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Characterization of a Novel *CDC* Gene (*ORC1*) Partly Homologous to *CDC6* of *Saccharomyces cerevisiae*

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A novel cell cycle gene was identified by a computer search for genes partly homologous to known *CDC* genes, *CDC6* of *Saccharomyces cerevisiae* and *CDC18* of *Schizosaccharomyces pombe*, using the nucleotide sequence data base for *S. cerevisiae* produced by the Yeast Sequencing Project. The protein sequence coded by the cloned gene was found to be identical to that of purified *ORC1* protein. Disruption of the gene and subsequent tetrad analysis revealed that the gene was essential for growth. The function of the gene product was analyzed by depleting the protein from the cell using a mutant haploid strain containing the disrupted *ORC1* gene on the chromosome and a galactose-inducible gene coding for HA-tagged *ORC1* protein on a single copy plasmid. The HA-tagged protein was expressed during growth in the presence of galactose but began to decrease rapidly upon depletion of galactose. Analysis of the cell cycle progression of the mutant cells by FACS after the removal of galactose from the medium, and microscope observations of cells and their nuclei revealed that the normal progression of 2N cells was immediately impeded as the *ORC1* protein started to decrease. This was blocked completely in the cells that had progressed to the S phase under conditions deficient in *ORC1* protein followed by cell death. Two-dimensional gel analysis of the replication intermediates after the galactose removal revealed that the depletion of *ORC1* protein caused a decrease in the frequency of initiation of chromosomal replication, eventually resulting in the inhibition of replication as a whole. The function of the *ORC1* protein in the cell cycle progression of *S. cerevisiae* is discussed in light of current information on *ORC*.

INTRODUCTION

The systematic sequencing of *Saccharomyces cerevisiae* chromosomes has identified numerous open reading frames whose functions should be analyzed systematically to understand the overall picture of gene function in a single eukaryotic cell. One way to accomplish this is to search for genes homologous to known essential genes to collect the complete set of gene families involved in various essential cell functions.

We have chosen the *CDC6* gene as a test gene as we have been interested in the regulation of initiation of chromosomal replication in *S. cerevisiae*. *CDC6* has been known to be essential for the G1/S transition of the yeast cell cycle and proposed to be involved in the activation of an initiation-replication complex composed of *ORC*, a member of the *MCM* family of proteins, and other regulatory proteins (Bueno and Russell, 1992; Hogan and Koshland, 1992; Bell *et al.*, 1993; Li and Herskowitz, 1993; Fox *et al.*, 1995; Liang *et al.*, 1995; Loo *et al.*, 1995). The function of *CDC* genes, however, as well as the mechanism of activation of the initiation complex still remains to be investigated. It is also possible that there are missing components essential for constructing the complexes involved in the initiation of chromosomal replication and its regulation still remains to be discovered.

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The aim of this paper is to identify, clone, and characterize the function of a novel *CDC* gene partly homologous to *CDC6*. The gene was found to be identical to *ORC1* by comparison with the amino acid sequence of purified *ORC1* protein (Bell *et al.*, 1995).

MATERIALS AND METHODS

Strains and Media

All strains used in this study are derivatives of W303–1A and W303–1B. Strain SKY303 (*MATa/α ade2–101 ura3–1 leu2–3, 112 trp1–1 his3–11 can1–100*) was constructed by crossing the strain W303–1A (*MATa ade2–101 ura3–1 leu2–3, 112 trp1–1 his3–11 can1–100*) with W303–1B (*MATα ade2–101 ura3–1 leu2–3, 112 trp1–1 his3–11 can1–100*). Using this diploid as the parental strain, YHY002 (*MATa/α ade2–101 ura3–1 leu2–3, 112 trp1–1 his3–11 can1–100 ORC1/orc1::hisG-URA3-hisG*), YHY004 (*MATa/α ade2–101 ura3–1 leu2–3, 112 trp1–1 his3–11 can1–100 ORC1/orc1::hisG*), YHY009a (*MATa ade2–101 ura3–1 leu2–3, 112 trp1–1 his3–11 can1–100 orc1::hisG* pYH009), and SKY007a (*MATa ade2–101 ura3–1 leu2–3, 112 trp1–1 his3–11 can1–100 orc1::hisG* pKS007) were constructed. YHY002 was constructed by transforming SKY303 with an *ORC1*(5′)-hisG-*URA3*-hisG-*ORC1*(3′) fragment amplified from pNKY51 (Alani *et al.*, 1987) using a primer containing 20 nucleotides of pNKY51 vector sequence and 50 nucleotide sequences flanking the *ORC1* open reading frame. YHY004 was derived from YHY002 by selecting against the URA3 gene on 5′ FOA media. YHY004 was transformed by pYH009 or pKS007 and its haploid derivatives were YHY009a and SKY007a, respectively. Transformation of all yeast strains was carried out using the Li-Ac method (Schiestl and Gietz, 1989). Yeast cells were grown in SC (synthetic complete) medium with or without uracil, supplemented with 2% raffinose or 2% galactose + raffinose instead of 2% glucose. An *Escherichia coli* strain DH5α [*F⁻supE44ΔlacU169 (f80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*] was used for plasmid preparation. Transformation of *E. coli* was done by electroporation.

Plasmid Construction

A 3.9-kb *ORC1* containing a DNA fragment in which 5′ *Sac*I and 3′ *Xba*I sites were introduced by long polymerase chain reaction (PCR; Perkin-Elmer, Norwalk, CT) was inserted at the *Sac*I–*Xba*I site of YCplac33 (Gietz and Sugino, 1988) generating pKS007. To tag *ORC1* at the N terminus, a 2.8-kb *ORC1*-containing fragment in which the 5′ *Xba*I and 3′ *Xba*I sites had been introduced by PCR was inserted at the *Xba*I site of pGT-HA. The pGT-HA was constructed by inserting synthetic oligonucleotides encoding the HA epitope at the *Xba*I site of pGT5 situated immediately upstream of the initiation ATG codon.

Neutral/Neutral Two-dimensional Gel Electrophoresis

Yeast cell cultures were mixed with an equal volume of the toluene stop solution (95% EtOH, 3% toluene, 20 mM tris (hydroxymethyl) aminomethane [Tris], pH 7.4) followed immediately by the addition of 0.25 M EDTA to a final concentration of 10 mM (Johnston and Williamson, 1978). The suspension was swirled for 1 min and placed on ice or immediately centrifuged at 4°C. The cells were then washed three times with a sterilized SE buffer (100 mM EDTA, 75 mM NaCl, pH 7.5). Then the yeast cells were encapsulated into agarose beads according to a modified version (Overhauser and Radic, 1987) of the method of Jackson and Cook (1985) as follows. Cells were suspended in SE buffer at the density of 5×10^8 and equilibrated to 37°C and mixed with an equal volume of 1% low melting point agarose in the SE buffer and 4 volumes of liquid paraffin at 37°C in a 300-ml Erlenmeyer flask. The mixture was vigorously shaken by hand for 1 min. The emulsion was poured into 200 ml of the SE buffer cooled on ice and the mixture was stirred for several minutes. DNA was isolated from an aliquot of the cells encapsulated in agarose beads and its quantity was determined as previously described (Shinomiya and Ina, 1993). Usually the DNA concentration of the agarose bead suspension is 8–10 μg/ml. Five milliliters of agarose bead suspension was digested by appropriate restriction enzymes (Takara Shuzo, Kyoto, Japan; 100 U per 1 μg DNA) for 60 min at 37°C. The resultant digested DNA was retrieved from beads as previously described (Shinomiya and Ina, 1994). Ten micrograms of DNA was regularly used for one two-dimensional analysis. Neutral/neutral two-dimensional agarose gel electrophoresis was carried out essentially by the method of Brewer and Fangman (1987), and DNA was blotted to nylon filters (Stratagene, La Jolla, CA). Radiolabeled probes were prepared as previously described (Shinomiya and Ina, 1994). After hybridization, filters were washed and exposed to a Fuji imaging plate for analysis with a BioImage Analyzer BAS2000 (Fuji Photo Film, Tokyo, Japan).

Western Blot Analysis

Whole cell extracts were prepared by vortexing the yeast cell with glass beads as described previously (Diffley *et al.*, 1994) except that the lysis buffer contained 10 mM Tris-Cl (pH 7.4), 10 mM $MgCl^2$, 0.5 M NaCl, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 1 μg/ml pepstatin A, and 5 mM 2-mercaptoethanol. Proteins from whole-cell extracts of 10^7 cells were separated by 10% SDS-PAGE electrophoresis and transferred to a polyvinylidene fluoride membrane (Amersham, Arlington Heights, IL) using a semi-dry transfer apparatus in transfer solution (100 mM Tris, 192 mM glycine, and 0.025% SDS, pH 8.3) at 150 mA for 90 min. The following procedures were carried out at room temperature. The membrane was washed for 5 min with Tris-buffered saline (TBS; 30 mM Tris, 200 mM NaCl, pH 7.5) + 0.05% Tween 20 (TTBS) and blocked with a blocking solution (TBS + 3% bovine serum albumin, and 0.1% skim milk) for 30 min, followed by incubation with 10 μg/ml 12CA5 antibody (Boehringer Mannheim, Indianapolis, IN) in the blocking solution for 1 h. After the incubation, the membrane was washed with TTBS five times, and incubated with 1:5000 diluted peroxidase-conjugated secondary antibody (goat anti-mouse IgG antibody; Bio-Rad, Richmond, CA) in the blocking solution for 1 h. The unbound antibody was washed out with TTBS five times and the HA-tagged *ORC1* signals were detected with an ECL detection reagent (Amersham).

DNA Manipulations and Genetic Techniques

Cloning and plasmid DNA isolation from *E. coli* were performed essentially as described (Sambrook *et al.*, 1989). Other standard yeast genetic techniques were used as described (Kaiser *et al.*, 1994).

Other Techniques

The flow-cytometric determination of cellular DNA content was performed as previously described (Epstein and Cross, 1992) on a Becton Dickinson FACScan (Mountain View, CA).

RESULTS

Identification and Cloning of a Gene Related to CDC6

We have carried out a thorough computer search for *CDC6* homologues in a nucleotide sequence data base of *S. cerevisiae* registered in GenBank by the EU Yeast Sequencing Project. No open reading frames were

found homologous to *CDC6* over the whole sequence. However one open reading frame, SC9745 8 (a hypothetical protein coding frame in cosmid 9745 from *S. cerevisiae* chromosome XIII, GenBank accession number Z38114) was identified that had only a portion of its sequence homologous to *CDC6*. Interestingly, the open reading frame shares sequences homologous, in part, to other cell cycle genes, *CDC18* (the *CDC6* homologue of *Schizosaccharomyces pombe*) (Kelly *et al.*, 1993) and also to *SIR3*, a silent information regulator gene (Figure 1) (Herskowitz *et al.*, 1992). In addition, two ATP/GTP binding sites were recognized in the open reading frame (Figure 1). These results suggested that the open reading frame represented a gene involved in cell cycle regulation.

Since no intron sequences were found in the open reading frame, we amplified the reading frame from a genomic DNA library by long PCR using primers designed to include several hundred basepairs in both the 5′ and 3′ regions outside the reading frame. The PCR product was then cloned in a YCplac33 plasmid (Geitz and Sugino, 1988) to generate pKS007. The cloned DNA was 3.9 kb in size and contained 792 bp of 5′-noncoding sequence, a 2742-bp open reading frame, and 317 bp of 3′-noncoding sequence. The gene is located on chromosome XIII and encodes a protein of 914 amino acids with a calculated molecular mass of 104 kDa. The amino acid sequence deduced from the nucleotide sequence of the open reading frame was identical with that of the frame SC9745 8 reported in the data base. Therefore we tentatively named the gene SC9745 8. It was later found that the protein sequence of SC9745 8 was identical to the amino acid sequence of the purified protein of *ORC1* (Bell *et al.*, 1995). Accordingly, we named the cloned gene *ORC1*. The *ORC1* protein is very hydrophilic and rich in charged amino acids with a calculated isoelectric point of 5.6. The sequence revealed no specific motifs other than two nucleotide binding sites.

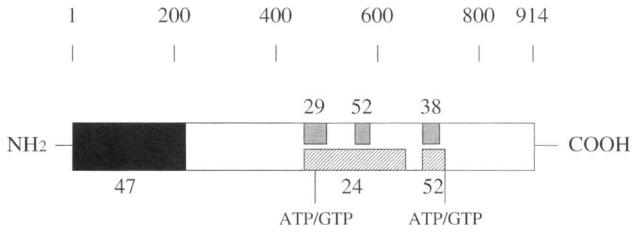
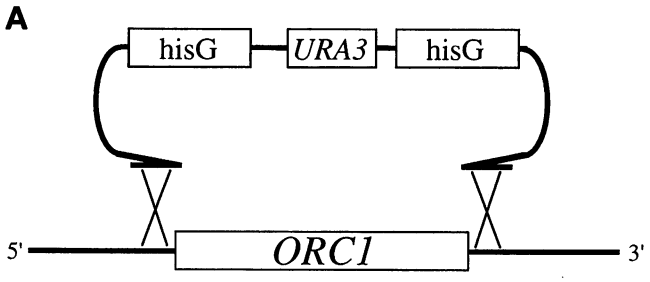


Figure 1. Schematic presentation of the structure of *ORC1*. The black box represents the domain whose amino acid sequence is similar to the *SIR3* sequence. The three gray boxes represent domains homologous to *CDC6* and the two shaded boxes represent domains homologous to *CDC18*. The positions of amino acids are shown at the top. Values below or above the boxes are the percentage of identical amino acids in each domain. Two ATP/GTP binding sites are indicated.

The ORC1 Gene Is Essential for Growth as Revealed by Gene Disruption

The chromosomal *ORC1* gene was disrupted by replacing the intact gene by a modified gene containing a 52-bp 5′-terminal coding sequence, a hisG-URA3-hisG cassette, and a 49-bp 3′-terminal coding sequence in this order (Figure 2A). A diploid strain, YHY002, containing a heterogeneous allele in the *ORC1* gene locus was selected by the *URA3* marker. The *URA3* marker was then deleted by intrachromosomal recombination during growth in the presence of 5′FOA to give rise to the strain YHY004. The mutant diploid strain showed no visible defect in growth compared with the parent strain. However, it produced only two viable spores in tetrad analysis indicating that the gene was essential for growth (Figure 2B). Colonies that grew up from viable spores all contained the intact *ORC1* gene as confirmed by PCR. Close observation revealed that nonviable spores grew two to three generations and then died.



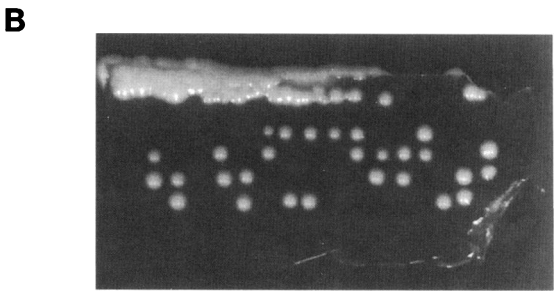


Figure 2. Disruption of the genomic *ORC1* gene. (A) The scheme of the replacement of the *ORC1* gene with the hisG-*URA3*-hisG fragment. (B) Tetrad analysis showing growth of two of four spores in each lane derived from a diploid *ORC1* disrupted strain (YHY004). Tetrads were grown on YPDA plates.

Construction of a Strain with a Disrupted Chromosomal ORC1 Gene but Containing a Galactose-inducible ORC1 Gene

To investigate the function of the *ORC1* gene, a strain of *S. cerevisiae* was constructed in which the expression of the *ORC1* gene was dependent solely on a galactose-inducible gene located on a single copy plasmid. To be able to identify and determine the cellular content of the *ORC1* protein, a HA-tag was fused to the *ORC1* open reading frame in the correct frame and placed downstream of a *GAL 1–10* promoter (pYH009, Figure 3). The plasmid was introduced into the heterozygous strain YHY004 and haploid spores containing the disrupted *ORC1* gene and the plasmids were selected for their growth dependency on galactose. The haploid cell containing the plasmid was named YHY009a. The presence of both the disrupted gene on the chromosome and the HA-tagged gene on the plasmid was confirmed by Southern blot analysis. To ascertain that the HA-tagged *ORC1* protein was indeed expressed in the galactose-containing medium and to examine its fate after the removal of galactose in YHY009a cells, the cellular content of HA-tagged *ORC1* protein was determined during incubation in the absence of galactose by Western blotting using a monoclonal antibody against HA-tag. Figure 4 shows that the protein was produced in the mutant cells in the presence of galactose and its content began to decrease immediately after the galactose depletion with the half-time of approximately 40 min.

Effects of Depletion of ORC1 Protein on Growth and Cell Cycle Progression

As expected from the selection method, the *ORC1*-disrupted mutant strain containing galactose-inducible *ORC1* gene grew normally in media containing galactose. No growth, however, occurred in the absence of galactose. This suggests that the HA-tagged *ORC1* protein is indeed induced by galactose and can replace the essential function of the native *ORC1* protein. If the normal growth of the mutant strain depends on the HA-tagged *ORC1*, analysis of growth of cells after depleting galactose from the growth medium should give insight into the specific function of *ORC1*. Raffinose was used as the carbon source in the *ORC1* depletion experiments to minimize the effects of the change of carbon source on cell growth.

The mutant haploid cell as well as the parental one grew with a doubling time of 3 h in the medium with raffinose + galactose. Removal of galactose from the medium during exponential growth resulted in temporary cessation of growth followed by growth at the same rate as before the removal of galactose (Figure 5).

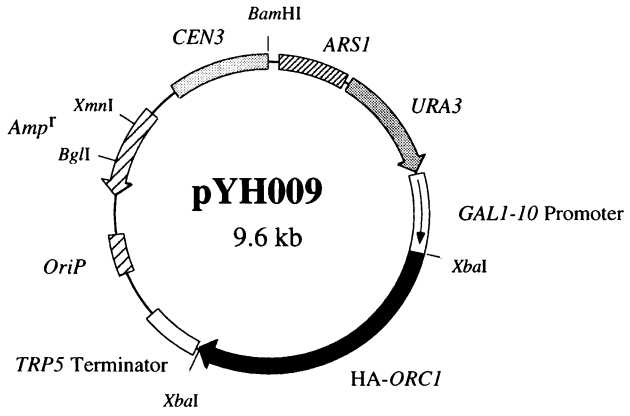


Figure 3. Structure of the expression vector used for HA-tagged *ORC1* gene expression. The construction of the plasmid is described in MATERIALS AND METHODS and in the text.

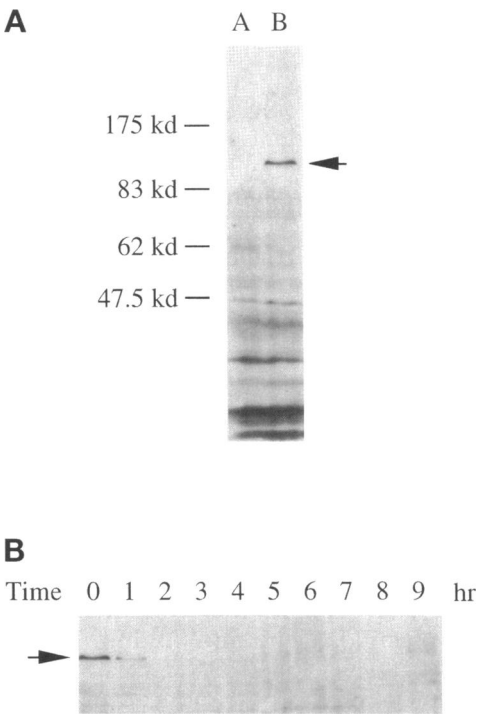


Figure 4. Determination of HA-tagged *ORC1*. An *ORC1*-disrupted mutant strain, YHY009a, containing an inducible HA-tagged *ORC1* gene fused to the *GAL1–10* promoter on a plasmid, and its isogenic parental cell (W303–1A) were grown exponentially in a raffinose + galactose medium. (A) Detection of HA-tagged *ORC1* by a monoclonal antibody against HA. A crude cell extract of wild type (A) and YHY009a (B) growing exponentially in raffinose + galactose medium was prepared, separated by electrophoresis, and Western blotted as described in MATERIALS AND METHODS. The arrowhead indicates the position of HA-tagged *ORC1*. (B) The cellular contents of the HA-tagged *ORC1* of the cells grown in the absence of galactose. The mutant strain cell extracts were prepared at the times indicated after the removal of galactose from the medium. The HA-tagged *ORC1* was detected for each sample. The position of the HA-tagged *ORC1* is indicated by the arrow. The relative densities of the HA-tagged *ORC1* times 0, 1, 2, and 3 h are 100, 50, 10, and less than 0.5%, respectively.

No significant difference in the pattern of growth determined by turbidity after the galactose depletion was observed between the mutant and parental cells (Figure 5). A remarkable difference was observed, however, when the viability of the cell was measured during the growth in the absence of galactose. In the mutant cell culture, viable cells increased slowly for 10 h to about 1.8-fold of the initial value, and then abruptly began to die with a half-life of about 2 h (Figure 5). The parental haploid cell containing the intact *ORC1* gene with or without the plasmid continued to increase viable cells during the incubation without galactose (Figure 5).

To analyze the fate of cells during growth in galactose-depleted medium, the progression of the cell cycle of mutant haploid cells containing the galactose-inducible HA-*ORC1* gene was compared by FACS to wild-type haploid cells (Figure 6). In the wild-type cell culture, a somewhat synchronous growth took place for the first 3 h after the removal of galactose, during which temporary cessation of the increase of turbidity was observed (Figure 6). Thus, 2N cells continued to proceed while the progression of G1 cells to the S phase seemed to be suppressed resulting in accumulation of 1N cells. This was probably due to the shift of carbon source from galactose + raffinose to raffinose alone. Resumption of cell cycle progression was observed by 4 h followed by partially synchronized growth until 8 h. By 12 h the cell cycle became randomized to give rise to a normal exponentially growing cell population. The mutant cell culture showed a pattern of cell cycle progression entirely different from that of the wild-type cell culture (Figure 6). There was no change in the relative amount of 1N cells to 2N cells up to 5 h after the removal of galactose. The population with a varied amount of DNA between 1N to 2N gradually increased and most of the 1N cell population disappeared by 12 h when cell death began to take place. These observations together with the fact that viable cell growth increased 1.8-fold during this period (Figure 5), would suggest that cells only at the G2 phase of cell cycle at the time of the galactose removal underwent a further slower cell cycle and then stopped at various times at the next S phase, while cells at the G1-S phase at time 0 proceeded to the S phase and stopped there in the first cell cycle. Further incubation in the absence of galactose resulted in a broader distribution of DNA content with the peak position at 2N in parallel with the rapid decrease in viability (Figure 6) suggesting that progression through the cell cycle does not reach the G2 phase. These results suggest that as soon as the *ORC1* protein starts to decrease in the cell, the rate of the progression of 2N cells in the first cell cycle is reduced, and that those cells that have entered into the S phse after the decrease in the protein content are no longer able to proceed through the S phase.

The shape of mutant cells and the morphology of their nucleus were observed under the microscope stained with DAPI during growth in the absence of galactose (Figure 7). At time 0, the time of galactose depletion, cells at various stages in the cell cycle were observed to mostly contain one nucleus of normal morphology (Figure 7B). At 6 h after the galactose depletion, an increase in the population of dumbbell-shaped cells with only one nucleus appeared (Figure 7C). Twelve hours after the depletion, most of the cells in the culture were dumbbell shaped and contained only one nucleus (Figure 7D). These observations are consistent with the notion that the loss of *ORC1* protein causes a defect in the progression of the cell cycle

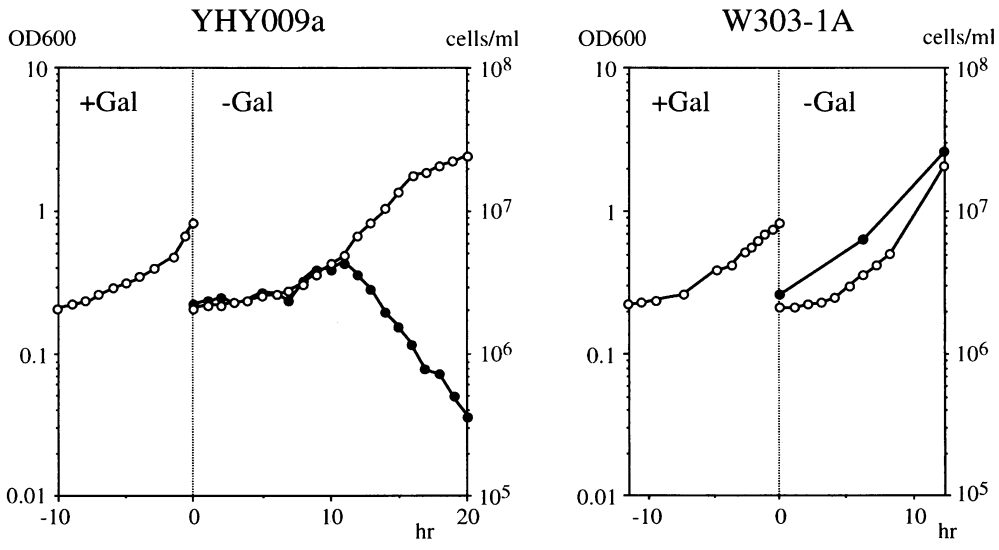


Figure 5. Effects of *ORC1* depletion on cell growth. At time 0, cells were transferred to a raffinose medium without galactose, and viable cell numbers (closed circle) and absorption at OD600 (open circle) were measured at the times indicated. All cells were grown at 28°C. The same culture samples as those in Figure 4 were used for this analysis.

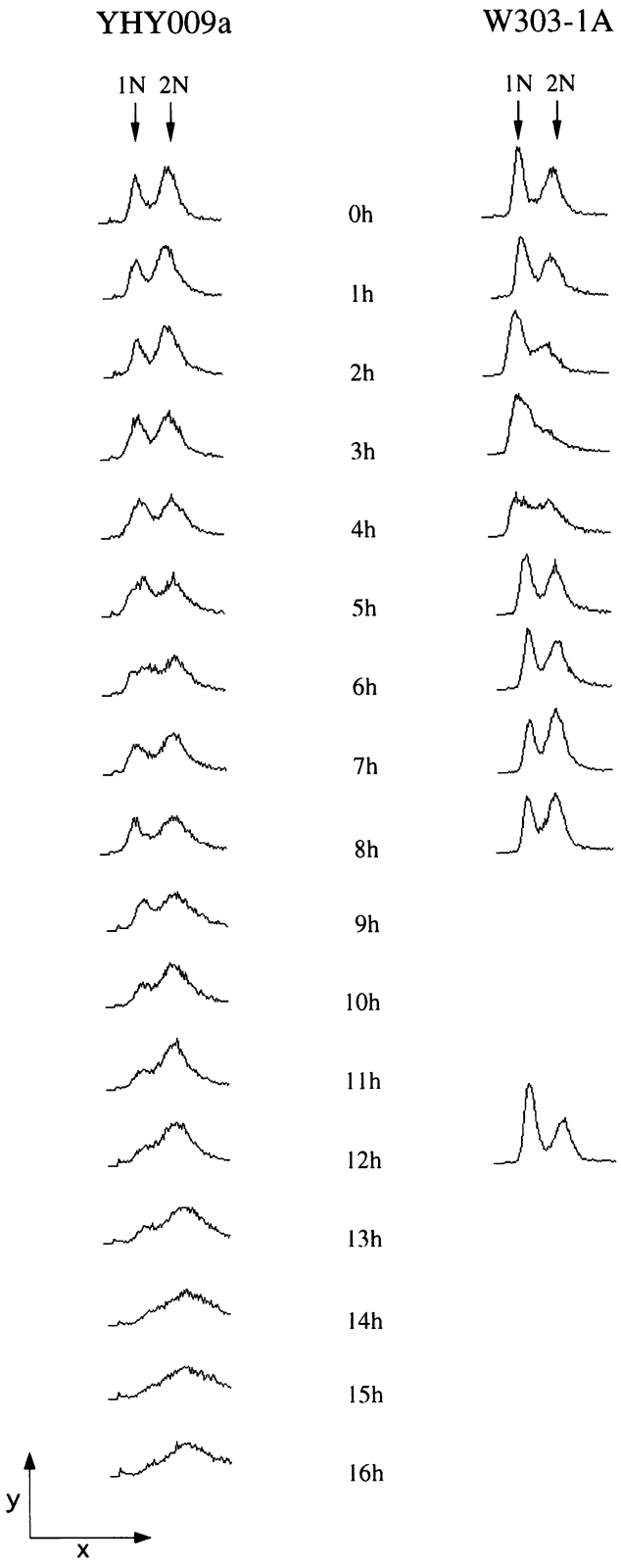


Figure 6.

at the S phase, most probably due to an abnormality in DNA replication.

Effects of Depletion of ORC1 Protein on Chromosomal Replication

ORC1 protein is the largest subunit protein of the complex (*ORC*) of six component proteins that is known to bind specifically to *ARS* (Bell and Stillman, 1992), sequences essential for initiation of replication from the replication origins of chromosomes of *S. cerevisiae*. Depletion of the *ORC1* protein may lead to the depletion of active *ORC*s resulting in decrease in the frequency of initiation of chromosomal replication. To test this possibility the mode of chromosomal replication was analyzed during the depletion of the HA-tagged *ORC1* protein in the mutant strain by the neutral/neutral two-dimensional gel electrophoresis method (Brewer and Fangman, 1987) (Figure 8). Two replication origins from the chromosome VI, *OriSC606* and *OriSC607*, whose ARS are active as replication origins both on mini-chromosomes (Shirahige *et al.*, 1993) and the chromosome VI were used (unpublished data). Only bubble arcs were observed in both origins at time 0, indicating that these two origins were initiated once in every cell cycle in the mutant strain as in wild-type cells in the presence of galactose. After the removal of galactose, arcs corresponding to simple Y form appeared in the two origin regions at 5 h, the proportion of bubble arcs to Y-arcs decreased at 10 h and both bubble and Y-arcs disappeared at 15 h (Figure 8). No changes in the arc patterns occurred in the wild type after the galactose removal from the medium (Figure 8). These results show clearly that the depletion of *ORC1* protein caused a decrease in the frequency of initiation of chromosomal replication eventually resulting in the inhibition of replication as a whole.

DISCUSSION

We have identified and cloned a novel gene essential for yeast cell growth and involved in chromosomal replication. The amino acid sequence deduced from the nucleotide sequence of the cloned gene was identical with that of purified *ORC1* protein. The gene was

Figure 6 (cont). Effects of *ORC1* depletion on cell cycle progression. The mutant strain, YHY009a, and its parental strain (W303–1A) were grown in a raffinose + galactose medium and then transferred to raffinose alone medium at time 0 as in Figure 4. At various times during the incubation in the galactose-depleted medium, aliquots of culture were withdrawn to measure the DNA content of each strain by FACS as described in the MATERIALS AND METHODS. In each panel, the x-axis represents DNA content and the y-axis represents the number of cells. Positions of 1N and 2N cells are shown respectively. The same culture samples as in Figure 4 were used for this analysis.

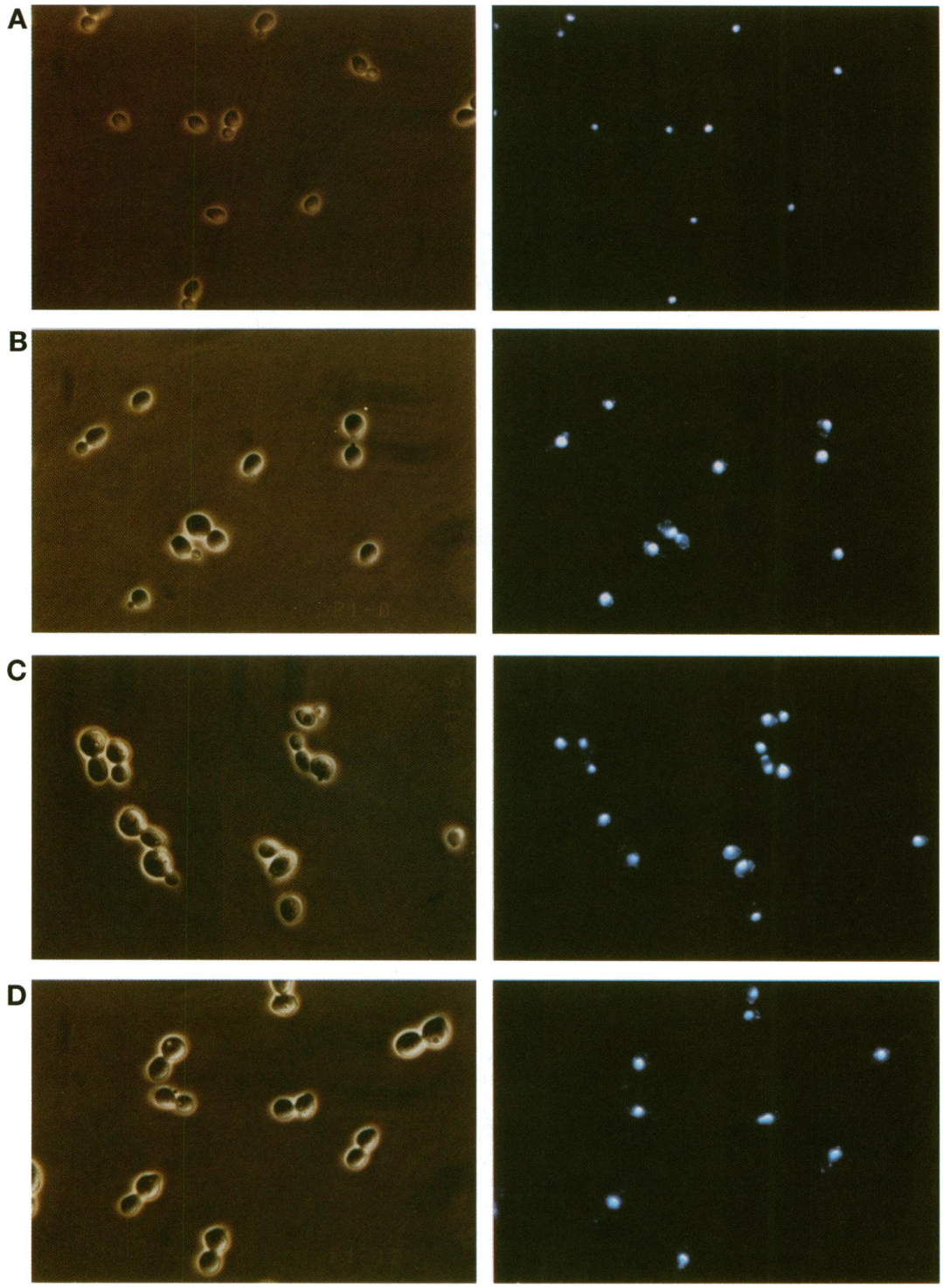


Figure 7. Effects of *ORC1* depletion on cell and nucleus morphology. The same culture samples as in Figure 4 were used. Cells were stained with DAPI and photographed using Normarski optics (the left panel) for cell shape analysis and under UV fluorescent illumination (the right panel) to detect DAPI-stained nuclei. (A) Wild-type strain (W303–1A) grown in raffinose + galactose medium (time 0 in Figure 6), (B) YHY009a grown in raffinose + galactose medium (time 0 in Figures 4 and 6), (C) YHY009a incubated for 6 h in raffinose medium alone (time 6 h in Figures 4 and 6), and (D) YHY009a incubate for 12 h in raffinose medium (time 12 h in Figure 6).

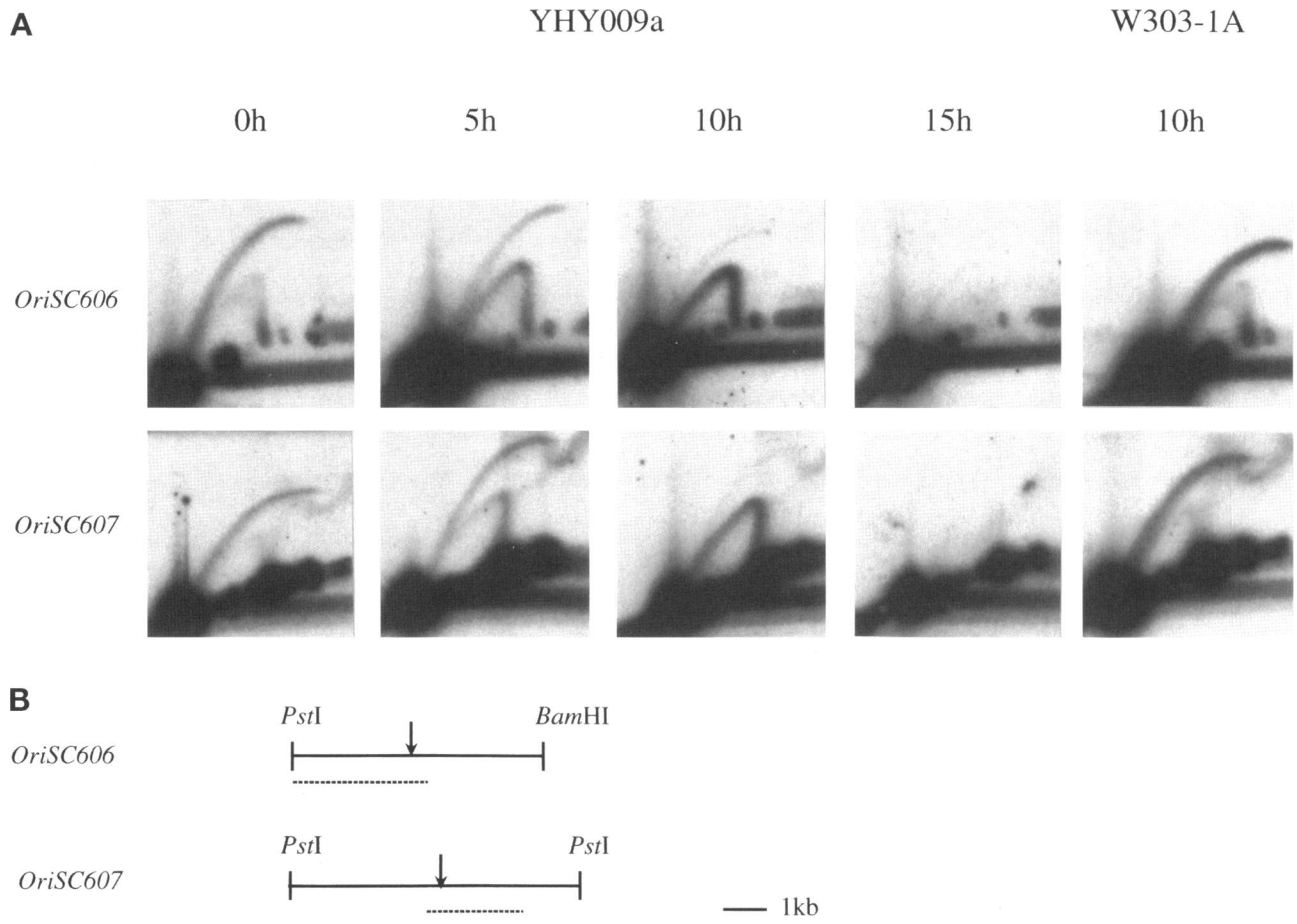


Figure 8. Two-dimensional gel electrophoresis of replication intermediates of chromosomal fragments containing *OriSC606* or *OriSC607*. W303–1A or YHY009a cells were grown in the raffinose + galactose medium or in the raffinose medium as in Figure 4 and culture samples withdrawn at indicated times were used for neutral/neutral two-dimensional gel analysis as described in MATERIALS AND METHODS. (A) The 5.9-kb *Bam*HI–*Pst*I fragment and 6.7-kb *Pst*I fragment containing *OriSC606* and *OriSC607*, respectively, were detected on two-dimensional gels by hybridization to P^{32}-labeled probes shown in panel B. Ten micrograms of DNA was used for 0 h, 5 h, and wild-type (10 h) samples, while 15 μg of DNA were used for 10 h and 15 h mutant samples. (B) The physical map of the *OriSC606* and *OriSC607* regions of chromosome VI. Positions of ACS are indicated by vertical arrows. Probes used to detect the replication intermediates of *OriSC606* and *OriSC607* are shown by dotted lines.

identified by a computer search for genes related to *CDC6*, a regulatory gene involved in the G1-S transition of the *S. cerevisiae* cell cycle (Bueno and Russel, 1992; Piatti *et al.*, 1995). Apart from local homologous regions with known cell proliferation-related genes, *CDC6*, *SIR3*, and *CDC18*, the amino acid sequence of the *ORC1* protein does not show any special structural features other than two binding sites for ATP and GTP.

Haploid spores in which all of the *ORC1* coding region was replaced by a foreign gene could not grow, indicating that the gene product is essential for growth. To analyze the function of the *ORC1* gene, a galactose-inducible gene for the HA-tagged *ORC1* protein was constructed in a plasmid and introduced into the haploid spores in which the chromosomal *ORC1* gene had been disrupted. As expected, the growth of the spore is absolutely dependent on galactose. The HA-tagged protein was produced in the presence of galactose, but began to decrease immediately after the depletion of galactose from the growth medium containing raffinose as the sole carbon source. *ORC1* is the largest subunit protein of the complex (*ORC*) of six component proteins that is known to bind specifically to ACS (Bell and Stillman, 1992), sequences essential for autonomous replication of ARS containing circular minichromosomes in *S. cerevisiae* (reviewed by Rowley *et al.*, 1994). The rapid degradation, with a 40-min half-life, of the *ORC1* protein was not anticipated from the known properties of *ORC* in the cell, which are formed at the replication origins of the chromosomes and maintained stably throughout the cell cycle (Diffley and Cocker, 1992; Diffley *et*

al., 1994). The unstable property observed of the galactose-inducible *ORC1* protein may be that of an artificial HA-tagged protein. On the other hand, nothing is known about the turnover of the *ORC* and its component proteins. *ORC* may be in a dynamic state, constantly exchanging its protein components with newly formed proteins during the cell cycle.

The effect of the decrease in HA-*ORC1* protein by the galactose depletion on the cell cycle progression appeared immediately. It should be noted that the shift of the carbon source from galactose + raffinose to raffinose alone caused a suppression of the G1 to S progression even in the wild-type strain, resulting in the accumulation of G1 phase cells. No effect of the carbon shift on G2 cell progress was observed in the wild type. The FACS pattern of the mutant strain was entirely different from that of the wild type. No change in the pattern was observed for the first 5 h except for a slight decrease in the 2N population. Since G1 to S phase progression must be suppressed like in the wild-type cell due to the medium shift, the pattern shows that the rate of cell cycle progression of the mutant 2N cell was greatly reduced immediately after the shift. The exact fate of 2N cells present at the time of galactose depletion is difficult to estimate by the FACS analysis alone. However, a slow increase of viable cells to 1.8 fold at 10 h after galactose depletion suggests that 2N cells at time 0 underwent cell division slowly. The G1 to S progression occurred after the lag period as observed in wild-type cells, but very slowly in mutant cells and eventually give rise to the accumulation of cells with a variable amount of DNA between 1N to 2N. The mutant strain cells are of dumbbell shape with one nucleus of irregular morphology. From these results we assumed that the primary effect of the decrease in *ORC1* protein is the suppression of cell cycle progression in the S phase. This is proved directly by two-dimensional gel analysis of replication intermediates of the two most active replication origins of the chromosome VI. The data in Figure 8 show clearly that the initiation of chromosomal replication is preferentially affected by the depletion of the *ORC1* protein without affecting elongation. The decrease in initiation frequency of the replication eventually caused the cessation of replication and may result in incompletely replicated chromosomes. Temperature-sensitive mutants of *ORC2* and *ORC5* proteins, other components of the *ORC*, have been reported to result in G2 arrest at restriction temperatures (Bell *et al.*, 1993; Loo *et al.*, 1995). The apparent difference from our observation of *ORC1* protein depletion may be due to the difference between the effects of ts mutant proteins and the depletion of the essential protein.

Since *ORC1* protein is a component of the *ORC*, the effect of depletion of the *ORC1* protein on the cell cycle progression should be discussed in relation to the dynamics of *ORC* during cell cycle (Figure 9). It has been proposed that the *ORC* remains bound to the replication origins throughout the cell cycle of *S. cerevisiae* cells (Diffley and Cocker, 1992; Diffley *et al.*, 1994). When chromosomes are replicated, the *ORC* of the parental chromosomes must be redistributed to two daughter chromosomes and new *ORC*s should be made to occupy all newly formed replication origins. This process may take place during the S phase, G2 phase, or in the G1 phase of the next cell cycle. However, the results presented above suggest that the con-

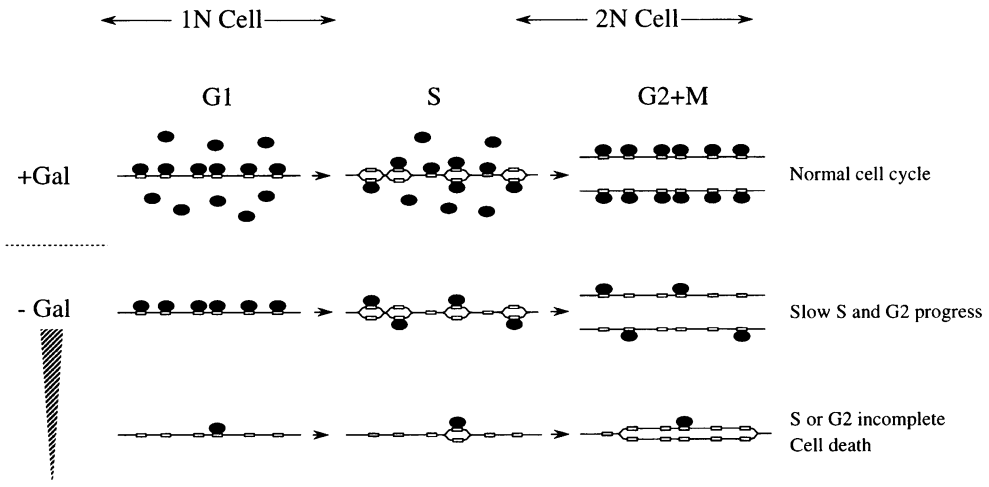


Figure 9. A model of *ORC* dynamics in the *S. cerevisiae* cell cycle. A model of the formation of the *ORC*-origin complex in the cell cycle of the mutant cells expressing HA-tagged *ORC1* is presented. First, we assume that during the S-phase, replication of a chromosome of multiple replicons takes place from origins bound with *ORC* (black ovals). Second, old *ORC* remained attached to origins, but no new *ORC*-origin complexes are formed until the late S-phase (observed as 2N cells). All of the newly replicated origins were bound by *ORC* only in 2N cells either in late S phase or during the G2 + M phase. In the presence of galactose (+Gal), cells can undergo an almost normal cell cycle with sufficient amounts of *ORC* containing HA-tagged *ORC1* provided by a galactose inducible gene. In the absence of galactose, cells containing 2N chromosomes with a fewer number of bound *ORC* are formed. Such chromosomes may still undergo mitosis and subsequent replication from the smaller number of active origins, but may result in the formation of incomplete chromosomes or complete replicated chromosomes with too few *ORC*. These cells are incapable of progressing to the M phase and eventually die.

struction of new *ORC*-origin complexes as well as their turnover occurs in cells during S and G2 phase. Therefore the absence of any component proteins would become rate limiting of S and G2 phase progression. Partial deficiency in the *ORC1* protein may give rise to a smaller number of intact *ORC*s per chromosome, which would still permit a slow progression of the cell cycle into another cycle. Replication of chromosomes may be possible with the fewer number of active origins than those functioning in normal growth. However, when the concentration of *ORC1* becomes lower than a threshold value the cell cycle progression would arrest at various stages in the S phase.

In this paper we have adopted a method in which the cellular content of a protein essential for cell growth can be controlled by an inducible expression system. In this way the function of the protein can be investigated in conditions more physiological than those using conditional mutations. The approach of depleting a subunit component from the *ORC* is particularly useful in the analysis of the dynamic aspects of the functional complex in the cell.

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