The ORC1 homolog orp1 in fission yeast plays a key role in regulating onset of S phase

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In a screen for new cell-cycle genes in Schizosaccharomyces pombe we have isolated cdc30, which is identical to orp1, a putative homolog of the Saccharomyces cerevisiae ORC1 gene. Analysis of the temperature-sensitive orp1-4 and the orp1A mutants indicates that orp1 is required at the onset of S phase for an early step of DNA replication. Orp1p is found in the nucleus and is present at a constant level throughout the cell cycle. Genetic interactions occur between orp1 and cdc18 and cdc21 (an MCM homolog). Orp1p forms protein complexes with both cdc18p and cdc21p in vivo, suggesting that interactions between these proteins and ORC are important for controlling the initiation of DNA replication at the onset of S phase. The orp1 gene is also required for the control that prevents entry into mitosis in the absence of DNA replication, suggesting a role for ORC in this checkpoint pathway.

[Key Words: ORC1 homolog; fission yeast; S-phase onset; checkpoint]

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Control of the G1→S-phase transition is an important regulatory point in the eukaryotic cell cycle, because successful replication of the chromosomes is a prerequisite for producing two normal daughter cells at division. Understanding how S phase is regulated requires characterization of the molecular mechanism that carries out DNA replication, and also a description of the regulatory processes that ensure that replication occurs once and only once every cell cycle. Here we characterize a fission yeast gene that may be part of the link between the mechanism of DNA replication and overall cell-cycle control.

Much progress has been made in describing the components of the eukaryotic DNA replication machinery using in vitro model systems, primarily SV40, and genetic systems, primarily budding yeast. One component of particular interest for understanding the initiation of replication is the complex of six proteins called the origin recognition complex (ORC) purified from budding yeast (Bell and Stillman 1992). The genes encoding these proteins have been cloned (Bell et al. 1993, 1995), and it has been demonstrated that the frequency of initiation events decreases in orc mutants (Fox et al. 1995; Liang et al. 1995). The ORC complex binds origins throughout the cell cycle (Diffley and Cocker 1992; Diffley et al. 1994), and therefore changes in origin binding of the six proteins making up the ORC complex seem unlikely to control the initiation of replication. The genomic footprint on replication origins changes prior to replication as a result of the formation of a prereplicative complex (Diffley et al. 1994). Components of this prereplicative complex additional to the six proteins making up the ORC have yet to be defined. Homologs of two components of the ORC complex have been identified in a number of organisms: ORC1 homologs have been found in Kluyveromyces lactis, human cells, and Schizosaccharomyces pombe (Gavin et al. 1995; Muzi-Falconi and Kelly 1995), and ORC2 homologs in Arabidopsis thaliana, Caenorhabditis elegans, human cells (Gavin et al. 1995), Xenopus laevis (Carpenter et al. 1996), fruit fly (Gossen et al. 1995), and S. pombe (Leatherwood et al. 1996). The existence of these homologs suggests that the ORC complex is likely to be conserved during evolution.

In fission yeast cdc18 is a crucial regulator of the initiation of DNA replication. The levels of cdc18 mRNA and of p65cdc18 encoded by cdc18 both increase prior to entry into S phase (Kelly et al. 1993; Nishitani and Nurse 1995; Muzi-Falconi et al. 1996). In the absence of cdc18 the cells cannot replicate their DNA, blocking initially with a 1C DNA content and then proceeding to mitosis. This last observation indicates that p65cdc18 is also necessary to prevent mitosis until S phase is complete (Kelly et al. 1993). Importantly, overexpression of the cdc18 gene is sufficient to drive cells into S phase in the absence of mitosis and without continuing protein synthesis (Nishitani and Nurse 1995), indicating that p65cdc18 is likely to play a rate determining role in the onset of S phase.
The regulatory processes governing DNA replication ensure that S phase is confined to a specific phase of the cell cycle and is coordinated with other major cell-cycle events. Two main control mechanisms are emerging that have been proposed to limit DNA replication to once per cell cycle. One is the global cell-cycle control acting over entry into S phase that involves cyclin-dependent kinases (CDKs). Identification of a role of CDKs in ensuring that there is only one S phase per cell cycle was made in fission yeast. Deletion of the cdc13 gene, which encodes the major mitotic B cyclin in fission yeast, leads to repeated rounds of replication without intervening mitoses, suggesting that the presence of the cdc2/cyclinB protein kinase prevents a G2 cell from undergoing another S phase [Hayles et al. 1994]. Overexpression of the rum1 gene also results in repeated rounds of replication [Moreno and Nurse 1994] as a consequence of p25rum1 acting as an inhibitor of the cdc2/cyclinB complex [Correa-Bordes and Nurse 1995; Martin-Castellanos et al. 1996]. The second control mechanism has emerged from experiments with Xenopus extracts that have identified an activity required to "license" DNA, such that replication occurs only once in each cell cycle. Several lines of evidence suggest that proteins of the MCM family may have a role in this licensing control. MCM genes were first identified in budding yeast in genetic screens for mutants that lose minichromosomes at high frequency [Maine et al. 1984]. The MCM proteins show a cell-cycle-dependent localization in the yeast nucleus [Hennessy et al. 1990; Yan et al. 1993; Dalton and Whitbread 1995], and have been identified in a large number of organisms [for review, see Chong et al. 1996]. In multicellular eukaryotes, MCM proteins are generally found to be nuclear throughout the cell cycle, although they show cell cycle-dependent chromatin binding [Kimura et al. 1994; Chong et al. 1995; Kubota et al. 1995; Madine et al. 1995; Todorov et al. 1995; Cou4 et al. 1996; Fujita et al. 1996; Krude et al. 1996]. The exact role of MCMs and the mechanism by which they contribute to proper regulation of S phase is as yet unclear.

In this paper we describe the isolation of cdc30 from fission yeast and show that it is identical to orp1, a gene proposed to encode a homolog of ORC1. Analysis of the phenotypes of temperature-sensitive and deletion mutants of orp1 indicates that the gene is required at an early step of DNA replication and is also necessary to restrain mitosis until DNA replication is complete. We show genetic and physical interactions between orp1 and cdc18, and between orp1 and cdc21, an MCM4 homolog in fission yeast, and propose that these interactions are important for linking the initiation of DNA replication to overall cell-cycle control.

Results

Isolation of mutants acting at G1/S

To identify new genes acting at the G1/S-phase [G1/S] control in fission yeast we screened a bank of 2000 temperature-sensitive lethal mutants for those that arrested at the G1/S transition. We isolated 172 mutants displaying an elongated phenotype at the restrictive temperature, and 65 of these were found by DAPI staining to arrest with a single interphase nucleus. These were subjected to FACS analysis to identify those which arrested with a 1C DNA content or close to a 1C DNA content, and 12 such mutants were isolated. Complementation analysis between these 12 mutants and mutants of previously described genes required at the G1/S transition identified novel alleles of cdc6, cdc23, and cdc20 and five new genes. The mutants in the five new genes showed various phenotypes: Two arrested with a 1C DNA content, two showed S-phase defects, and one arrested with a mixed population of cells having 1C and 2C DNA contents. One of the mutants that arrested with a 1C DNA content, cdc30-4, was chosen for further study because it showed uniform first cell-cycle arrest. Cell division stopped after a cell number doubling, and cells became elongated with a uniform 1C DNA content (Fig. 1).

cdc30-4 arrests early in the cell cycle

To map the cell-cycle arrest point of the cdc30-4 mutant more accurately we carried out a reciprocal shift experiment with a hydroxyurea block. DNA replication in cdc30+ and cdc30-4 cells was inhibited using hydroxyurea at 25°C, the permissive temperature for cdc30-4. The cells were then shifted to the restrictive temperature 36°C, and the hydroxyurea removed. The two cultures underwent cell division with the same kinetics [Fig. 2A], indicating that cdc30-4 functions either upstream or at the hydroxyurea block. The same result was obtained when the cells were kept at 36°C during the last hour of the hydroxyurea block to ensure that the temperature-sensitive protein was fully denatured before re-

Figure 1. cdc30-4 arrests with a 1C DNA content. (A) cdc30-4 cells after 5 hr at the restrictive temperature. (B) Cell number increase of cdc30-4 cells upon shift to 36°C. (C) FACS analysis of cdc30-4 cells at permissive temperature and 2, 4, and 5 hr after shift to the restrictive temperature.
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Figure 2. cdc30-4 arrests at an early stage of S phase. (A) Cell number increase in a hydroxyurea reciprocal shift experiment. (A) cdc30-4 cells; (●) cdc30+ cells. Cells were arrested with 12 mM hydroxyurea at the permissive temperature of 25°C, then shifted to the restrictive temperature of 36°C and the hydroxyurea removed. (B) cdc30-4 cells can conjugate from the arrest point. cdc30-4, cdc10-129, cdc25-22, and leu1-32 cells were mixed with mat2-102 ade6-4 cells to determine the conjugation frequency as described in Materials and Methods. Open bars: incubated at 25°C, hatched bars: shifted to 36°C for 4 hr and then to 32.5°C to allow conjugation. (C) Level of cdc18 message in cdc30-4. RNA was isolated from cells shifted to 36°C for 0, 3, 4, and 5 hr. ura4 transcript level was measured as loading control. (D) Tyrosine phosphorylation of cdc2 in cdc30-4. Protein extracts were prepared from cells shifted to 36°C for 0, 3, 3.5, 4, 4.5, and 5 hr for Western blotting with a-cdc2 [upper lanes] and α-tyrosine phosphorylated cdc2 [lower lanes]. Panel on left shows cdc30-4 extracts, panel on right shows cdc25-22 [lane 1] and cdc10-129 [lanes 2,3] incubated at 36°C for 0, 4, and 4 hr, respectively, to demonstrate the specificity of the antibody for the tyrosine phosphorylated form. (E) PFGE of chromosomes from orpl-4 [cdc30-4] cells 0, 3, 4, and 5 hr after shift to 36°C. [F] Plasmid stability in orpl-4. leu1-32 and leu1-32 orpl-4 cells were transformed with a plasmid carrying one copy of ARS1 and a LEU2 marker [see text]. The histogram shows the number of leu+ cells as a percentage of total cells at the time of shift back to 25°C.

removal of the hydroxyurea (data not shown). This excludes the possibility that the observed replication and cell division were due to residual activity of the temperature-sensitive protein following shift-up. The reciprocal experiment was also performed when cdc30-4 was incubated at 36°C for 4 hr before shift to 25°C in the presence and absence of hydroxyurea. However, even the cells shifted to 25°C in the absence of hydroxyurea did not undergo cell division within a 5-hr incubation [data not shown], indicating that the cdc30-4 cells cannot rapidly recover from the 36°C arrest. This lack of recovery pre-

vents a distinction being made as to whether cdc30+ functions upstream or at the hydroxyurea block. From these experiments we conclude that cdc30+ functions early in the cell cycle, either before or at the start of S phase.

Cells become committed to the mitotic cycle at the G1/S boundary at the control called START. In fission yeast three genes have been found to operate at or before START, cdc2, cdc10, and res1 [Nurse and Bissett 1981; Tanaka et al. 1992; Caligiuri and Beach 1993]. To determine whether the cdc30-4 mutant is committed to the cell cycle at its arrest point, we asked whether such arrested cells were able to conjugate. The conjugation efficiency of cdc30-4 was compared with that of cdc10-129, which is able to conjugate from its arrest point in G1 [Nurse and Bissett 1981]. Arrested cdc30-4 cells form about half as many diploids as cdc10-129, and far more than those formed with the G1-arrested cdc25-22 cells [Fig. 2B]. Therefore cdc30-4 appears to arrest before or at the point of commitment to the mitotic cell cycle.

cdc10 is required for the transcriptional activation of genes necessary for the initiation of S phase [Lowndes et al. 1992], including transcription of the cdc18 gene [Kelly et al. 1993]. cdc30-4 arrests with cdc18 mRNA present at a level comparable to that seen in exponentially growing cells [Fig. 2C], suggesting that the cdc30-4 mutant arrests later than cdc10-129. Consistent with this conclusion is the observation that the tyrosine phosphorylated form of p34cdc2 accumulates in cdc30-4 cells 4.5-5 hr after the shift to 36°C [Fig. 2D, lower left] when they have all arrested in G1 [Fig. 1C]. This result is in contrast to a cdc10-129 mutant 4 hr after the shift to 36°C when no tyrosine phosphorylated form is detectable [Fig. 2D, lower right, Hayles and Nurse 1995].

Overall, these experiments establish that cdc30 functions early in the cell cycle, close to the point of cell cycle commitment at START and to the onset of S phase.

cdc30 encodes a homolog of ORC1

The cdc30 gene was cloned by complementation of the temperature-sensitive lethal phenotype using a fission yeast genomic library. The gene encodes a putative protein of 707 amino acids that is 31% identical to the product of the S. pombe cdc18 gene and 30% identical to the product of the S. cerevisiae ORC1 gene. Recently the gene has been isolated independently by Gavin et al. [1995] and Muzi-Falconi and Kelly [1995] as orpl. Furthermore, a temperature-sensitive mutant has been isolated by Suto, H. Murakami, and H. Okayama [pers. comm.], which also defines the same gene. Given that the name orpl has already been used for this gene, from now on we shall use the name orpl instead of cdc30.

The orpl-4 mutant is defective in DNA replication

The early arrest point of the orpl-4 [originally cdc30-4] mutant suggests a role at the G1/S transition. Given the sequence similarity to the S. cerevisiae ORC1, we inves-
tigated whether DNA replication is defective in this mutant. DNA was prepared for pulsed field gel electrophoresis from orp1-4 cells grown at permissive and restrictive temperatures. When the cells were grown at the permissive temperature, the three chromosomes entered the gel and could be separated as three distinct bands. In contrast, when the cells were grown at the restrictive temperature, the chromosomes could not enter the gel (Fig. 2E). This indicates that the topology of the DNA is altered, suggesting that some early stages of the initiation process have occurred at the nonpermissive temperature. Since these cells have an approximately 1C DNA content, this indicates that the orp1-4 mutant has a defect at an early stage of DNA replication.

Several *S. cerevisiae* mutants defective in DNA replication have been reported to lose episomal plasmids at high frequency (Hogan and Koshland 1992; Foss et al. 1993; Micklem et al. 1993; Loo et al. 1995). We examined whether this is also true for the orp1-4 mutant. orp1 + and orp1-4 cells were transformed with a plasmid carrying one copy of ARS1 and a selectable marker. The transformants were shifted to the restrictive temperature and selection for the plasmid relaxed. After 4 hr at 36°C the cells were shifted to the permissive temperature and plated onto appropriate media to determine the rate of plasmid loss. While the temperature shift had no effect on plasmid loss in orp1 + cells, less than a quarter of the orp1-4 cells retained the plasmid after 4 hr incubation at 36°C (Fig. 2F). This result indicates that the orp1 gene function is required for efficient replication of plasmids.

**Synthetic checkpoint defects in orp1-4 cdc18-K46 and orp1-4 cdc21-M68 double mutants**

Between 3 to 5 hr after shifting the orp1-4 mutant to the restrictive temperature, the cell number increase reached a plateau, and after this time, from 5 to 8 hr, cell number increased again [Fig. 3Aa]. This increase was due to mitosis and cell division as judged by spindle formation and the appearance of postanaphase arrays [Fig. 3Ab,c]. Despite undergoing mitosis, the cells had still failed to undergo DNA replication, and divided with a 1C DNA content, producing daughter cells with an uneven distribution of DNA [Fig. 3Ac]. This so-called “cut” phenotype was eventually exhibited by up to 50% of the population [Fig. 3Aa]. This suggests that in addition to being required for DNA replication, the orp1 gene also may have some role in the checkpoint pathway that prevents mitosis until S phase is complete.

This G1 arrest and cut phenotype of the orp1+ mutant is reminiscent of that observed with cdc18Δ. Therefore we asked whether there was a genetic interaction between orp1 and cdc18. We constructed a double mutant carrying both the temperature-sensitive cdc18-K46 and orp1-4 alleles. The temperature-sensitive allele cdc18-K46 is leaky, and unlike the full deletion of cdc18 it arrests with an approximately 2C DNA content and fails to enter mitosis (Nasmyth and Nurse 1981). While the orp1-4 mutant arrests at G1/S and only then proceeds slowly to mitosis and cell division, the double mutant proceeds rapidly to mitosis and cell division [Fig. 3B]. Overexpression of cdc18 in the orp1-4 mutant rescues the checkpoint defect [Fig. 3B]. These observations sug-
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uggest that orp1 and cdc18 might interact in the process that prevents mitosis in the absence of DNA replication.

In addition to the synthetic checkpoint defect of cdc18 and orp1, a genetic interaction was also observed between orp1 and cdc21, which encodes the fission yeast homolog of MCM4 (Coxon et al. 1992). The cdc21-M68 and orp1-4 mutants are synthetically lethal, at 29°C the double mutant is unable to form colonies, unlike the single mutants. Furthermore, the checkpoint phenotype of the cdc21-M68 orp1-4 double mutant at 36°C is enhanced, although only a smaller percentage of 1C cells undergo mitosis [Fig. 3C] compared with the cdc18-K46 orp1-4 double mutant.

These results establish that orp1 plays a role in preventing mitosis when S phase is incomplete, and are suggestive that orp1 may interact with cdc18 and the MCM4 homolog cdc21 in bringing about this checkpoint control.

Characterization of orp1Δ and orp1 overexpressing strains

We deleted the region coding for amino acids 195–607 of orp1 and replaced it with the ura4+ gene; the deletion removes the region most highly conserved between cdc18 and ORC1. One of the wild-type copies of the gene was replaced with the deletion construct in a diploid strain homozygous for ura4-D18. When the diploid was sporulated and tetrads analyzed, only two spores were able to form colonies. Both of these were ura4−, showing that the orp1 gene is essential. To analyze the phenotype of the deletion mutant, the diploid was sporulated and the spores inoculated into medium that allows germination of only the spores carrying the deletion. The orp1Δ spores germinated and underwent the first cell cycle with a similar kinetics to orp1+ spores [Fig. 4A], but at later timepoints they underwent cell-cycle arrest. However, unlike the temperature-sensitive orp1-4 mutant, the deletion mutant cells arrested with a 2C DNA content. Furthermore, only a few percent of the cells showed aberrant nuclear morphology indicative of a premature entry into mitosis, even 18 hr after inoculation [Fig. 4A]. These results are similar to the findings of Muzi-Falconi and Kelly (1995).

We were concerned that the phenotype of the temperature-sensitive mutant was different from that of the orp1 deletion. The spores carrying the deletion underwent at least one complete normal cell cycle after germination, indicating that the spores might have contained residual orp1 protein carried over from the parental diploid into the spore, masking expression of the deletion phenotype. We therefore constructed a diploid strain heterozygous for the deletion and the temperature-sensitive orp1-4 allele. Spores derived from this diploid were germinated at the restrictive temperature to inactivate any residual temperature-sensitive orp1 protein. The spores carrying the orp1Δ arrested in G1, and then proceeded to mitosis primarily with a 1C DNA content, with over 90% of cells displaying the cut phenotype 10 hr after inoculation [Fig. 4B]. This result confirmed our conclusions drawn from the phenotype of the temperature-sensitive mutant.

We next tested the effect of overexpressing orp1 to high levels in cells. The orp1 gene was expressed using the full-strength nmt1 promoter. Cells became elongated with a 2C DNA content and the nuclei showed an unusual, enlarged, diffuse morphology [Fig. 4C]. The cells cannot form colonies and thus high levels of the orp1 gene product are lethal.

Figure 4. Deletion and overexpression of the orp1 gene. (A) FACS analysis of orp1+ [left] and orp1Δ [right] spores germinating at 30°C. DAPI-stained cells are shown 18 hr after inoculation. The arrowheads point at rare cells with aberrant nuclear morphology. Bar, 10 μm. (B) FACS analysis of orp1+ [left] and orp1Δ spores carrying the temperature-sensitive orp1 protein [right], germinating at 36°C. Smearing of the FACS profile is often seen in germinating spores undergoing cutting. DAPI-stained cells are shown 10 hr after inoculation. Arrowheads point at cells showing the cut phenotype. Bar, 10 μm. (C) Overexpression of the orp1+ gene from the nmt1 promoter. [Left] FACS of cells grown in the presence of thiamine [promoter off]. [Middle] FACS analysis of cells 22 hr after induction. [Right] DAPI-stained cells 22 hr after induction. Bar, 10 μm.
Regulation of orp1 transcription, protein level, and intracellular localization

To investigate whether orp1 is regulated at the transcriptional level during the cell cycle, the orp1 message level was measured in cells arrested at various cell-cycle stages, using cell-cycle mutants and hydroxyurea. The stage of the cell-cycle block is shown in brackets in the following list. The level of the orp1 transcripts was the same in cells arrested with the cdc10-V50 mutant (G1), cdc25-22 mutant (G2/M), hydroxyurea (S), nda3-KM311 mutant (metaphase), and dis3-54 mutant (anaphase) [Fig. 5A], indicating that there was unlikely to be any periodic changes in transcript level during the cell cycle. To follow the protein level during the cell cycle the orp1 gene was tagged on its carboxyl terminus with a triple HA tag. The orp1 gene carrying the HA tag under control of the orp1 promoter was introduced into the chromosome by gene replacement, and this strain is referred to as orp1-HA [Fig. 5B]. The apparent molecular weight of the tagged protein is 90 kD. The protein level was monitored during the cell cycle in a cdc25 mutant block and release experiment to synchronize the cells. The level of orp1 protein did not change significantly during the cell cycle as cells proceed through mitosis and S phase, either in soluble [Fig. 5C, top] or insoluble [Fig. 5C, bottom] fractions (see Materials and Methods) of protein extracts.

To investigate the intracellular localization of orp1p, exponentially growing orp1-HA cells were fixed and examined by immunofluorescence. Consistent with orp1p being a component of the ORC complex, the protein was localized primarily to the nucleus with some weak staining in the cytoplasm [Fig. 5D]. However, we did not detect the orp1 protein in cells undergoing mitosis [Fig. 5D, arrowed cells]. This may be a result of an altered exposure of the epitope, possibly because of chromatin condensation, or a result of relocalization of the protein from the nucleus into the cytoplasm during mitosis. As a control, wild-type cells lacking orp1-HA were examined in a similar way by immunofluorescence, and although the cytoplasmic staining was detected and so is presumably nonspecific, no distinct nuclear staining was observed [Fig. 5D, inserts].

orp1p interacts physically with cdc18p and cdc21p

To investigate whether the genetic interactions between the cdc18, cdc21, and orp1 genes result from direct protein–protein interactions, protein extracts prepared from protoplasts were immunoprecipitated with α-HA antibody and Western blotted with α-cdc18p [gift of H. Nishitani, Imperial Cancer Research Fund, London, UK] and α-cdc21p antibodies [gift of D. Maiorano and S. Kearsey, Oxford University, UK]. The orp1-HA cells were used, and wild-type and cig2-HA cells used as controls. Extracts from all three stains were Western blotted using the α-cdc18p [Fig. 6A, lanes 5–7] and α-cdc21p [Fig. 6B, lanes 5–7] antibodies. Both antibodies detected proteins of the correct molecular weight, confirming previous characterization carried out by Nishitani and Nurse [1995] and Maiorano et al. [1996]. The extracts were also Western blotted with α-HA antibodies that detected proteins of the correct molecular weight in extracts from the orp1-HA and cig2-HA stains but not wild-type [Fig. 6C, lanes 5–7].

The orp-HA protein was immunoprecipitated from extracts of the orp1-HA stain, and this immunoprecipitation could be competed by excess HA peptide [Fig. 6, lanes 1,2]. Both cdc18p [Fig. 6A, lane 2] and cdc21p [Fig. 6B, lane 2] could be detected in the orp1-HA immunoprecipitation but neither was present in the HA peptide.
The orp1 protein interacts with the cdc18 and cdc21 proteins. Protein extracts were prepared from exponentially growing orp1-HA (lanes 1,2,5) untagged wild-type (lanes 3,6) and cig2-HA (lanes 4,7) cells. α-HA immunoprecipitates from 10-μg soluble extracts were run in lanes 1–4. 50 μg soluble extracts were run in lanes 5–7. The orp1 protein was immunoprecipitated both in the presence (lane 1) and in the absence (lane 2) of HA peptide. The samples were run on SDS-PAGE gel, Western blotted, and probed with α-cdc18 [A], α-cdc21 [B], and α-HA [C] antibodies.

**Figure 6.** The orp1 protein interacts with the cdc18 and cdc21 proteins. Protein extracts were prepared from exponentially growing orp1-HA (lanes 1,2,5) untagged wild-type (lanes 3,6) and cig2-HA (lanes 4,7) cells. α-HA immunoprecipitates from 10-μg soluble extracts were run in lanes 1–4. 50 μg soluble extracts were run in lanes 5–7. The orp1 protein was immunoprecipitated both in the presence (lane 1) and in the absence (lane 2) of HA peptide. The samples were run on SDS-PAGE gel, Western blotted, and probed with α-cdc18 [A], α-cdc21 [B], and α-HA [C] antibodies.

**Discussion**

In this paper we characterize a gene that we propose has a role at the onset of S phase in fission yeast. The gene, initially called cdc30, was found to be identical to the recently described orp1 gene that encodes a protein homologous to S. cerevisiae ORC1 (Gavin et al. 1995; Muzi-Falconi and Kelly 1995). Given that orp1 already has been described, and the name is more consistent with the nomenclature in budding yeast where the ORC complex is best characterized, we use the name orp1 instead of cdc30.

We have presented several lines of evidence that orp1 acts at the G1/S-phase transition. Analysis of temperature-sensitive and deletion mutants of orp1 show that they arrest with a 1C DNA content, prior to bulk DNA synthesis. This result is consistent with the fact that the arrest point is at or before the arrest point of the DNA synthesis inhibitor, hydroxyurea. The chromosomes prepared from the temperature-sensitive orp1-4 mutant incubated at the restrictive temperature fail to enter the gel during pulsed field gel electrophoresis, indicating that at the arrest point some topological changes of the DNA have occurred that prevent entry into the gel. The orp1-4 mutant also fails to maintain autonomously replicating episomal plasmids, indicating a defect in DNA replication. These various observations establish that orp1 acts at the G1/S-phase transition at a stage close to the initiation of DNA replication. Given the homology of orp1 to ORC1, we conclude that an equivalent of the ORC complex is present in fission yeast and that this has an important role in the initiation of DNA replication. Similar conclusions have been suggested by others (Gavin et al. 1995; Muzi-Falconi and Kelly 1995; Leatherwood et al. 1996).

We also have presented evidence that orp1 interacts genetically with both cdc18 and cdc21, the latter showing homology to the MCM4 gene from budding yeast. The associations between orp1, cdc18, and cdc21 indicated by these genetic interactions have been confirmed by immunoprecipitation experiments. Immunoprecipitates of HA-tagged orp1p from cell extracts contain the proteins encoded by both cdc18 and cdc21. In these experiments all three genes are controlled by their endogenous promoters and thus the three proteins are expressed at normal levels. Therefore the observed interactions are not a result of an artefact of overexpression. We conclude that orp1p forms complexes in vivo with both p65cdc18 and the MCM homolog cdc21p, suggesting that these latter two proteins become associated with the ORC complex. Expressing p65cdc18 to high levels is sufficient to drive cells into DNA synthesis, and the level of p65cdc18 is periodic during the cell cycle, peaking at the G1- to S-phase transition (Nishitani and Nurse 1995; Muzi-Falconi et al. 1996). In contrast, we have shown here that the levels of orp1p are constant during the cell cycle; orp1p is also found in the nucleus, although it may undergo some change during mitosis that makes it less easy to detect by immunofluorescence. Given the physical association between orp1p and p65cdc18 reported here and the interaction between orp2p and p65cdc18 reported by Leatherwood et al. (1996), we propose that the increase in level of p65cdc18 at G1/S results in p65cdc18 becoming associated with orp1p and orp2p, transiently activating a putative ORC and bringing about the initiation of DNA replication. Sometime toward the end of S phase, p65cdc18 levels drop, and thus further activation of ORC complexes is prevented. The MCM4 homolog cdc21 encodes a protein that also appears to be complexed with orp1p. Given its role in other systems, cdc21p may be one of the components that distinguish G1 chromosomes from G2 chromosomes, allowing G1 but not G2 cells to undergo DNA replication. The biochemical mechanisms involved in these processes are unclear, but orp1p contains a nucleotide binding site and so its action may involve ATP or GTP hydrolysis.

The proposal that associations between p65cdc18
cdc21p, and ORC are important steps in the initiation of DNA replication can be incorporated into models of S-phase control presented by ourselves and others (Liang et al. 1995; Nishitani and Nurse 1995; Cocker et al. 1996, Wuarin and Nurse 1996). The association between p65cdcl8 and orplp may be necessary to generate a pre-replicative complex similar to that proposed for budding yeast. The formation of this complex during G1 of budding yeast requires the CDC6 gene function (Cocker et al. 1996), which is a close homolog of cdcl8, and other genes may also be involved such as those encoding the MCM proteins. This process would license the DNA for replication. The generation of this complex would be the first stage in a two-step process for the initiation of DNA replication, with the second step being the onset of DNA replication. The fall in p34cdc2 protein kinase activity as cells exit mitosis and enter G1 would allow the association to occur and for the prereplicative complex to form on DNA origins. The complex could be similar to that found in *Xenopus* nuclei when p34cdc2 is inactivated (Adachi and Laemmli 1994). After this first step is completed the complex requires phosphorylation by p34cdc2 to bring about DNA replication, this process being the second step in the initiation of DNA replication. The rise in late G1 of p34cdc2 protein kinase activity or of other S-phase promoting CDKs would bring about this second step and automatically prevent the formation of any further prereplicative complexes. As a consequence DNA would only be replicated once in any S phase. Such a model (Wuarin and Nurse 1996) could explain licensing and also links a number of molecules implicated in this control, including p34cdc2, p65cdcl8 [CDC6], orplp [ORC1], orplp2 [ORC2], and cdc21p [MCM4].

In addition to being required for initiation of DNA replication, the orpl gene is also required for the checkpoint pathway that prevents entry into mitosis until S phase is complete. The phenotypes of both the orpl temperature-sensitive and deletion mutants are similar to that of cdcl8A, and several other mutations such as cdcl8Δ, cdt1Δ, cut5/rad4, and pol1Δ, which not only block DNA replication but also allow the cells to enter mitosis with unreplicated DNA (Saka and Yanagida 1993; Hofmann and Beach 1994; D’Urso et al. 1995; Maiorano et al. 1996). We have also shown that the cdcl8ts orplts and cdc21ts orplts double mutants show a synthetic checkpoint defect, and that the corresponding proteins interact physically within the cell. These observations suggest that a complex involving these proteins is necessary to generate a signal that S phase is in progress and as a consequence onset of mitosis should be blocked. The effects of the orplts, cdcl8ts, and cdc21ts single mutants are less severe than the double mutants or the corresponding null mutants. This may be because the single temperature-sensitive mutants have a less severe effect on the formation of the complex, which allows some defective DNA replication to take place and still generates a checkpoint signal that partially blocks mitosis. More severe defects produced by the double temperature-sensitive or null mutants might disturb formation of the complex more profoundly, blocking DNA replication and preventing the checkpoint signal from being generated.

We have also shown that the orpl-4 mutant is not yet committed to the mitotic cycle at its arrest point. Unlike the situation for previously identified mutants arresting pre-START, in orpl-4 cells DNA replication has been initiated at the arrest point as judged from the inability of the chromosomes to enter the gel during pulsed-field gel electrophoresis (PFGE). This observation indicates that initiation of DNA replication does not require complete commitment to the mitotic cycle because cells can still be diverted away from the mitotic cycle to undergo conjugation. The window during which cells are able to undergo conjugation appears to extend into S phase. This finding suggests that the traditional view of START as an event in late G1 that commits the cell to the mitotic cycle might require some re-evaluation. START is perhaps best considered as a window early in the cell cycle, during which cells can be diverted to undergo the alternative developmental pathway of conjugation. As cells proceed further toward S phase they complete more and more events required for progression through the cell cycle. Eventually sufficient events are completed for the cell to be put in a state that is incompatible for conjugation. Our experiments indicate that this window may not be closed until sometime just after the initiation of DNA replication. It is also possible that cells blocked using orpl-4 are initially in a committed state for the mitotic cycle but that this state is reversible and cells can eventually slip back to the pre-START state from which they can conjugate. However, operationally it is very difficult to distinguish between these different interpretations.

We conclude that orplp is required for the onset of S phase and plays a role at an early step in the initiation of DNA replication. Orplp is localized to the nucleus, consistent with a role in DNA replication, but its level remains unchanged through the cell cycle. However, orplp is found physically associated with both p65cdcl8 and cdc21p, and so a periodic change during the cell cycle in these protein associations could be important for controlling the onset of S phase. Orplp is also required to signal to the cell that S phase is in progress, implicating this early step in the initiation of DNA replication in this checkpoint control. Given that orplp is likely to be a component of ORC, these results indicate an important role for interactions between cdc18p, cdc21p, and ORC in controlling the initiation of DNA replication at the onset of S phase and in the associated checkpoint controls.

**Materials and methods**

*S. pombe* strains and methods

All strains were derived from the wild types L972 h- and L975 h+. Media and growth conditions were as described by Moreno et al. (1991). The permissive and restrictive temperatures used for the heat sensitive mutants cdcl8-k46, cdc21-M68 [Nasmyth and Nurse 1981], cdc25-22 [Fantes 1979], cdc10-129 [Nurse et al. 1976], and cdc10-V50 [Marks et al. 1992] were 25°C and 36°C.
respectively. The permissive and restrictive temperatures used for the cold-sensitive mutants nda3-RM311 (Hiraoka et al. 1984) and fix3-44 (Ohkura et al. 1988) were 32°C and 20°C, respectively. Standard genetic techniques, 4’, 6-diamidino-2-phenylindole (DAPI) staining, and cell number counting were carried out as described previously [Moreno et al. 1991]. Cells were prepared for FACS analysis as described previously [Sazer and Sherwood 1990]. Mating assays were carried out as described previously [Nurse and Bissett 1981] with the following modifications: Cells were grown to mid-log phase in minimal medium containing 2 mg/ml NH₄Cl. They were then washed and shifted to 36°C in minimal medium without a N-source. Samples were taken for FACS analysis at the time of shift to check that the cells were not accumulating in G₁ due to N starvation. After 4 hr the mating partners were mixed and shifted to 32.5°C to allow conjugation for 7 hr, then plated onto appropriate media to determine the number of diploid cells. Immunofluorescence was carried out as described [Moreno et al. 1991]. Cells were fixed with methanol. TAT1 antibodies (gift from Keith Gull, Manchester University, UK) and CY3 conjugated goat antimouse secondary antibodies (Sigma) were used from Keith Gull, Manchester University, UK) and CY3 conjugated goat antimouse secondary antibodies (Sigma) were used for tubulin staining. 12CA5 antibodies (Boehringer) and CY3 conjugated goat antimouse secondary antibodies were used to examine the localization of orpl-HA.

**Cloning and sequencing**

The genomic clone of orpl + was obtained by transforming the orpl-4 ura4-D18 h+ strain with a genomic library in pJR19 provided by A.M. Carr [Barbet et al. 1992]. Transformants were plated on minimal medium, incubated at 25°C overnight, and then shifted to 36°C. Plasmids were reisolated from 12 colonies which contained three overlapping clones. Sequencing was carried out using the Sequenase II and ATaq cycle sequencing kits United States Biochemical. Alignments were carried out using the GAP program of the GCG package of sequence software.

**PFGE**

Agarose plugs were prepared as described by Kelly et al. [1993]. PFGE was carried out in 0.6% chromosomal grade agarose gel (BioRad), using a BioRad CHEF-DRII. The gel was run for 72 hr at 50 V with a switch time of 30 min, in 0.5× TAE.

**Deletion and spore germination**

A fragment carrying the orpl::ura4 + construct with 1 kb of flanking sequence was transformed into a diploid strain ade6-M210/ade6-M216 ura4-D18/ura4-D18 h+ /h- . Stable transformants were isolated and gene replacement was confirmed by Southern analysis. Diploid cultures of the strain carrying the orpl::ura4 + construct were sporulated on rich medium at 30°C, then sporulated in minimal medium lacking a nitrogen source. Spores were germinated in minimal medium at 30°C or 36°C (orpl1 / orpl1Δ).

**HA tagging of orpl**

A restriction site for Not1 was introduced before the STOP codon of the orpl ORF and was used to insert a fragment encoding three copies of the HA tag. A genomic fragment containing orpl-HA was transformed into the orpl-4 mutant and transformants were incubated at 36°C. Integration was confirmed by Southern analysis.

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**Preparation of protein extracts, immunoprecipitations, and Western blots**

The soluble and insoluble fractions were prepared as described previously [Nishitani and Nurse 1995]. For immunoprecipitations the cells were protoplasted with Novozyme in 0.65 M KCl, washed in 1 M sorbitol and lysed in HB buffer. HB buffer contains 60 mM β-glycerophosphate, 15 mM p-nitrophenylphosphate, 25 mM MOPS (pH 7.2), 1% Triton, 15 mM MgCl₂, 15 mM EGTA, 1 mM DTT, 0.1 mM sodium-vanadate, 1 mM PMSF, 20 mg/ml leupeptin, and 20 mg/ml aprotinin. The lysate was incubated on ice for 30 min, then centrifuged at 15,000 rpm using an Eppendorf microfuge for 20 min at 4°C. Ten milligrams of the supernatant was used for immunoprecipitations. orpl-HA was immunoprecipitated with 12CA5 Mab cross-linked to Dynabeads coated with α-mouse secondary antibody [Dynal]. Protein extracts and immunoprecipitates were electrophoresed using an 8% SDS-polyacrylamide gel. Dilutions of the antibodies were 1:2000 for α-cdc2, 1:500 for α-cdc21, and 1:1000 for α-HA, and proteins were detected using ECL (Amersham).

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