

Regulation of DNA Replication during the Yeast Cell Cycle

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One central event that must occur exactly once per cell division cycle is the replication of the genomic DNA. One defining observation of the eukaryotic cell cycle is that the nuclear DNA replicates during a short period (the S phase) and that during that period each part of every chromosome is entirely replicated. Although there are many origins of DNA replication per chromosome, and it is known that not all of them are required to act in every cycle, nevertheless reinitiation in the same cycle at any one of them is never observed. For these reasons, it has long been assumed that the initiation of DNA replication is under very stringent control.

When Hartwell et al. (1970) began systematic isolation of cell-division-cycle (*cdc*) mutants more than 20 years ago, it was expected that mutants specifically defective in DNA replication, especially those involved in initiation of DNA replication, would be prominent among the mutants that exhibit *cdc* phenotypes. Growing yeast cells show a morphology characteristic for the position they have reached in the cell cycle: The bud emerges just about the time that S phase begins and has reached its full size at the time that mitosis begins. Thus conditional-lethal mutants defective in DNA elongation were expected to arrest, under nonpermissive conditions, with a large bud. Indeed, temperature-sensitive mutations in genes specifying DNA polymerases (*cdc2*, *cdc17*; Budd and Campbell 1987; Sitney et al. 1989) and DNA ligase (*cdc9*; Johnston and Nasmyth 1978) have this arrest phenotype, as do mutations that affect DNA precursor metabolism (*cdc8*, *cdc21*; Bisson and Thorne 1977; Sclafani and Fangman 1984) and wild-type cells treated with hydroxyurea, a specific inhibitor of DNA replication (Pringle and Hartwell 1981). Indeed, as shown more recently by Hartwell and his colleagues (Hartwell and Weinert 1989; Brown et al., this volume), this phenotype is reflective of a regulatory "checkpoint" mechanism, an essential component of which is the *RAD9* gene product, which acts to arrest the cell cycle when DNA replication has not successfully been completed.

Although the DNA elongation functions were represented among the *cdc* mutants more or less as expected, it has been puzzling that so few of the *cdc* mutants displayed phenotypes expected for failure of initiation of DNA synthesis. The complexity of the enzymology, especially in bacteria (for review, see Newlon 1988; Stillman 1989; Matson and Kaiser-Rogers 1990), certainly suggests that many gene products will be specifi-

cally involved in DNA initiation in eukaryotic organisms as well. Furthermore, as pointed out by Hartwell and Weinert (1989), there are strong reasons to suggest that a regulatory checkpoint mechanism might act at this step. Yet the only mutations among the classic *cdc* mutant collections (Pringle and Hartwell 1981) that block the cell cycle after spindle-pole-body duplication with largely unreplicated DNA (i.e., G₁ DNA content) are *cdc4* and *cdc7*, neither of which has yet been shown to be involved directly in DNA initiation.

We have identified four interacting genes (*CDC45*, *CDC46*, *CDC47*, and *CDC54*) whose phenotypes, summarized below, turn out to be consistent with a role in initiation of DNA replication (Hennessy et al. 1990, 1991). The mutations defining these genes were all isolated many years ago (Moir and Botstein 1982; Moir et al. 1982), and the group was classified (wrongly, as it turns out) as acting later than DNA synthesis. As we suggest below, the reason for this misclassification is most likely the intrinsically leaky phenotype of one of these mutations, attributable to the fact that there are many origins of replication on each chromosome, any one of which suffices to initiate replication of at least a part of that chromosome. That DNA initiation mutants might not have the phenotype expected of them was anticipated very early by Tye (Sinha et al. 1986).

We have also found (see below and Hennessy et al. 1990) that the product of one of these genes, *CDC46*, is synthesized periodically during the cell cycle and accumulates in the cytoplasm until mitosis is being completed, whereupon it is rapidly mobilized into the nucleus, where it remains until just after the cells have become committed to another cell cycle (i.e., another round of DNA synthesis), after which it rapidly disappears from the nucleus. We believe that this behavior may provide the molecular basis for the cell-cycle-dependent regulation of DNA replication, possibly (but not necessarily) along the lines suggested by the detailed model of Blow and Laskey (1988).

Genetic Interactions

The mutations that define the *CDC45* and *CDC54* genes were isolated as cold-sensitive lethal mutations that display, after shift to a nonpermissive temperature, a large-bud arrest phenotype (Moir et al. 1982; Hennessy et al. 1991). The mutations that define the *CDC46* and *CDC47* genes were isolated as suppressors of cold-sensitive *cdc45* and *cdc54* alleles that not only

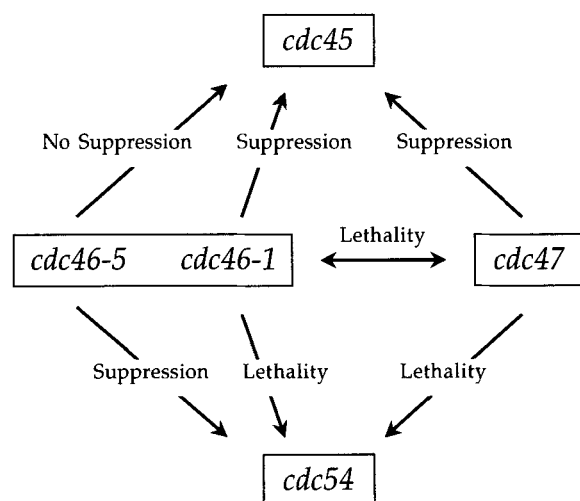


Figure 1. Four interacting genes required for completion of the cell cycle. Two genes were identified as cold-sensitive cell cycle mutants (*cdc45* and *cdc54*), and *cdc46* and *cdc47* were isolated as second-site suppressors of the two cold-sensitive mutants. Mutant alleles of each were crossed to the other mutants within this group, and the phenotypes were determined. Some combinations create lethal phenotypes that neither parent exhibits individually. Alternatively, the *cdc46-5* and *cdc54* combination is both temperature-sensitive and cold-sensitive. The other *cdc46* combinations suppress the cold-sensitive mutations as originally isolated.

suppressed the cold sensitivity, but also simultaneously conferred a new phenotype, heat sensitivity for growth (Jarvik and Botstein 1975; Moir et al. 1982). The suppressor mutations, when crossed away from the original cold-sensitive mutations, retained their heat sensitivity; the phenotype after shift to nonpermissive temperature was once again cell cycle arrest with a large bud. Thus, all four genes are defined by conditional-lethal alleles that show the same *cdc* phenotype. Further investigation of the genetic interactions among these genes revealed, in addition to the suppression relationships, synthetic lethality among some combinations of mutations in these genes (Hennessy et al. 1991). These relationships are summarized in Figure 1.

Evidence for Involvement of the *CDC46* Group in DNA Initiation

As mentioned above, the original mutants defining the *CDC46* group of genes were classified as being defective in steps after DNA replication initiation (Moir and Botstein 1982; Moir et al. 1982). Most of the evidence for this conclusion involved the cold-sensitive *cdc45* mutation tested at 16°C, a temperature at which mutant cells arrest with a large bud in the first cycle after temperature shift. Our reexamination of the mutant phenotypes was prompted by our observations of cell-cycle-dependent changes in localization of the product of the *CDC46* gene (see below; Hennessy et al. 1990), and we applied new technology not available to us in 1982 (flow cytometry and pulsed-field gel electrophoresis). Our results can be summarized as follows:

1. *The DNA phenotype of cdc45-1 is different at 11°C and 15°C.* Flow cytometry analysis (Fig. 2) of *cdc45-1* cells shifted to 15°C shows a nearly normal G₂ (replicated) DNA content, consistent with the previous conclusion of Moir et al. (1982); yet when shifted to 11°C, cells from the same culture arrest with a G₁ (unreplicated) DNA content. Despite this difference, cells arrest with a single large bud in the first cycle after shift at both nonpermissive temperatures.
2. *The heat-sensitive phenotypes of cdc46 mutants are consistent with a block at DNA initiation.* Flow cytometry analysis and reciprocal-shift ordering experiments (Hereford and Hartwell 1974; Hartwell 1976) relative to hydroxyurea suggest that *cdc46* acts at the beginning of S, leaving a G₁ DNA content (Fig. 2) (Hennessy et al. 1991). A particularly revealing experiment involves a new assay for replication status of individual chromosomes based on pulsed-field (CHEF) gel electrophoresis. Figure 3 shows the CHEF replication assay, which is based on the observation that actively replicating chromosomes are unable to migrate into the gel, presumably because of topological constraints imposed by the replication bubbles. Growing cultures of *Mata cdc46-1* cells at permissive temperature were arrested in a variety of ways: α -factor treatment (resulting in G₁ arrest; Pringle and Hartwell 1981); hydroxyurea treatment (resulting in arrest during S phase; Slater 1973); nocodazole treatment (resulting in arrest at mitosis; Huffaker et al. 1988); and 37°C (resulting in the *cdc46* arrest). The chromosomes were separated on a CHEF gel, blotted, and probed serially with probes from the relatively short chromosome V (~600 kb) and the longest chromosome IV (>2000 kb). The results suggest that in the *cdc46* arrest some (but not all) of the larger chromosomes IV may have initiated replication, but that most of the smaller chromosomes V had not.
3. *Arrest at the cdc45, cdc46, and cdc54 blocks results in DNA damage.* Assay of mitotic chromosome loss and recombination after cell cycle arrest was introduced by Hartwell and Smith (1985) as a way of deciding whether DNA replication is complete, producing undamaged chromosomes, at the time of cell cycle arrest in mutants. When this test is applied to *cdc45*, *cdc46*, or *cdc54* mutants, it is clear that the chromosomes are damaged enough to stimulate strongly mitotic recombination (data in Hennessy et al. 1991). This result, seen in the context of the observation that the chromosomes are largely unreplicated, suggests the possibility that some (possibly abortive) initiation has occurred on the yeast chromosomes at the nonpermissive temperature—enough to cause damage, but not enough to cause much replication.
4. *The DNA sequence of the CDC46 gene helps define a new family of genes, some of which are functionally associated with origins of DNA replication.* The DNA sequence of the *CDC46* gene (Hennessy et al.

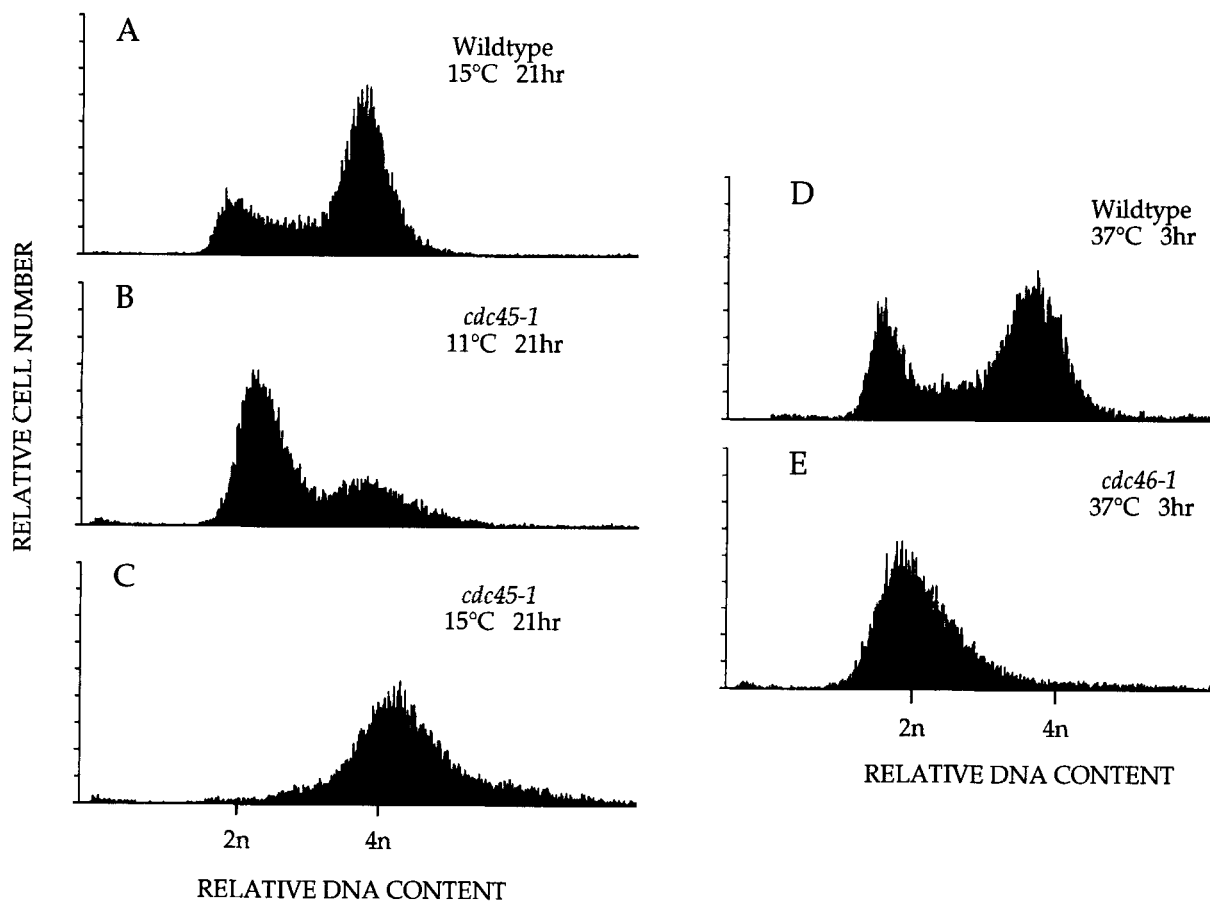


Figure 2. *CDC45* and *CDC46* are required for DNA replication. The DNA content of individual *cdc45* cells was determined by flow cytometry. Cultures of both wild type (A) or *cdc45* diploid cells were shifted from a permissive temperature of 30°C to the nonpermissive temperatures of either 15°C (B) or 11°C (C). Both conditions resulted in over 95% arrest of the *cdc* culture with large buds. In a separate experiment, the DNA content of *cdc46-1* (E) and wild type (D) homozygous diploid strains was determined after the indicated times at 37°C. Once again the *cdc* culture contained over 90% large-bud arrested cells after incubation at the restrictive temperature.

1991) shows that it is substantially homologous (60% over 200 residues at the amino acid level) to *MCM2* and *MCM3*, yeast genes isolated originally as differentially required for maintenance of artificial chromosomes carrying different origins of replication (Maine et al. 1984; Gibson et al. 1990; Yan et al. 1991). The *mcm* mutant phenotypes strongly resemble the leakier (15°C) phenotype of the cold-sensitive *cdc45* and *cdc54* mutants.

The weight of the evidence summarized above suggests that the *CDC46* group of genes is involved in the initiation of DNA replication. The phenotypes are consistent with the notion that initiation of DNA replication requires action at many hundreds of origins of DNA replication. Some of the genes we have identified might be required to act at many or all of the origins, whereas others (as might be expected for the MCM gene products) might be required at only a subset of the origins of replication. As suggested previously (Sinha et al. 1986), this situation is biased in the direction of leaky mutant phenotypes, because if failure to replicate means complete absence of function at all of the origins

of replication, a mutant must allow no residual function at all. Conditional-lethal mutants, by their very nature, tend to be leaky, allowing partial function of the order of a few percent of normal at the nonpermissive temperature. Thus, the result that many mutants in the *CDC46* group show some replication, especially at intermediate temperatures, might have been expected.

The observation that mutants in genes of the *CDC46* group, when arrested at their nonpermissive temperatures, result in damage to the chromosomes can be explained by abortive initiation at only a few origins of replication. The observation of damage during arrest also helps to explain why mutants like the *cdc45* mutants showed up with a phenotype of large bud arrest in the first cycle with a G_2 (replicated) DNA content. If damage occurs during arrest, then one might expect the RAD9-dependent checkpoint mechanism (Hartwell and Weinert 1989) to halt the cell cycle before mitosis is attempted.

We can thus define better our expectation of the phenotype of mutations involved specifically in initiation of DNA replication: They are indeed cell cycle mutants, with terminal arrest with a large bud, but with

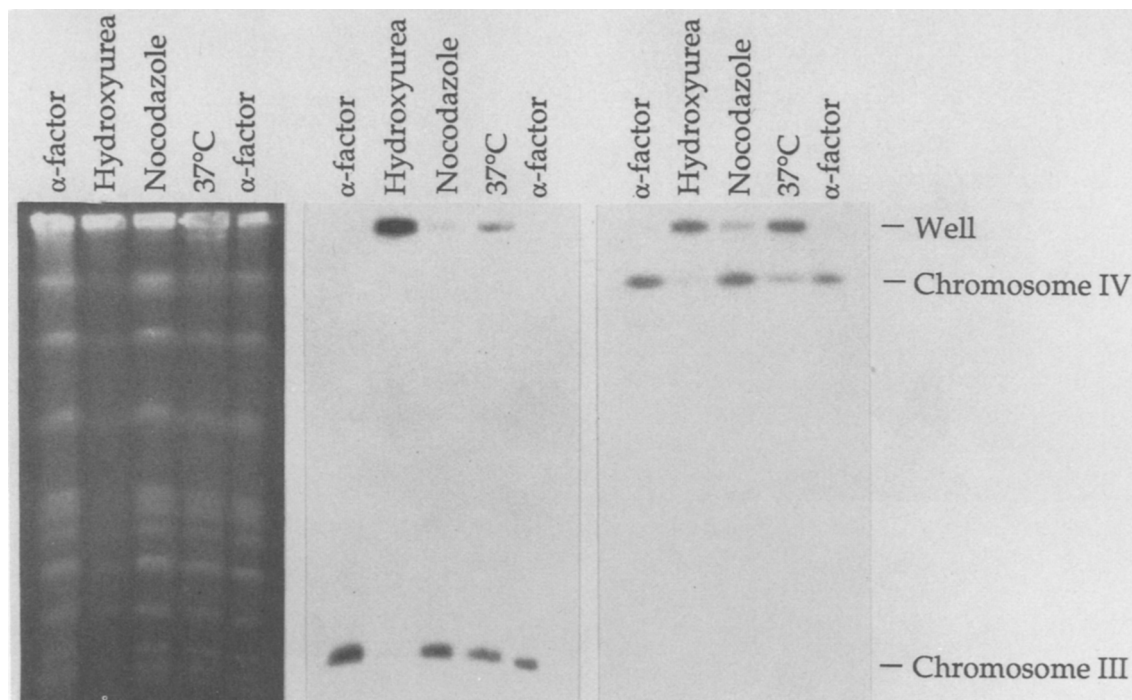


Figure 3. Chromosome abnormalities in *CDC46* mutants. Aliquots of an actively growing *cdc46-1* strain have been arrested at various points in the cell cycle using chemical inhibitors. Another aliquot was held at the nonpermissive temperature of *cdc46-1*, 37°C, for 3 hr. The chromosomes of each aliquot were separated by CHEF and stained with EtBr (right panel). The DNA was then acid nicked, blotted, and hybridized with a ³²P-labeled fragment of the *SAC6* gene, which resides on chromosome IV (left panel), or a labeled fragment of the *LEU2* gene on chromosome III (middle). The top row of bands are where the sample was loaded and contains the residual material that was not able to migrate into the gel.

either a G₁ or G₂ DNA content, depending on the degree of loss of function of the mutation. We might speculate that as little as a few percent residual function could result in a G₂ DNA content. We can also expect chromosomes to be damaged by cell cycle arrest in these mutants, although again the degree of damage might depend on the extent of residual and/or abortive DNA initiation that can occur under nonpermissive conditions. Given this new expectation, based on the *CDC46* group and the *MCM* genes, it seems quite possible that there are many more such genes to be found.

Cell-cycle-dependent Nuclear Localization of the *CDC46* Gene Product

An unexpected result was obtained when the subcellular localization of the *CDC46* protein was studied using affinity-purified antibodies (Hennessy et al. 1990). Prominent staining of the nucleus was seen in some cells, and staining of the cytoplasm (and apparently not the nucleus) was seen in others. As shown in Figure 4 and summarized in Figure 5, the staining of the nucleus turned out to be cell-cycle-dependent: The protein appears in the nucleus sometime toward the end of mitosis, coincident with the breakdown of the spindle but before cytokinesis, and disappears from the nucleus

at about the time of bud emergence (i.e., at about the time of the beginning of the S period).

The immunofluorescence results were supported by cell fractionation experiments, which showed that cells arrested at G₁ with α factor had most of their *CDC46p* in the nuclear fraction, whereas cells arrested at M by shifting a cold-sensitive *tub2* (β -tubulin) mutant had most of their *CDC46p* in the cytoplasmic fraction (data in Hennessy et al. 1990). The overall amount of *CDC46p* seems not to vary substantially over the cell cycle, as judged from immunoblotting experiments. Finally, immunofluorescence staining experiments using standard cell cycle mutants at their characteristic arrest points were consistent with the pattern shown in Figure 5: The only new information obtained from this set of experiments was that the disappearance of the protein from the nucleus is not yet apparent in cells arrested at the *cdc4* block but clearly has begun in cells arrested at the *cdc7* block. However, the reappearance has not been observed in any of the "late" arresting *cdc* mutations (i.e., *cdc5*, *cdc15*, *cdc14*), suggesting that these mutations arrest during mitosis rather than post-mitotically.

Finally, measurements of *CDC46* mRNA (Hennessy et al. 1990) in synchronized cells showed that the mRNA varies with the cell cycle also, being maximal just at the time that the protein disappears from the nucleus. An interesting feature of these experiments is

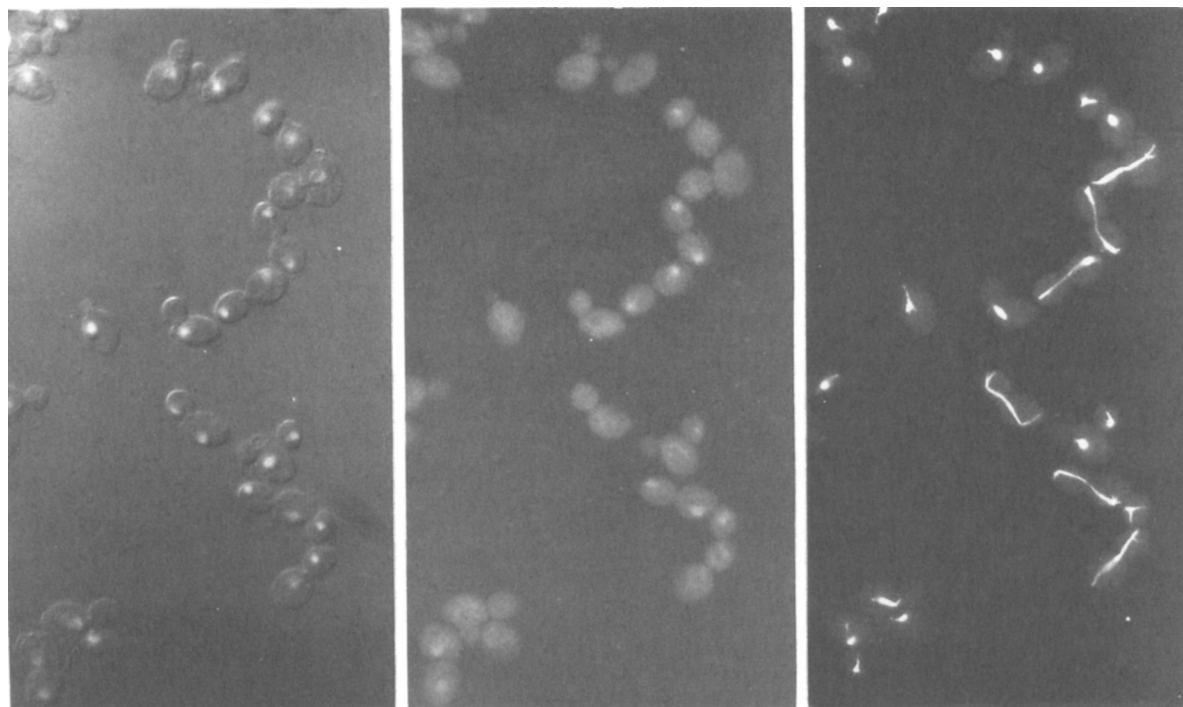


Figure 4. Localization of the *CDC46* product is cell-cycle-dependent. Affinity-purified antibodies to *CDC46* were reacted with a population of wild-type *Saccharomyces cerevisiae* cells and visualized by indirect immunofluorescence (*middle*). In addition, the cells were stained with an anti-tubulin monoclonal to determine the cell cycle position of each cell (*right*) as well as DAPI and DIC (*left*).

the observation that the peak in *CDC46* mRNA is distinctly earlier in the cell cycle than the peak in the histone mRNA, which also varies periodically with the cell cycle.

The experimental results concerning localization of *CDC46p* suggest a scenario as follows: *CDC46* mRNA

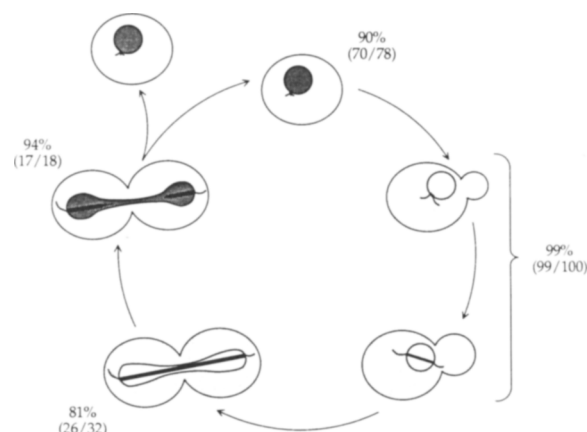


Figure 5. Fraction of nuclear-staining cells during each stage of the cell cycle. Two hundred cells stained as in Fig. 4 were grouped by their position in the cell cycle and then scored as either *CDC46* nuclear-staining positive or negative on photos. The shaded areas schematically summarize one model to explain the results. The number (and fraction) of cells with *CDC46* localized in the schematically represented subcellular location is indicated for each portion of the cell cycle: (1) unbudded, (2) small budded, (3) large budded with an intact spindle, or (4) large budded with mitosis completed.

is synthesized beginning during the S period, with protein product accumulating in the cytoplasm. Toward the end of mitosis, the protein enters the nucleus, where it performs a function (possibly involving initiation of DNA synthesis), after which it is removed from the nucleus (probably degraded). The constant level in the cells is thus a balance between synthesis of new *CDC46p* in the cytoplasm early in S phase coincident with degradation of the old *CDC46p* in the nucleus.

Essentially the same pattern of cell-cycle-dependent synthesis and localization has been observed for one other yeast protein, the product of the *SWI5* gene, a gene controlling in mating type switching and not, so far as is known, in DNA replication (Nasmyth et al. 1990). K. Nasmyth and colleagues (pers. comm.) have, for their protein, acquired more direct evidence for degradation in the nucleus, and they have preliminary evidence for posttranslational modifications associated with the translocation into the nucleus.

Model for the Role of *CDC46p* in Regulation of DNA Replication

Our studies have identified a set of yeast genes involved in an early step of nuclear DNA replication, possibly at the level of initiation of DNA synthesis at origins of DNA replication. The product of one of these genes (*CDC46*) is synthesized periodically early during the S period of the cell cycle and accumulates in the cytoplasm until just as mitosis is being completed, whereupon it is rapidly mobilized into the nucleus,

where it remains until just after the cells have become committed to another cell cycle (i.e., another round of DNA synthesis), after which it rapidly disappears from the nucleus.

The changes in localization of CDC46p suggest a way in which a DNA initiation factor might be able to act, as it must, just once in a cell cycle. The factor enters the nucleus, where it must be to act, and where it has a very limited lifetime. One could speculate that the protein either immediately becomes part of an initiation complex or is degraded. Factor in complexes is degraded as soon as the complex disassembles when the function is complete. Newly synthesized factor can, in this scheme, never act to reinitiate synthesis in the same cycle in which it is made because it remains in the cytoplasm until mitosis has ended. An essentially identical model was suggested by Blow and Laskey (1988), who invoked a cytoplasmic "licensing factor" that could only enter the nucleus during the breakdown of the nuclear membrane in higher eukaryotes and thus be responsible for the failure to see reinitiation of DNA replication before mitosis in *Xenopus* eggs.

One might ask, since *CDC46* is a member of an extensive gene family sharing considerable sequence homology, whether all members share the same cell-cycle-dependent nuclear localization. It might be that *CDC46* is a member of a regulatory subset of DNA initiation proteins. In view of all the evidence for genetic interactions, it will be of interest to see whether the products of *CDC45*, *CDC47*, and *CDC54* are periodically synthesized and translocated to the nucleus in a cell-cycle-dependent way.

In conclusion, we believe that we have identified a number of interacting genes whose function is early in nuclear DNA replication, possibly initiation of DNA chains at origins of DNA replication. One of these displays a cell-cycle-dependent localization phenotype consistent with a regulatory role in initiation of DNA replication exactly once in every cell cycle.

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