MEIOTIC RECOMBINATION AND DNA SYNTHESIS IN A NEW CELL CYCLE MUTANT OF *SACCHAROMYCES CEREVISIAE*

YONA KASSIR AND GIORA SIMCHEN

Department of Genetics, The Hebrew University, Jerusalem, Israel

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ABSTRACT

Vegetative cells carrying the new temperature-sensitive mutation *cdc40* arrest at the restrictive temperature with a medial nuclear division phenotype. DNA replication is observed under these conditions, but most cells remain sensitive to hydroxyurea and do not complete the ongoing cell cycle if the drug is present during release from the temperature block. It is suggested that the *cdc40* lesion affects an essential function in DNA synthesis. Normal meiosis is observed at the permissive temperature in *cdc40* homozygotes. At the restrictive temperature, a full round of premeiotic DNA replication is observed, but neither commitment to recombination nor later meiotic events occur. Meiotic cells that are already committed to the recombination process at the permissive temperature do not complete it if transferred to the restrictive temperature before recombination is realized. These temperature shift-up experiments demonstrate that the *CDC40* function is required for the completion of recombination events, as well as for the earlier stage of recombination commitment. Temperature shift-down experiments with *cdc40* homozygotes suggest that meiotic segregation depends on the final events of recombination rather than on commitment to recombination.

MEIOSIS is a complex cellular process consisting of several events that alter the structural and chemical composition of the cell and redistribute its genetic contents. The genetic changes, which are the outcome of recombination and haploidization, are unique to meiotic cells. Most of the differences between meiosis and mitosis can be related to these genetic changes. Meiosis as defined here begins at G_1, prior to premeiotic DNA replication, and ends when four haploid G_1 nuclei are produced. Most mitotic functions are also required in meiosis, as is evident from the meiotic defects of cell-division cycle (*cdc*) mutants in yeast (SIMCHEN 1974). Thus, the events of DNA synthesis and nuclear division that were defined by the *cdc* lesions in mitotic cells seem to occur in meiotic cells as well. Superimposed on these shared functions, meiosis-specific functions are present, as evident from the occurrence of meiotic mutants that do not affect mitotic cells (ESPOSITO and ESPOSITO 1969; ROTH and FOGEL 1971; SIMCHEN, PIÑON and SALTS 1972).

Meiotic cells of *S. cerevisiae* become committed to the recombination process at an early stage, during the premeiotic DNA replication (ESPOSITO and ESPOSITO 1974b). Commitment to sporulation takes place only later, after the completion

of premeiotic DNA synthesis (SIMCHEN, PIÑON and SALTS 1972). Commitment to a meiotic process is defined as the ability to complete the process after transfer to vegetative media. Mutant strains in which meiosis is arrested prior to premeiotic replication do not show recombination commitment or haploidization (ROTH 1973; BAKER *et al.* 1976; SHILO, SIMCHEN and SHILO 1978; J. HIRSCHBERG, Y. KASSIR and G. SIMCHEN, unpublished), whereas mutant strains that are blocked after replication is completed usually show normal commitment to recombination and arrest at later stages (ESPOSITO and ESPOSITO 1974a; J. HIRSCHBERG, Y. KASSIR and G. SIMCHEN, unpublished). A study of meiosis in *cdc4* homozygotes shows that recombination commitment may require only the early stages of DNA replication and not its completion (SIMCHEN and HIRSCHBERG 1977). In the present paper, we report the effects on meiosis of a new nuclear division mutation, *cdc40*. Diploids homozygous for this mutation are defective in sporulation at the restrictive temperature. Under these conditions premiotic DNA replication occurs, but no recombination is attained.

MATERIALS AND METHODS

Strains

425: a diploid strain resulting from a mating of the haploid strain 325 (HARTWELL's 473—HARTWELL *et al.* 1973) with our standard α strain 309 (SIMCHEN 1974). It has the following genotype: **a**/α, *cdc5/CDC5, ade1/ADE1, ade2/ade2R-8, ura1/URA1, gal1-4/GAL1, his7-1/HIS7, lys2-2/LYS2, tyr1-2/TYR1, metx/METX, can1-11/CAN1.*

425-9: a diploid isolated as a temperature-sensitive colony following UV irradiation of strain 425. Its genotype is: **a**/α, *cdc40/cdc40, cdc5/CDC5, ade1/ADE1, ade2/ade2R-8, ura1/URA1, gal1-4/GAL1, his7-1/HIS7, lys2-2/LYS2, tyr1-2/TYR1, metx/METX, can1-11/CAN1.*

391: a haploid segregant isolated from a tetrad of 425-9, with the following markers: α, *cdc40, ade2, ura1, his7-1, leux, metx, can1-11.*

490: a diploid strain constructed by mating strain 391 to another haploid progeny of strain 425-9. Strain 490 contains the following markers: **a**/α *cdc40/cdc40, ade1/ADE1, ade2/ade2R-8, his7-1/his7-1, tyr1-1/TYR1, lys2-2/LYS2, ura1/URA1, leux/LEUX, metx/METX, can1-11/CAN1.*

Media

YEPD and PSP2 are the glucose and acetate vegetative media, respectively, and SPM is the sporulation medium (for details see SIMCHEN, PIÑON and SALTS 1972). MIN is a minimal glucose medium. —AD serves to detect adenine prototrophs. CAN medium supports the growth of canavanine resistant colonies only. (For details see SIMCHEN, IDAR and KASSIR 1976.) Media were solidified with 1.5% Difco agar.

Vegetative growth and sporulation

Cultures were grown overnight in liquid PSP2 to which adenine (40 μg/ml) and histidine (10 μg/ml) were added, as required by the strains. Cells were grown while shaking at 25° to a titer of approximately 10^7/ml, harvested, washed once in water and resuspended in SPM. The culture was incubated at 25° and at 30 to 60 min a subculture was transferred to 34°. By this procedure, the exposure to restrictive temperature in the meiotic conditions (sporulation medium) came after the cells had completed the ongoing mitotic cell cycle.

DNA measurements

Radioactive label in DNA was measured by prelabeling cells in the vegetative PSP2 medium. The medium contained adenine (40 μg/ml), uracil (3 μg/ml) and ^{14}C-2-uracil (0.8 μci/ml,

60 mci/mM; A.E.C. Nuclear Research Center, Negev). No radioactive label was added to the sporulation medium (SPM). Samples of 0.5 ml (in triplicate) were removed at various times from the vegetative culture or from the culture in sporulation medium; equal volumes of 1 N NaOH were added, and the mixtures were incubated overnight (18–24 hours) at 36°. The next morning, 1 ml 20% TCA was added to each sample, followed by a 20 min precipitation on ice. The TCA precipitate was collected and washed on GF/C filters, as described by SIMCHEN, PIÑON and SALTS (1972).

Commitment to intragenic recombination and haploidization

The diploid strains used are heteroallelic at the *ade2* locus. Intragenic recombination may result in a prototrophic cell which can grow on —AD plates.

The parental diploids are also heterozygotes for *can1-11*. This canavanine-resistant allele is recessive, and therefore the diploid will not grow on CAN plates. Canavanine resistant colonies arise normally in meiosis by one of two processes: (1) crossing over between the locus and the centromere, giving rise to diploid colonies, (2) meiotic segregation resulting in two canavanine-resistant haploid spores in every tetrad.

Samples from cultures in SPM that were incubated at 25° or 34° were spread at different times on plates of the following media: YEPD (viable counts), —AD (for *ADE* prototrophs) and CAN (for canavanine-resistant colonies). The plates were incubated at 25° and colonies were scored after three to five days of incubation. At each time, five plates of each kind were spread and values were calculated from the average colony counts. Thus, frequencies of ADE prototrophs and of canavanine-resistant colonies were based on the number of viable, colony-forming cells (on YEPD) in the culture.

Haploidization was also monitored among *ADE* prototrophs, and among colonies that grew on YEPD, by mating samples of these with two mating-type testers. The frequency of maters was usually comparable to the frequency of canavanine-resistant colonies.

Double shifts with hydroxyurea (HU) (following HARTWELL *1976)*

Cells of strain 425–9 were grown in liquid YEPD medium at 25°, sonicated to separate cell clumps, and diluted with YEPD to a titer of 1.5×10^6 cells/ml. Experiment I examined the fate of cells that were first exposed to the restrictive temperature and later to the HU treatment. The first incubation was with YEPD liquid medium, either at 25° (culture FI) or at 36° (cultures AI and CI). After three hr incubation, cells were sonicated and cultures AI and FI were plated on YEPD agar slabs containing 0.3 M hydroxyurea (Matheson). Culture CI was similarly plated on YEPD agar slabs without HU. The agar slabs were incubated at 25°. Experiment II was designed to exposed cells first to HU and later to the restrictive temperature. Cells were incubated for three hr in liquid YEPD, either with 0.1 M HU (cultures AII and CII) or without HU (culture FII). Following a double wash in water, cells were sonicated and plated on YEPD agar slabs. The incubation of the agar slabs was either at 36° (cultures AII and FII) or at 25° (culture CII). The agar slabs were scored for number of cells in aggregates at three and eight hrs. Additional control experiments (as described by HARTWELL 1976) indicated that the temperature and hydroxyurea blocks were effective and reversible on both solid and liquid media.

RESULTS

Origin of cdc40

The *cdc40* mutation was induced in a diploid strain that was heterozygous for the late nuclear division mutation, *cdc5*. This strain, designated 425, was subjected to ultraviolet irradiation (SIMCHEN 1974) in order to obtain homozygous temperature-sensitive colonies of *cdc5/cdc5* constitution. One such ts colony was designated 425–9. Strain 425–9 was arrested in vegetative growth at the restric-

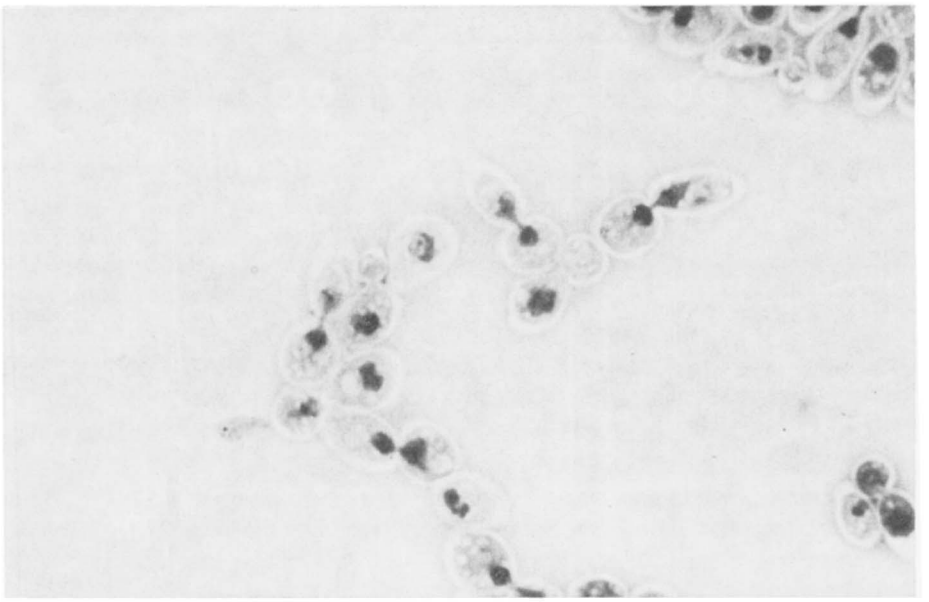

FIGURE 1.—Terminal phenotype of strain 425–9. Cells of strain 425–9 were grown in YEPD at 25° to a titer of about 5×10^6 cells/ml. The culture was transferred to 36° and after 5.5 hr cells were fixed and stained with Giesma (× 1500).

tive temperature of 36° with a terminal phenotype of medial nuclear division (Figure 1), *i.e.*, with an undivided nucleus placed in the isthmus between the mother cell and the large bud. This phenotype was unlike the one reported by HARTWELL *et al.* (1973) for strains carrying *cdc5*, which is a late nuclear division mutation (extended mother-cell and bud with stretched out, dividing nucleus). Sporulation was induced in strain 425–9 at 25° and asci were dissected. Five tetrads with four viable spores each and 29 asci with three viable spores were obtained, all spores giving rise to temperature-sensitive colonies. The haploid segregants were mated to *cdc5* testers of mating types **a** or α. Each of the five tetrads with four viable progeny contained only two *cdc5* segregants, and the 29 tetrads with three viable progeny contained one or two *cdc5* segregants. The other temperature-sensitive segregants of these tetrads carried another ts mutation as shown by the normal growth at 36° of the diploids produced by matings of the latter segregants with *cdc5* testers. The new temperature-sensitive mutation was designated *cdc40*. Random-spore analysis of 425–9 produced 450 haploid segregants, all being temperature sensitive. From the above tetrads, 13 *cdc5* segregants were mated to *cdc40* haploids that did not carry *cdc5*. All 13 *cdc5* segregants proved to be double mutants, namely *cdc5 cdc40*. Thus, we concluded that strain 425–9 had the constitution *CDC5/cdc5*, *cdc40/cdc40*. Tetrad analysis of the parental strain 425 revealed its constitution to be *CDC5/cdc5 CDC40/CDC40*, thus suggesting that the new mutation, *cdc40*, and its homozygotization might have resulted from a single UV-induced event. Similar cases have been recorded

elsewhere (HOPPER and HALL 1975, WILLIAMS 1976). It is not surprising that the *cdc5/cdc5* homozygote was not obtained by mitotic recombination, because *cdc5* is very tightly linked to the centromere of chromosome *XIII* (HARTWELL *et al.* 1973). Mapping of *cdc40* was not attempted, but a small-scale tetrad analysis of a diploid constructed by mating strain 391 to a standard **a** strain gave 2:2 segregation in 22 asci and no hints of tight linkage between *cdc40* and the centromere.

Mitotic terminal phenotype and execution point

The diploid strain 425–9 was grown in YEPD medium at 25° to a titer of 5×10^6 cells/ml. The culture was then transferred to the restrictive temperature of 36°, and at hourly intervals samples were sonicated, fixed with 4% formaldehyde and stained with Giemsa (procedure as described by ROBINOW and MARAK 1966 and modified by HARTWELL, CULOTTI and REID 1970). At the time of transfer, 32% of the cells were unbudded and 14.5% were in stages of nuclear division. After six hrs at 36°, 90% of the cells were arrested as budded cells, with the following nuclear morphologies: 55% were arrested in the middle of nuclear division (Figure 1), 20% were arrested as budded cells with only one nucleus in the mother cell, and 15% were budded cells with a nucleus in the mother cell and a nucleus in the bud. (The remaining phenotypes were 6% unbudded cells and 4% multinucleate ones.) We regard the typical terminal phenotype as a budded cell with the undivided nucleus located in the isthmus (Figure 1), but the multitude of phenotypes may be a genuine feature of the *cdc40* mutation (see DISCUSSION). Incubation at 34° (the restrictive sporulation temperature) gave similar proportions and the same terminal phenotype. In a comparable experiment with a haploid which carries *cdc40* (strain 391), essentially the same results were obtained.

The execution point of a cell-cycle temperature-sensitive mutation is defined as the last point in the cycle at which temperature sensitivity prevents the completion of that cycle. The execution point can be determined in an experiment in which an asynchronous population is transferred to the restrictive temperature (HARTWELL *et al.* 1973). Cells that have not reached the execution point at the time of transfer will arrest before the end of the cycle, showing the terminal phenotype, while cells that have passed this point will complete the cycle and arrest in the next cell generation as two adjacent dividing cells, both with terminal phenotypes. Such an experiment was performed with strain 425–9, which was grown in YEPD medium at 25° to a titer of 1.5×10^6 cells/ml, at which stage it was sonicated, plated on agar slabs and incubated at 36°. The results are given in Table 1; similar frequencies to those obtained at five hrs were also found after 24 hrs incubation at 36°, this being an indication that the mutation is not leaky. In order to calculate the execution point from the frequencies of cells that are arrested within one cycle or in the second cycle, the age distribution of the cells at various stages in the cycle must be taken into account (HARTWELL *et al.* 1973). In this computation, we ignore the cells that were arrested at the unbudded

TABLE 1

Arrest of an asynchronous culture of strain 425–9 following incubation at 36°

Time at 36°	Percent Unbudded cells	Budded cells	Aggregates of 3 or 4 cells	Aggregates of more than 4 cells
Experiment 1				
0 hrs	31.0	53.5	11.0	4.5
5 hrs	6.5	43.25	35.25	15.0
Experiment 2				
0 hrs	34.5	58.0	4.5	2.5
5 hrs	5.5	45.0	38.5	7.0
24 hrs	6.25	46.5	39.75	7.5

Note: each sample consisted of 400 aggregates, including budded or unbudded cells.

state, as well as those that were arrested as groups of cells with more than four cells (columns 2 and 5, respectively). The latter probably result from groups of three or more cells at time 0. Thus, in Experiment I (Table 1) at five hrs, the proportion of cells completing the ongoing cell cycle and arresting in the next one is $0.3525/(0.3525 + 0.4325) = 0.45$. This value is modified according to the age distribution, and therefore the execution point of *cdc40* in diploid cells in YEPD medium is 0.46. Estimates of the execution point based on the results at five and 24 hrs in Experiment 2 are both around 0.47.

DNA synthesis was examined at the permissive and restrictive temperatures (25° and 36°), as was viability of the mutant strains at the restrictive temperature (Figure 2). After shift to the high temperature, there was a twofold increase in DNA content. These results indicate that another round of DNA replication occurs in most cells of the culture upon being transferred to 36°, and suggest that the arrested cells are at the G_2 state of the cell cycle. Vegetative *cdc40* cells incubated at 36° die within a few hours.

Double shift experiments with hydroxyurea (HU)

HARTWELL (1976) sequenced several *cdc* functions in relation to DNA synthesis and its sensitivity to hydroxyurea. Each mutant was arrested at the restrictive temperature and then transferred to the permissive temperature in the presence of hydroxyurea. Strains that traversed the hydroxyurea-sensitive stage of the cell cycle (*i.e.*, the DNA synthetic period) at the restrictive temperature were able to complete the cycle following the shift. Each mutant was also subjected to the reciprocal shift, namely from hydroxyurea arrest at the permissive temperature to drug-free medium at the restrictive temperature. Again, if cells have traversed the temperature-sensitive stage in the presence of HU, they could complete the cycle following the shift. The *cdc* mutants were characterized as follows (HARTWELL 1976): the DNA initiation genes *CDC4* and *CDC7*, as well as *CDC28*, appeared to function before the HU-sensitive stage, the DNA elongation genes *CDC8* and *CDC21* appeared to function concurrently with HU, and the nuclear division genes (*CDC9*, *CDC13*, *CDC16*, *CDC23*, *CDC5* and *CDC15*)

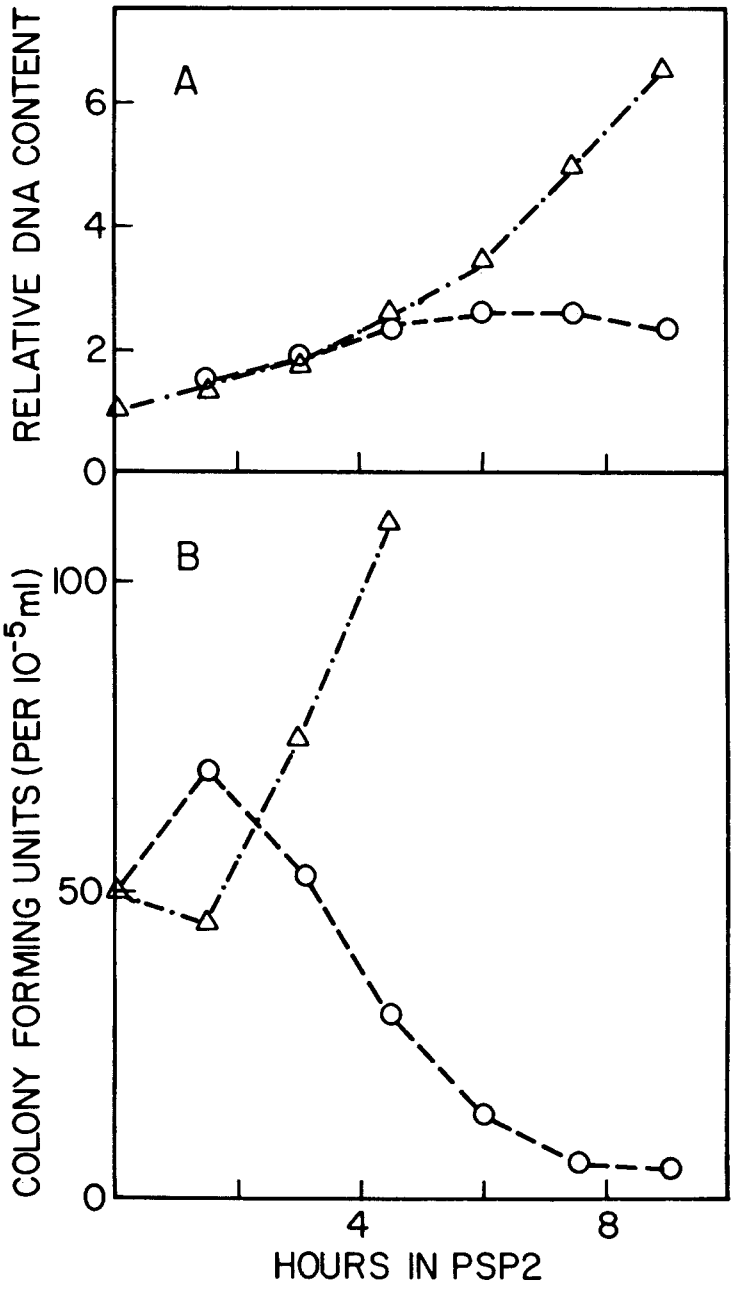

FIGURE 2.—Vegetative DNA synthesis and viability of strain 425–9. Cells of 425–9 were grown in PSP2 + adenine (40 μg/ml) + uracil (3 μg/ml) + 14-C-2-uracil (0.8 μci/ml), at 25° to a titer of 5×10^6 cells/ml. At time 0, half of the culture was transferred to 36°. At hourly intervals samples from both temperatures were taken for an assay of DNA content (see MATERIALS AND METHODS) and also spread on YEPD plates in order to assess viability. (Δ = 25°, ○ = 36°)

appeared to function after the HU-sensitive stage of the cell cycle. The only grossly exceptional behavior was that of *cdc2* and *cdc6*, which went through a round of DNA replication at the restrictive temperature, but nevertheless could not traverse the cell cycle in the presence of HU following such an incubation. The DNA synthesis observed at the restrictive temperature in these two mutants is believed not to be completed, and therefore cannot serve the cycle without further incubation at the permissive temperature in the absence of HU.

We analysed our new mutation, *cdc40*, by the methodology of HARTWELL (1976). The experiments are described in MATERIALS AND METHODS. In order to compare the various treatments, the proportion of cells that completed at least one cell cycle during the second incubation period was computed in each case. This proportion, designated Q, represents the cells that were released from the first point of arrest. The computation is based on the percentages of single and budded cells at three hr and eight hr (Table 2). Thus, for treatment CI in Table 2, $Q = 1-14.5/(14.0-4.0 + 66.5) = 0.811$. It is assumed that the 4% of cells that remained single at eight hrs were atypically arrested, perhaps even dead. The cells that were aggregated at three hrs despite the sonication were also not taken into account.

TABLE 2

Arrest of cells of strain 425–9 following double shifts of high temperature and hydroxyurea

Type	Time	Number of cells and buds in aggregates 1	2	3	4	>4	Q
		Experiment I (temperature shift-up followed by hydroxyurea)					
CI	3 hrs	14.0	66.5	10.5	6.5	2.5	
	8 hrs	4.0	14.5	8.0	26.5	47.0	0.811
AI	3 hrs	14.0	66.5	10.5	6.5	2.5	
	8 hrs	5.0	54.3	15.5	18.0	7.2	0.281
FI	3 hrs	11.5	76.5	3.0	7.5	1.5	
	8 hrs	1.0	26.2	18.3	41.0	13.5	0.699
		Experiment II (hydroxyurea followed by temperature shift-up)					
CII	3 hrs	16.5	78.0	3.0	2.5	0.0	
	8 hrs	2.0	3.5	7.0	15.5	72.0	0.962
AII	3 hrs	16.5	78.0	3.0	2.5	0.0	
	8 hrs	5.0	31.5	18.2	32.0	13.3	0.648
FII	3 hrs	11.5	76.5	3.0	7.5	1.5	
	8 hrs	2.7	19.3	18.8	41.0	18.2	0.774

Notes: Each sample consisted of 400 aggregates. Values in columns 3 to 7 are given in percentages.
Q = the proportion of cells that completed at least one cycle during the second incubation.
CI = 36° → 25° without HU.
AI = 36° → 25° with HU.
FI = 25° → 25° with HU.
CII = 25° with HU → 25° without HU.
AII = 25° with HU → 36° without HU.
FII = 25° without HU → 36° without HU.
The first incubation lasted from zero to three hr, the second from three to eight hr.

The results of Experiment 1 (Table 2—for details see MATERIALS AND METHODS) indicate that following an arrest of *cdc40* at 36°, many cells are sensitive to HU and cannot complete the cycle in the presence of the drug (*Q* values of 0.281 and 0.699 for rows AI and FI respectively). The results of Experiment II were less clear (*Q* values of 0.648 and 0.774 for AII and FII), but reproducible. From Experiment I, we may conclude that the *CDC40* gene function is required during or prior to the HU-sensitive period, and Experiment II suggests that it is probably required during the period in which the cell is sensitive to the drug, namely concurrently with DNA synthesis.

We would like to point out that the *Q* value of culture F could give an estimate for the execution point of the cause of the cycle arrest. This value for the lesion of *cdc40* (Experiment II) is quite similar to the value obtained for the HU treatment (Experiment I), supporting the idea that the two agents act at the same stage of the cell cycle. However, the data presented in Table 2 are not suitable for obtaining reliable estimates of execution points, as at time 0 of that experiment (three hr), only 11.5% of the cells were unbudded. Normal asynchronous cultures should give about 30% unbudded cells. A lower frequency of unbudded cells was, however, more suitable for the double-shift experiment. Nevertheless, the data provide a comparison between the two different arrests. Other experiments in which culture F exhibited the expected percent of unbudded cells at three hr gave essentially the same result, but were not as clear with regard to the double shifts.

Allelism of cdc40 *with other* cdc *mutations*

As the *cdc40* mutation appeared to inhibit nuclear division at the restrictive temperature, we mated a haploid α *cdc40* with all the *cdc* mutants available in our laboratory that were known to be deficient in nuclear division and/or DNA replication and had a terminal phenotype resembling the one exhibited by *cdc40*. The mutants examined for complementation with *cdc40* were: *cdc2*, *cdc4*, *cdc5*, *cdc6*, *cdc7*, *cdc8*, *cdc9*, *cdc13*, *cdc14*, *cdc15*, *cdc16*, *cdc17*, *cdc20*, *cdc21*, *cdc23*. All the diploids grew normally at 36°. We conclude that *cdc40* is not allelic with the above-mentioned *cdc* genes (unless it shows intragenic complementation). In another study (JOHNSTON and GAME 1978), *cdc40* was found not to be allelic with seven mutations that showed depressed DNA synthesis.

Meiotic characterization

Diploids homozygous for *cdc40* are able to sporulate at the permissive temperature (25°), but not at the restrictive temperature (34°). Characteristic landmarks in yeast meiosis—premeiotic DNA synthesis, genetic recombination and haploidization—were examined for the *cdc40* homozygote strain 425–9 at 25° and 34°. Premeiotic DNA synthesis occurred with similar kinetics at both temperatures, but neither commitment to recombination nor segregation took place at the restrictive temperature. (Experimental details are given in MATERIALS AND METHODS.) The results in Figure 3 relate to intragenic recombination at the *ade2* locus, but we also found that intergenic recombination between *can1–11* and the

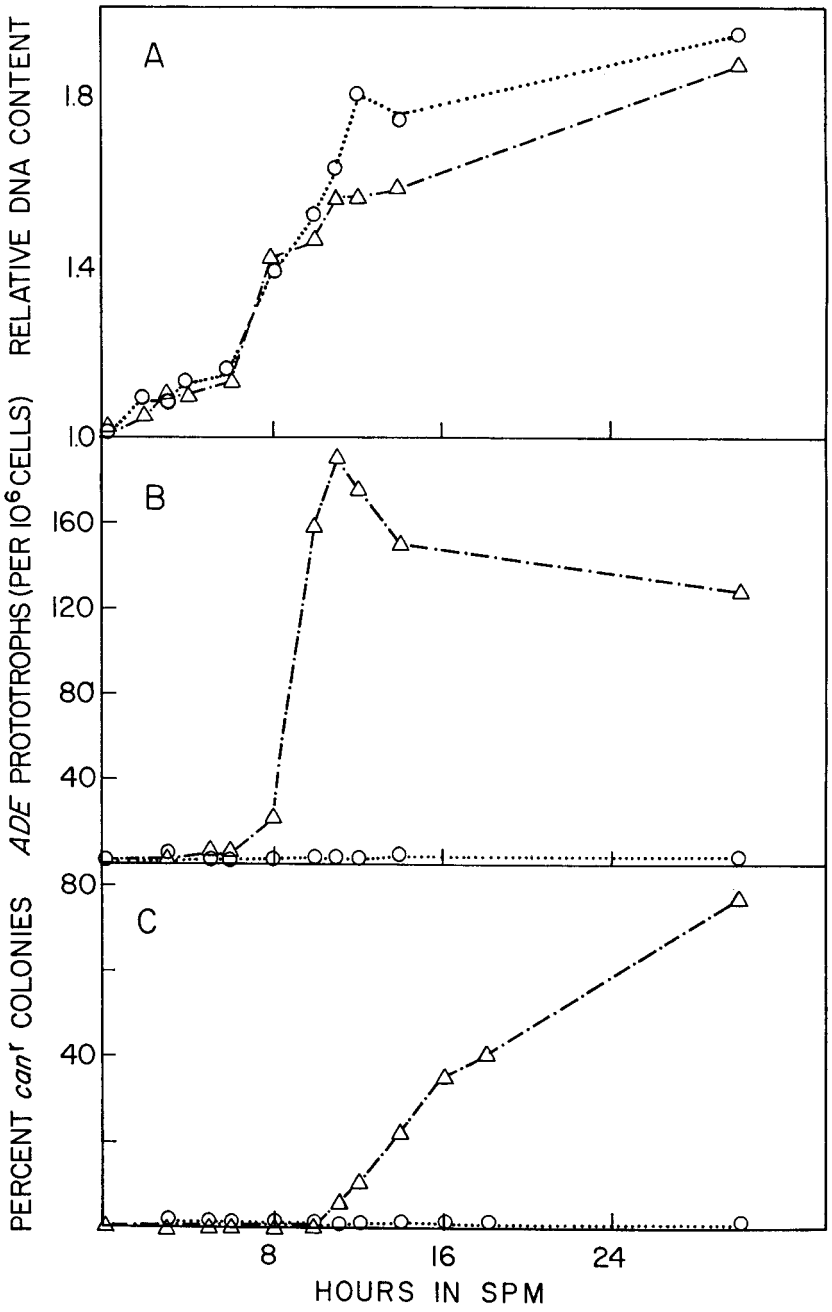

FIGURE 3.—Premeiotic DNA synthesis, commitment to recombination and haploidization in strain 425–9 at the permissive and restrictive temperatures. Cells of strain 425–9 were grown at 25° in PSP2 + adenine (40 μg/ml) + uracil (3 μg/ml) + 14-C-2-uracil (0.8 μci/ml), to a titer of 1×10^7 cells/ml. Cells were washed once in water, resuspended in SPM and incubation continued at 25°. At 45 min a subculture was transferred to 34°. At hourly intervals samples were taken from both cultures for DNA content assays and were spread on YEPD, —AD and CAN plates. (A)—DNA content, (B)—recombination commitment (C)—haploidization. (Δ = 25°, ○ = 34°)

centromere was blocked in *cdc40* homozygotes at the restrictive temperature (data not shown). Viability of these strains remained high up to 24 hr incubation at the restrictive temperature in sporulation medium, in contrast to the situation in vegetative medium (*e.g.*, Figure 2B).

Premeiotic DNA synthesis in yeast differs from vegetative (premitotic) DNA synthesis with regard to its source of precursor nucleotides (SIMCHEN, PIÑON and SALTS 1972). Cells labeled in PSP2 medium with ^{14}C-2-uracil and transferred to "cold" SPM medium (sporulation medium) double their label content in DNA under meiotic conditions, but do not show much increase in label when transferred to "cold" PSP2 medium (vegetative conditions). By this criterion, DNA synthesis in sporulation medium shown by strain 425–9 at both permissive and restrictive temperatures was premeiotic. At both temperatures, the amount of radioactive label in DNA almost doubled after 11 to 12 hrs in SPM. The increase consisted mostly of nuclear DNA replication rather than mitochondrial, as was evident from equilibrium sedimentation in CsCl gradients (data not shown).

Meiotic execution points

The above observations indicate that the *CDC40* gene product is needed at an early stage of meiosis, at the time of commitment to recombination. It may, however, also be required at later times. In order to study execution points in meiosis of the temperature-sensitive mutation, we have performed temperature-shift experiments as discussed by ESPOSITO *et al.* (1970) and SIMCHEN and HIRSCHBERG (1977). Cells of strain 490 grown in PSP2 to a titer of 1×10^7 cells/ml were washed once in water, resuspended in SPM (time 0) and incubated in a shaker at 25°. At hourly intervals, aliquots were transferred to 34°, and at 33 hrs (from time 0) sporulation parameters were assessed. Figure 4A shows the percentage of asci in the subcultures at 33 hr as a function of the time of transfer. Asci were observed in the subcultures only when shift-ups were done from 11 to 12 hr onwards; therefore, the last execution point (for 50% of the sporulating cells) is estimated to be around 15 hr. Figure 4A also gives the time of commitment to haploidization during incubation at 25°, as measured by the percentage of colonies formed by cells spread on CAN plates at various times during sporulation (see MATERIALS AND METHODS). It is evident that the last time in meiosis (at 25°) at which *CDC40* is required for sporulation is approximately two to three hrs after commitment to haploidization. The shift-up subcultures were also assessed for canavanine-resistant colonies at 33 hr, and the results are also given in Figure 4A. The latter curve represents the last time (at 25°) at which the *CDC40* function is required for haploidization; it is about one hour before the last occasion on which the *CDC40* function is required for spore formation.

Recombination commitment in yeast occurs during the premeiotic DNA synthesis (ESPOSITO and ESPOSITO 1974b). There are indications that the recombination events are realized only later, at the time of meiotic prophase (SILVA-LOPEZ, ZAMB and ROTH 1975; SALTS, SIMCHEN and PIÑON 1976). As the *cdc40* lesion affects commitment to recombination, it was of interest to examine whether later steps in the recombination process were also affected. Thus, the last execution point for recombination was measured in the same way described above for

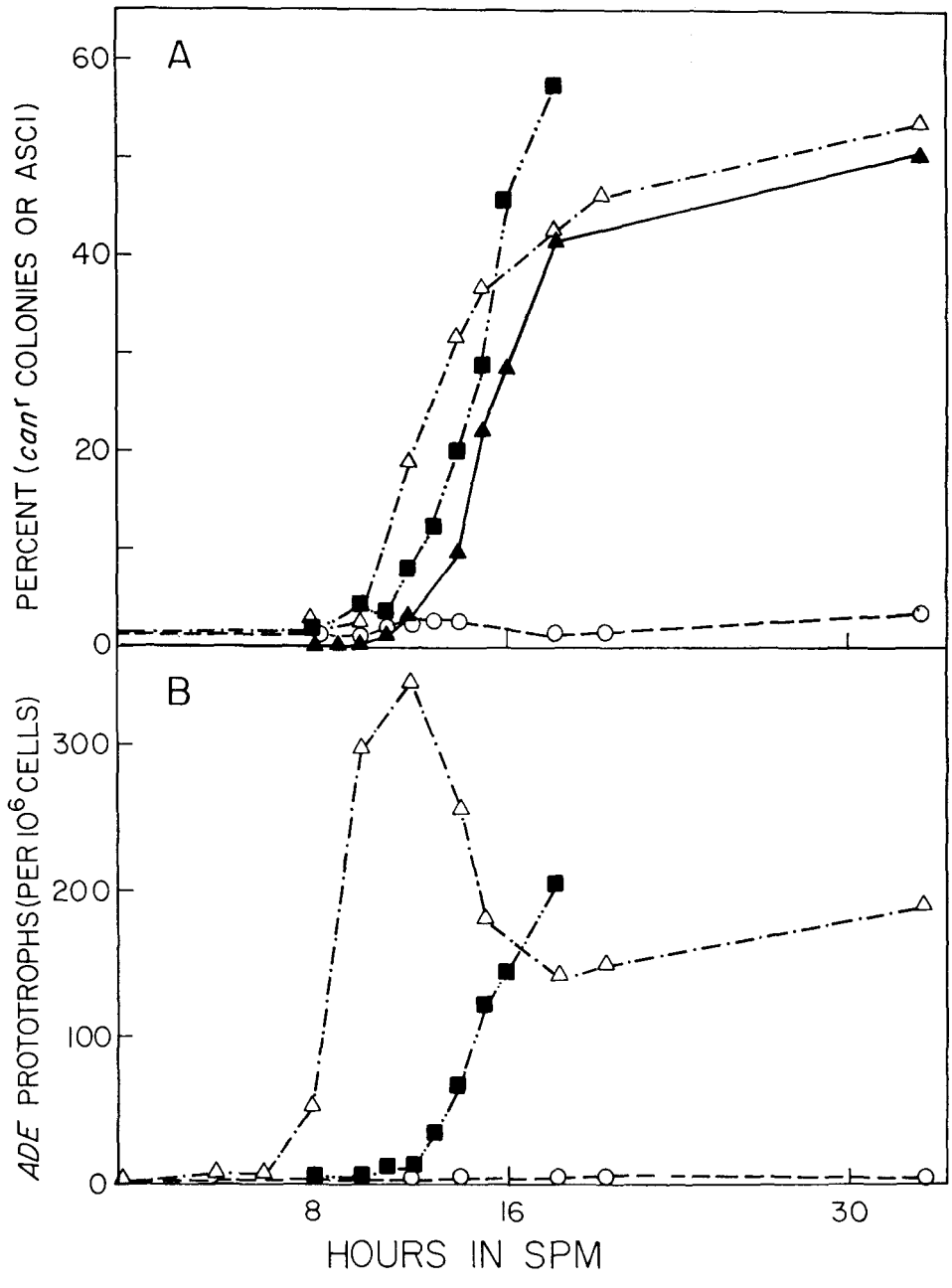

FIGURE 4.—Meiotic execution points and other parameters in shift-up experiments. Strain 490 was grown in PSP2 + adenine (40 μg/ml) + histidine (10 μg/ml) at 25°, to a titer of 1×10^7 cells/ml, washed once in water, resuspended in SPM and incubated at 25°. At hourly intervals 5 ml subcultures were shifted to 34°. At the time of transfer, and at 33 hr, samples from all subcultures were spread on YEPD, —AD and CAN plates. At 33 hr sporulation was assessed in all the subcultures. Note that except for the control cultures, the abscissa represents

spore formation and haploidization. We scored the shift-up subcultures of strain 490 after 33 hr in SPM for the frequency of *ADE* prototrophs and plotted them as a function of the time of transfer of the subcultures from 25° to 34° (Figure 4B). We expected that if the *cdc40* lesion were limited to the commitment to recombination, the frequencies of prototrophs in the subcultures at 33 hr would be equal to their frequencies at the time of transfer to 34°. Results for commitment to recombination at the times of transfer are therefore included in Figure 4B. Recombination commitment begins at seven to eight hr in SPM (25°) and reaches a maximum at 12 hr. The subsequent decline in prototroph frequency is due to 50% loss of haploid *ade1* segregants at the time when haploidization starts (the diploid is heterozygous for *ADE1*). The curve that describes prototroph frequency as a function of time of transfer to 34° does not overlap with the commitment curve. At a time when cells are almost fully committed to recombination (eight to 12 hr), but before segregation (compare with Figure 4A), a shift-up to the restrictive temperature causes a drastic reduction in the frequency of prototrophs. As indicated in MATERIALS AND METHODS, recombination and haplodization values at each point were based on numbers of colonies formed on selective media and on the regular growth medium, YEPD. The latter also provided us with estimates of viability in the controls and in the shift-up cultures, from which we concluded that viability was not significantly reduced throughout the experiment.

In order to follow the loss of recombinants in shift-up subcultures, strain 490 was grown as described earlier, transferred to SPM at 25° and at various times subcultured to 34°. At various times, samples were taken from the subcultures, spread on YEPD, -AD, and CAN plates, and incubated at 25°. It is evident (Figure 5) that, at the restrictive temperature, commitment to recombination continues to rise for about three to four hr, and that the ability to form recombinants is lost only later. The rise and decline in canavanine resistant colonies at the 13 hr shift-up subculture probably represent commitment to recombination between *can1* and the centromere and the loss of commitment to recombination, respectively. Again, no marked reduction in viability was evident.

The results indicate that the product of *CDC40* is required for recombination at a time comparable to meiotic prophase, in addition to its earlier role in commitment to recombination (see above). The results prove also that recombination

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the time of transfer from 25° to 34°. Time 0 represents a culture shifted to 34° at 30 min and scored at 33 hr. Time 33 represents a culture incubated throughout the period at 25° and scored at 33 hr.

△—commitment to haploidization (A) or recombination (B) in a control subculture incubated at 25°.

○—commitment to haploidization (A) or recombination (B) in a control subculture incubated at 34°.

▲—percent asci at 33 hr in subcultures, as a function of time of transfer to 34°.

■—percent canavanine-resistant colonies (in A) or frequency of *ADE* prototrophs (in B) at 33 hr in the subcultures, as a function of time of transfer to 34°.

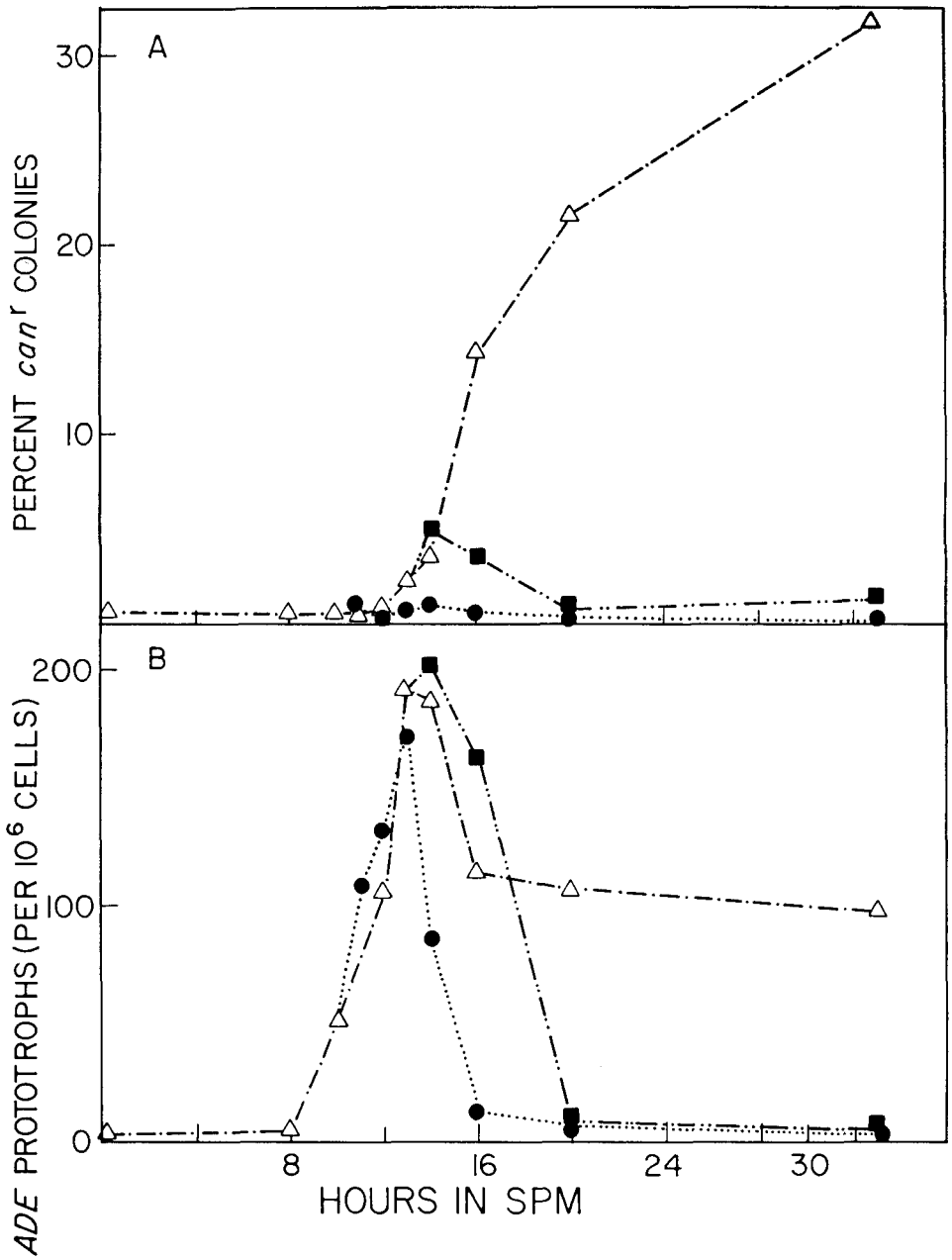

FIGURE 5.—Commitment to recombination and haploidization in strain 490 at 25° and in two shift-up subcultures. Growth and sporulation conditions as in legend to Figure 4.

△—recombination commitment or haploidization at 25°.

●—subculture shifted from 25° to 34° at ten hr (*i.e.*, ten hr after the transfer from PSP2 to SPM). At hourly intervals samples were taken for plating.

■—Subculture shifted from 25° to 34° at 13 hr. At hourly intervals samples were taken for plating.

needs to be realized after the completion of premeiotic DNA synthesis, at a time when segregation starts.

Temperature shift-down

Meiosis in yeast is a complex process, spread, at 25°, over a period of about 16 hr. As we have shown, the *CDC40* gene product is required at least twice in meiosis, first for commitment to recombination, and later for realization of the recombination events and for meiotic segregation. At the restrictive temperature, diploid strains homozygous for *cdc40* are able to traverse the meiotic process normally up to the first point when the gene function of *CDC40* is required. Incubation at the restrictive temperature for the period prior to the time at which the gene product is required, followed by incubation at the permissive temperature, is not expected to affect sporulation parameters. A prolonged incubation

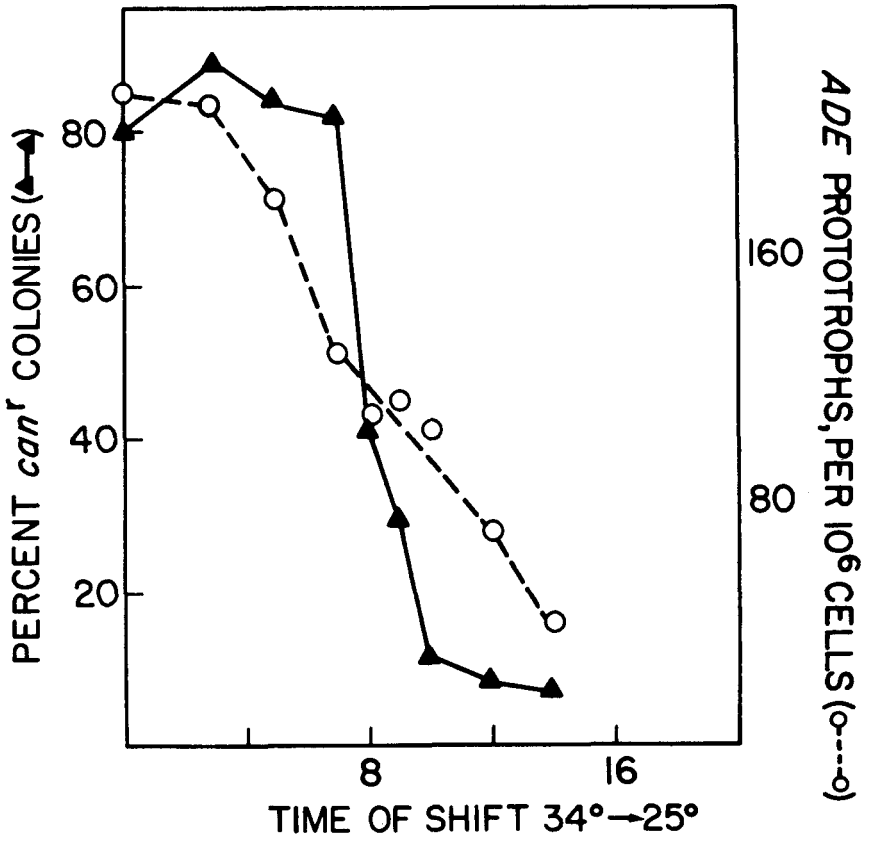

FIGURE 6.—Recombination and haploidization at 30 hr in shift-down subcultures of strain 425-9. Strain 425-9 was grown at 25° in PSP2 + adenine (40 μg/ml) to 1×10^7 cells/ml, washed once in water, resuspended in SPM, and incubated at 25°. At 30 min the culture was transferred to 34° and at intervals subcultures were transferred back to 25°. At 30 hr the subcultures were examined for recombination (ADE prototrophs)—○; and haploidization (canavanine resistant colonies)—▲. The results were plotted as a function of the time of shiftdown.

at the restrictive temperature beyond the time the gene product is required may have one of two consequences. Depending on the length of incubation at the restrictive temperature and on the nature of the deficient gene function, the arrest can be either reversible or irreversible. In the first situation cells can return to the normal sequence of meiotic events upon shift-down to the permissive temperature, while in the latter this is not possible. Cells of 425–9 were grown in PSP2 to a titer of 1×10^7 cells/ml, were harvested, washed once in water, resuspended in SPM and incubated at 25°. At 30 min the culture was shifted to 34°, and at intervals subcultures were transferred back to 25°. Sporulation parameters were assessed at 30 hr, which is ample time for a meiotic cell to complete the process in which it is engaged (time 0 is the time of transfer to SPM). Figure 6 summarizes results on the reversibility of the recombination and haploidization processes with respect to the *CDC40* function. The parameters for each subculture are plotted as a function of the time of shift-down to 25°. When a subculture was kept at 34° for as long as seven hr, the percent of haploidization was the same as in the control (time 0). After that time, the haploidization process was affected irreversibly and a drastic decline in haploidization was observed. Recombination commitment was affected upon very short incubations at 34°; in comparison, haploidization was irreversibly affected only after considerably longer incubations at the restrictive temperature.

DISCUSSION

We have characterized the behavior of a new cell cycle mutation, *cdc40*, in the mitotic cell cycle and in meiosis. Following incubation at the nonpermissive temperature, most cells of *cdc40* strains (haploids or homozygous diploids) arrest with a terminal phenotype of medial nuclear division. There is also an approximate doubling in DNA content following the transfer from permissive to restrictive conditions, suggesting that one round of DNA replication can occur at the high temperature and that the arrest occurs later, during nuclear division. The execution point, which was estimated to be at 0.46 of the cell cycle, is in agreement with execution points of other medial nuclear-division mutants (HARTWELL *et al.* 1973). Further characterization of the lesion conferred by *cdc40* was achieved by double-shift experiments with hydroxyurea (HU), following HARTWELL (1976). We find that most *cdc40* cells arrested at 36° have not yet traversed the HU-sensitive stage of the cell cycle, indicating that the replication of DNA at the restrictive temperature is not complete. The deficiency in his round of replication is not clear, but its curing at the permissive temperature depends on the absence of hydroxyurea.

The new mutation *cdc40* showed complementation in diploids with 15 known DNA synthesis or nuclear division *cdc* mutations that have somewhat similar terminal phenotypes. (The possibility of intragenic complementation has not been ruled out, however). Thus, *cdc40* appears to occur in a gene not represented in the original collection of 148 *cdc* mutants, which defined 32 loci (HARTWELL *et al.* 1973). One possible explanation of the absence of *cdc40* in HARTWELL's

collection lies in the fact that only 55% of the cells carrying this mutation arrest at the typical nuclear phenotype when incubated at the restrictive temperature. In contrast, a temperature-sensitive mutant was retained as belonging to the *cdc* group only if 80% or more of its cells (HARTWELL *et al.* 1973) arrested at a typical terminal phenotype. However, it might be inherent in the *cdc40* lesion that not more than 55% of asynchronously dividing cells may arrest with the typical medial nuclear division phenotype. This could be related to the apparent involvement of the *CDC40* function in normal nuclear DNA synthesis (see the double-shift experiment with hydroxyurea), which extends over a period of a quarter of the cell cycle in *S. cerevisiae* (WILLIAMSON 1965). Thus, it is possible that cells that are transferred to the restrictive temperature during the earlier parts of the S phase arrest with an atypical terminal phenotype, for instance, as budded cells with single undivided nuclei. Indeed this might be one reason why DNA synthesis mutants are poorly represented in the *cdc* collection. The only ones that appear to affect DNA replication throughout the S period, concurrently with the hydroxyurea sensitivity, are *cdc8*, *cdc21* and *cdc2* (HARTWELL 1976).

The effects of the *cdc40* mutation on meiosis are as follows. Homozygotes for *cdc40* traverse meiosis normally at 25° and reach complete sporulation, but at 34° meiotic cells arrest mostly before the first nuclear division, apparently after a full round of the premeiotic DNA replication. At the restrictive temperature, recombination commitment and haploidization are not achieved, apparently demonstrating that the premeiotic replication is not a sufficient condition for recombination commitment, and that an additional event, which is defective in *cdc40/cdc40* at 34°, is also required for recombination commitment. One must remember, however, the previously discussed double-shift experiments with hydroxyurea, which demonstrate that the vegetative DNA replication at the restrictive temperature is somewhat defective. It is therefore likely that the same defect occurs also in the premeiotic DNA replication, and it is tempting to relate it to the inability of *cdc40* cells to become committed to recombination at 34°.

Temperature-shift experiments present additional information about the role of the *CDC40* gene in meiosis. The shift-up experiments (Figure 4) show that even cells that have undergone full commitment to recombination at the permissive temperature cannot finalize the recombination events at the restrictive temperature. Thus, in addition to the role of the *CDC40* gene product during premeiotic replication (its requirement for recombination commitment), it is required later for the realization of recombination events. The latter requirement also affects haploidization, which cannot occur in cells in which recombination was inhibited (HOPPER, KIRSCH and HALL 1975; SALTS, SIMCHEN and PIÑON 1976). The shift-down experiments (Figure 6) show that quite early during premeiotic S the defect imposed by *cdc40* at 34° becomes irreversible with respect to recombination commitment, and a few hours later it becomes irreversible with respect to haploidization. The two meiotic processes become irreversible with different kinetics, thus suggesting that recombination commitment at 34° is affected by a different mechanism than the one affecting haploidization. It is

evident from the subculture that was transferred from 34° to 25° at seven hr (Figure 6) that a 50% reduction in recombination commitment does not interfere with haploidization. Since haploidization is reduced by means of interference with realization of recombination (SALTS, SIMCHEN and PIÑON 1976), we may conclude that irreversibility with respect to recombination commitment occurs separately from irreversibility with respect to realization of recombination events. It is further suggested that realization of meiotic recombination, rather than early events in recombination commitment, is the event essential for normal meiotic segregation.

As the molecular nature of the *cdc40* lesion, we may consider a recent suggestion (HARTWELL 1976) that mutants that go through a round of DNA synthesis at the restrictive temperature, but are still sensitive to hydroxyurea inhibition of the ongoing cell cycle, might have a primary defect in the enzyme DNA ligase. According to this suggestion, the DNA newly replicated at the restrictive temperature might be defective, and if the affected synthesis is the premeiotic replication, it is not difficult to envisage why commitment to recombination does not take place under these conditions. It is even easier to see how the inability of a defective ligase to function at 34° prevents the finalization of meiotic recombination events. Similar considerations might point to some proteins other than ligase that might have a role in stabilizing DNA. In any case, we may conclude that such a function during premeiotic replication is necessary for recombination commitment and is again essential for the finalization of the recombination events later, in the meiotic prophase.

To summarize our understanding of the process of commitment to recombination, we know (1) that it occurs concurrently with premeiotic replication (SHERMAN and ROMAN 1963; ROTH 1973; ESPOSITO and ESPOSITO 1974b), and that different regions of the genome might become committed to recombination at slightly different times during the premeiotic S (ESPOSITO and ESPOSITO 1974b; SIMCHEN, IDAR and KASSIR 1976); (2) that recombination commitment is not attained in the absence of the premeiotic DNA replication in mutants (BAKER *et al.* 1976; ROTH 1973) or in the presence of hydroxyurea (SIMCHEN, IDAR and KASSIR 1976); (3) that recombination commitment might be attained without the completion of the premeiotic S but not without its early steps (SIMCHEN and HIRSCHBERG 1977); (4) that it might be reversed by experimental interference, at the time of meiotic prophase, with hydroxyurea (SILVA-LOPEZ, ZAMB and ROTH 1975), UV irradiation (SALTS, SIMCHEN and PIÑON 1976) or shifts to 34° of *cdc40* homozygotes (present report); (5) that commitment to recombination is dependent on the normal functioning of the *CDC40* gene product during premeiotic DNA synthesis, which might possibly be a DNA ligase or a DNA stabilizing protein; and (6) that single-strand nicks in DNA appear during the premeiotic DNA replication (JACOBSON *et al.* 1975), but were shown not to be the physical manifestation of recombination commitment, as meiotic *cdc40* cells contained such nicks when incubated at either permissive or restrictive temperatures (KASSIR and SIMCHEN, unpublished).

A similar summary of our understanding of the realization of meiotic recombination events in *S. cerevisiae* reveals (1) that it occurs normally in meiotic prophase, after the completion of premeiotic DNA synthesis; (2) that it depends on the *CDC40* gene product; (3) that finalization of recombination events might be essential for normal meiotic segregation in *S. cerevisiae* (see above for discussion); and (4) that meiotic recombination takes place without meiotic segregation if the cells are transferred to a vegetative medium after recombination commitment, but before commitment to haploidization has occurred (SHERMAN and ROMAN 1963, ESPOSITO and ESPOSITO 1974b), thus giving rise to diploid products of meiotic recombination.

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Corresponding editor: F. SHERMAN