Cdc28 and Ime2 Possess Redundant Functions in Promoting Entry Into Premeiotic DNA Replication in *Saccharomyces cerevisiae*

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Manuscript received July 9, 2001
Accepted for publication September 28, 2001

ABSTRACT

In the budding yeast *Saccharomyces cerevisiae* initiation and progression through the mitotic cell cycle are determined by the sequential activity of the cyclin-dependent kinase Cdc28. The role of this kinase in entry and progression through the meiotic cycle is unclear, since all *cdc28* temperature-sensitive alleles are leaky for meiosis. We used a "heat-inducible Degron system" to construct a diploid strain homozygous for a temperature-degradable *cdc28-deg* allele. We show that this allele is nonleaky, giving no asci at the nonpermissive temperature. We also show, using this allele, that Cdc28 is not required for premeiotic DNA replication and commitment to meiotic recombination. *IME2* encodes a meiosis-specific hCDK2 homolog that is required for the correct timing of premeiotic DNA replication, nuclear divisions, and asci formation. Moreover, in *ime2*Δ diploids additional rounds of DNA replication and nuclear divisions are observed. We show that the delayed premeiotic DNA replication observed in *ime2*Δ diploids depends on a functional Cdc28. *Ime2*Δ *cdc28-4* diploids arrest prior to initiation of premeiotic DNA replication and meiotic recombination. Ectopic overexpression of Clb1 at early meiotic times advances premeiotic DNA replication, meiotic recombination, and nuclear division, but the coupling between these events is lost. The role of Ime2 and Cdc28 in initiating the meiotic pathway is discussed.

SACCHAROMYCES cerevisiae cells induced for meiosis can return to the mitotic cell cycle during and following completion of premeiotic DNA synthesis (Kupiec *et al.* 1997), suggesting that in both cycles DNA synthesis uses the same structural and regulatory elements. In addition, the *CDC* genes that are required for the mitotic cell cycle are required for the meiotic cycle (Simchen 1974; Kupiec *et al.* 1997). Nevertheless, fundamental differences exist between the two divisions. Entry into the meiotic cycle is not accompanied by bud emergence. Separation of the duplicated spindle pole body (SPB) is delayed in meiotic cells and occurs only after premeiotic DNA replication is complete. During the premeiotic S-phase, cells become committed to meiotic recombination, and with its completion chromosomes synapse and meiotic recombination occurs. Finally, two successive nuclear divisions, a reductional and an equational, follow a single round of DNA replication (for review see Kupiec *et al.* 1997). These regulated events are expected to use meiosis-specific regulators rather than mitotic ones. Indeed, in *Schizosaccharomyces pombe*, the Mcm proteins that are essential for DNA replication in the mitotic cell cycle are not required for premeiotic DNA replication (Forsburg and Hodson 2000). Furthermore, specific meiotic genes regulate the duration of the premeiotic S-phase (Cha *et al.* 2000). In this report, the term meiosis is used in a broad manner to describe the developmental pathway that initiates at G1, continues with premeiotic DNA replication and nuclear divisions, and ends with the formation of four ascospores.

Progression through the mitotic cell cycle is regulated by the successive activation of a single cyclin-dependent kinase (CDK), Cdc28, by the G1 (Cln1–3) and the B-type (Clb1–6) cyclins (for review see Lew *et al.* 1997). There is no conclusive evidence for the role of Cdc28 in initiation of premeiotic DNA replication. Temperature-sensitive *cdc28* alleles arrest in meiosis at pachytene, while they arrest in the mitotic cell cycle at G1 (Hartwell 1971; Shuster and Byers 1989). Furthermore, cells deleted for the three G1 cyclins, *CLN1–3*, are sporulation proficient (Dirick *et al.*; 1998 Colomina *et al.* 1999), suggesting that Cdc28 is not required for entry into the meiotic cycle. However, cells deleted for *CLB5–6* arrest at G1 (Dirick *et al.* 1998; Stuart and Wittenberg 1998; Smith *et al.* 2001), pointing to a possible role of Cdc28 in premeiotic DNA replication. Moreover, the various *cdc28* temperature-sensitive alleles give rise to low levels of asci (10–20%), suggesting that these mutants are leaky for meiosis and that Cdc28 is in fact required for entry into premeiotic DNA replication (Dirick *et al.* 1998; Stuart and Wittenberg 1998).

IME2 encodes a meiosis-specific protein kinase that shows 58.9% similarity and 37% identity to the human cyclin-dependent kinase *hCDK2* (Kominami *et al.* 1993). Interestingly, within the T-loop region that associates with the cyclin molecule (Jeffrey *et al.* 1995), *IME2* shows 52.3% identity to *hCDK2*. Ime2 is inactive in vege-

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TABLE 1

Yeast strains used

Strain	Relevant genotype
Y422	*MAT***a**/*MAT*α *ade2-1/ade2-R8*
Y752	*MAT***a**/*MAT*α *ura3-52/ura3-52 ade2-1/ade2-R8 ime2::LEU2/ime2::LEU2*
Y1094	*MAT***a**/*MAT*α *ade2-1/ade2-R8 cdc28-4/cdc28-4*
Y1100	*MAT***a**/*MAT*α *ade2-1/ade2-R8 cdc28-4/cdc28-4 ime2*Δ*::URA3/ime2*Δ*::URA3*
Y1314	*MAT***a**/*MAT*α *ade2-1/ade2-R8 pCUP1-UBI-DHFRts-HA-CDC28::URA3/ pCUP1-UBI-DHFRts-HA-CDC28::URA3*
Y1315	*MAT***a**/*MAT*α *ade2-1/ade2-R8 pCUP1-UBI-DHFRts-HA-CDC28::URA3/CDC28*

tative cells since its transcription is fully dependent on the meiosis-specific transcriptional activator Ime1 (Smith and Mitchell 1989; Yoshida *et al.* 1990), and its activity is inhibited by nutrients (glucose and nitrogen) through the function of the small G-protein Gpa2 (Donzeau and Bandlow 1999). Ime2 is a positive regulator of meiosis that is required for multiple meiotic events: the correct timing and high level transcription of early meiosis-specific genes (Mitchell *et al.* 1990; Yoshida *et al.* 1990); the transcription of middle and late genes (Mitchell *et al.* 1990; Yoshida *et al.* 1990); the correct timing of entry into premeiotic DNA replication, meiotic recombination, and nuclear division (Foiani *et al.* 1996); and ascus formation (Smith and Mitchell 1989; Yoshida *et al.* 1990; Foiani *et al.* 1996). In addition to its positive role, Ime2 is also a negative regulator that is required at a number of stages for the following purposes: transient transcription of *IME1* (Smith and Mitchell 1989; Yoshida *et al.* 1990); limit of premeiotic DNA replication and nuclear division to one and two rounds, respectively (Foiani *et al.* 1996); and reduction of the steady-state level of Sic1 (Dirick *et al.* 1998), Pol1, and Pol12 (Foiani *et al.* 1996).

We reexamine the role of Cdc28 in promoting premeiotic DNA replication, using the heat-degradable Cdc28-Degron protein (Dohmen *et al.* 1994). We show that unlike *cdc28-4* cells, diploid cells carrying the *cdc28-deg* allele are sporulation deficient at 34°. Using this allele we show that Cdc28 is not essential for premeiotic DNA replication and meiotic recombination. However, in the absence of Ime2, Cdc28 is required for these processes. The double mutant *cdc28-4 ime2*Δ arrests at 34° prior to premeiotic DNA replication, whereas at 25° these cells are proficient in both premeiotic DNA replication and commitment to recombination. We show that early expression of Clb1 in *ime2*Δ diploid cells leads to premature initiation of premeiotic DNA replication, meiotic recombination, and nuclear division. The role of Cdc28 and Ime2 in the initiation of premeiotic DNA replication is discussed.

MATERIALS AND METHODS

Strains: The relevant genotype of strains is given in Table 1. All strains used are isogenic and based on the mating of *MAT***a** and *MAT*α derivatives. For Y1314, the *CDC28* allele in the parental haploids of this strain was replaced by the *pCUP1-UBI-DHFRts-HA-CDC28* allele following transformation with pPW66R (Dohmen *et al.* 1994) digested with *Msc*I. Two criteria were used to confirm the genotype: the temperature sensitivity of the resulting haploid strains and the detection of Cdc28 by Western analysis using antibodies directed against HA. Strain Y1315 was constructed by mating the wild-type *MAT***a** strain to the *MAT*α *pCUP1-UBI-DHFRts-HA-CDC28* derivative. For Y752, the *IME2* allele in the parental haploids of this strain was replaced by the *ime2*Δ*::LEU2* as previously described (Foiani *et al.* 1996). For Y1100, the *IME2* allele in the parental haploids of strain Y1094 was replaced by the *ime2*Δ*::URA3* following transformation with P1930 digested with *Ssp*I.

Plasmids: pPW66R carries *pCUP1-UBI-DHFRts-HA-5′ cdc28* on a *URA3* vector (Dohmen *et al.* 1994). P1365 carries p*IME1*(−1365 to −31) on a bluescript vector. This plasmid was constructed by inserting a 1.3-kb *Hin*dIII fragment of *IME1* into bluescript. P1930 carries *ime2* from −268 to −108 and from +1318 to +2231 [+1 is the start of the *IME2* open reading frame (ORF)]. This plasmid was constructed in two steps. First, a 2.38-kb *Bgl*II fragment, carrying *IME2* (from pMW1; Yoshida *et al.* 1990), was inserted into pUC119 cut with *Bam*HI. Then, a *URA3* gene blaster (Alani *et al.* 1987) on a 4.2-kb *Pvu*II fragment was inserted into the resulting plasmid cut with *Eco*RV. P2049 carries the *CLB1* ORF on pUC19. This plasmid was constructed by inserting a 1.5-kb *Bam*HI fragment carrying the CLB1 ORF (supplied by D. Lew) into pUC19. YEp2053 carries *pIME1-CLB1* on a pBR322 2μ *URA3* vector. This plasmid was constructed by three-piece ligation between a 1358-bp *Sal*I-*Eco*RI fragment from P1365, a 1497-bp *Sal*I-*Kpn*I fragment from P2049, and as a vector YEplac195 (Gietz and Sugino 1988) cut with *Kpn*I and *Eco*RI.

Media and genetic techniques: PSP2 (minimal acetate medium) and SPM (sporulation medium) have been described (Kassir and Simchen 1991). SD (synthetic dextrose) and SG (synthetic galactose) have been described (Sherman 1991). Meiosis was induced as follows. Cells were grown in PSP2 supplemented with the required amino acids to 1×10^7 cells/ml, washed once with water, and resuspended in SPM. Intragenic meiotic recombination was measured as described (Kassir and Simchen 1991). Yeast transformation with LiOAc was done as described (Gietz *et al.* 1995). Standard methods for DNA cloning and transformation were used (Sambrook *et al.* 1989).

Antibodies: Mouse monoclonal antibodies directed against the hemagglutinin (HA) epitope (12CA5) were purchased from Boehringer Mannheim (Mannheim, Germany). Rabbit polyclonal antibodies directed against Cdc2 (PSTAIRE) were purchased from Santa Cruz Biotechnology.

Preparation of yeast protein extracts and Western analysis: Protein extracts were prepared from trichloroacetic acid-treated cells as described previously (Foiani *et al.* 1995). Western procedure was essentially as described (Foiani *et al.* 1994).

TABLE 2

Cdc28 is essential for sporulation

Growth conditions		Percentage			
PSP2	SPM	Tetrads	Dyads	Monads	Asci
25° + Cu	25° + Cu	18	3	—	21
25° + Cu	34° + Cu	0	0	0	0
25°	25° + Cu	10.8	1.5	0.7	12
25°	25°	2.6	5.3	3.9	11.8
25°	34°	0	0	0	0
25° + 2 hr 37°	25°	0.5	1.0	0.5	2
25° + 2 hr 37°	34°	0	0	0	0

Strain used was Y1314. Cu, addition of 0.1 mM copper sulfate. Sporulation was determined following 72 hr incubation. Between 400 and 1000 cells were counted. 0 asci reflects no spores in >10,000 cells.

Immunoprecipitation and *in vitro* kinase assay: These methods were applied essentially as described by Bowdish *et al.* (1994). The culture (15 ml at 1×10^7 cells/ml) was pelleted, washed with ice-cold water, and frozen in a dry ice/ethanol bath. Cell lysates were prepared by vortexing cell suspensions in an extraction buffer in the presence of glass beads (10 × 37-sec bursts). Lysates were cleared by two spins. All samples were normalized to contain the same amount of total proteins (Bradford 1976). The lysate (500 μg) in immunoprecipitation (IP) buffer was incubated with 2 μg of anti-HA antibodies for 2 hr at 4° with gentle shaking. Immune complexes were collected on protein A Sepharose beads by gentle shaking at 4° for 1 hr. Beads were pelleted by gentle centrifugation and washed twice with IP buffer, once with IP buffer without ovalbumin, and once with kinase buffer. For kinase assay 5 μg histone H1 (Sigma, St. Louis) and 2.5 μl of [γ-^{32}P]ATP (6000 Ci/mmol; New England Nuclear, Boston) were added, and the reaction mix was incubated at room temperature (~22°) for 1 hr. Reactions were terminated by the addition of an equal volume of elution buffer (125 mM Tris pH 6.8, 10% β-mercaptoethanol, 4% SDS, 20% glycerol, 25 mM EDTA, and 46% urea). Proteins were separated from the radioactive ATP on P-6 micro-bio-spin chromatography columns (Bio-Rad, Richmond, CA). Following the addition of the sample buffer, proteins were separated on SDS-PAGE. The gel was dried and exposed to X-ray film.

Fluorescence-activated cell sorter analysis: Cells were analyzed for DNA content by fluorescence-activated cell sorter (FACS) analysis as previously described (Foiani *et al.* 1994), using a Beckton Dickinson (Franklin Lakes, NJ) FACScan analyzer.

4′,6-Diamidino-2-phenylindole staining: Cells were prepared and stained as described (Rose *et al.* 1990).

RESULTS

High levels of Cdc28 are required in the mitotic cycle preceding meiosis: As described above, the evidence indicating that Cdc28 is not required for premeiotic DNA replication is based on the use of temperature-sensitive mutations in *CDC28* that are leaky for meiosis. The "heat-inducible Degron system" provides an alternative method for constructing temperature-degradable alleles (Dohmen *et al.* 1994; Labib *et al.* 2000). We used this method to reexamine the role of Cdc28 in initiation of premeiotic DNA replication. A diploid strain homozygous for *pCUP1-UBI-DHFRts-HA-CDC28* (Y1314) is temperature sensitive for both growth and meiosis. It gave 21% asci following 72 hr incubation at 25° and 0 asci at 34° (Table 2, lines 1 and 2). The isogenic *CDC28/pCUP1-UBI-DHFRts-HA-CDC28* heterozygote strain (Y1315) gave 67% asci when incubated at 34° for 24 hr. A restrictive temperature of 34° rather than 37° was used to avoid the deleterious effect of high temperature on sporulation. Thus, this allele, to be designated *cdc28-deg*, unlike the reported *cdc28-ts* alleles, is nonleaky for meiosis.

The availability of Cdc28 in the homozygous *cdc28-deg* strain is also subject to transcriptional regulation; its expression is induced by copper sulfate. Incubation with copper sulfate was not required for cell growth, although microscopic analysis revealed that cells grown without copper sulfate were large, with an elongated bud (data not shown). Western analysis revealed that the steady-state level of Cdc28-deg was reduced dramatically in the absence of the inducer (Figure 1A, compare lanes 1 and 2). Pregrowth without the inducer reduced the level of sporulation 12% compared to 21% (Table 2, compare lines 1 and 3). In both cases copper sulfate was added to the sporulation media, and consequently a high level of Cdc28 was attained [data not shown; Figure 1, A (lane 7) and B (lane 10), shows the level and activity, respectively, of Cdc28 in SPM following 2 hr induction with copper sulfate]. We conclude, therefore, that proper entry into the meiotic cycle depends on the presence of sufficient levels of active Cdc28 under the pregrowth conditions. When copper sulfate was also omitted from SPM, the level of sporulation did not decline, but the level of four-spored asci declined, and cells formed mainly two-spored and one-spored asci (Table 2, compare lines 3 and 4). This result suggests that high levels of Cdc28 in meiosis are required to promote two meiotic divisions. In agreement, Western analysis, using antibodies directed against the PSTAIRE sequence in CDKs, revealed a constitutive level of Cdc28 throughout the meiotic pathway of a wild-type diploid (Y422; Figure 1C).

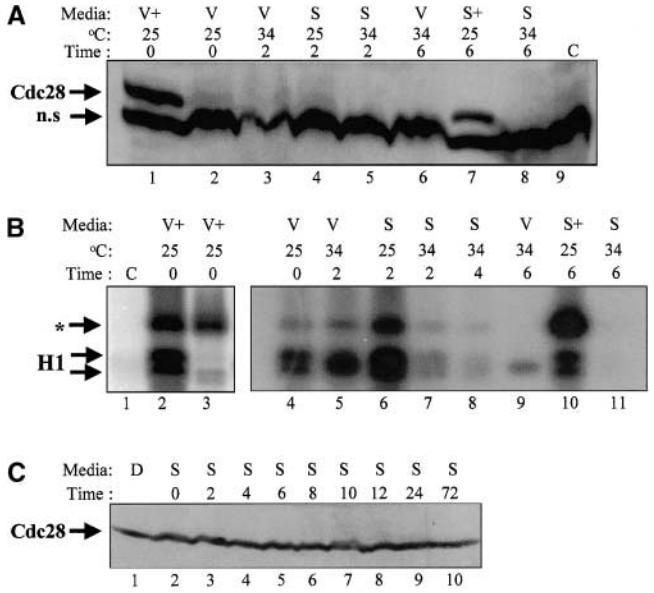

FIGURE 1.—The Cdc28-Degron fusion protein is gradually degraded at 34°. Western analysis was performed using antibodies directed against either HA (A) or Cdc2-PSTAIRE (C). (B) Histone H1 kinase activity following immunoprecipitation, using antibodies directed against HA. Histone H1 (5 μg) was added to the reaction mix (B, lanes 1, 2, and 4–11). Strains used were as follows: Y1314 (A, lanes 1–8; and B, lanes 2–11) and Y422 (A, lane 9; B, lane 1; and C, lanes 1–10). Cells were grown at 25° in PSP2 with copper sulfate (final concentration 0.1 mM; A, lane 1, and B, lanes 1–3) or without it (A, lane 2; and B, lane 4). Cells grown at 25° in PSP2 (without copper sulfate) to a titer of 1×10^7 cells/ml either were shifted to 34° for 2 (A, lane 3; and B, lane 5) and 6 hr (A, lane 6; and B, lane 9) or washed once in water and resuspended in SPM. Cells in SPM were incubated either at 25° for 2 (A lane 4; and B, lane 6) and 6 hr (A, lane 7; and B, lane 10) or at 34° for 2 (A, lane 5; and B, lane 7), 4 (B, lane 8), and 6 hr (A, lane 8; and B, lane 11). Copper sulfate (final concentration 0.1 mM) was added at 4 hr in SPM (A, lane 7; and B, lane 10). Cells were grown at 30° in SD (C, lane 1) to a titer of 1×10^7 cells/ml. Cells grown at 30° in PSP2 to a titer of 1×10^7 cells/ml were washed once in water and resuspended in SPM for the indicated times (C, lanes 2–10, and A, lane 9, is a sample taken at 6 hr in SPM). C, control; D, vegetative cultures grown in SD; V, vegetative cultures grown in PSP2; S, SPM. +, Addition of copper sulfate. n.s., nonspecific; H1, histone H1; *, a physiological substrate.

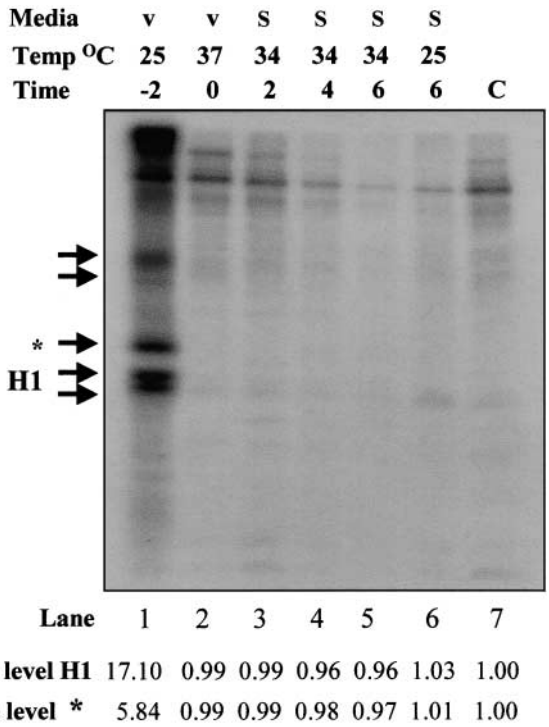

FIGURE 2.—The Cdc28-Degron kinase activity is eliminated in meiotic cultures following preincubation at 37°. Histone H1 kinase activity was determined following immunoprecipitation, using antibodies directed against HA. Cells of strain Y1314 grown at 25° in PSP2 to a titer of 1×10^7 cells/ml (lane 1) were shifted to 37° for 2 hr (lane 2). Then cells were washed, resuspended in SPM, and incubated at either 25° for 6 hr (lane 6) or 34° for 2 (lane 3), 4 (lane 4), and 6 hr (lane 5). C, control: cells of strain Y422 grown at 25° in PSP2 to a titer of 1×10^7 cells/ml (lane 7). Level of radioactivity in bands was quantified using a Fujix Bas 1000 phosphoimager. Relative levels in relation to the control (lane 7) are given. Arrows mark various phosphorylating substrates.

To promote an immediate degradation of Cdc28 at the nonpermissive temperature, such that the presence of the protein at early meiotic times would be precluded, in the experiments reported below cells were grown without copper sulfate. Following 2 hr incubation at 34° Cdc28 was not detected in cells incubated under either vegetative or meiotic conditions (Western analysis, Figure 1A, lanes 3, 5, 6, and 8). A more sensitive method for detecting Cdc28 is to determine its *in vitro* activity as a kinase on proteins that coimmunoprecipitate with it, as well as on a supplemented substrate, such as histone H1. Figure 1B shows phosphorylation of specific proteins that depends on the presence of the HA-tagged Cdc28 (Figure 1B, compare lanes 1 and 2). The phosphorylated histone H1 protein was identified by comparing reactions with and without histone H1 (Figure 1B, compare lanes 2 and 3). The assay identified a specific substrate of Cdc28 with apparent molecular weight of ~40 kD (marked with an asterisk), probably Sic1 (NUGROHO and MENDENHALL 1994). A shift to SPM at 34° led to a gradual decline in both histone H1 kinase activity and phosphorylated p40 (Figure 1B). Following 2 and 4 hr incubation at the nonpermissive temperature, low activity was observed (Figure 1B, lanes 7 and 8), while at 6 hr histone H1 kinase activity was not detected on either substrate (Figure 1B, lane 11). This procedure does not furnish the data required to enable a resolution of the issue whether Cdc28 is, or is not, essential for premeiotic DNA replication, since it is possible that the low activity of Cdc28 at early meiotic times suffices to promote premeiotic DNA replication.

Previously it was reported that efficient and immediate degradation of Cdc28-deg occurs at 37° (DOHMEN

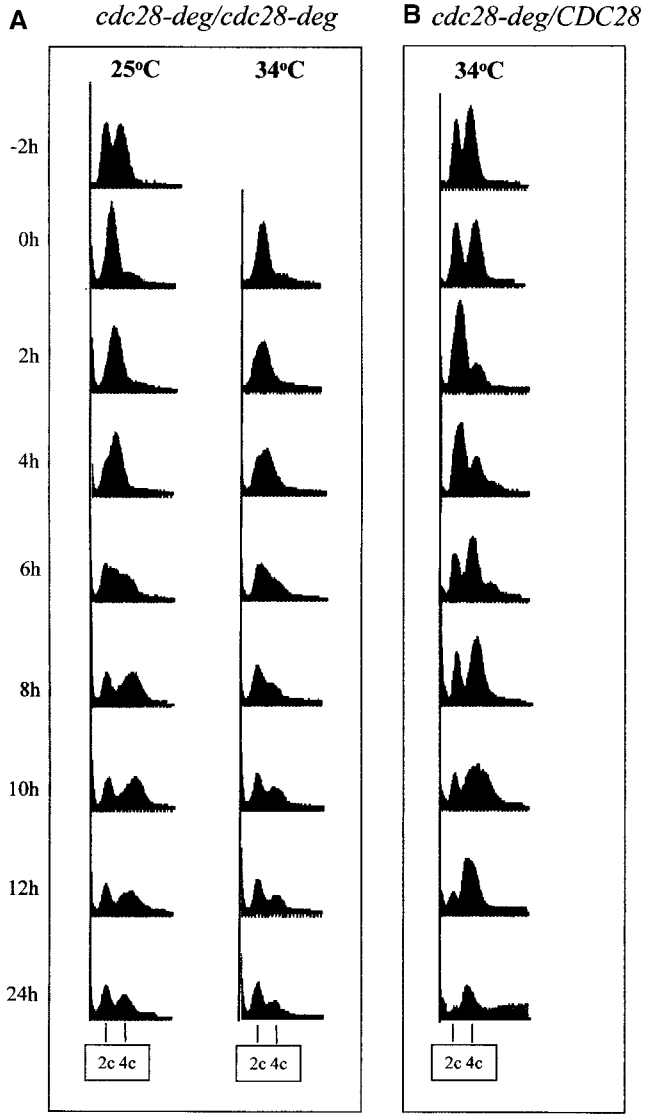

FIGURE 3.—Cdc28 is not essential for premeiotic DNA replication. Diploid cells homozygous for *cdc28-deg* (Y1314; A) or heterozygous (Y1315; B) were grown at 25° in PSP2 to a titer of 1×10^7 cells/ml (−2h) and shifted to 37° for 2 hr (0h). Then cells were washed, resuspended in SPM, and incubated at either 25° or 34°. Samples were taken at the indicated times to process for FACS analysis.

et al. 1994). This being so we examined whether, prior to the shift to sporulation medium, incubation at 37° for 2 hr would suffice for complete degradation of Cdc28 and thus would enable us to induce meiosis at 34° in Cdc28-depleted cells. As expected, no activity of Cdc28 was detectable using this method, as judged by the levels of phosphorylated histone H1 and the additional physiological substrates (Figure 2, compare lane 1 to lanes 2–5; histone H1 and the various coimmunoprecipitated substrates are marked with arrows). Moreover, this treatment led to G1 arrest as judged by the accumulation of unbudded cells (>90%) with a 2C DNA content (Figure 3A, time 0). We conclude that in our strain background a shift to 37° results in complete degradation of Cdc28-deg. Nevertheless, it is possible that an undetected amount of Cdc28 activity remained in these cells. We note that a shift to 25° did not result in a pronounced increase in histone H1 kinase activity, and only a slight increase above background (without histone) was observed (Figure 2, compare lane 6 to lane 7). The low activity of Cdc28 gave rise, at 25°, to low levels of sporulation, 2%, but at 34°, without any Cdc28 activity, asci were not formed (0 asci in >10,000 cells; Table 2, lines 6 and 7).

Cdc28 is not required for premeiotic DNA replication and meiotic recombination: FACS analysis revealed that following 2 hr incubation at 37° vegetative diploid cells homozygous for *cdc28-deg* arrested at G1, with a 2C DNA content (Figures 3A and 4B, time 0). These arrested cells were transferred to a sporulation medium and incubated at either 25° or 34°. At both the permissive and nonpermissive temperatures, premeiotic DNA replication took place (Figure 3A). Quantitative analysis of the results presented in Figure 3 revealed that in the absence of any Cdc28 activity DNA replication was initiated at the same time (between 4 and 6 hr in SPM) at both the permissive and nonpermissive temperatures (Figure 4B). At 10 hr in SPM, 67% of the cells incubated at 25° had a 4C DNA content, whereas at 34°, 39% had a 4C DNA content, reaching a maximum of 41.5% at 12 hr (Figure 4B). At 24 hr in SPM, at both temperatures, cells with 4C as well as with 2C DNA content were observed (Figure 3A), suggesting that DNA replication was either not complete or totally absent in a fraction of the cells. As a control, the same protocol was applied for the heterozygote isogenic strain. The 2-hr incubation at 37° did not cause cell cycle arrest (Figure 3B). When these cells were shifted to SPM and incubated at 34°, cells completed the mitotic cycle, and, at 2 hr, most cells had a 2C DNA content. At 4 hr, premeiotic DNA replication was initiated, and at ~12 hr it was completed. Only a small fraction of cells remained in G1 (Figures 3B and 4B).

Depletion of Cdc28 was deleterious, causing a gradual loss of the ability of cells to form colonies when plated on YEPD supplemented with copper sulfate and incubated at 25° (Figure 4A). This decrease in viability was independent of growth conditions: A similar decrease was observed for cells incubated in synthetic acetate or SPM (Figure 4A). Moreover, a similar loss of viability was observed for cells incubated in SPM at either 25° or 34° (Figure 4A). By contrast, the heterozygote *CDC28/cdc28-deg* strain did not lose viability when subjected to the same procedure (Figure 4A), indicating that the loss of Cdc28 rather than the temperature was responsible for this effect. Unlike the *cdc28-deg* allele, in the *cdc28-ts* alleles a shift to the nonpermissive temperature causes a reversible cell cycle arrest at G1, without any loss of viability (HARTWELL 1971; SHUSTER and BYERS 1989). We suggest that complete depletion of Cdc28 is deleterious and that the residual activity of Cdc28 in

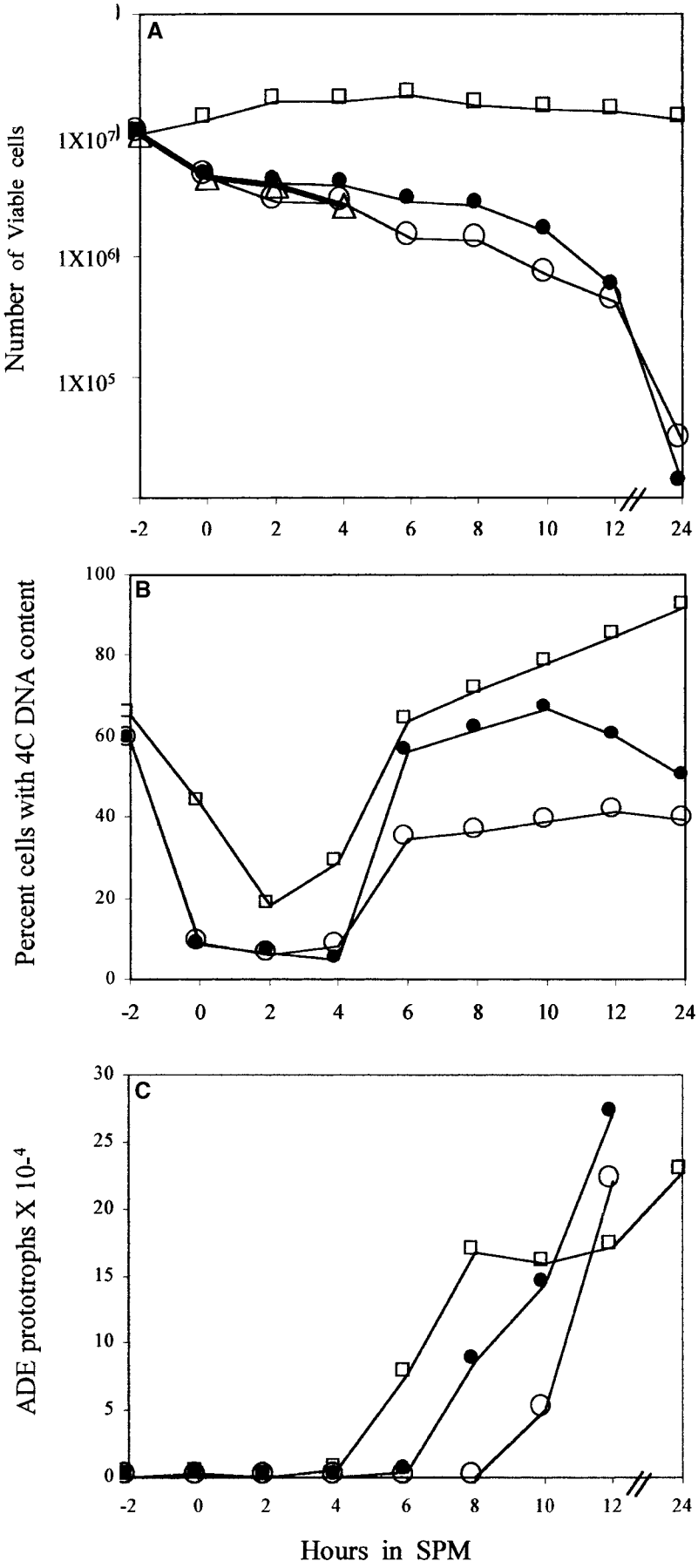

FIGURE 4.—Cdc28-Degron is not required for premeiotic DNA replication and meiotic recombination but is required for the ability of cells to form viable colonies. Cells of strains Y1314 (*cdc28-deg/cdc28-deg*; circles) or Y1315 (*cdc28-deg/CDC28*; squares) grown at 25° in PSP2 to a titer of 1×10^7 cells/ml (time, −2 hr) were shifted to 37° for 2 hr (time 0), and then cells were either shifted to 34° (open triangles, thick line) or washed, resuspended in SPM, and incubated at either 25° (solid circles) or 34° (open circles and squares). At various times samples were plated on YEPD and −ADE plates supplemented with 0.1 mM copper sulfate (final concentration) and incubated at 25° to measure their ability to form colonies (A) and commitment to intragenic recombination at *ADE2* (C). The frequency of ADE^+ prototrophs per 10^4 viable cells is given. DNA content was measured by FACS analysis, and the percentage of cells with 4C DNA content calculated from Figure 3 is given (B).

the *cdc28-ts* alleles is most probably responsible for the faithful cell cycle arrest.

Meiotic recombination is completely dependent on DNA replication and can be assayed even in cells that do not complete meiosis (Kassir and Simchen 1991). Samples from the above experiment were plated at various times on YEPD and −ADE plates supplemented with copper sulfate, and plates were incubated at 25°. Strains Y1314 and Y1315 carry two different *ADE2* alleles, *ade2-1* and *ade2-R8*, that upon intragenic recombination give rise, in this return to growth method, to *ADE2* prototrophs. The level of meiotic recombination was similar for the heterozygote and the homozygote isogenic strains, regardless of the temperature of incubation, 25° or 34° (Figure 4C). As a consequence of the delay in premeiotic DNA synthesis, initiation of meiotic recombination was similarly delayed (compare Figure 4B and 4C). At the nonpermissive temperature meiotic recombination was further delayed (Figure 4C), probably reflecting the increased deleterious effect of the mutation by continuous incubation at the nonpermissive temperature. Our results demonstrate that premeiotic DNA replication, as well as meiotic recombination, can take place in the absence of Cdc28. However, Cdc28 is required in the mitotic cycle preceding meiosis for efficient meiosis.

The role of Ime2 in promoting initiation of premeiotic DNA replication: The above results imply either that Cdc28 is not required for initiation of premeiotic DNA replication or that, in its absence, another kinase promotes this process. Sequence homology between *IME2* and *hCDK2* suggests that Ime2 might be the kinase that promotes the initiation of premeiotic DNA replication in the absence of Cdc28. The involvement of Ime2 in the initiation of premeiotic DNA replication is evident from the observation that in diploid cells deleted for *IME2* premeiotic DNA replication is delayed and a second round of DNA replication occurs at late meiotic times (Foiani *et al.* 1996). This hypothesis suggests that, in the absence of both Ime2 and Cdc28, cells will arrest prior to premeiotic DNA replication and meiotic recombination.

Isogenic *cdc28-4* (Y1094) and *ime2*Δ *cdc28-4* (Y1100) diploid cells grown in PSP2 to 1×10^7 cells/ml were shifted to SPM and incubated at either 25° or 34°. At various times samples were taken to measure DNA content. As reported, *cdc28-4* cells enter and progress through the premeiotic S-phase at both 25° and 34° (Shuster and Byers 1989 and data not shown). However, in the double mutant *ime2*Δ *cdc28-4*, premeiotic DNA replication took place only at the permissive temperature (Figure 5). At 25°, this mutant exhibited the same phenotypes as reported for the single *ime2*Δ diploid strain incubated at either 25° or 34° (Foiani *et al.* 1996 and data not shown). First, there was a delay in initiation of premeiotic DNA replication. Only at 12 hr in SPM was an increase in cells with 4C DNA content observed. Second, there was a defect in the entry into S-phase. Many cells remained in G1. Third, a second round of DNA replication was initiated at 48 hr in SPM (Figure 5). By contrast, at 34°, cells of the double mutant accumulated with a DNA content corresponding to 2C (Figure 5). We conclude that, in the absence of Cdc28, Ime2 promotes premeiotic DNA replication. We used the *cdc28-4* allele for this experiment, because in diploid cells that carry the *cdc28-4* allele the level of sporulation at the permissive temperature was high, and at the non-

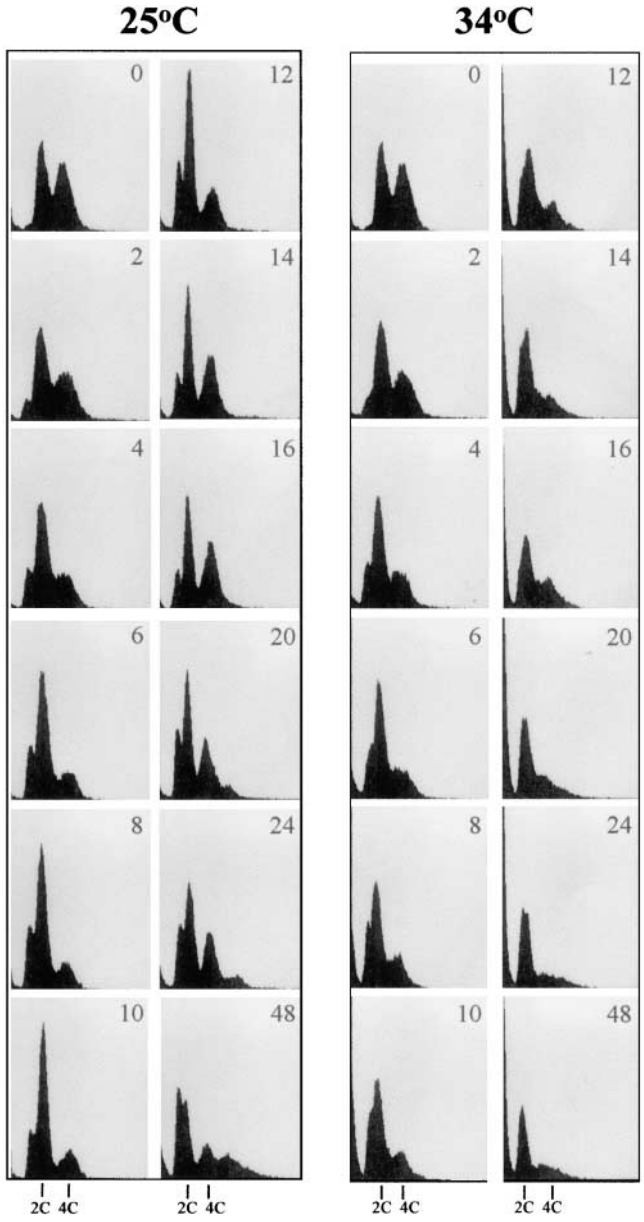

Figure 5.—In the absence of Ime2, Cdc28 is required for premeiotic DNA replication. Diploid cells homozygous for *ime2*Δ *cdc28-4* (Y1100) grown at 25° in PSP2 to a titer of 1×10^7 cells/ml were washed, resuspended in SPM, and incubated at either 25° or 34°. Samples were taken at the indicated times to process for FACS analysis.

permissive temperature most cells entered premeiotic DNA replication. On the other hand, the level of sporulation decreased in diploid cells carrying the *cdc28-deg* allele, and only a fraction of the cells entered the premeiotic S-phase.

At both temperatures completion of the mitotic cycle, which occurred upon nitrogen depletion, was defective, as is evident from the presence of cells with 4C DNA content throughout the early meiotic times (2–10 hr in SPM; Figure 5). At 34° this peak persisted, although its level declined at late meiotic times (20 hr and onward; Figure 5). Since the isogenic *ime2*Δ diploid is certainly capable of responding to SPM and accumulating in G1 (data not shown and Foiani *et al.* 1996), we suggest that this phenotype is due to *cdc28-4*, which is probably partially defective even at 25°. We further suggest that this phenotype reflects the need of Cdc28 for completing the mitotic nuclear division, as suggested in previous sections.

To confirm the conclusion that both Cdc28 and Ime2 can promote premeiotic DNA replication, we determined the level of intragenic meiotic recombination at *ADE2* in the *cdc28-4* (Y1094) and *ime2*Δ *cdc28-4* (Y1100) isogenic diploids. The frequency of ADE^+ prototrophs was increased in the same manner, and to the same extent, in *cdc28-4* cells incubated at 25° or 34°, as well as in *ime2*Δ *cdc28-4* cells incubated at 25° (Figure 6). However, in the latter strain, at 34°, the level of ADE^+ prototrophs did not increase significantly (Figure 6). We conclude that meiotic recombination is completely dependent on the function of at least one of these proteins, either Cdc28 or Ime2, although neither one of them is essential for commitment to meiotic recombination (Figure 4C and Shuster and Byers 1989; Foiani *et al.* 1996).

As noted above, Cdc28 and Ime2 can promote premeiotic DNA replication. Still, it is not clear why, in the absence of Ime2, premeiotic DNA replication takes place with a substantial delay (Figure 5 and Foiani *et al.* 1996). Since Cdc28 is an abundant protein throughout the meiotic cycle (Figure 1C), we assumed that at early meiotic times Cdc28 is not active. Lack of activity might result from an increase in the level of Sic1, the Cdc28 inhibitor (Dirick *et al.* 1998), or late activation by the B-type cyclins, transcription of which is induced at a late meiotic time (Grandin and Reed 1993; Chu and Herskowitz 1998). Thus, we surmised that early expression of a B-type cyclin would advance meiotic events in *ime2*Δ diploid cells. Early expression of a B-type cyclin was accomplished by fusing *CLB1* to the *IME1* promoter. The decision to express cyclin Clb1 rather than Clb5, which is required for entry into premeiotic DNA replication (Dirick *et al.* 1998; Stuart and Wittenberg 1998), was based on the report that Clb5 can substitute for the G1 cyclins in promoting bud emergence and entry into the mitotic cell cycle (Epstein and Cross 1992).

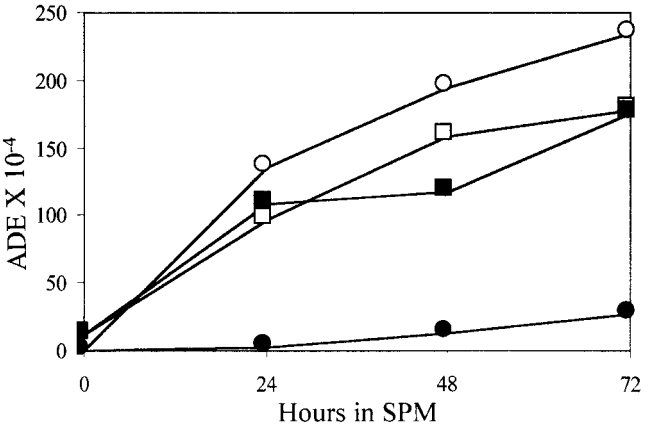

Figure 6.—In the absence of Ime2, Cdc28 is required for meiotic recombination. Diploid cells homozygous for either *cdc28-4* (Y1094; squares) or *ime2*Δ *cdc28-4* (Y1100; circles) grown at 25° in PSP2 to a titer of 1×10^7 cells/ml were washed, resuspended in SPM, and incubated at either 25° (open circles and squares) or 34° (solid circles and squares). Samples were taken to plate on −ADE and YEPD at the indicated times. The frequency of ADE^+ prototrophs per 10^4 viable cells is given.

An *ime2*Δ diploid strain (Y752) or a transformant carying *pIME1-CLB1* on a 2μ plasmid (YEp2053) was shifted to sporulation conditions. At various times samples were taken to study the following meiotic events: (1) completion of the mitotic cell cycle, which was determined by measuring the percentage of budded cells (Figure 8A); (2) DNA replication, which was determined by FACS analysis (Figures 7 and 8B); (3) intragenic meiotic recombination, which was determined by plating on −ADE and YEPD (Figure 8C); and (4) nuclear division, which was determined by 4′,6-diamidino-2-phenylindole (DAPI) staining and microscopic analysis (Figure 8D). All the meiotic parameters examined, namely, DNA synthesis, meiotic recombination, and nuclear division, were advanced in the *ime2*Δ diploid strain expressing Clb1 from the *IME1* promoter in comparison to cells expressing only the endogenous *CLB1* (Figures 7 and 8). We conclude, therefore, that in *ime2*Δ cells Cdc28 is activated at late meiotic times and that this activation is responsible for the delayed premeiotic DNA synthesis occurring in *ime2*Δ diploid cells. Careful examination of the results demonstrates that ectopic overexpression of Clb1 in cells starved for nitrogen leads to uncoupling between DNA synthesis and cell division. Upon becoming nitrogen depleted, wild-type, as well as *ime2*Δ, diploid cells complete DNA replication and mitotic division and accumulate as unbudded cells with 2C DNA content (Figure 7, Figure 8, A and B, and Foiani *et al.* 1996). Similarly, early expression of *CLB1* had no effect on the ability of the cells to respond to nitrogen depletion and accumulate as unbudded cells (Figure 8A). However, this cell cycle arrest was not accompanied by the accumulation of cells with 2C DNA content. On the contrary, the number of cells with 4C DNA content increased

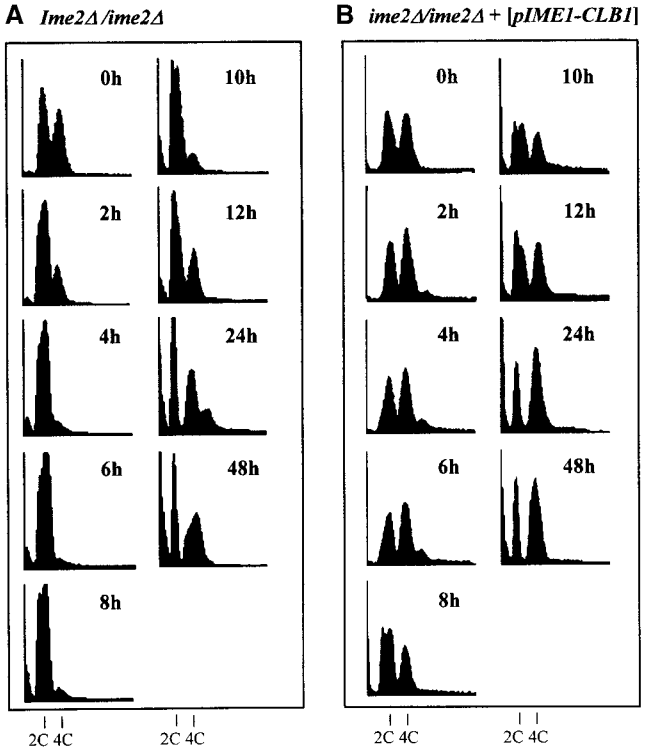

FIGURE 7.—Overexpression of Clb1 at early meiotic times advances premeiotic DNA replication in *ime2*Δ diploid cells. Diploid cells homozygous for *ime2*Δ (Y752; A) and Y752 carrying *pIME1-CLB1* on a 2μ plasmid (P2053; B) were grown at 30° in PSP2 to a titer of 1×10^7 cells/ml. Cells were washed, resuspended in SPM, and incubated at 30°. Samples were taken at the indicated times to process for FACS analysis.

(Figures 7 and 8B). Moreover, FACS analysis revealed that, between 2 and 6 hr in SPM, cells with >4C DNA content were observed (Figure 7B), whereas at 8 hr in SPM and onward this fraction of cells disappeared, and an increase in the number of cells with 2C DNA content was observed (Figure 7B). Since cells with two nuclei were observed beginning at 6 hr in SPM (Figure 8D), it is possible that cell division restored the DNA content in these cells. A second wave of DNA replication was detected in these cells following 12 hr incubation in SPM (Figures 7B and 8B). Uncoupling was also observed between meiotic recombination and nuclear division. In wild-type as well as *ime2*Δ diploid cells nuclear division follows meiotic recombination (Figure 8 and FOIANI *et al.* 1996), whereas overexpression of Clb1 inverted these events (Figure 8).

DISCUSSION

Cdc28 is not essential for initiation of premeiotic DNA replication: In this report, using a nonleaky temperature-degradable *CDC28* allele (*cdc28-deg*), we determined the requirement of Cdc28 for initiation and progression through meiosis. We showed that although Cdc28 is completely required for asci formation (Table 2), cells deprived of Cdc28 can enter the meiotic cycle; *i.e.*, these cells initiate and progress through both premeiotic DNA replication and meiotic recombination (Figures 3 and 4). To ensure that undetectable remainders of Cdc28 were not responsible for promoting entry into the meiotic cycle, we used severe conditions. Cells were grown without copper sulfate and incubated at 37° for 2 hr prior to the shift to the meiotic conditions at either 25° or 34°. Two criteria were used to determine the complete degradation of Cdc28: Western analysis and Cdc28 kinase activity (Figures 1 and 2). Under these conditions, vegetative cultures did not enter S-phase. Our results confirm previous reports that were based on "leaky" *cdc28* temperature-sensitive alleles (SHUSTER and BYERS 1989; C. WITTENBERG, personal communication; N. GUTTMANN-RAVIV, unpublished data). We conclude that Cdc28 is not essential for initiation of premeiotic DNA replication or commitment to meiotic recombination.

Ime2 and Cdc28 are interchangeable in promoting initiation of premeiotic DNA replication: We showed that in the absence of the hCdk2 homolog, *IME2*, Cdc28 is required for entry into premeiotic DNA replication. The double mutant *ime2*Δ *cdc28-4* arrests at the nonpermissive temperature prior to premeiotic DNA replication and meiotic recombination, whereas at the permissive temperature this mutant exhibits the same phenotype as a single *ime2*Δ diploid; namely, these processes occur, but with a substantial delay (Figures 5 and 6). As discussed above, the single *cdc28-4* mutant is proficient in both premeiotic DNA replication and commitment to meiotic recombination (Figure 6, data not shown, and SHUSTER and BYERS 1989). Thus, Ime2 and Cdc28 may possess overlapping functions.

MADHANI *et al.* (1997) suggest that kinase specificity can, in some cases, be determined by using a "kinase dead" point mutation rather than a deletion allele. It is assumed that in the absence of the protein an imposter kinase will substitute for it (MADHANI *et al.* 1997). However, a kinase dead mutation in *IME2* (*ime2K97A*) exhibits the same phenotypes as the deletion allele, with respect to premeiotic DNA replication and meiotic recombination (data not shown). Furthermore, in this study we used a temperature-sensitive point mutation in *CDC28*, *cdc28-4*. Thus, this methodology did not help in determining whether Ime2 and/or Cdc28 are normally responsible for entry into premeiotic DNA replication.

What might be the kinase that promotes entry into premeiotic DNA replication under normal conditions? Three possible models can be envisioned: (1) Cdc28 alone is required for initiation of premeiotic DNA replication, (2) the activity of both Cdc28 and Ime2 is required for initiation of premeiotic DNA replication, and (3) Ime2 alone suffices for initiation of premeiotic DNA replication. The first model is the least appealing because it does not explain the DNA replication phenotypes associated with either the *ime2*Δ or *ime2*Δ *cdc28-4*

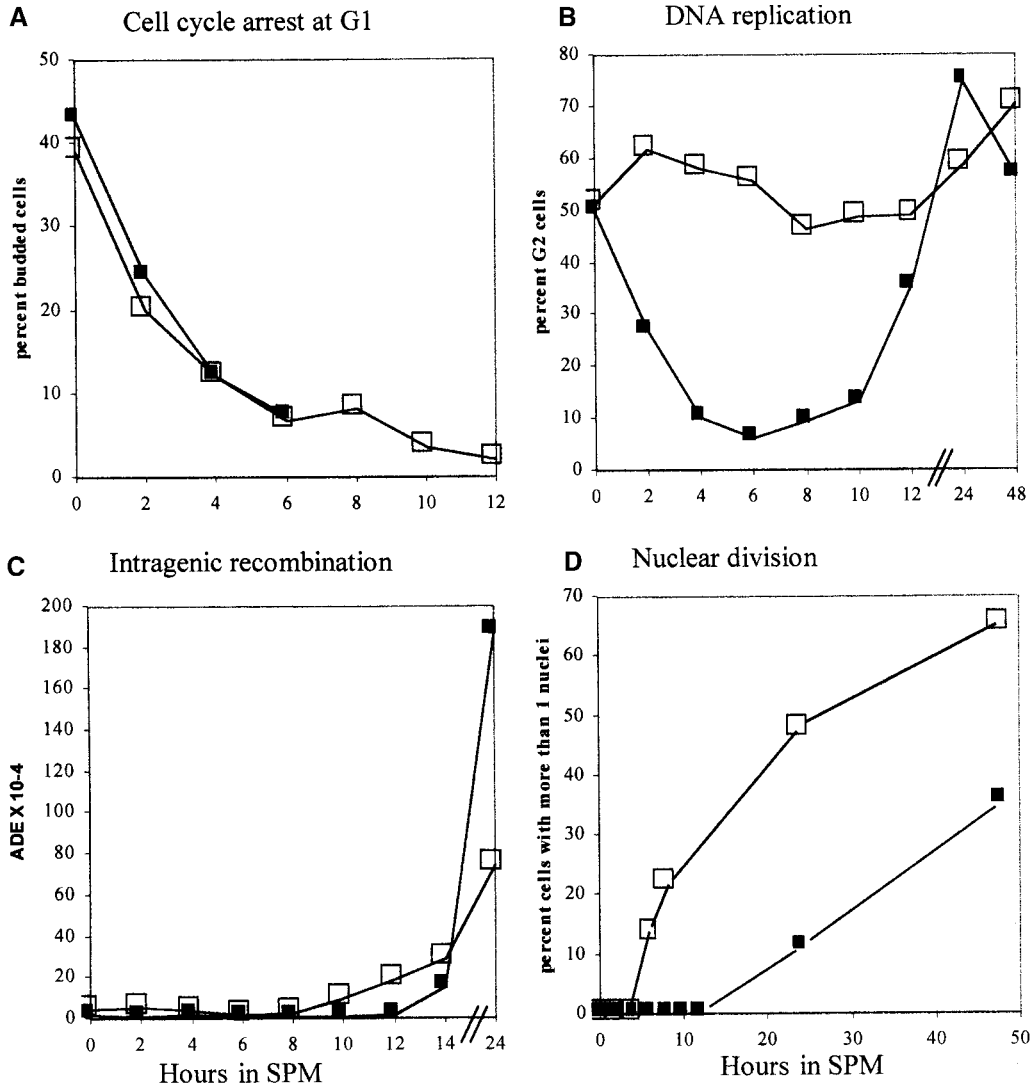

FIGURE 8.—Overexpression of Clb1 at early meiotic times advances premeiotic DNA replication, meiotic recombination, and nuclear division in an *ime2*Δ diploid. Diploid cells homozygous for *ime2*Δ (Y752; solid squares) carrying *pIME1-CLB1* on a 2μ plasmid (P2053; open squares) were grown at 30° in PSP2 to a titer of 1×10^7 cells/ml. Cells were washed, resuspended in SPM, and incubated at 30°. Samples were taken at the indicated times to measure the percentage of budded cells (A), to process for FACS analysis and calculate (from Figure 7) the percentage of cells with 4C DNA content (B), to plate on YEPD and −ADE plates to measure the frequency of ADE^+ prototrophs (C), and to stain by DAPI to measure the percentage of cells with more than one nuclei (D).

mutants. The second and third models are based on the observation that the kinase activity of Ime2 is required for its role in promoting premeiotic DNA replication. The second model assumes that although Ime2 and Cdc28 can substitute for one another, these kinases possess specific functions. For example, Ime2 phosphorylates Sic1, whereas Cdc28/Clb5,6 phosphorylates additional proteins required for DNA synthesis. Phosphorylation of Sic1 by the Cdc28/Cln1,2 kinase, and, similarly, its phosphorylation by Ime2, might target it for degradation, thus relieving its inhibiting effect on the activity of the Cdc28/Clb complexes (SCHWOB *et al.* 1994). This model is supported by the following observations: (1) In *ime2*Δ diploid cells the level of Sic1 is increased (DIRICK *et al.* 1998); (2) *clb5*Δ *clb6*Δ diploids arrest prior to premeiotic DNA replication (DIRICK *et al.* 1998; STUART and WITTENBERG 1998); and (3) overexpression of Clb1 at early meiotic times advances meiotic events (Figure 8). The third model assumes that initiation of premeiotic DNA replication depends only on Ime2. This model is supported by the following observations: (1) Cdc28 is not required for premeiotic DNA replication; (2) when entry into the meiotic cycle is promoted only by Cdc28 (*ime2*Δ cells), DNA replication is not restricted to a single round (Figure 5A and FOIANI *et al.* 1996); (3) the B-type cyclins, *CLB1*, *-3*, *-4*, *-5*, and *-6*, are middle-meiosis-specific genes, the transcription of which is induced following the completion of premeiotic DNA synthesis (CHU and HERSKOWITZ 1998); and (4) ectopic overexpression of Clb1 in *ime2*Δ cells leads to uncoupling between DNA synthesis and cell division, reinitiation of DNA synthesis, and the accumulation of cells with >4C DNA content (Figure 8). These results disagree with the second model, which assumes that the delay in premeiotic DNA replication is due to the inhibitory effect of Sic1. According to this model, early activation of Cdc28 by Clb1 is expected to result in either inhibition to DNA synthesis or proper entry into the meiotic cycle, as observed for ectopic expression of Clb2 in the mitotic cycle (AMON *et al.* 1994; DETWEILER and

Li 1998). Consequently, we favor the third model, which assumes that the requirement for Clb5/6 (Dirick *et al.* 1998; Stuart and Wittenberg 1998) for entry into the meiotic cycle might reflect the requirement for Cdc28 in the mitotic cell cycle preceding meiosis. We further suggest that in the absence of Ime2, Cdc28 promotes entry into meiosis, but with a delay, because the expression of the B-type cyclins is induced at late meiotic times.

High activity of Cdc28 is essential for the second meiotic division: Using the *cdc28-deg* allele we showed that Cdc28 is essential for meiosis, that in its absence meiosis was not completed, and that asci were not formed (Table 2). High and effective levels of Cdc28 throughout meiosis are required for completing two meiotic divisions. In cells incubated in SPM without copper sulfate, only 22% of the asci had four spores, compared to 90% in the presence of the inducer (Table 2). Under these conditions the percentage of asci did not decline, suggesting that attenuated activity of Cdc28 suffices for completing one nuclear division. Previously it was reported that Cdc28 is required at two meiotic stages, at pachytene and at meiosis II, for separation of the duplicated SPB (Shuster and Byers 1989). *Cdc28-ts* diploids form mainly dyads (within a total of ~10% asci), and genetic analysis, using centromere markers, has shown that these asci carry diploid spores that result from a single meiotic division, the reductional (Shuster and Byers 1989). Similarly, McCarroll and Esposito (1994) have shown that *cdc28-1* cells incubated at 30° give ~30% asci, of which 90% are two-diploid spores that have gone through meiosis I (MI) and not MII. These results suggest that Cdc28 might not be essential for the first meiotic division. On the other hand, the observation that diploid cells deleted for *CLB1*, *-3*, and *-4* give rise to mainly one-spored asci (Grandin and Reed 1993) suggests that Cdc28 might be required for both meiotic divisions. In this report, we focused on the role of Cdc28 in promoting premeiotic DNA replication and meiotic recombination. Further work is required to determine the point of arrest of *cdc28-deg* diploids in relation to spindle formation and nuclear division.

CONCLUSION

We have demonstrated that Cdc28 and Ime2 have redundant functions in relation to premeiotic DNA replication and meiotic recombination. Nonetheless, it cannot be determined from our results whether it is Cdc28 or Ime2 that normally promotes entry into premeiotic DNA replication. We favor the hypothesis that it is Ime2, because the use of a specific regulator for entry into meiosis might ensure that this pathway is an alternative to mitosis. Moreover, this hypothesis might explain how, in meiosis, two successive nuclear divisions follow a single round of DNA replication. This hypothesis is supported by the observation that in cells deleted for *IME2*, regulation of DNA replication and nuclear division is lost, and an additional round of DNA synthesis and nuclear division is observed (Foiani *et al.* 1996). It is possible that, in the absence of Ime2, the activity of Cdc28 leads to a coupling between DNA replication and nuclear division; namely, in a fashion similar to the mitotic cell cycle, following nuclear division an additional round of DNA replication and nuclear division takes place. In agreement with this, prior to the second round of DNA replication the B-subunit of the DNA polymerase α-primase complex Pol12 is dephosphorylated, whereas in wild-type diploids following completion of premeiotic DNA replication, it is degraded (Foiani *et al.* 1996). We suggest that in *S. cerevisiae* the use of different CDKs for progression through the cell cycle and meiosis is the normal mode to control these divisions, as appears to be the case in higher eukaryotes (Morgan 1997).

We thank K. Benjamin, M. Foiani, and I. Herskowitz for helpful discussions and critical reading of the manuscript. We thank J. Diffley, B. Futcher, N. Kleckner, M. Johnston, D. Lew, A. Sugino, and Y. Yamashita for kindly providing plasmids. This work was supported by a grant from the Israel Science Foundation and the United States-Israel Binational Science Foundation.

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Communicating editor: B. J. Andrews