

Addition of extra origins of replication to a minichromosome suppresses its mitotic loss in *cdc6* and *cdc14* mutants of *Saccharomyces cerevisiae*

EILEEN HOGAN AND DOUGLAS KOSHLAND

Department of Embryology, Carnegie Institute of Washington, 115 West University Parkway, Baltimore, MD 21210

Communicated by Alan Spradling, December 13, 1991 (received for review July 29, 1991)

ABSTRACT Many cell division cycle (*cdc*) mutants of *Saccharomyces cerevisiae* exhibit elevated mitotic loss of pDK243, a 14-kilobase minichromosome with a centromere and one autonomous replicating sequence (ARS). Tandem copies of different ARSs were added to pDK243. The addition of these ARS clusters to pDK243 had no effect on its mitotic loss in *cdc7* (protein kinase), *cdc9* (DNA ligase), or *cdc16* or *cdc17* (DNA polymerase) mutants. However, in *cdc6* and *cdc14* mutants, the mitotic loss of pDK243 with an ARS cluster was suppressed by a factor of 6–8 compared to pDK243 without the cluster. This suppression was dependent upon the number of ARSs in the cluster and the integrity of the ARS consensus sequence in each ARS of the cluster. ARSs are known to be DNA replication origins. Therefore, the suppression of minichromosome loss by ARSs in *cdc6* and *cdc14* mutants suggests that these mutants are defective in the initiation of DNA replication. Since the CDC6 protein appears to act at the G₁/S phase transition, the CDC6 protein may be a factor required at the beginning of S phase to initiate DNA replication at origins. In contrast, the CDC14 protein acts after mitosis. We suggest that the CDC14 protein performs a function late in the cell cycle that may be required for efficient initiation of DNA replication during S phase of the next cell cycle.

Many of the proteins that mediate the initiation of eukaryotic chromosome replication have not been identified. One approach to isolating these proteins is to assume that a subset of them will bind to DNA replication origins. In the yeast, *Saccharomyces cerevisiae*, specific nucleotide sequences [the autonomous replicating sequences (ARSs)] that promote autonomous replication of artificial chromosomes have been identified (1, 2). These ARSs have been shown to be DNA replication origins on endogenous and artificial chromosomes (3–6). A comparison of the nucleotide sequence of ARSs revealed a conserved central core of 11 base pairs (bp); 3' to the thymidine-rich strand of the core are ≈100 bp of A+T-rich DNA (7). The mitotic loss rate of artificial chromosomes with a single wild-type or mutant ARS has been used as a measure of the relative ability of various ARSs to act as origins (8–11). By using this assay, the core sequence and its 3' flanking sequence have been shown to be required for the origin function of all ARSs whereas sequences 5' to the core are important for maximal origin function in some ARSs. Several proteins that bind to the core sequence or flanking sequences have been identified by genetic or biochemical methods (12–15). To date, however, a direct role for these proteins in DNA replication has not been demonstrated.

The products of the cell division cycle (CDC) genes are also potential candidates for proteins that are involved in the initiation of DNA replication. These genes are defined by temperature-sensitive mutants that arrest at distinct stages of

the cell cycle when shifted to the nonpermissive condition (16). When *cdc* mutants are grown at temperatures that partially inactivate their products, a subset has elevated mitotic loss of endogenous chromosomes and artificial minichromosomes (17, 18). Hartwell and Smith (17) suggested that the proteins encoded by this subset of CDC genes might be involved in some aspect of normal chromosome metabolism such as DNA replication or chromosome segregation. In fact, three of the CDC genes in this subset encode DNA polymerases (*CDC2* and *CDC17*) (19–21) and DNA ligase (*CDC9*) (22). Another two genes in this subset, *CDC6* and *CDC7*, encode proteins that function at a step in the cell cycle just prior to DNA elongation. The time of action of these two proteins makes them candidates for regulating or participating directly in the initiation of DNA replication. *CDC7* protein is a serine threonine kinase (23, 24), whereas the function of *CDC6* protein is unknown.

In this report, we investigated the role of CDC proteins in the initiation of DNA replication. Specifically, we asked whether minichromosome loss induced in a subset of *cdc* mutants could be suppressed by adding multiple ARSs to the minichromosome. We chose to analyze four *cdc* mutants that arrest in S phase, the *cdc6* and *cdc7* mutants and two mutants known to be defective in DNA elongation, *cdc17* and *cdc9*. In addition, we analyzed two other *cdc* mutants, *cdc16* and *cdc14*, that also exhibit a dramatic increase in minichromosome loss (18). The *CDC16* and *CDC14* proteins appear to function at steps in the cell cycle after the completion of DNA replication (25, 26). We found that the addition of multiple ARSs to a minichromosome suppressed its loss in *cdc6* and *cdc14* mutants but not in *cdc7*, *cdc9*, *cdc16*, or *cdc17* mutants. These observations are discussed in light of our knowledge of the properties of these CDC proteins and their time of function in the cell cycle.

MATERIALS AND METHODS

Plasmid Construction. A 374-bp restriction fragment that contained the wild-type H4 ARS had been isolated and subjected to *in vitro* mutagenesis (9, 11). *EcoRI*–*HindIII* restriction fragments containing the various ARS derivatives were ligated with *EcoRI*/*HindIII*-digested pDK302, a vector with a yeast centromere (*CEN3*), a yeast selectable marker (*URA3*), and an *Escherichia coli* selectable marker and origin of replication (27). Alternatively, these wild-type and mutant H4 ARS fragments were introduced in either pUC8 or pIC19H (28). A *Xho* I restriction site was introduced into the polylinker of these plasmids by ligating a *Xho* I linker into the *Sma* I site of the polylinker. These vectors were digested with *Xho* I and *Sal* I, which released a 380-bp *Xho* I–*Sal* I restriction fragment that contained all of the H4 ARS DNA and a few extra bases from the polylinker. These fragments

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: *cdc* or CDC, cell division cycle; ARS, autonomous replicating sequence.

were isolated and ligated to the vector pDK243 (29) cut with *Sal*I (see Fig. 1). The ligations were done with excess fragment to increase the probability of obtaining multiple inserts. The recombinant molecules were recovered in the *E. coli* DB6329 by selecting for complementation of the *leuB* marker of DB6329 by the yeast *LEU2* gene on the vector (29). Plasmid DNA was recovered from several independent isolates and digested with *Nru*I and *Eco*RI. The *Sal*I site of pDK243 lies within a *Nru*I–*Eco*RI restriction fragment. The number of ARS fragments inserted into the *Sal*I site of recombinant molecules was determined by taking the difference between the molecular weights of the *Nru*I–*Eco*RI fragments of the recombinant and parental plasmids and dividing this difference by the molecular weight of the ARS restriction fragment. By digestion of plasmids with *Sal*I and *Eco*RI, recombinant clones were identified in which all of the ARS inserts were in a directly repeated array and the orientation of the array relative to flanking plasmid sequences was the same.

Mitotic Loss of Minichromosomes with Various ARS Derivatives in *cdc* Mutants. Recombinant plasmids derived from pDK243 were transformed into a series of congenic strains containing *leu2 ade2 ade3 his7* markers and various *cdc* mutations (18). To test the effect of different numbers of wild-type and mutant ARSs on minichromosome transmission, transformants of each *cdc* strain were used to inoculate 5 ml to 50 ml of YPD medium (30) supplemented with adenine (10 μ g/ml) and grown overnight at 23°C. When cells in the cultures reached a density of 3×10^6 to 1×10^7 cells per ml, a sample was diluted and spread onto YPD plates. The cultures were then shifted to 36°C and incubated for 3 h. At the end of 3 h, >90% of the cells were arrested at the appropriate stage of the cell cycle, indicating that the CDC gene product in the majority of cells had been inactivated. Arrested cells were diluted and spread onto YPD plates at 23°C where they reentered the cell cycle. The dilution and plating were done rapidly to ensure that arrested cells did not complete a cell division before they were plated. Cells plated prior to or after the pulse at 36°C were allowed to form colonies and develop color. To determine the total rate of minichromosome loss in these cells, ≈ 500 colonies were scored for half-sectoring colony morphology (18, 31). The average loss rate for each minichromosome prior to or after the pulse at 36°C was determined from the loss rates observed for four to eight trials using several transformants.

Origin Function of Wild-Type and Mutated H4 ARSs. To test the relative amount of origin function for the wild-type and mutant ARSs, these ARSs were inserted into pDK302, a plasmid that contained a centromere and selectable marker but lacked a canonical ARS (27). Derivatives of pDK302 containing a single copy of these ARSs were transformed into 4513-216 (*MATa his3 leu2 ade2 ade3 ura3 can1 sap3*) (27). Four transformants were picked from selective plates and inoculated into 5 ml of YPD medium supplemented with adenine. Immediately after inoculation (t_0) and after seven to nine generations of growth (t_f), cells from the YPD cultures were diluted and spread onto plates that were selective or nonselective for the minichromosome. By counting the col-

onies, we determined the total number of cells in the cultures at t_0 and t_f and the fraction of those cells with the minichromosome. These values were used to calculate the mitotic loss rates of the plasmids as described (27, 29). The average mitotic loss rate for each plasmid and the standard deviation was determined from four trials.

RESULTS

Rationale for Experimental Design. If a protein is involved in the initiation of DNA replication, then a reduction in the quality or quantity of its function might lead to a reduced efficiency of initiation of DNA replication and to subsequent chromosome loss. Under limiting CDC function, addition of extra DNA replication origins to the chromosome might improve its probability of establishing an active origin that would result in a reduction in chromosome loss. To test this possibility, we examined the mitotic loss rate of a minichromosome, pDK243, with and without extra ARSs in various *cdc* mutants. pDK243 is an artificial circular chromosome containing a single origin of replication (*ARS1*), a centromere (*CEN3*), a selectable marker (*LEU2*), and a marker (*ADE3-2p*) for following changes in chromosome ploidy (Fig. 1). Clusters of origins were added to pDK243 by ligating restriction fragments with ARSs into the unique *Sal*I site of this plasmid (see Fig. 1). These plasmids were transformed into congenic strains that contained different *cdc* mutations. The mitotic loss rates of pDK243 with and without additional ARSs were measured in *cdc* cells incubated at the permissive temperature only or in *cdc* cells incubated for 3 h at the nonpermissive temperature and thus temporarily depleted for the function.

Mitotic Loss of pDK243 and pDK368-7 Minichromosomes in Various S-Phase *cdc* Mutants. pDK368-7 is a derivative of pDK243 that contains seven copies of a 374-bp restriction fragment that includes the H4 ARS. We examined the mitotic loss of this plasmid and pDK243 in *cdc6*, *cdc7*, *cdc9*, or *cdc17* mutants. The mitotic loss of pDK243 was 7- to 30-fold greater in these *cdc* mutants after brief exposure to the nonpermissive temperature than in *CDC*⁺ cells (Fig. 2) or in these *cdc* mutants grown at their permissive temperature only (data not shown). Therefore, as we reported (18), the mitotic loss rate of pDK243 was induced by conditions that reduced the function of these four CDC proteins. The mitotic loss of pDK368-7 was 7.5-fold less than pDK243 in *cdc6* cells incubated at the nonpermissive temperature, 1.4-fold less in *cdc7* cells, and no different in *cdc9* and *cdc17* cells. Therefore, the addition of multiple H4 ARS inserts to pDK243 suppressed its loss dramatically in *cdc6* cells but poorly or not at all in *cdc7*, *cdc9*, or *cdc17* mutants.

Origin Function of H4 ARS Inserts and Suppression of Mitotic Loss in *cdc6* Mutants. Only part of the H4 ARS restriction fragment is devoted to origin function (9, 11). Therefore, it was necessary to determine whether the origin function of the fragment was responsible for its ability to suppress pDK243 mitotic loss in *cdc6* cells. First, we asked whether a restriction fragment that contained another yeast

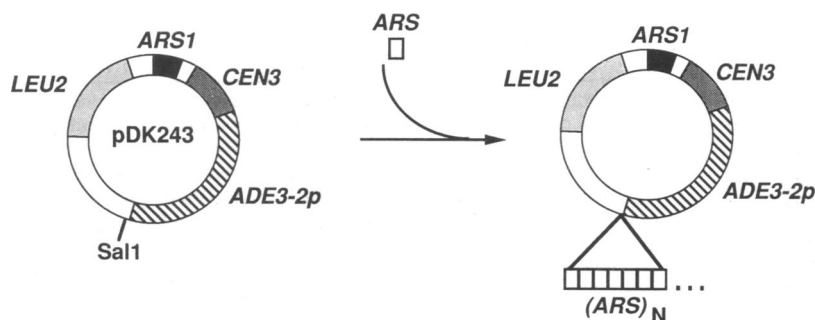


FIG. 1. Plasmid pDK243 contains the *LEU2* gene for selection, the centromere DNA from chromosome III (*CEN3*), an origin of replication (*ARS1*), and the *ADE3-2p* gene, a color marker for following minichromosome loss during mitotic cell divisions (29). A 374-bp restriction fragment that contained the H4 ARS or derivatives of this fragment (see Fig. 3) were ligated into the unique *Sal*I site. Ligations were done under conditions where multiple tandem inserts of the H4 ARS restriction fragments were obtained. The number of restriction endonuclease digestion.

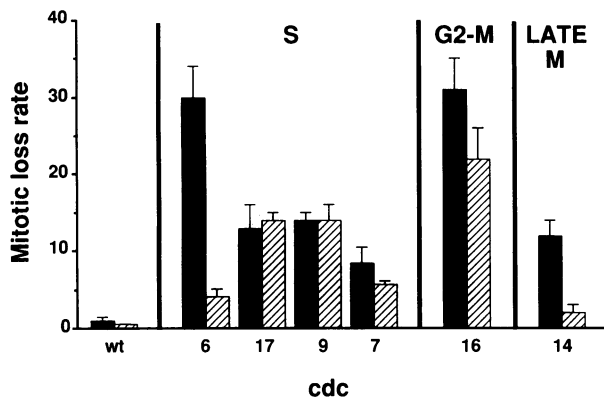


FIG. 2. Comparison of mitotic loss rate of pDK243 and pDK368-7 in *cdc* mutants. pDK243 and pDK368-7 (pDK243 with seven wild-type H4 ARS inserts) were transformed into a wild-type strain and a congenic series of *cdc* mutants. The mitotic loss rate of these plasmids was measured in cell divisions where the *cdc* mutant had been incubated at the 36°C (the nonpermissive temperature) for 3 h and then returned to 23°C (the permissive temperature). The ordinate indicates the *cdc* genotype of the strain. The abscissa is the rate of mitotic loss of pDK243 (solid bars) or pDK368-7 (hatched bars) expressed as the percent of cell divisions where a mitotic loss event was observed. The mutants are grouped by the stage of the cell cycle (S, G₂/M, or late M) where they arrest. Error bars indicate standard deviations obtained from three or more trials.

origin, *ARS1* (1), was also able to suppress the mitotic loss of pDK243 in *cdc6* cells. A derivative of pDK243 was constructed that contained seven copies of a 500-bp *ARS1* restriction fragment. The mitotic loss rate of this derivative was suppressed 5-fold relative to pDK243 in *cdc6* cells incubated at the nonpermissive temperature (data not shown). The ability of both the H4 ARS and *ARS1* restriction fragments to confer similar amounts of suppression was consistent with their origin function being responsible for suppression, especially since origin function is the only biological function that these two fragments are known to share.

Second, we asked whether the same nucleotides of the H4 ARS restriction fragment are required for origin function and for suppression of the loss of pDK243 in *cdc6* cells. Derivatives of the H4 ARS restriction fragment were obtained that had nucleotide substitutions or deletions (Fig. 3). To assess the amount of origin function associated with these restriction fragments, we introduced a single copy of each into pDK302. This plasmid contained a centromere and selectable marker but lacked a canonical ARS sequence (27). The recombinant molecules were transformed into a CDC⁺ strain and their mitotic loss rates were determined (Table 1). The parental plasmid pDK302 was lost in 26% of cell divisions. The ability of this plasmid to be transmitted in yeast (albeit poorly) in the absence of a canonical ARS was probably due to the presence of weak ARS activity associated with CEN3 (15). Addition of the wild-type H4 ARS restriction fragment to pDK302 reduced the mitotic loss rate to 2% per cell divisions, a rate typical for small minichromosomes with a single well-functioning origin (29, 32). In contrast, plasmids had the same high rate of mitotic loss as pDK302 when they contained an H4 ARS restriction fragment with a substitution of the central core (89/32) or deletions of the core and either 3' (L60) or 5' (R9) flanking sequences. Therefore, these three mutations abolish origin function of the H4 ARS as expected from their characterization (9, 11). Finally, the restriction fragment with a deletion of sequences 5' to the core (R8) had a mitotic loss rate intermediate between pDK302 with and without the wild-type H4 ARS insert, indicating that the R8 restriction fragment had intermediate origin function.

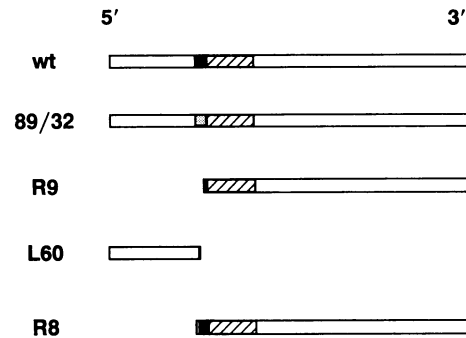


FIG. 3. Wild-type and mutated H4 ARS restriction fragments. Wild-type (wt) H4 ARS restriction fragment is 374 bp. Solid box represents the 11-bp core. The 5' and 3' flanking sequences are to the left and right of the box, respectively. The 3' sequences essential for origin function are indicated by the hatched area (9). The isolation and nucleotide sequence of these mutated derivatives of the H4 ARS restriction fragment have been reported (9, 11). Mutation 89/32 contains a 12-bp substitution for the central core. R9 contains the 3' flanking sequences and 6 bp of the core. L60 contains the 5' flanking sequences and 6 bp of the core. R8 contains the 3' flanking sequence, the entire core, and 2 bp of 5' flanking sequence. The A+T content was 70% for the wt, R9, and R8 inserts and 68% for the 89/32 insert.

Seven copies of each of the mutated H4 ARS restriction fragments were inserted in tandem into the unique *Sal* I site of pDK243 (Fig. 1). These recombinant plasmids were transformed into *cdc6* cells. After brief exposure of the *cdc6* cells to the nonpermissive temperature, the mitotic loss rate for pDK398-7 was identical to pDK243 (Table 2); thus a substitution of just the H4 ARS central core abolished both the ability of the H4 ARS restriction fragment to suppress the mitotic loss of pDK243 and its origin activity. Similarly, deletions of the H4 ARS restriction fragment that abolished (L60 or R9) or reduced (R8) its origin function also abolished or reduced its ability to confer suppression (Tables 1 and 2). Finally, mutant inserts that failed to suppress had either the same length or A+T content as the wild-type insert; thus neither of these two properties of the wild-type insert could account for its ability to suppress. Therefore, the insert's origin function was apparently necessary for it to suppress the mitotic loss of pDK243 in *cdc6* cells.

Suppression of Mitotic Loss of Minichromosomes as a Function of the Number of H4 ARS Inserts. The previous experiments suggested that an insert must have origin function to suppress the mitotic loss of pDK243 in *cdc6* cells. Next, we determined whether the amount of suppression was depen-

Table 1. Mitotic loss in CDC⁺ cells for minichromosomes with a single wild-type or mutant ARS

Minichromosome	ARS	Allele	Rate of minichromosome loss
302	H4	—	28 (4)
403	H4	wt	2 (1)
404	H4	89/32	27 (5)
385	H4	R9	34 (4)
405	H4	L60	24 (3)
384	H4	R8	7 (4)
370	ARS1	wt	1 (1)

Derivatives of pDK302 that contained a single copy of one of the H4 ARS or ARS1 restriction fragments were constructed and transformed into a CDC⁺ strain, 4513-216. The H4 ARS alleles in these minichromosomes are presented in Fig. 3. The fraction of cells that retained these minichromosomes after seven to nine generations of nonselective growth was determined for four transformants. These values were used to calculate the average mitotic loss rate of each minichromosome. The standard deviation for each measurement is in parentheses. A greater origin function of the ARS correlates with a lower mitotic loss rate. wt, Wild type.

Table 2. Suppression of minichromosome loss in *cdc6* and *cdc14* mutants by the addition of multiple ARS inserts

Minichromosome	H4 ARS insert		Rate of minichromosome loss	
	No.	Allele	<i>cdc6</i>	<i>cdc14</i>
243	0	—	30 (4)	12 (1)
368-7	7	wt	4 (1)	2 (1)
398-7	7	89/32	30 (3)	11 (2)
391-7	7	R9	34 (9)	15 (1)
397-7	7	L60	27 (3)	10 (1)
390-7	7	R8	21 (1)	2 (0.5)

Derivatives of pDK243 were constructed by insertion of seven tandem copies of a particular H4 ARS restriction fragment at the *Sal* I site (see Fig. 1). The mitotic loss rates of pDK243 and these derivatives were measured in cell divisions where the *cdc6* and *cdc14* proteins were temporarily inactivated by growing cells briefly at the nonpermissive temperature (see Fig. 2). Mitotic loss rates are expressed as the percent of cell divisions where a mitotic loss event was observed. Standard deviations are in parentheses. wt, Wild type.

dependent upon the number of inserts with origin function. For this purpose, we constructed minichromosomes with different numbers of wild-type H4 ARS inserts. These minichromosomes were then introduced into *cdc6* cells, and the rates of loss of these plasmids were determined in cells that were exposed briefly to the nonpermissive temperature (Fig. 4). A greater number of H4 ARS inserts in pDK243 correlated with a lower mitotic loss in *cdc6* cells. Therefore, the amount of suppression of the mitotic loss of pDK243 in *cdc6* cells was dependent upon the number of inserts with origin function.

Mitotic Loss of Minichromosomes in *cdc14* and *cdc16* Cells. We also examined the mitotic loss of pDK243 with and without extra origins in *cdc16* and *cdc14* mutants. After exposure of *cdc16* cells to the nonpermissive temperature, the mitotic loss rate of pDK368-7 was only 1.3-fold less than pDK243 (Fig. 2). Therefore, the mitotic loss of pDK243 in the *cdc16* mutant could only be weakly suppressed by addition of extra wild-type H4 ARS sequences. In contrast, the mitotic loss rate of pDK243 with inserts of wild-type H4 ARS (Fig. 3), R8 H4 ARS (Fig. 3), or ARS1 (data not shown) was 6-fold less than pDK243 in *cdc14* mutants (Table 2). These inserts all have origin function (Table 1). No suppression was observed for pDK243 with R9, 89/32, and L60 inserts (Table 2). These inserts all lacked origin function (Table 1). Therefore, the suppression of mitotic loss of pDK243 in *cdc14* cells apparently required that the insert in pDK243 have origin function. The amount of suppression in *cdc14* cells also increased with the number of wild-type H4 ARS inserts (Fig. 4). Unlike the *CDC6* gene, multiple alleles of the *CDC14* gene have been isolated. Minichromosome loss and ARS suppression were similar for the *cdc14-1* allele (Figs. 2-4 and Table 2) and the *cdc14-8* allele (data not shown), suggesting that the ARS suppression was not confined to a single allele of *CDC14* gene. In conclusion, the ARS suppression of pDK243 loss was very similar in *cdc6* and *cdc14* cells. However, the R8 H4 ARS insert with partial origin function was capable of suppressing the mitotic loss rate of pDK243 in *cdc14* cells to a much greater extent than in *cdc6* cells (Table 2).

DISCUSSION

In this report we showed that the mitotic loss of pDK243, a minichromosome with a single ARS, was induced in *cdc6*, *cdc7*, *cdc9*, *cdc14*, *cdc16*, and *cdc17* mutants. Tandem copies of restriction fragments with either the H4ARS or ARS1 were inserted into pDK243. The addition of a wild-type ARS cluster to pDK243 had no effect on its mitotic loss in *cdc7* (protein kinase), *cdc9* (DNA ligase), or *cdc16* or *cdc17* (DNA

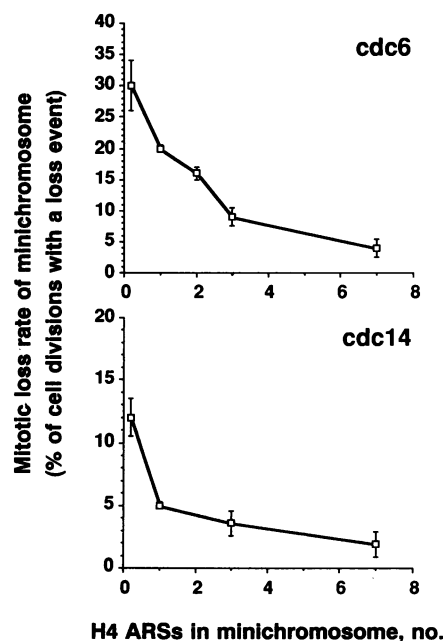


FIG. 4. Mitotic loss rate of minichromosomes with various numbers of wild-type H4 ARS inserts in *cdc6* and *cdc14*. Plasmids were constructed that contained various numbers of the wild-type H4 ARS inserts in the unique *Sal* I site of pDK243 (see Fig. 1). These plasmids were transformed into *cdc6* and *cdc14* cells. The mitotic loss rates of pDK243 and these derivatives were measured in cell divisions where the *cdc6* and *cdc14* proteins were temporarily inactivated by growing cells briefly at the nonpermissive temperature (see Fig. 2). Mitotic loss rates are expressed as the percent of cell divisions where a mitotic loss event was observed. Error bars indicate the standard deviations obtained from three or more trials.

polymerase) mutants. In contrast, in *cdc6* and *cdc14* mutants, the mitotic loss of pDK243 with ARS clusters was suppressed by a factor of 6-8 compared to pDK243. This suppression was dependent upon the number of ARSs in the cluster and the integrity of the ARS consensus sequence in each ARS of the cluster.

ARSs are known to be DNA replication origins (3-6). Therefore, one explanation for the ability of ARS clusters to suppress pDK243 loss in *cdc6* and *cdc14* mutants is that the *CDC6* and *CDC14* proteins are involved in the initiation of DNA replication. In this case, a reduction in the quality or quantity of their function might lead to a reduced efficiency of initiation of DNA replication and to subsequent minichromosome loss. The addition of extra DNA replication origins to the minichromosome might improve its probability of establishing an active origin, which would be reflected in the suppression of minichromosome loss. Alternatively, *CDC6* and *CDC14* could participate in DNA elongation. For example, under conditions of limiting *CDC6* or *CDC14* function, a replication fork might occasionally terminate prematurely. Addition of an extra origin to the minichromosome might create a second replication fork that would complete the replication of the molecule.

Of these two hypotheses, we favor that the *CDC6* and *CDC14* proteins may be involved in the initiation of DNA replication rather than DNA elongation. (i) The addition of ARS clusters to minichromosomes did not suppress their loss in *cdc17* and *cdc9* mutants, which are defective in activities specifically required for DNA elongation (19-22). (ii) The fact that suppression in *cdc6* and *cdc14* mutants is dependent upon the number of ARSs in the cluster is difficult to reconcile with these mutants being defective in DNA elongation.

Several published observations are also consistent with the hypothesis that the *CDC6* and *CDC14* proteins are involved in

DNA initiation rather than DNA elongation. (i) The CDC6 and CDC14 proteins act at stages of the cell cycle distinct from known DNA elongation proteins (see below). (ii) The type of minichromosome loss in *cdc6* and *cdc14* mutants is distinct from other *cdc* mutants including those affecting DNA elongation. Two types of chromosome loss events can be distinguished by a visual assay for chromosome ploidy (29, 31); the two daughter cells of a division either receive 1 and 0 (1:0) or 2 and 0 (2:0) copies of a chromosome. Out of 13 *cdc* mutants tested, only the *cdc6* and *cdc14* mutants exhibited elevated levels of 1:0 minichromosome loss events exclusively (18). The 1:0 events are the expected outcome for cells that fail to initiate minichromosome replication because they are depleted of a DNA initiation factor. In contrast, in cells depleted for DNA elongation activities such as polymerases or ligase, both 1:0 and 2:0 minichromosome loss events are induced significantly (18). Defects in DNA elongation induce missegregation (2:0 events) of circular minichromosomes because they apparently stimulate sister chromatid recombination (18). Therefore, the induction of only 1:0 minichromosome loss events in *cdc6* and *cdc14* mutants is consistent with the CDC6 and CDC14 proteins being required for DNA initiation but not elongation.

A priori, one would expect that proteins required for the initiation of DNA replication might act at the beginning of S phase. The *cdc6* mutant was found to incorporate significant amounts of radioactive nucleotides into its DNA at the nonpermissive condition. However, reciprocal shift experiments between the *cdc6* mutant and hydroxyurea, the DNA elongation inhibitor, suggest that the CDC6 protein apparently functions at a step in the cell cycle before DNA elongation (25). To reconcile these two observations, Hartwell (25) suggested that the incorporation of nucleotides in the *cdc6* mutant results from unscheduled repair synthesis that occurs in the absence of proper initiation. Consistent with this conclusion, cells depleted of CDC6 function arrest with a G₁ phase content of DNA as determined by fluorescence-activated cell sorting (R. E. Palmer and D.K., unpublished results). Therefore, the apparent G₁/S function of CDC6 protein coupled with ARS suppression of minichromosome loss in the *cdc6* mutant (this study) suggests that the CDC6 protein may participate directly in the initiation of DNA replication. The CDC6 gene has been cloned and sequenced (33). The predicted primary amino acid structure of CDC6 has revealed that it has a consensus nucleotide binding site (33, 34) and some similarity to α subunit of guanine nucleotide binding proteins (34). Clearly, more information on function of the CDC6 protein will be necessary before one can understand the significance of these structural motifs in its putative role in initiation of DNA replication.

In contrast to the G₁/S function of the CDC6 protein, several observations suggest that the CDC14 protein acts at a step late in the cell cycle. (i) The terminal arrest phenotype of *cdc14* mutants is a telophase-like state (16), and (ii) reciprocal shift experiments between a *cdc14* mutant and the microtubule inhibitor nocodazole suggest that the CDC14 protein functions after mitosis (26). One might suggest that the late cell cycle function of the CDC14 protein precludes its involvement in the initiation of DNA replication, and therefore, the ARS suppression of minichromosome loss in the *cdc14* mutants must reflect some spurious effect. However, a more exciting hypothesis is that the late cell cycle function of the CDC14 protein is required for the efficient use of origins of DNA replication in the next cell cycle. Interestingly, the analysis of DNA replication in *Xenopus laevis* extracts (35) and the nuclear localization of a putative yeast replication factor (36) have led to the proposal that events late in the cell cycle might be important for DNA replication in the next cell cycle. Since the function of the CDC14 protein is unknown, we can only speculate on its potential role in DNA replication. It may act either in a global process (i.e., chromosome packaging or

assembly of various nuclear structures) or a specific replication event (i.e., assembly of preinitiation complexes between origins and replication factors) that is required for the efficient initiation of DNA replication in the next cell cycle.

The observation that the H4 ARS with deletion of 5' flanking sequences was able to suppress minichromosome loss in the *cdc14* but not *cdc6* mutant suggests that the mode of suppression cannot be completely identical in the two mutants. Although this result is intriguing, it seems premature to speculate on the molecular explanation for this difference until more is known about the molecular function of the CDC6 and CDC14 proteins.

We thank Mitchell Smith for providing us with the wild-type and mutant H4 ARSs. We thank Tim Formosa, Bob Umek, Andy Fire, Ayumu Yamamoto, Vincent Guacci, and Bino Palmer for critical reading of the manuscript. We thank Susan Fox for help in preparation of the manuscript. D.K. is a Lucille P. Markey Scholar and this work was supported in part by a grant from the Lucille P. Markey Charitable Trust.

1. Stinchcomb, D. T., Struhl, K. & Davis, R. W. (1979) *Nature (London)* **282**, 39–43.
2. Hsiao, C. L. & Carbon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 3829–3833.
3. Brewer, B. J. & Fangman, W. L. (1987) *Cell* **51**, 463–471.
4. Huberman, J. A., Spotila, L. D., Nawotka, K. A., El-Assouli, S. M. & Davis, L. R. (1987) *Cell* **51**, 473–481.
5. Huberman, J. A., Zhu, J., Davis, L. R. & Newlon, C. S. (1988) *Nucleic Acids Res.* **16**, 6373–6384.
6. Ferguson, B. M., Brewer, B. J., Reynolds, A. E. & Fangman, W. L. (1991) *Cell* **65**, 507–515.
7. Broach, J. R., Li, Y. Y., Feldman, J., Jayaram, M., Abraham, J., Nasmyth, K. A. & Hicks, J. B. (1983) *Cold Spring Harbor Symp. Quant. Biol.* **47**, 1165–1173.
8. Celniker, S. E., Sweder, K., Srienc, F., Bailey, J. E. & Campbell, J. L. (1984) *Mol. Cell. Biol.* **4**, 2455–2466.
9. Bouton, A. H. & Smith, M. M. (1986) *Mol. Cell. Biol.* **6**, 2354–2363.
10. Srienc, F., Bailey, J. E. & Campbell, J. L. (1985) *Mol. Cell. Biol.* **5**, 1676–1684.
11. Holmes, S. G. & Smith, M. M. (1989) *Mol. Cell. Biol.* **9**, 5464–5472.
12. Umek, R. M., Linskens, M. H. K., Kowalski, D. & Huberman, J. A. (1989) *Biochim. Biophys. Acta* **1007**, 1–14.
13. Schmidt, A. M. A., Herterich, S. U. & Krauss, G. (1991) *EMBO J.* **10**, 981–985.
14. Hofmann, J. F.-X. & Gasser, S. M. (1991) *Cell* **64**, 951–960.
15. Campbell, J. L. & Newlon, C. S., eds. (1991) in *The Molecular and Cellular Biology of the Yeast Saccharomyces: Genome Dynamics, Protein Synthesis, and Energetics* (Cold Spring Harbor Lab., Cold Spring Harbor, NY), Vol. 1.
16. Pringle, J. R. & Hartwell, L. H. (1981) in *The Molecular Biology of the Yeast Saccharomyces: Life Cycle and Inheritance*, eds. Strathern, J. N., Jones, E. W. & Broach, J. R. (Cold Spring Harbor Lab., Cold Spring Harbor, NY), pp. 97–142.
17. Hartwell, L. H. & Smith, D. (1985) *Genetics* **110**, 381–395.
18. Palmer, R. E., Hogan, E. & Koshland, D. (1990) *Genetics* **125**, 763–774.
19. Sitney, K. C., Budd, M. E. & Campbell, J. L. (1989) *Cell* **56**, 599–605.
20. Carson, M. J. (1987) Ph.D. thesis (Univ. of Washington, Seattle).
21. Boulet, A., Simon, M., Faye, G., Bauer, G. A. & Burgers, P. M. J. (1989) *EMBO J.* **8**, 1849–1854.
22. Barker, D. G. & Johnston, L. H. (1983) *Eur. J. Biochem.* **134**, 315–319.
23. Hollingsworth, R. E., Jr., & Sclafani, R. A. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 6272–6276.
24. Yoon, H. J. & Campbell, J. L. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 3574–3578.
25. Hartwell, L. H. (1976) *J. Mol. Biol.* **104**, 803–817.
26. Wood, J. S. & Hartwell, L. H. (1982) *J. Cell Biol.* **94**, 718–726.
27. Koshland, D., Rutledge, L., Fitzgerald-Hayes, M. & Hartwell, L. H. (1987) *Cell* **48**, 801–812.
28. Marsh, J. L., Ertke, M. & Wykes, E. (1984) *Gene* **32**, 481–485.
29. Koshland, D., Kent, J. C. & Hartwell, L. H. (1985) *Cell* **40**, 393–403.
30. Sherman, F., Fink, G. R. & Hicks, J. B. (1986) *Methods in Yeast Genetics* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
31. Koshland, D. & Hieter, P. (1987) in *Methods in Enzymology*, ed. Wu, R. (Academic, New York), pp. 351–372.
32. Hieter, P., Mann, C., Snyder, M. & Davis, R. W. (1985) *Cell* **40**, 381–392.
33. Zhou, C., Huang, S. & Jong, A. Y. (1989) *J. Biol. Chem.* **264**, 9022–9029.
34. Lisiewicz, J., Godany, A., Agoston, D. V. & Kuntzel, H. (1988) *Nucleic Acids Res.* **16**, 11507–11520.
35. Blow, J. J. & Laskey, R. A. (1988) *Nature (London)* **332**, 546–548.
36. Hennessy, K. M., Clark, C. D. & Botstein, D. (1990) *Genes Dev.* **4**, 2252–2263.