. The cells were resuspended in medium at time 0. Since the smallest cells were selected, they underwent a lengthy G1 phase which ended with the initiation of budding, signaling entry into S phase, at ca. 100 min. Cytokinesis occurred at ca. 190 min as evidenced by the decrease in percent budded cells and the increase in cell number. Finally, the cells underwent a second round of budding, reaching a peak of percent budded cells at 260 min.

The 12 lanes in the autoradiograph (Fig. 5B) represent hybridization data from RNA extracted from the corresponding culture samples indicated directly above the lanes in panel A. The changes in *CDC9* mRNA levels were quantitated by densitometric scanning, and these results are plotted in Fig. 5A. It can be seen that the level of *CDC9* mRNA is low in the first four samples and high in the following lane, at 85 min. This high point occurs shortly before bud initiation, just as in the α factor-induced synchrony experiment (Fig. 2). The two samples from cells at 135 and 155 min, in which most of the cells are in late S or G2 phase, show low levels of *CDC9* mRNA. The following two samples, at 175 and 195 min, represent cells in cytokinesis and the G1 phase of the ensuing cell cycle; these show an unmistakable increase in *CDC9* mRNA. The *CDC9* mRNA levels fall in the following lanes which correspond to RNA from cells entering the subsequent S phase. These results confirm the conclusion that the level of *CDC9* mRNA increases to high levels at a point in late G1. In contrast, the levels of *CDC36* and *URA3* mRNAs vary little throughout the cell cycle. The variations in *CDC9* mRNA level are not as pronounced as with the α factor experiment, indicating that the synchrony achieved was not as good. Also, it may be noticed in Fig. 5 that the first *CDC9* peak is of lesser magnitude than the second peak. This may be explained by sample bracketing of an actual peak or by perturbations of the cells by the selection procedure. Despire the quantitative differences, essentially the same pattern of cell cycle modulation was obtained. The fact that the same qualitative result was found with both induction and selection synchrony argues that the intracellular steady-state level of *CDC9* mRNA is indeed cell cycle regulated.

Levels of *CDC9* mRNA increase after UV irradiation. UV light induces the formation of pyrimidine dimers in DNA, which are subsequently photoreactivated or removed by excision repair (30, 43, 44) or bypassed during DNA replication by a postreplication repair mechanism(s) (31). Since DNA ligase is required in excision repair (45) and would be needed for the final ligation step in postreplication repair, we asked whether the level of *CDC9* mRNA would increase after UV irradiation. Briefly, log-phase cells of *S. cerevisiae* B-635 (Cdc^+ Rad^+; Table 1) were UV irradiated and then incubated under growth conditions in the absence of photoreactivating light. At various times after UV irradiation, cell samples were removed and total RNA was prepared. Equal amounts of the RNA preparations were electrophoresed through formaldehyde-containing agarose gels, blotted to Gene Screen (New England Nuclear Corp.), and probed with the same radiolabeled 2.7-kbp *Sac*I (*CDC9* and *CDC36*) and 1.1-kbp *Hin*dIII (*URA3*) DNA fragments described previously.

The resulting autoradiograph is shown in Fig. 6. The first two lanes correspond to RNA from unirradiated cells and cells sampled at 0 min post-UV; there is a low basal level of *CDC9* mRNA seen in these log-phase asynchronous cells. The following three lanes correspond to the RNA extracted

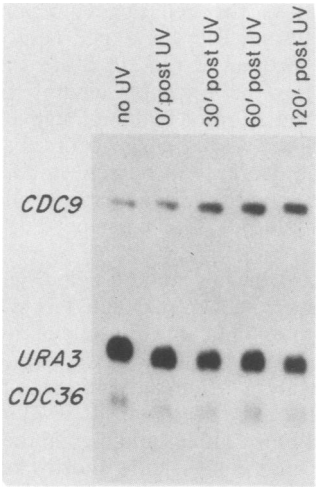

FIG. 6. Increase in *CDC9* mRNA levels after UV irradiation. Autoradiograph of RNA blot displaying RNA prepared from UV-irradiated cells and hybridized with the same radiolabeled probes used in Fig. 2 to detect *CDC9*, *CDC36*, and *URA3* mRNAs. RNA was prepared from cells at the indicated times after UV irradiation as described in the text.

from cell samples taken at 30, 60, and 120 min post-UV irradiation, respectively. It is apparent that the intracellular *CDC9* mRNA level is increased significantly in these UV-irradiated cells.

These results were quantitated by densitometric scanning of the bands (Table 3). The amount of *CDC9* mRNA in each lane was normalized to the amount of *URA3* mRNA which is not expected to vary under these conditions. Inspection of Table 3 shows that the normalized intracellular level of *CDC9* mRNA is increased about sixfold at 30 and 60 min post-UV irradiation and about eightfold at 120 min, relative to the level in unirradiated cells. The levels of *CDC9* mRNA also are elevated when cells are grown in the presence of methylmethanesulfonate (MMS), a monofunctional alkylating agent (data not shown). Thus, exposure of cells to both UV light and MMS results in an increase in the intracellular levels of *CDC9* mRNA.

DISCUSSION

We present evidence that we have cloned *CDC9*, identified and mapped its transcript, and observed regulation of the steady-state levels of *CDC9* mRNA by both progression through the cell cycle and UV irradiation. *CDC9* was recently independently cloned and shown to be the structural gene for a DNA ligase (1). Comparisons of the restriction maps and preliminary sequence data indicate that our *CDC9* DNA segment is identical with that isolated by Barker and Johnston, who similarly cloned *CDC9* by selecting for complementation in a strain carrying a *cdc9* mutation (1). Our subcloning analysis shows that *CDC9* is in fact a transcribed sequence previously known as *TSL36*, which is adjacent to the start gene *CDC36* (3). The R-looping data indicate that the *CDC9* transcript is ca. 2,500 nucleotides in length (3). A transcript this size can potentially encode a polypeptide with a molecular weight of 100,000, a size consistent with most reported sizes of eucaryotic DNA ligases (37).

It must be emphasized that our transcriptional studies examined the steady-state levels of *CDC9* mRNA, which are dependent both on the frequency of transcription of *CDC9* and on the stability of the resulting mRNA. In the case of *CDC9*, control is exerted at either or both of these levels, but our experiments do not distinguish between them. Cell cycle regulation of yeast histone mRNA occurs at both transcriptional and posttranscriptional levels (10, 13). Although from the point of view of economy we favor transcriptional control of *CDC9*, we have no evidence to exclude a posttranscriptional component. However, for the purposes of this discussion we shall at times refer to the observed increases in the steady-state levels of *CDC9* mRNA as the induction of *CDC9*.

Cell cycle regulation of *CDC9*. In three independent experiments with α factor-synchronized wild-type cells, in two experiments involving release from α factor arrest to subsequent mutationally imposed cell cycle blocks, and in a synchronous culture prepared from size-selected cells, we have consistently observed cell cycle periodicity in the steady-state level of *CDC9* mRNA. The kinetics and extent of *CDC9* induction vary somewhat among the experiments, but these differences can be attributed to growth rate variations of the different strains under the various experimental conditions and to the degrees of synchrony achieved in each case. Nevertheless, each experiment supports the conclusion that the steady-state level of *CDC9* mRNA is cell cycle regulated and reaches a maximum near the G1-S phase boundary. We view this result as significant for two main reasons. First, it places previously characterized cell cycle-specific transcription into a wider context, and it may be useful in future research as a molecular marker for different cell cycle stages. Second, it calls into question the commonly held view that enzyme regulation in *S. cerevisiae* is not effected at the level of synthesis of gene products (8). As such, it may be the first example of a possibly general phenomenon, the accelerated synthesis (due to increased transcription) of the enzymes required for DNA replication just before its onset.

Our data from α factor-synchronized cell cultures demonstrate that *CDC9* mRNA is present at relatively low levels in cells arrested in late G1 at start. Shortly after completion of start, but before bud emergence and S phase, the *CDC9* mRNA level increases significantly. The level of induction seen in our experiments varies from 4- to 10-fold, and we think these differences result from differences in the degree of synchrony achieved in each case. The actual level of induction may be much greater than 10-fold, since our results are limited by the difficulty in achieving very high synchrony, the narrow window of induction, and the rapid turnover of the ligase mRNA. The induction is followed by a rapid decrease in *CDC9* mRNA towards basal levels, with an apparent half-life of ca. 20 min. The level remains low

TABLE 3. Intensities of hybridizing bands in Fig. 5[a]

Sample	CDC9	URA3	% (*CDC9*/*URA3*)	Fold increase in CDC9
No UV	10	155	6.5	1.
0 min post-UV	10	105	9.5	1.5
30 min post-UV	25	58	43	6.6
60 min post-UV	37	85	44	6.8
120 min post-UV	32	53	58	8.2

[a] The intensities of the hybridizing band in Fig. 5 were quantitated by densitometric scanning and are expressed as arbitrary units in the first two columns. In the third column, the level of *CDC9* mRNA is normalized to the level of *URA3* mRNA in each lane to correct for loading inaccuracies, since the *URA3* mRNA level is expected to remain constant throughout the experiment. In the fourth column, the fold increase in *CDC9* mRNA level is determined by the ratio of the normalized *CDC9* mRNA levels to the unirradiated value.

throughout the rest of the cell cycle. The same pattern of *CDC9* mRNA periodicity was seen in synchronous cells prepared by the fundamentally different technique of sedimentation selection of small cells (Fig. 5). Therefore, we are confident that the periodicity we see represents cell cycle regulation and not an α factor-induced perturbation.

In addition to examining the levels of *CDC9* mRNA in synchronized cultures, it is important to determine whether the induction of *CDC9* is causally dependent upon events occurring in G1 and S phase. From the experiments with α factor-synchronized cells it is clear that completion of the α factor-sensitive step is required for *CDC9* induction. When temperature-sensitive *cdc7* cells were synchronized with α factor and then shifted to the restrictive temperature without α factor, it was found that *CDC9* mRNA begins to accumulate shortly after release from α factor, and significant, albeit declining, *CDC9* mRNA levels are maintained throughout the extensive incubation (Fig. 3). We can conclude that the *cdc7*-sensitive step is not required for *CDC9* induction. When α factor-synchronized cells were similarly released to the *cdc8* block, we found induction of *CDC9*, followed by a rapid decay towards basal levels (Fig. 4). Again we can conclude that the *cdc8*-sensitive step is not required for induction. *cdc7* and *cdc8* arrest cells in late G1 phase (after the α factor-sensitive step) and in S phase, respectively. Therefore, these results are consistent with the observed induction of *CDC9* at the G1-S phase boundary in Cdc^+ cells. To the unknown extent that *CDC9* regulation in *cdc7* and *cdc8* cells held at their restrictive temperatures resembles that in cycling wild-type cells, the results may be interpreted to mean that the mechanisms controlling *CDC9* mRNA level effect high levels of *CDC9* mRNA at the *cdc7* step and low levels at the *cdc8* step.

Hereford and co-workers have shown that histone mRNA levels are controlled by transcriptional and posttranscriptional mechanisms which cause an accumulation of histone mRNA at the G1-S phase boundary (10, 13). In several of our experiments we probed the same RNA blots with both *CDC9* and histone probes to compare the kinetics of accumulation. We found that, with the exception of some minor differences which may or may not be significant, the behavior of *CDC9* mRNA accumulation parallels that of the histone message. Additionally, the products of the homothallism gene *HO* also accumulate at the G1-S phase boundary (24). Another similarity to the histone and *HO* genes lies in our finding of a sequence with weak *ars* activity ca. 3 kbp from the 5′ end of *CDC9* (data not shown). It has been postulated that *ars* sequences are involved in prereplicative transcriptional regulation of the histone and *HO* genes (24, 28).

It seems reasonable that the *CDC9* mRNA which accumulates in late G1 is translated to produce the DNA ligase required in the ensuing S phase. However, two observations suggest that DNA ligase may be present in considerable excess, which, if true, calls into question the need for a prereplicative transcriptional induction of *CDC9*. The first observation is that extracts from *cdc9*(Ts) mutants grown at the permissive temperature have very low levels of DNA ligase activity in vitro (17). One possible explanation is that the temperature-sensitive mutation also may render the enzyme hypersensitive to inactivation during extraction, and so the in vitro assay may not reflect the full activity in vivo. The second observation comes from experiments with hybrid cells containing *cdc9* nuclei and cytoplasm containing normal levels of wild-type ligase (B. Byers and L. Sowder, J. Cell Biol. **87**:6a, 1980). It was found that these cells could complete several cell cycles at the restrictive temperature, suggesting that the original hybrid cell contained an excess of *CDC9* gene product which was sufficient for several additional cell cycles. Although this experiment demonstrates that cells may have sufficient ligase to complete several cycles, this amount may be much less than that required for continued optimal growth.

DNA ligase activity has been shown to undergo variation during naturally synchronous meiotic divisions in microsporocytes (Eli Lilly & Co.), reaching a point of maximal activity coincident with DNA synthesis (14). Additionally, cell cycle periodic activity fluctuation has been observed in another enzyme involved in DNA replication, DNA topoisomerase. In both mammalian cells induced to synchrony (34) and naturally synchronous sea urchin embryonic nuclei (29), maximal DNA topoisomerase activity was found in the S phase of the cell cycle. In both systems, mixing experiments gave no indication of any endogenous inhibitor or activator of DNA topoisomerase activity. These examples are precedents for cell cycle regulation of activity of enzymes involved in DNA replication. For *S. cerevisiae*, our results suggest that the basis of the regulation of DNA ligase lies at the level of synthesis of the gene product.

This is further supported by the finding that protein synthesis is required for initiation, but not completion, of nuclear DNA replication in yeasts (11). Additionally, cells synchronized in G1 by α factor cannot initiate DNA synthesis upon removal of α factor in the presence of cycloheximide unless they are permitted a short incubation in the absence of this inhibitor of protein synthesis (11). Taken together, these results argue that some proteins required for DNA replication are synthesized before S phase, and at least some of those required are synthesized in the short period in late G1 between start and S phase. Our results would place DNA ligase in this latter group.

As mentioned above, previous studies of some 200 yeast proteins gave almost no indication of periodic synthesis (7). However, these studies examined only the most abundant proteins, which are most likely to comprise structural proteins, glycolytic enzymes, and other major species, and it is therefore not surprising that no periodic synthesis was detected. Such studies necessarily overlook the larger class of less-prevalent yeast proteins, including many enzymes and regulatory proteins which would be more probable candidates for cell cycle-regulated synthesis.

Regulation by UV light and MMS. The levels of *CDC9* mRNA increase significantly when cells are exposed to UV light or MMS. The response is rapid, as evidenced by the sixfold increase in *CDC9* mRNA seen 30 min after UV irradiation. It is likely that this increased *CDC9* mRNA level results in increased synthesis of DNA ligase needed for DNA repair. In this regard, several studies have demonstrated the requirement for *CDC9* in repair processes in *S. cerevisiae* (16, 17, 23, 45). It has recently been suggested that DNA ligase activity is dormant under physiological conditions but is stimulated by poly(ADP-ribose) which is synthesized in situ in response to DNA breakage (6, 27). For yeasts, this model is inconsistent with our results showing induction of the DNA ligase structural gene by DNA-damaging agents.

The nature of the inducing signal(s) is presently unclear. The repair-associated induction elicited by UV irradiation and MMS may represent part of a generalized SOS response or induction by an agent produced during the repair process itself. One candidate is DNA ends and nicks, the ligase substrate, which are generated during DNA repair. This

hypothesis may be tested by asking whether repair-associated *CDC9* induction occurs in incision-defective *rad* mutant strains (45). Although nicked DNA also is a product of replication, it is unlikely to be the inducing agent for the cell cycle induction of *CDC9*, since the latter occurs before S phase. Thus, it seems likely that the regulation of *CDC9* mRNA levels is achieved by at least two distinct mechanisms.

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