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Saccharomyces cerevisiae cdc2 Mutants Fail to Replicate Approximately One-Third of Their Nuclear Genome

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Chromosomal DNA replication was examined in temperature-sensitive mutants of *Saccharomyces cerevisiae* defective in a gene required for the completion of S phase at the nonpermissive temperature, 37°C. Based on incorporation of radioactive precursors and density transfer experiments, strains carrying three different alleles of *cdc2* failed to replicate approximately one-third of their nuclear genome at 37°C. Whole-cell autoradiography experiments demonstrated that 93 to 96% of the cells synthesized DNA at 37°C. Therefore, all cells failed to replicate part of their genome. DNA isolated from terminally arrested cells was of normal size as measured on neutral and alkaline sucrose gradients, suggesting that partially replicated DNA molecules do not accumulate and that DNA strands are ligated properly in *cdc2* mutants. In addition, electron microscopic examination of the equivalent of more than one genome's DNA from arrested cells failed to reveal any partially replicated molecules. The sequences which failed to replicate at 37°C were not highly specific; eight different cloned sequences replicated to the same extent as total DNA. The 2-μm plasmid DNA and rDNA replicated significantly less well than total DNA, but approximately one-half of these sequences replicated at 37°C. These observations suggest that *cdc2* mutants are defective in an aspect of initiation of DNA replication common to all chromosomes such that a random fraction of the chromosomes fail to initiate replication at 37°C, but that once initiated, replication proceeds normally.

Several cell division cycle (*cdc*) mutants of *Saccharomyces cerevisiae* exhibit temperature-sensitive defects in DNA replication which are neither simple blocks to entry into S phase nor simple blocks to propagation of the replication fork. *cdc2*, *cdc6*, *cdc9* (8, 19), and *cdc40* (23) mutants initiate S phase at the restrictive temperature (37°C) and synthesize a considerable quantity of DNA, but do not appear to complete S phase, since cells which have been incubated at 37°C will not divide if shifted to the permissive temperature (23°C) in the presence of the DNA synthesis inhibitor hydroxyurea (HU). This paper describes the analysis of DNA synthesis in *cdc2* mutants.

Four alleles of *cdc2* cause arrest in the first cycle after a shift to 37°C, producing cells with large buds; three other alleles do not cause immediate arrest and are capable of producing 2 to 25 cells at 37°C (20). The *cdc2* mutation has an execution point at about 0.25 in the cell cycle, near the beginning of S phase, and *cdc2* cells incubated at 37°C arrest at an early stage of nuclear division (8) with a completely formed but not fully elongated spindle (4). Culotti and Hartwell (8) found that at 37°C *cdc2* strains were able to synthesize an amount of DNA roughly equivalent to a full round of replication. However, this study did not discriminate between nuclear and mitochondrial DNA synthesis, which led to an overestimate of nuclear DNA synthesis because mitochondrial DNA synthesis continues at 37°C for at least 6 h (C. S. Newlon, unpublished data).

Reciprocal shift experiments by Hartwell (19) indicated that DNA synthesis in *cdc2* mutants at 37°C was incomplete or defective, since cells preincubated at 37°C failed to divide when returned to 23°C in the presence of HU, and cells preincubated in HU failed to divide when placed at 37°C. We have analyzed DNA replication in *cdc2* mutants and have confirmed the prediction of reciprocal shift experiments by finding that approximately one-third of the nuclear genome fails to replicate at 37°C. Attempts to detect partially replicated molecules have been unsuccessful, suggesting that the mutation interferes with an initiation process. There is not a high degree of specificity to the DNA sequences which fail to replicate, suggesting that the *cdc2*

mutation affects the replication of all DNA sequences. A model which explains the DNA replication defect is presented.

MATERIALS AND METHODS

Strains. *S. cerevisiae* strains are listed in Table 1 and were supplied by the authors listed or constructed by standard procedures. Strains lacking mitochondrial DNA were generated by treatment with ethidium bromide (15). To permit density labeling of DNA with bromodeoxyuridine monophosphate (BrdUMP), a *tup* (TMP uptake) derivative was isolated from strain 19041-1 as described by Leff and Lam (26).

Bacterial strains used for the growth of plasmids have been described by Devenish and Newlon (10).

Media and culture conditions. Most experiments were conducted with cultures grown in Y minimal medium (pH 5.8) (35) supplemented with (per liter): 0.5 g of yeast extract, 10 mg of adenine, 2 mg of uracil, and 50 mg each of histidine, lysine, tyrosine, and methionine, plus other amino acids as required.

To allow more economical use of α-factor, some cultures were grown in Y minimal medium (pH 3.5), prepared as described above, but with 0.75 g of NaOH per liter.

Density transfer experiments were conducted with cultures grown in YSAT medium containing (per liter): 6.7 g of yeast nitrogen base without amino acids, 10 g of succinic acid, 6 g of NaOH, 1 g of yeast extract, 6 g of sulfanilamide, and 50 mg each of histidine, lysine, tyrosine, and methionine. In early experiments, dTMP was added to a concentration of 25 μg/ml (YSAT-25). Later it was found that cultures grown in concentrations of 50 μg/ml (YSAT-50) developed a higher degree of synchrony.

The permissive temperature was 23°C, and the restrictive temperature was 37°C.

Cultures were synchronized by adding α-factor (prepared by the method of Duntze et al. [11]) for the equivalent of one generation to cause arrest in G1. The cells were then centrifuged, washed, and suspended in fresh medium.

Growth of bacterial plasmids was carried out as described by Devenish and Newlon (10).

Isotopes, enzymes, and chemicals. [6-^{3}H]uracil, [2-^{14}C]uracil, [8-^{3}H]adenine, [2-^{14}C]adenine, and [α-^{32}P]dATP and [α-^{32}P]dCTP were purchased from Amersham Corp., Arlington Heights, Ill.

Restriction endonucleases were purchased from New England Biolabs (Beverly, Mass.), Bethesda Research Laboratories (Gaithersburg, Md.), or Boehringer Mannheim Biochemicals (Indianapolis, Ind.); buffers were as specified by the supplier. *Escherichia coli* DNA polymerase I was from Boehringer Mannheim. Glusulase was from Endo Laboratories (Garden City, N.Y.); Zymolyase-60,000 was from Miles Laboratories, Inc. (Indianapolis, Ind.). BrdUMP was prepared by the method of Michelson et al. (29) or purchased from Sigma Chemical Co. (St. Louis, Mo.). After addition of BrdUMP, cultures were grown in foil-wrapped flasks. Manipulations of BrdUMP-substituted DNA were carried out in red light.

Measurement of DNA synthesis. DNA synthesis was monitored by the incorporation of [^{3}H]uracil into alkali-stable, trichloroacetic acid-precipitable material, essentially as described by Hartwell (18). All strains were uracil auxotrophs.

Whole-cell autoradiography. DNA-specific whole-cell autoradiography was performed as described by Rivin and Fangman (38). DNase-treated control cells were also treated with S1 nuclease (1,000 U/ml in 10 mM sodium acetate [pH 4.8], 0.25 M NaCl, and 1 mM $ZnCl_2$) for 2 h at 37°C.

Sucrose gradients. Neutral sucrose gradients were as described by Petes and Fangman (36). Alkaline gradients were 15 to 30% sucrose in 0.9 M NaCl and 0.04 M EDTA with NaOH added to give a pH of 12.3.

Spheroplasts were prepared by the method of Forte and Fangman (12) and lysed on sucrose gradients by the addition of Sarkosyl (36). Neutral gradients were centrifuged in an SW50.1 or SW65 rotor at 4°C and 10,000 rpm for 26 h or at 8,000 rpm for 72 h in an SW40 rotor. Alkaline gradients were centrifuged at 10,000 rpm for approximately 36 h in an SW50.1 or SW65 rotor. Fractions (approximately 0.2 ml) were collected from the bottom of the tube, the RNA in the fraction was hydrolyzed, and the DNA was precipitated with trichloroacetic acid as described by Newlon and Fangman (34).

Analysis of the replication of specific sequences. Cultures of 19041-1T1 were synchronized with α-factor and then density labeled with BrdUMP at 37°C. Spheroplasts were suspended in SCE (1 M sorbitol–

TABLE 1. *S. cerevisiae* strains used

Strain	Genotype	Source or reference
A364A	*MATa ade1 ade2 his7 tyr1 lys2 ura1 gal1*	(17)
APE5	A364A [*rho*^{0}]	This laboratory
370	*MATa ade1* or *ade2 ura1 cdc2-1*	(20)
370.2.3	*MATa leu1 ura1 cdc2-1*	This laboratory
370.2.3-1	370.2.3 [*rho*^{0}]	This laboratory
336	*MATa ade1 ade2 his7 tyr1 lys2 ura1 gal1 cdc2-2*	(20)
336-1	336 [*rho*^{0}]	This laboratory
19041	*MATa ade1 ade2 his7 tyr1 lys2 ura1 gal1 cdc2-3*	(20)
19041-1	19041 [*rho*^{0}]	This laboratory
19041-1T1	19041-1 *tup*	This laboratory
C23-9B	*MATa ade1* or *ade2 lys2 ura1 trp1-289 gal*? *cdc2-3*	This laboratory
C23-9B-1	C23-9B [*rho*^{0}]	This laboratory
X2180-1B	*MATα SUC2 mal1 gal2 CUP1*	Yeast Genetic Stock Center, Berkeley, Calif.

TABLE 2. Plasmids used

Plasmid	Yeast sequence	Reference
pYe(*LEU2*)10	*LEU2*	37
pYe*HIS4*	*HIS4*	21
pCS-IV	*CYC1* (sequence 3′ to iso-1-cytochrome *c*)	39
pYe*CYC7*	*CYC7* (iso-2-cytochrome *c*)	32
YIp5-Sc4137	*CEN4* (centromere 4)	40
YRp16-Sc4301	*CEN3* (centromere 3)	40
pLC544	*TRP1*	24
p27A5	*K2* (distal to rDNA)	42
pBD4	rDNA (rRNA)	. 1
p82-6B	2-μm DNA	16

0.1 M sodium citrate–0.06 M EDTA [pH 5.8]) and lysed by the addition of Sarkosyl (5% [wt/vol] in SCE) to a final concentration of 1%. Approximately 50 μg of proteinase K was added per ml, and the lysate was incubated at 37°C for 1 to 2 h. Lysate (0.5 ml) was added to 4.0 ml of 64.8% (wt/wt) CsCl and 0.5 ml of 0.1 M Tris-chloride–0.1 M EDTA (pH 8). Water was added to adjust the refractive index to 1.4020.

CsCl gradients were centrifuged at 33,000 rpm in a type 50 or type 65 fixed-angle rotor or at 40,000 rpm in a VTi 65 rotor for 48 to 60 h at 20°C.

DNA in fractions of the CsCl gradients was denatured and immobilized on nitrocellulose filters as described by Brewer et al. (3).

Preparation of ^{32}P-labeled probes. Cloned *S. cerevisiae* DNA fragments (Table 2) were excised from their vectors with the appropriate restriction enzymes and separated by electrophoresis on agarose gels. In most cases, the entire yeast insert was used. The exceptions were the *LEU2* probe, which consisted of the 3.4- and 3.6-kilobase (kb) *Eco*RI fragments of pYe(*LEU2*)10, and the 1-kb K2 fragment excised from p27A5.

Restriction fragments were isolated from agarose gels (10) and labeled by nick translation with [α-^{32}P]dATP or [α-32P]dCTP according to the method of Maniatis et al. (30). Hybridization conditions have been described (10). The filters were exposed to Kodak XAR5 film. Filters hybridized with single-copy probes were exposed for 3 days to 3 weeks with an intensifying screen at −70°C. Filters hybridized with 2-μm DNA or rDNA probes were exposed for from several hours to several days without an intensifying screen.

Quantitation of hybridizations. Autoradiograms were scanned with a Transidyne RFT II densitometer (wavelength, 550 nm; slit width, 0.2 mm; slit length, 1 cm; speed, 1cm/sec). The area under each peak was used as a measure of the amount of [^{32}P]DNA that was hybridized. The linear response range of the film was determined by scanning autoradiograms of filters spotted with serial dilutions of [^{32}P]DNA and by comparing the densitometer measurements with the amount of ^{32}P in each spot determined by liquid scintillation counting.

To calculate the efficiency of replication of a sequence homologous to a particular probe, the ratio of percent [^{32}P]DNA bound to the HL (replicated) fractions/percent HL DNA on the filter was multiplied by the percent HL DNA in the CsCl gradient used to prepare the filters, thus normalizing each filter to the CsCl gradient from which it was prepared to correct for differences in binding of [^{3}H]DNA to different filters. This percentage was then converted to the percentage of a given sequence replicated by taking into account that the mass of a given sequence doubles when it replicates (see Fig. 3).

Finally, the efficiency of replication of a given sequence is expressed by the ratio: percent probe DNA replicated/percent total DNA replicated, referred to as the replication index (RI).

RESULTS

Quantitation of DNA synthesized at 37°C. The first set of experiments examined DNA synthesis in *cdc2* strains lacking mitochondrial DNA. The incorporation of ^{3}H from uracil into DNA was monitored in a synchronous S phase at 23 and 37°C (Fig. 1). At 23°C, there was a doubling of DNA content, with a second round of DNA synthesis initiated soon after the completion of the first. At 37°C, there was only a single round of DNA synthesis, and the amount of DNA made was only about 70% of that made at 23°C. After prolonged incubation at 37°C, a decline in the level of DNA was occasionally observed, presumably due to cell lysis.

Because at 23°C a second round of DNA synthesis was initiated soon after the end of the first, there was some difficulty in precisely determining the amount of DNA made in the first round. Experiments in which α-factor was add-

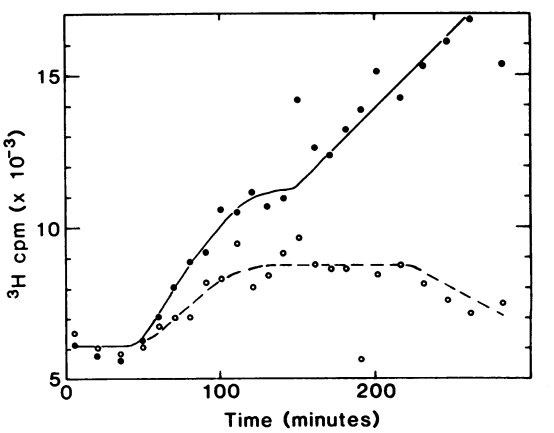

FIG. 1. DNA synthesis in *cdc2* at 23 and 37°C. Strain 370.2.3-1 was grown overnight at 23°C to a density of 2.5 × 10^{6} cells per ml in Y minimal medium plus supplements (pH 3.5), containing 5 μCi of [^{3}H]uracil per ml. α-Factor was added for 3.5 h (slightly over one generation), and at t = 0 the culture was centrifuged, washed, and suspended in fresh medium containing [^{3}H]uracil at the same specific activity. The incorporation of ^{3}H into alkali-stable, acid-precipitable material was monitored at 23°C (●) and 37°C (○).

TABLE 3. Amount of DNA synthesized at 37°C

Strain	Allele	Expt no.	% Doubling
370.2.3-1	*cdc2-1*	1	78
		2	60
		3	77
		4	59
336-1	*cdc2-2*	1	61
19041-1	*cdc2-3*	1	66
		2	64
C23-9B-1	*cdc2-3*	1	64

ed to prevent initiation of a second cell cycle yielded similar results (data not shown).

The results of studies conducted with several *cdc2* strains are summarized in Table 3. In each of the strains carrying three first-cycle arrest alleles of *cdc2*, the amount of DNA made at 37°C was 60 to 70% of that made a 23°C.

The amount of DNA synthesized at 37°C was also measured in density transfer experiments with a *tup cdc2* strain. BrdUMP was used to density label DNA synthesized in synchronous cultures at 37 and 23°C in control experiments. Measurements of precursor incorporation demonstrated that S phase in synchronized cells in BrdUMP medium at 23°C is complete approximately 3.5 h after release from the α-factor block (data not shown). Therefore, all DNA in a culture shifted to BrdUMP medium at 23°C upon release from α-factor block should have replicated and be of hybrid density after 3.5 h. Figure 2C shows the profile of DNA isolated from such a culture 3.25 h (0.6 doublings) after release from an α-factor block. Of the DNA present at time 0 (t_0), 16% was still of light density. The bulk of the DNA which had not replicated after 3.25 h still had not replicated 5.5 h later (Fig. 2D). At this time, although molecules of fully heavy density which must have replicated twice were present, 12% of the DNA present at t_0 was still of light density. The DNA which had not replicated after more than 1.5 generations in dense medium was presumably from dead cells (see below). We conclude from this experiment that at least 95% of the DNA capable of replicating in a *cdc2* strain grown in BrdUMP medium shifts to hybrid density during the first S phase in synchronized cells at 23°C.

In contrast, about one-third of the DNA remained in the light-density fractions after the equivalent of one generation at 37°C (Fig. 3). This represents about one-half of the DNA present in the culture before the shift to 37°C. Longer incubations at 37°C did not significantly increase the amount of DNA which shifted from light to hybrid density (see Fig. 8). In five such experiments, the average fraction of DNA which replicated was 58.8 ± 6.3% (see Table 4). In control experiments conducted with a TS^+ revertant of the *cdc2 tup* strain, virtually all of the DNA shifted away from the LL density at both 23 and 37°C after approximately one generation in BrdUMP (data not shown). Thus, the density transfer experiments are consistent with the [^{3}H]uracil incorporation experiments in demonstrating that DNA synthesis at 37°C is incomplete in *cdc2* strains.

One trivial explanation for the DNA synthesis deficit is that a fraction of the cells in the population fail to synthesize DNA at all at 37°C. This hypothesis was tested by performing DNA-specific whole-cell autoradiography on *cdc2* cells which had been labeled with [^{3}H]uracil

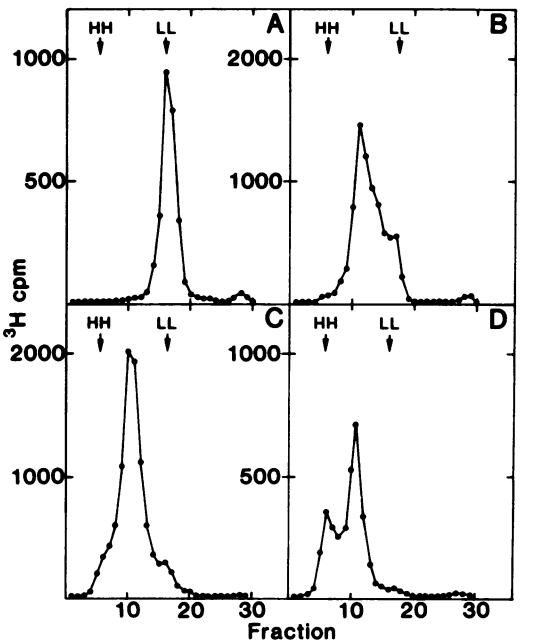

FIG. 2. Density labeling of a *cdc2 tup* strain at 23°C. Strain 19041-1T1 was grown for 22 h (about four generations) in YSAT-25. [^{3}H]adenine (10 μCi/ml) was included throughout the experiment. The culture was incubated with α-factor for 5.2 h and then the cells were centrifuged, washed, and suspended in fresh radioactive medium. At t = 0, 25 μg of BrdUMP per ml was added, and at intervals portions were withdrawn and lysates were prepared for centrifugation on CsCl gradients. Marker DNA was prepared by using asynchronous cultures labeled with [^{14}C]adenine. Light-density (LL) marker was prepared from a culture that did not contain BrdUMP. Heavy (HH) marker was prepared from a culture grown for 30 h in BrdUMP. CsCl gradients were centrifuged in a type 65 rotor at 33,000 rpm and 20°C for 60 h. A, t = 0; B, t = 2 h; C, t = 3.25 h; D, t = 8.75 h.

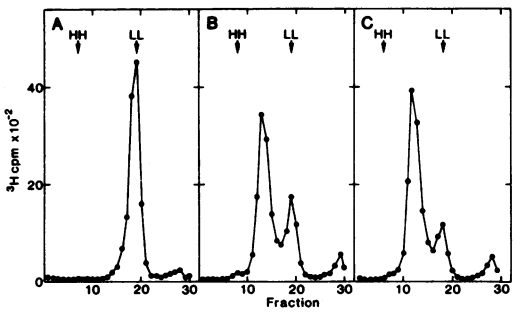

FIG. 3. Density labeling of a *cdc2 tup* strain at 37°C. Strain 19041-1T1 was grown for 36 h at 23°C to a density of 3×10^6 cells per ml in YSAT-25 containing [^{3}H]adenine at 10 μCi/ml. α-Factor was added for 5 h and then the cells were centrifuged, washed, and suspended in fresh radioactive medium. BrdUMP was added to 25 μg/ml, and the culture was incubated at 37°C. At intervals, portions were taken, and lysates were prepared and centrifuged in CsCl gradients as described in Fig. 2. The fraction of the genome which failed to replicate was determined by summing the radioactivity in the light-density peak and correcting for the doubling of mass (radioactivity) when a DNA molecule replicates. Percent unreplicated = 2(LL)/[2(LL) + (HL)]. A, t = 0 h; B, t = 2.5 h; C, t = 5.5 h.

TABLE 4. Replication efficiency of specific sequences

Chromosome	Probe	Expt	DNA replicated[a]		RI[b]
			% Total	% Probe	
III	*HIS4*	1	59	61	1.03
	LEU2	2	57	57	1.00
		2	67	72	1.08
	CEN3	2	57	60	1.05
		3	67	57	0.85
IV	*TRP1*	1	59	20	0.34
		2	57	61	1.07
		3	67	75	1.12
	CEN4	2	57	62	1.09
V	*CYC7*	2	57	55	0.96
X	*CYC1*	2	57	57	1.00
		3	67	63	0.94
XII	K2	2	57	53	0.93
		3	67	75	1.12
	rDNA	1	59	37	0.63
		2	57	19	0.33
		3	67	57	0.85
		4	61	53	0.87
2-μm DNA	2-μm DNA	1	59	47	0.80
		3	67	45	0.67
		4	61	48	0.79

[a] Calculated as described in the text.
[b] RI = percent probe DNA replicated/percent total DNA replicated.

during a synchronous S phase at 37°C. If the deficit were due to cells which failed to enter S phase, a significant fraction (30 to 40%) of unlabeled cells would be found in the autoradiograms.

The results of such an experiment are shown in Fig. 4. The experimental sample has a mean of 6.15 grains per cell which are effectively removed by DNase treatment. The experimental distribution was tested against a Poisson distribution with the same mean and was found to be significantly different ($\chi^2 = 17.56$; 9 df; $0.025 < P < 0.05$). In the experimental population, 7% of the cells were unbudded and, therefore, had not reached the *cdc2* terminal phenotype. These cells had not incorporated tritium into their DNA. When they were subtracted from the distribution, the remaining sample (Fig. 4C) showed no significant difference from the expected ($\chi^2 = 3.61$, 9 df; $P > 0.90$). Thus, although a small fraction of the DNA synthesis deficit can be ascribed to cells which failed to bud and did not enter S phase at 37°C, it appears that all of the cells that did bud made DNA. Arrested cultures were always monitored for

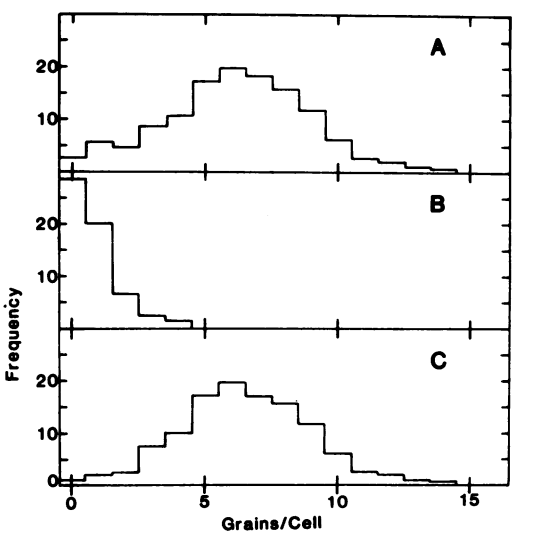

FIG. 4. Autoradiography of whole cells. Strain 19041-1 was grown overnight in Y minimal medium plus supplements (pH 5.8) to midexponential phase and blocked with α-factor for 3 h. The culture was then suspended at 37°C in fresh medium containing 20 μCi of [^{3}H]uracil per ml and 1 μg of cold uracil per ml. The culture was harvested after 3 h and prepared for autoradiography as described in the text. Slides were examined at ×1,250 with an oil immersion objective. The distributions of grains per cell are presented for: (A) cells not treated with DNase; (B) cells extensively treated with DNase; and (C) the experimental cells with the unbudded cells subtracted from the distribution.

budding, and in no case was the fraction of unbudded cells greater than 7%; usual values were about 4% unbudded. Therefore, not more than one-quarter of the deficit in DNA synthesis can be due to a fraction of cells in the population which fail to synthesize DNA.

DNA synthesis during recovery from *cdc2* arrest. Assuming that all of the cells in a *cdc2* culture recovered from an incubation at 37°C, the amount of DNA made during recovery would be equal to the amount of DNA left unreplicated at 37°C.

The experiment shown in Fig. 5 was designed to detect DNA synthesis during recovery from the *cdc2* block and to characterize the size of the molecules into which nucleotides were incorporated during this period. A culture of *cdc2* was pregrown, synchronized with α-factor, and arrested at 37°C in [^{14}C]uracil-containing medium. [^{3}H]uracil was then added, and the culture was split into four portions, with portions being maintained at 37°C or shifted to 23°C, with or without the addition of 0.2 M HU for 3 h. The sedimentation properties of DNA from the four aliquots were examined.

As shown in Fig 5, all four cultures incorporated ^{3}H into low-molecular-weight material (fractions 22 through 30). Based on its density in CsCl and its resistance to boiling in 10% trichloroacetic acid (data not shown), this material is not DNA. Only the culture that was shifted to 23°C in the absence of HU incorporated ^{3}H into high-molecular-weight DNA (Fig. 5B). This DNA synthesis cannot be attributed to cells which had divided and were in the S phase of the succeeding cell cycle because there was no increase in cell number in any of the cultures. The DNA synthesized by this culture is equivalent to 48% of the DNA present in the Gl culture. The culture maintained at 37°C (Fig. 5A) did not synthesize DNA after the addition of [^{3}H]uracil, indicating that the DNA synthesis had ceased by the time the temperature was shifted from 37 to 23°C. Addition of 0.2 M HU effectively prevented DNA synthesis during recovery from the *cdc2* block (Fig. 5C).

Three different experimental protocols show that one-third to one-half of the nuclear genome fails to replicate in *cdc2* strains at the nonpermissive temperature.

Sedimentation analysis of DNA from arrested cells. A number of defects in DNA synthesis could permit initiation of DNA replication but not allow its completion. A low supply of a nucleotide, if insufficient to allow completion of S phase, would cause accumulation of partially replicated DNA molecules. One can also speculate that certain regions of the chromosomes such as centromeres or telomeres might require special functions for their replication, and the loss of these specialized functions would give rise to diagnostic intermediates (X-shaped molecules or large circular molecules) at the nonpermissive temperature. Incompletely replicated molecules would be expected to sediment faster than nonreplicating DNA in a neutral sucrose gradient.

DNA from *cdc2*-arrested cells labeled with ^{3}H

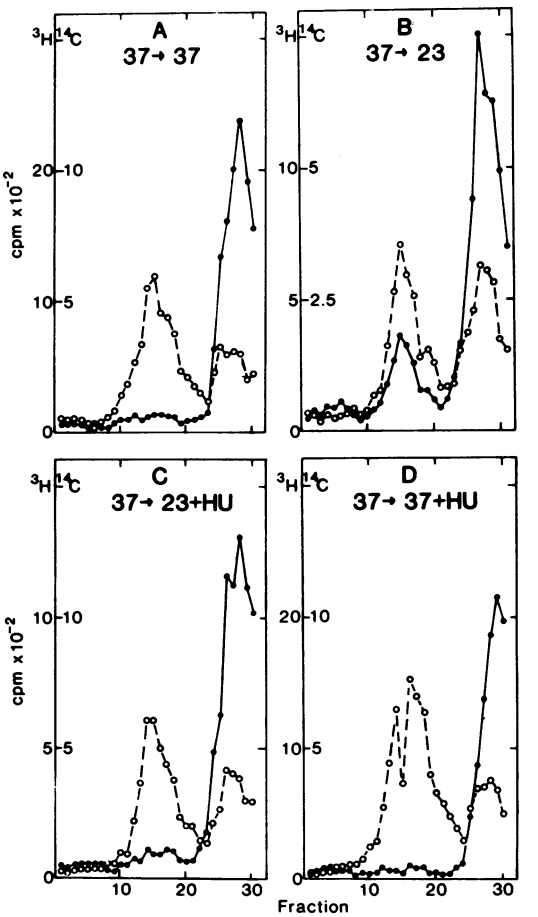

FIG. 5. Sucrose gradient analysis of DNA synthesized during recovery from the *cdc2* block. Strain 370.2.3-1 was pregrown at 23°C in Y minimal medium plus supplements (pH 5.8), containing 1 μCi of [^{14}C]uracil per ml. The culture was synchronized with α-factor and then suspended in fresh medium with 1 μCi of [^{14}C]uracil per ml and shifted to 37°C. After 3 h at 37°C, 30 μCi of [^{3}H]uracil per ml was added, and the culture was divided into four portions: (A) maintained at 37°C; (B) shifted to 23°C; (C) shifted to 23°C with the addition of 0.2 M HU; or (D) maintained at 37°C with the addition of 0.2 M HU. After an additional 3 h, a portion was taken from each culture, converted to spheroplasts, and lysed on neutral sucrose gradients. There was no increase in cell number in any of the cultures, indicating that cell division did not occur. The gradients were centrifuged in an SW40 rotor for 70 h at 8,000 rpm and 4°C Symbols: (●) ^{3}H, (○) ^{14}C.

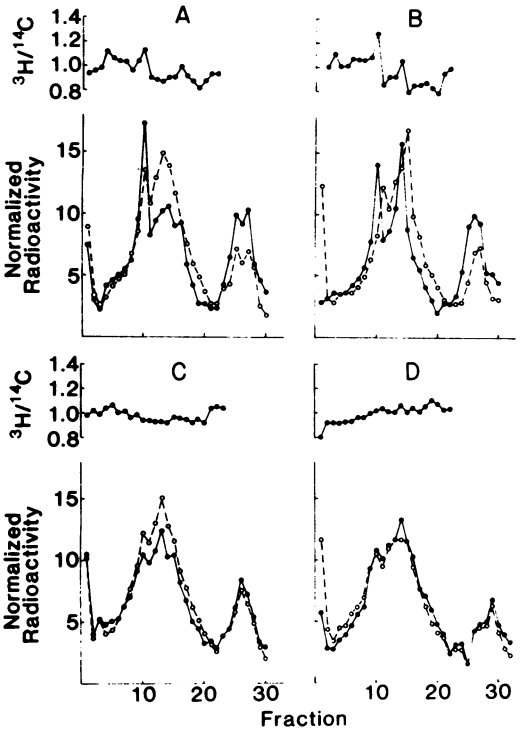

FIG. 6. Comparison of the size of DNA molecules from S-phase and *cdc2*-arrested cells with DNA from G1 cells. A culture of strain 370.2.3-1 was grown at 23°C in nonradioactive medium, arrested with α-factor, and then suspended in fresh medium containing 9 μCi of [^{3}H]uracil per ml. Part of the culture was incubated at 23°C, and the remainder was shifted to 37°C. Portions were withdrawn from the 23°C culture at 50, 65, and 80 min after release from the α-factor block, HU was added to a final concentration of 0.2 M, and the samples were kept on ice until spheroplasts were prepared. The 37°C culture was shaken for 3 h. Spheroplasts from the ^{3}H-labeled samples were mixed with spheroplasts from a culture of strain 370.2.3-1 which had been grown at 23°C to a density of 3.5×10^6 cells per ml in Y minimal medium plus supplements (pH 5.8) containing 2 μCi of [^{14}C]uracil per ml and then incubated with α-factor for 3 h to cause arrest in G1 and lysed on neutral sucrose gradients. The gradients were centrifuged in an SW40 rotor at 4°C and 8,000 rpm for 72 h. Symbols: (●) ^{3}H, (○) ^{14}C. To simplify comparison, the amount of ^{3}H and ^{14}C in each fraction is expressed as a fraction of the total number of counts per minute in the gradient. Ratios of the normalized ^{3}H to normalized ^{14}C are presented above each gradient. Since other experiments indicated that much or all of the low-molecular-weight material was not DNA, the ratios are not presented for the top fractions of the gradients. (A) [^{3}H]DNA from 50 min after release from the α-factor block. Total counts per minute: ^{3}H, 3,085; ^{14}C, 6,848. (B) [^{3}H]DNA from 65 min after release of the α-factor block. Total counts per minute: ^{3}H, 4,615; ^{14}C, 6,881. (C) [^{3}H]DNA from 80 min after release of the α-factor block. Total counts per minute: ^{3}H, 6,795; ^{14}C, 7,776. (D) [^{3}H]DNA from terminally arrested *cdc2* cells. Total counts per minute: ^{3}H, 24,019; ^{14}C, 7,776.

was sedimented with ^{14}C-labeled DNA from α-factor-arrested cells which were blocked in G1 (11) to look for size differences. To demonstrate that DNA molecules taken from S-phase cells do sediment faster than nonreplicating molecules, [^{3}H]DNA was also isolated from *cdc2* cells grown at 23°C during a synchronous S phase and centrifuged with [^{14}C]DNA from the cells blocked with α-factor.

The DNA profiles of the sucrose gradients are shown in Fig. 6. The slow-sedimenting material (fractions 21 through 30) is almost certainly not DNA (see above). The average molecular weight of [^{14}C]DNA, calculated from Freifelder's equation (13), is approximately 1,000 kb, within experimental error of the value of 875 to 900 kb expected and reported for intact chromosomal DNA (36). [^{3}H]DNA (S phase) sedimented slightly faster than [^{14}C]DNA (G1). The ^{3}H/^{14}C ratios were detectably higher on the high-molecular-weight side of the nuclear DNA peak and lower on the low-molecular-weight side (Fig. 6A and B). The failure to find two distinct peaks for [^{3}H]- and [^{14}C]DNA is probably attributable to asynchrony between cells, as well as between DNA molecules within the same cell. When DNA from terminally arrested *cdc2* cells is compared with G1 DNA (Fig. 6D), the profiles are practically identical; the ^{3}H/^{14}C ratio remains constant throughout the gradient. Assuming that most chromosomal DNA molecules are one-half to two-thirds replicated in terminally arrested cells and that the Freifelder equation applies to replicating molecules, the expected shift in sedimentation is three to four fractions. Thus, we conclude that *cdc2* mutants do not accumulate a large fraction of DNA molecules larger than G1 chromosomal DNA and that the defect in *cdc2* strains does not cause arrest of replication forks to produce partially replicated molecules.

Additional support for this conclusion was provided by electron microscopic examination of DNA from *cdc2*-arrested cells. Strains carrying an allele of *cdc2* (strains 370.2.3-1 or 19041-1) were synchronized with α-factor, washed, and suspended in fresh medium at 37°C. After 3 h (about one generation) at 37°C, DNA was prepared by centrifugation of lysates in sucrose or CsCl gradients. DNA was spread for electron microscopy by the formamide technique of Davis et al. (9). For each strain, more than 75 molecules with a minimum size of 75 kb and ranging up to several hundred kilobases in size were examined. No replication structures (double-stranded forks or bubbles) were seen in the DNA examined, which represents at least the equivalent of one genome. To demonstrate that replication structures can be visualized in DNA isolated from *cdc2* strains incubated at high

temperature, strain 370.2.3-1 was synchronized as described above, and DNA was prepared on sucrose gradients from cells harvested 1 h after release from the α-factor block. At this time, DNA synthesis was still in progress at 37°C (see Fig. 1). In this sample, 6% (5 of 81) of the molecules examined contained forks, bubbles, or both. Thus, although replicating molecules are readily detectable in DNA isolated from *cdc2* strains during S phase at 37°C, partially replicated molecules do not persist at 37°C. This suggests that molecules which initiate replication at 37°C are able to complete replication at high temperatures.

cdc9 mutants of *S. cerevisiae* are defective in ligation (22) and behave in a manner similar to *cdc2* mutants in reciprocal shift experiments (19). Incompletely ligated molecules can be detected by the presence of low-molecular-weight, single-stranded fragments in alkaline sucrose gradients.

To test the possibility that *cdc2* mutants are also defective in ligation, we compared the sedimentation properties of single-stranded DNA synthesized at 37°C with DNA made a 23°C. *cdc2* cells were grown and synchronized in the presence of [^{14}C]uracil and then washed and suspended at 37°C in fresh medium in which [^{3}H]uracil had been substituted for [^{14}C]uracil. After the equivalent of one generation at the nonpermissive temperature, DNA from the culture was sedimented in alkaline sucrose gradients (Fig. 7). The ^{3}H and ^{14}C profiles are virtually superimposable, indicating that there are no major size differences between the DNA strands made at 23°C and those made at 37°C. These results rule out the hypothesis that *cdc2* mutants are defective in ligation, since these strains do not accumulate significant quantities of the 200 to 400-nucleotide fragments expected if Okazaki fragments had not been ligated. These results also provide evidence against the hypothesis that the *cdc2* lesion prevents the fusion of replication forks. Assuming that the average distance between origins is 36 to 40 kb (33), failure of forks from adjacent origins to fuse would result in the presence of fragments with a number-average molecular weight of 40 kb (around fraction 20) that would not be present in the [^{14}C]DNA made at 23°C.

In summary, when the sedimentation properties of DNA from *cdc2*-arrested cells are compared with those of DNA from cells grown at the permissive temperature, no differences are detected in either the single-stranded or double-stranded molecular weight. Taken with the inability to find partially replicated molecules from *cdc2*-arrested cells in the electron microscope, these results suggest that those DNA molecules which replicate do so completely.

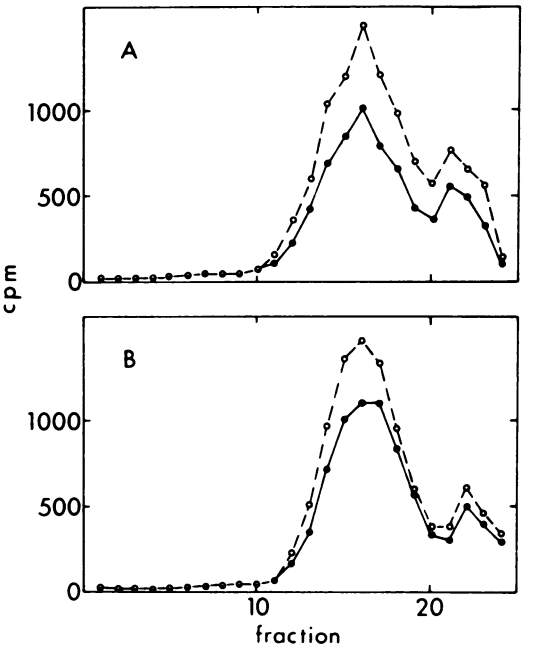

FIG. 7. Analysis of DNA synthesized by *cdc2* strains at 23 and 37°C on alkaline sucrose gradients. Strain 19041-1 (*cdc2-3*) was pregrown at 23°C for four to five generations in Y minimal medium plus supplements (pH 3.5), containing 2 μCi of [^{14}C]uracil per ml, to 3.5 × 10^{6} cells per ml and treated with α-factor for 3.5 h. The cells were then washed and suspended at 23 or 37°C in fresh medium in which the [^{14}C]uracil was replaced with 3 μCi of [^{3}H]uracil per ml. α-Factor was added again to the 23°C culture 100 min after release from the first α-factor block to prevent the start of a second cell cycle. At 180 min after release from the α-factor block, portions of each culture were converted to spheroplasts and lysed on alkaline sucrose gradients. Centrifugation was in an SW50.1 rotor at 4°C and 10,000 rpm for 38 h. (A) DNA prelabeled with [^{14}C]uracil and then labeled with [^{3}H]uracil during a synchronous S phase at 23°C. (B) DNA prelabeled with [^{14}C]uracil and then labeled with [^{3}H]uracil during a synchronous S phase at 37°C. Symbols: (●) ^{3}H; (○) ^{14}C.

Replication of specific sequences in *cdc2* mutants. All three *cdc2* alleles which we have examined prevent the replication of approximately one-third of the genome at 37°C. It is possible that the *cdc2* mutation prevents the replication of specific chromosomes or regions of chromosomes. If the *cdc2* mutation is highly specific in its action, a given region of the genome would be expected to replicate either fully or not at all at 37°C. On the other hand, if the *CDC2* function is involved in the replication of all DNA sequences and the residual DNA synthesis we observe occurs by an equally nonspecific alternate pathway a 37°C, all sequences would have the same probability of replicating at

37°C. To determine the extent of replication of specific DNA sequences in arrested *cdc2* cultures, DNA was density labeled during incubation at 37°C. Replicated DNA was separated from unreplicated DNA in CsCl gradients, and cloned DNA sequences were used as hybridization probes to determine the distribution of specific sequences in replicated and unreplicated DNA.

The results of such experiments, using two probes, are shown in Fig. 8. In control experiments (data not shown) we have demonstrated that the probes hybridize equally efficiently to BrdUMP-substituted and unsubstituted DNA. The *CYC7* probe hybridized to both the hybrid (HL)- and light (LL)-density peaks, closely following the profile of DNA in the gradient. rDNA hybridized more strongly to the light-density peak than would have been expected from the distribution of [^{3}H]DNA. The [^{32}P]rDNA profile was shifted slightly to the dense side of the ^{3}H peaks, as expected, since the guanosine- and cytosine-rich rDNA is more dense than the bulk of chromosomal DNA.

The results of hybridizations with different probe sequences are summarized in Table 4. The efficiency of replication of a given sequence is expressed by the RI. A sequence which replicates as efficiently as an average sequence, represented by total DNA, has an RI of 1. Sequences replicating less efficiently than total DNA have an RI less than 1; those sequences replicating more efficiently have an RI greater than 1. Probe sequences representing single-copy genes from five chromosomes exhibit RIs of approximately 1 (RI = 0.97 ± 0.20). This result rules out models which postulate a high degree of specificity to the sequences affected by *cdc2* mutations. However, it does appear that 2-μm DNA (RI = 0.75 ± 0.07) replicates less efficiently at 37°C than an average stretch of DNA. rDNA, even though more variable, also replicates less efficiently than average (RI = 0.67 ± 0.25). The RI of rDNA is significantly different from the mean RI of the single-copy sequences (t = 2.54; 16 df; $P < 0.05$); the mean RI of the 2-μm DNA is lower than the RI of the single copy chromosomal sequences, but at a lower level of significance (t = 1.83; 15 df; $P < 0.10$).

The fact that no sequence examined exhibited either full replication or total failure to replicate strongly suggests that the replication defect is not confined to a subset of the chromosomes or to specific regions of chromosomes. This conclusion is further strengthened by our observation that specific functional regions of chromosomes, i.e., centromeres of chromosomes III and IV, and the autonomously replicating segment (*ARS*) associated with *TRP1* (40) replicate to the same extent as total DNA. However, we cannot exclude the possibility that other sequences not represented in the probes we used replicate less efficiently than those studied.

In summary, most nuclear DNA sequences examined replicate with approximately the same efficiency as total nuclear DNA in *cdc2* strains at 37°C. Although rDNA and 2-μm DNA replicate

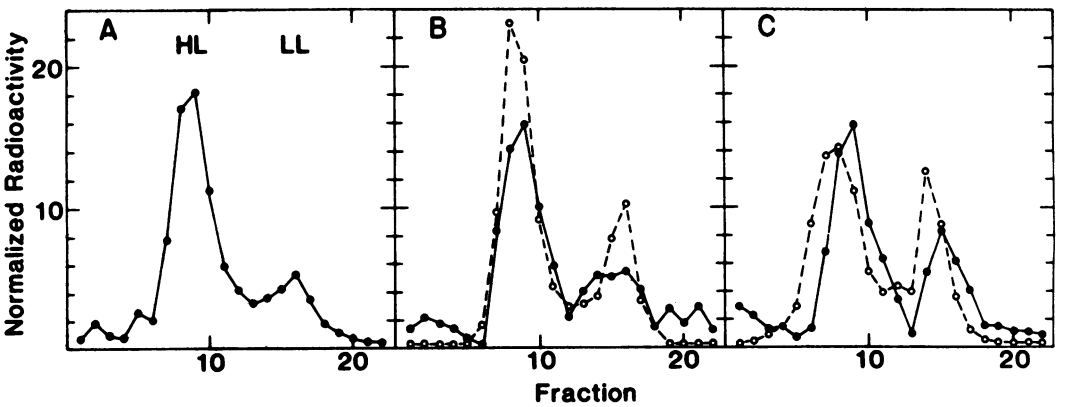

FIG. 8. Replication of *CYC7* DNA and rDNA in *cdc2* at 37°C. Strain 19041-1T1 was synchronized and incubated with BrdUMP as described in Fig. 3. After 8.5 h at 37°C, lysates were prepared, and DNA was centrifuged in CsCl gradients. DNA from 10-μl portions of gradient fractions was immobilized on nitrocellulose filters as described in the text, and the filters were hybridized to ^{32}P-labeled *CYC7* DNA or rDNA (Table 4). Quantitation of hybridization was as described in the text. Individual spots were then cut from the filters to determine the amount of ^{3}H in each spot by liquid scintillation counting. The amount of radioactivity in each spot is expressed as a fraction of the total (6,465 cpm of ^{3}H for A, 1,917 cpm of ^{3}H for B, and 2,160 cpm of ^{3}H for C). (A) The [^{3}H]DNA profile of the CsCl gradient used to prepare the filters; (B) *CYC7* probe; (C) rDNA probe. Symbols: (●) ^{3}H; (○) ^{32}P.

less efficiently than an average DNA sequence, they do not fail to replicate at all. The *cdc2* lesion, therefore, appears to affect the replication of all regions of the genome.

DISCUSSION

By measuring the incorporation of radioactive precursors into DNA and the fraction of DNA which shifts to hybrid density after incubation of cells with BrdUMP, we have shown that *cdc2* mutants fail to replicate approximately one-third of their nuclear DNA at 37°C. Autoradiography experiments demonstrated that this deficit results from individual cells failing to replicate one-third of their genomes. Despite the inability of *cdc2* strains to complete S phase, there was no significant accumulation of the faster-sedimenting DNA expected of partially replicated molecules. On alkaline sucrose gradients, there was no size difference between DNA strands made at 23°C and those made at 37°C, ruling out defects in the ligation of Okazaki fragments or fusion of replicon-sized strands. Results of the sedimentation studies were confirmed by electron microscopy of *cdc2* cell DNA. We failed to find replicating molecules in more than one genome equivalent of DNA from terminally arrested cells, even though replication intermediates could be easily visualized in DNA isolated from S-phase cells. Electron microscopy observations (M. N. Conrad and C. S. Newlon, submitted for publication) did reveal that DNA from *cdc2* strains incubated at 37°C contained small, denatured regions similar to structures seen in another *S. cerevisiae* DNA replication mutant, *cdc8* (25).

We examined the extent to which 10 different nuclear DNA sequences replicate at 37°C in *cdc2* cells. Sequences from five different chromosomes, including three sequences from chromosome III and two sequences from chromosome IV, replicate to the same extent as total DNA. This could be the result either of one-third of the copies of a chromosome in the population failing to replicate at all or of the arrest of replication forks at random sites after two-thirds of the chromosome had replicated. Our failure to detect partially replicated molecules is most consistent with the first alternative.

We propose that the *cdc2* mutants are defective in the activity of a factor which activates replication origins. This model does not necessarily imply that the *cdc2* mutation is in the structural gene for the initiation factor; the DNA synthesis defect in *cdc2* strains may be a pleiotropic effect. However, the *cdc2* mutation has no major effect on RNA synthesis, suggesting that the mutation is fairly specific for DNA replication (8). At the nonpermissive temperature, either this factor would function much less efficiently, or another, less efficient initiation system would substitute for the *cdc2* gene product. As a consequence, the number of origins activated would be reduced to the point where some chromosomes would fail to activate even a single origin and would therefore not replicate. Those that were successful in activating one or several origins would replicate fully, albeit from fewer origins than at 23°C.

This model accounts for a number of the properties of *cdc2* mutants. The execution point (the time in the cell cycle after which a shift to the nonpermissive temperature no longer prevents completion of the cell cycle) of the *CDC2* function is early in the cell cycle both in synchronous (8) and asynchronous cultures (20), roughly coincident with the onset of S phase and definitely earlier than the execution points of mutants defective in chain elongation, e.g., *cdc8* and *cdc21* mutants. The failure to detect partially replicated molecules suggests that the *cdc2* mutation affects an initiation process. The lack of pronounced sequence specificity implies that the *CDC2* function is required for the replication of all chromosomes.

Very little is known about proteins required for the initiation of replication on eucaryotic chromosomes, so it is difficult to speculate what the defect in *cdc2* mutants may be. Liu et al. (28, 29) have proposed that topoisomerase II may be required for the initiation of DNA replication in T4 DNA and DNA from eucaryotes, perhaps facilitating the opening of the helix at replication origins by catalyzing negative supercoiling of DNA in a site-specific way at the origins. It is possible that *cdc2* mutants could be defective in such an activity, and that in its absence, the initiation of replication is much less efficient and occurs only at origins which for some other reason are in the appropriate topological state.

The finding that 2-μm DNA and rDNA replicate less efficiently than the average sequence is not directly predicted by the model. The 2-μm DNA result is easy to understand because, in contrast to chromosomal DNA, which can potentially replicate with fewer than the normal number of origins, the replication of each 2-μm DNA molecule requires activation of an origin. Therefore, it might be expected that, if an initiation factor is limiting, 2-μm DNA would replicate less efficiently than chromosomal DNA molecules which have multiple origins.

The rDNA result is less easy to explain. The less efficient replication of rDNA (RI = 0.67 ± 0.3) may be the result of a low concentration of replication origins in the rDNA cluster. If each rDNA cistron replicated from its own origin, the entire cluster, about 100 tandem copies of an 8.4-kb repeat (6), could be replicated in several minutes. However, since the replication of

rDNA takes place over the entire S phase (3, 14), it is likely that rDNA is replicated from forks initiated from the outside of the cluster. Indeed, the K2 region, a single-copy segment immediately centromere-distal to the rDNA cluster (42), replicates as efficiently as an average sequence.

However, the differential replication of two sequences on the same chromosome implies that some fraction of the copies of chromosome XII arrest as partially replicated forms. These were not detected by sedimentation or electron microscopic analysis. If chromosome XII were the only one to behave in this manner, it is unlikely that we would have detected it.

The large size of chromosome XII and the presumed lack of replication origins in rDNA could account for its different properties. Chromosome XII is probably the largest *S. cerevisiae* chromosome. Genetically, the chromosome outside the rDNA cluster is 165 centimorgans (cM) long (42); by using a conversion of 2.7 kb/cM (41), the chromosome is at least 1,300 kb in length, including the rDNA. If *cdc2* mutants are forced to replicate their rDNA from an origin very distant from the cluster, the replication fork may stop before rDNA is completely replicated. Although incubation of the cells at 37°C for the equivalent of 1.5 to 2 generations should have been enough time to replicate even the longest yeast chromosome from a single origin, other factors may influence the completion of replication. rDNA might possess properties which slow the replication fork, or there may be a limited period of time (a "window") during which S phase must take place.

The three *cdc2* alleles examined in this study are remarkably uniform in preventing the replication of approximately one-third of the genome at 37°C. It is unlikely that three equally leaky mutations could have been selected unless one assumes that complete elimination of activity at 37°C is not possible without severely affecting growth at 23°C. The residual DNA synthesis at 37°C could be driven by a second, perhaps less efficient initiation pathway. Alternatively, the *cdc2* mutation may prevent the synthesis of new initiation factor at 37°C. Excess initiation factor remaining from the previous cell cycle could account for the residual synthesis.

Predictions of the model. The use of a reduced number of replication origins need not unduly prolong the length of S phase in *S. cerevisiae*. In synchronized cells at 23°C, the rate of fork movement is about 3.6 kb/min per fork, and the length of S phase is 100 min (38). In 100 min, a single pair of forks moving bidirectionally from an origin in the middle of a chromosome could replicate 720 kb, close to the 900-kb length of an average chromosome (36). Although the rate of fork movement has not been determined at 37°C in the medium employed in the density transfer experiments, we assume that the rate is roughly proportional to the generation time, as has been found with *S. cerevisiae* cultures grown on different nitrogen sources (38).

If one replication origin is sufficient to duplicate an entire chromosome, the number of chromosomal origins active in *cdc2* can be estimated. Assuming that replication has an equal probability of being initiated on every chromosome, one can calculate the mean of a Poisson distribution in which the probability of the zero class (chromosomes not initiating) is 0.3, obtaining a value of 1.2 activations per chromosome or about 20 active chromosomal origins per haploid genome. Only about one-half of the 2-μm DNA replicates at 37°C. Assuming 50 copies per cell, the observed level of 2-μm DNA synthesis would require an additional 25 active origins.

If our proposal that fewer origins are active in *cdc2* cells at the nonpermissive temperature is correct, the average distance between active origins should be greatly increased. Additionally, it is possible that manipulation of the number of replication origins could alter the amount of chromosomal DNA which can be made at 37°C. If the *CDC2* locus encodes or controls a factor necessary for initiation which is in limited supply, then increasing the number of replicons by transformation of a *cdc2* strain with a plasmid carrying an authentic replication origin would be expected to decrease the amount of chromosomal DNA replicated at 37°C, because the plasmid would compete with chromosomal origins for the initiation factor. By the same token, reducing the number of replicons per cell by curing strains of 2-μm DNA might be expected to increase the amount of DNA synthesized at 37°C by reducing competition for the factor. These experiments are in progress.

Implications. If *cdc2* mutants are, in fact, defective in an initiation factor, then our observations have several implications for the mechanism of eucaryotic DNA replication. If the number of active origins can be reduced in *S. cerevisiae*, then there must be no fixed termini of replication. Otherwise, we should have detected partially replicated molecules in terminally arrested cells. This conclusion is supported by observations in *Drosophila* sp. and *Triturus* sp. which demonstrated a change in origin spacing with developmental stage (2, 5). In addition, it appears that a single gene product may be involved in activating most, if not all, replication origins in the genome. Thus, there may be two classes of DNA replication initiation factors: those, like the *CDC2* gene product, which influence the activity of most origins, and other factors which influence only a subset of origins

and which are involved in activating additional origins in fast-growing cells (2, 5) or in the timing of replication of regions of chromosomes (7, 27). Alternatively, the change in origin spacing with the developmental stage could result from an increased supply of a single factor. The temporal order of replication in S phase could then be determined by the affinity of different regions of the genome for a single factor and the increase in supply of that factor throughout the course of S phase.

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