ORC-dependent and origin-specific initiation of DNA replication at defined foci in isolated yeast nuclei

Philippe Pasero, Diego Braguglia, and Susan M. Gasser
Swiss Institute for Experimental Cancer Research, CH-1066 Epalinges/Lausanne, Switzerland

We describe an in vitro replication assay from yeast in which the addition of intact nuclei to an S-phase nuclear extract results in the incorporation of deoxynucleotides into genomic DNA at spatially discrete foci. When BrdUTP is substituted for dTTP, part of the newly synthesized DNA shifts to a density on CsCl gradients, indicative of semiconservative replication. Initiation occurs in an origin-specific manner and can be detected in G₁- or S-phase nuclei, but not in G₂-phase or mitotic nuclei. The S-phase extract contains a heat- and 6-DMAP-sensitive component necessary to promote replication in G₁-phase nuclei. Replication of nuclear DNA is blocked at the restrictive temperature in an orc2-1 mutant, and the inactive Orc2p cannot be complemented in trans by an extract containing wild-type ORC. The initiation of DNA replication in cln-deficient nuclei blocked in G₁ indicates that the ORC-dependent prereplication complex is formed before Start. This represents the first nonviral and nonembryonic replication system in which DNA replication initiates in an ORC-dependent and origin-specific manner in vitro.

[Key Words: DNA replication; yeast; nuclear organization; replication foci; ORC]

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Replication of the genetic material must be achieved once per cell cycle, and with high fidelity and speed, to avoid deleterious consequences for the progeny of the cell. These important requirements imply a complex degree of coordination between cis-acting sequences and regulatory factors involved in this process. In eukaryotic cells, DNA replication initiates at multiple defined sites in the genome, called origins of replication (for review, see Coverley and Laskey 1994; Hamlin and Dijkwel 1995), at which the simultaneous engagement of polymerases ensures coordinated initiation events and a rapid replication of large chromosomes. Critical control mechanisms that are not yet understood fully, ensure that the genome is replicated once and only once per cell cycle (for review, see Muzi-Falconi et al. 1996; Donovan and Diffley 1996; Kearsey et al. 1996).

Thanks largely to yeast genetics, the sequences and factors that control the initiation of DNA replication have been extensively characterized. Two-dimensional gel techniques have shown that ARS (autonomously replicating sequence) elements allow cell cycle-controlled plasmid replication, and function as genomic origins of replication in budding yeast [Brewer and Fangman 1987; Huberman et al. 1987]. On average, ARS elements are found once per 36 kb (Campbell and Newlon 1991), yet chromosomal context and local chromatin structure also regulate initiation, because not all ARS elements are active origins in their genomic location (for review, see Fangman and Brewer 1991; Coverley and Laskey 1994). The origin recognition complex (ORC), a six-subunit complex that recognizes the 11-bp ARS consensus (Bell and Stillman 1992), is bound at both active and inactive origins throughout the cell cycle (Diffley et al. 1994). Strains that carry temperature-sensitive alleles of ORC2 and ORC5 show defects in the maintenance of ARS-containing plasmids, incomplete DNA replication at non-permissive temperature, and a reduced efficiency of origin firing, consistent with a role in the initiation of genomic DNA replication [Bell et al. 1993; Micklem et al. 1993, Fox et al. 1995; Liang et al. 1995]. Recent studies on the temperature-sensitive orp1-4 mutant of Schizosaccharomyces pombe, defective in the largest subunit of ORC, show that spores completely lacking orp1+ arrest with a 1N DNA content, confirming its role in the G₁/S transition [Grallert and Nurse 1996].

Because the ORC complex remains bound at origins throughout the cell cycle [Diffley et al. 1994], other events other than ORC binding are required to trigger the initiation of DNA replication. A two-step process for the activation of origins has been proposed [Coverley and Laskey 1994; Nasmyth 1996]. In yeast cells, the first step requires transformation of the postreplicative ORC footprint into a prereplicative state [Donovan and Diffley

1Present address: Gene Therapy Center, University of Lausanne Medical School, CHUV, CH-1011 Lausanne, Switzerland.
2Corresponding author.
E-MAIL sgasser@eliot.unil.ch; FAX 41 21 652 6933.
1996]. This requires the synthesis of Cdc6p, a protein that interacts with ORC, before activation of the Clb5/Cdk1 complex at the G1/S transition [Liay et al. 1995; Cocker et al. 1996; Piatti et al. 1996; for review, see Heichman 1996; Kearsey et al. 1996]. Consistent with this model, the initiation of DNA replication in Xenopus extracts also requires the Xenopus homolog of Cdc6p [Coleman et al. 1996]. Other candidate regulatory factors acting at origins of replication include the MCM [Mini-chromosome maintenance] family of genes, the mutation of which reduces the efficiency of replication from both genomic and plasmid-borne origins in yeast [for review, see Tye 1994; Romanowski and Madine 1996]. A requirement for both ORC and the XMcM3 protein was confirmed for the replication of sperm nuclei in Xenopus egg extracts [Coleman et al. 1996; Romanowski et al. 1996; Bowles et al. 1996]. The final stimulation of initiation at replication-competent origins is promoted by the activation of an S-phase promoting factor, which in yeast requires both the Clb/Cdk1 and Dbf4/Cdc7 kinase complexes [Jackson et al. 1993; Schwob and Nasmyth 1993].

Studies in higher eukaryotic cells suggest that nuclear organization may be a prerequisite for replication as well. In Xenopus eggs or egg extracts, the input DNA must be preassembled in a pseudonuclear structure to permit replication, and the integrity of the nuclear envelope is necessary for restricting DNA replication to one round only [Blow and Laskey 1986, 1988]. In reconstituted pseudonuclei, DNA synthesis was found to occur in discrete foci, each of which represents a clustering of ~300 replication forks, resembling the foci observed in replicating mammalian nuclei in vivo [Nakamura et al. 1986; Cox and Laskey 1991, Newport and Yan 1996].

To link the genetics of G1/S control—which is well characterized in yeast—with the structural observations of replication foci made in Xenopus or mammalian systems, we have developed an in vitro system for DNA replication based on yeast nuclear extracts. Using nuclei isolated from yeast cells synchronized in S-phase, we prepare nuclear extracts that support a low level of plasmid replication, as monitored by a base substitution and density shift analysis, the appearance of replication intermediates on neutral–neutral two-dimensional (2D) gels, and sensitivity to mutations in the replicative polymerases δ, α, and primase [D. Braguglia, P. Pasero, B. Duncker, P. Heus, and S. Gasser, in prep.]. However, the replication of a supercoiled plasmid in this soluble extract initiates at random sites within the plasmid in both the presence and absence of an intact ARS consensus, reminiscent of the sequence-independent initiation of plasmid replication observed in Xenopus extracts or in transformed mammalian tissue culture cells [Blow and Laskey 1986; Hyrien and Méchali 1992; for review, see Coverley and Laskey 1994].

To circumvent the random initiation that occurs on a naked plasmid, we have introduced isolated yeast nuclei into S-phase extracts, and have monitored replication of the genomic DNA in vitro. Derivatized dUTP is incorporated into genomic DNA in distinct foci, indicating that replication foci are not unique to metazoans. This occurs in G1- or S-phase yeast nuclei, but not in G2- or M-phase nuclei, and is aphidicolin-sensitive. Density-shift experiments confirm the presence of semiconservative DNA synthesis, whereas two-dimensional gel analyses indicate initiation events at or near the origin of replication (ARS) on plasmids carried in these nuclei. These criteria are consistent with bona fide replication of the yeast genomic DNA in vitro. Importantly, replication in G1-phase nuclei is entirely dependent on ORC, being temperature-sensitive in orc2-1 nuclei. The fact that we cannot complement the orc2-1 defect in template nuclei with a wild-type S-phase extract, suggests an important role for ORC in the organization of the prereplication complex (pRC) in G1-phase nuclei, whereas the immediate block to replication upon a temperature shift of preorganized orc2-1 nuclei, demonstrates the essential role of ORC in the initiation event.

Results

The incorporation of nucleotides occurs at discrete foci in isolated yeast nuclei

Our aim has been to establish an efficient in vitro system from yeast that supports both the initiation and elongation events of DNA replication, to analyze mutations that affect DNA replication on a biochemical level. We monitor the incorporation of a derivatized nucleotide, biotin–dUTP, in isolated yeast nuclei introduced into a S-phase nuclear extract, during and after 90 min at 25°C [see Materials and Methods]. Biotin–dUTP incorporation is followed by reaction with avidin–FITC after depositing the nuclei on a microscope slide. As shown in the laser scanning microscopy images [Fig. 1A], a fraction of these nuclei, isolated from a nonsynchronized population of cells, shows discrete yellow foci of the biotin–dUTP signal within the general DNA stain [ethidium bromide (EtBr) red]. The biotin–dUTP [Fig. 1A] can be replaced by digoxigenin-derivatized dUTP (DIG-dUTP) and detected with FITC-tagged F(ab) fragments with identical results [bottom nucleus, Fig. 1C–E], ruling out that the focal staining pattern results from the aggregation of multivalent molecules. The peaks of incorporated label are three- to 10-fold higher than the low background signal within the nucleus [see Fig. 1E], and are absent in the presence of aphidicolin, which inhibits the three DNA polymerases implicated in genomic DNA replication [Fig. 1B]. At longer incubation times, the incorporation of derivatized dUTP expands to produce a uniform FITC signal [e.g., upper nucleus, Fig. 1C–E], consistent with the hypothesis that these correspond to the replication foci seen in vertebrate nuclei [Nakamura et al. 1986; Cox and Laskey 1991]. To demonstrate that the foci are within the nuclear spheres, we show nine partially overlapping focal sections scanning from the bottom to the top of the nucleus, revealing the existence of 15–20 sites of incorporation per nucleus [Fig. 1, DIG–dUTP in G, DNA in F, and the merger in H]. If the yeast genomic origins are distributed equally among these...
foci, each should contain roughly 20 bidirectional replication forks.

The incorporation of derivatized dUTP can be quantified in two ways. First, the fraction of nuclei containing foci can be calculated over several randomly chosen fields (usually scoring 400 nuclei). Second, the ratio of total FITC signal [DIG-dUTP] to EtBr signal [total DNA] can be determined, indicating the level of incorporation relative to nuclear DNA (see Materials and Methods). In a typical incubation using S-phase nuclei (e.g., Fig. 4G, below), 60%-70% of the nuclei show strong replication foci, whereas the rest fail to incorporate any label at all based on fluorescence detection. This assay for replication foci allows us to eliminate any background from contaminating free DNA or sheared nuclei, as fields of intact nuclei are analyzed, and free DNA does not adhere to the microscope slide. In some instances, the yeast were rendered deficient for mitochondrial DNA to avoid signals attributable to contaminating organelles, although this proved unnecessary. The appearance of nuclear DIG-dUTP foci is identical in rho+ and rho0 cells (cf. Fig. 1 with Figs. 3,4,7, below).

Quantitation of the in vitro DNA replication reaction
In metazoans the appearance of replication foci has been shown to correlate with genomic replication [Newport and Yan 1996]. To correlate the observed yeast foci with genomic replication, we monitored DNA synthesis by an independent technique based on [α-32p]dATP incorporation, which is quantified easily. When yeast nuclei are incubated in an S-phase extract in the presence of [α-32p]dATP and analyzed by agarose gel electrophoresis, we observe the accumulation of label in high-molecular-weight DNA (e.g., Fig. 5C, below). To calculate the amount of DNA synthesized, we determined the internal nuclear pool of dATP, the dilution factor for the radiolabeled dATP, the moles of DNA synthesized and moles of template DNA added (see Materials and Methods; Table 1). As expected, the extent of label incorporation depends on both the period of incubation and the ratio of nuclei to nuclear extract (Fig. 4I, below; data not shown). Typically, for 100 ng of input genomic DNA (or 107 nuclei) and a 90-min incubation, between 20-30 ng of newly synthesized DNA comigrate with the band of genomic DNA on the agarose gel. Incorporation generally increases significantly after a further 90 min (see S-phase nuclei in Fig. 4I, below; Table 1, samples 6,7).

The values listed in Table 1 represent a minimal estimate of total DNA synthesis, as they do not include the background of label incorporated into DNA fragments running below genomic DNA, a fraction that is likely to reflect repair synthesis on fragmented chromosomes. The rapid isolation of yeast nuclei for use as template
Table 1. Quantitation of DNA synthesis

| Experiment | Nuclei | Reaction [min] | DNA synthesized pmol | ng |
|------------|--------|----------------|----------------------|----|
| 1          | S phase| 90             | 73                   | 24 |
| 2          | S phase| 90             | 83                   | 28 |
| 3          | S phase| 90             | 80                   | 27 |
| 4          | S phase| 90             | 75                   | 25 |
| 5          | S phase| 90             | 93                   | 31 |
| 6          | S phase| 90             | 57                   | 19 |
| 7          | S phase| 180            | 98                   | 32 |
| 8          | G1 phase| 90           | 73                   | 24 |
| 9          | G1 phase| 180          | 95                   | 32 |
| 10         | G1 phase| 90           | 88                   | 29 |

In each experiment, 100 ng equivalent of nuclei from GA-59 cells arrested in G1 phase by α-factor or released into S phase, were incubated in a wild-type S-phase extract for 90 or 180 min, as indicated. Samples 6 and 7, and samples 8 and 9 were sequential time points of one reaction. Replication assays and quantitation of [α-32P]dATP incorporation into the high-molecular-weight genomic DNA are described in Materials and Methods.

BrdUTP incorporation confers a density shift on replicated genomic DNA

To calculate the fraction of incorporated nucleotide that reflects semiconservative DNA replication, as opposed to patch- or gap-repair, we replaced dTTP by the heavy analog BrdUTP during the synthesis reaction, adding [α-32P]dATP as a tracer. After a 180-min incubation in extract, total DNA is isolated and analyzed by CsCl density gradient centrifugation, and the sedimentation of radiolabeled genomic DNA is determined. In the experiment using G1-phase nuclei shown in Figure 2A [open circles], 40% of radiolabeled DNA shifts to the position of fully substituted heavy–light DNA, a position confirmed by the sedimentation analysis of fully substituted M13 DNA [heavy–light peak, Fig. 2D]. This shift is absent in intact nuclei under conditions where replication foci are not formed, that is, in the presence of aphidicolin [Fig. 2A, filled circles] or in the absence of the S-phase extract [Fig. 2B]. When sheared genomic DNA is used as template, a very pronounced peak of light–light DNA is formed, but no heavy–light DNA is detected [Fig 2C]. These results indicate that neither the heavy–light peak nor the replication foci can be artefactually produced by the high repair activity that produces a peak at the light–light position.

In *Xenopus* egg extracts, in which the template DNA assembles spontaneously into membrane-bound nuclei, replication depends entirely on the integrity of the nuclear envelope [Blow and Laskey 1986; Leno et al. 1992]. Because our yeast nuclear extracts lack a significant lipid fraction and cannot repair or form nuclear membranes de novo, we are able to impair nuclear envelope integrity in vitro and monitor how this influences replication. Isolated nuclei were washed with 0.1% Triton X-100, a nonionic detergent that disrupts membranes, and were tested for replication and for their ability to exclude large FITC-derivatized dextran molecules (>150 kD). Control nuclei exclude the FITC-dextran and appear as dark spots in the background fluorescence [Fig. 2F], whereas the Triton-washed nuclei become permeable to FITC-dextran, and become uniformly fluorescent [Fig. 2G]. In the replication assay Triton-washed nuclei lose 75% of their heavy–light peak, whereas the light–light peak extends into a large shoulder of partially replicated DNA [representing 31% of overall incorporation; Fig. 2E]. The focal staining pattern of DIG–dUTP incorporation is also sensitive to pretreatment of the nuclei with detergent [data not shown]. In conclusion, an intact nuclear envelope stimulates semiconservative genomic replication, perhaps by preventing loss of a component essential for initiation. Although the gradients shown are inappropriate for the detection of a heavy–heavy peak, we have no indication from others that rereplication occurs [data not shown].

Replicated nuclei are not competent for reinitiation in S-phase extracts

Proper cell-cycle constraints on replication require that nuclei isolated from G1-phase cells can be induced to replicate, whereas G2- and M-phase nuclei cannot, even in an S-phase cytoplasm [Rao and Johnson 1970]. To examine whether our reconstituted system preserves this control, we monitored replication of pre- and post-S-phase nuclei using a cdcl6-1 mutant strain [GA-161] synchronized with α-factor at permissive temperature (25°C). From a portion of these cells, late G1-phase nuclei were isolated at permissive temperature [see Materials and Methods], while the rest of the culture was released from α-factor and allowed to accumulate in mitosis at 37°C, in response to the cdcl6-1 defect [arrested with short spindles, unseparated chromosomes, and high-Cdk1 activity; Sikorski et al. 1990]. FACs analysis confirmed that these cells have a 2N DNA content [data not shown], and that the nuclei do not undergo rereplication during the arrest, in contrast with an earlier report [Heichman and Roberts 1996]. M-phase nuclei were then isolated and tested for replication at permissive temperatures.

Figure 3B shows that G1-phase nuclei efficiently incorporate derivatized dUTP in subnuclear foci in wild-type S-phase extracts, whereas cdcl6-arrested nuclei fail to incorporate nucleotides [cf. Fig. 3, B and C]. Quantitation of DIG–dUTP in G1- and M-phase nuclei was calculated as the ratio of green to red fluorescent signals [DIG–dUTP to DNA, see Materials and Methods] on eight low-magnification fields of nuclei [Fig. 3D, darkly shaded...
was measured for each fraction and the incorpo-
rated [c^-32p]dATP was quantified on a Packard
liquid scintillation counter. The peak at fraction
4 corresponds to heavy-light DNA (HL) contain-
ting one strand fully substituted with BrdUTP,
wheras the peak at fraction 11 corresponds to
nonsubstituted DNA (LL). (B) An experiment
identical to that in A was performed in a G1
nuclear extract, resulting in no heavy-light peak.
(C) The same amount of sheared nuclei (genomic
DNA) was incubated in an S-phase extract in the
presence (O) or absence (©) of aphidicolin. DNA was sepa-
rated on a CsC1 gradient, the refractory index
was measured for each fraction and the incorpor-
ated [a-B2p]dATP and 20 μM BrdUTP replacing dTTP
(see Materials and Methods) in the presence (●)
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(C) The same amount of sheared nuclei (genomic
DNA) was incubated in an S-phase extract in the
presence of BrdUTP as described in above. A
large light-light, but no heavy-light peak is ob-
tained. (D) To monitor the sedimentation of
heavy-light DNA, M13mp18 DNA was primed
with M13 universal primer and elongated with
Sequenase in the presence of dTTP or BrdUTP,
and [α-32p]dATP. The two reaction products
were mixed and separated on CsCl gradients
(see Materials and Methods). (E, F) An equal
amount of intact G1-phase nuclei (open circles),
and the same nuclei washed for 15 min in 0.1%
Triton X-100 at 4°C before the replication reac-
tion (●) were assayed for BrdUTP incorporation
under the standard replication conditions. The
heavy-light peak is reduced by 75% by the de-
tergent treatment, whereas a shoulder of par-
tially replicated DNA is increased by the pretreatment. [F, G] To monitor nuclear membrane integrity, intact nuclei (stained with EtBr
in the left panel of F) or Triton-washed nuclei (stained with EtBr in the left panel of G) were incubated with 150-kD FITC-dextran
molecules (see Materials and Methods). Fluorescence images (right panels) show that intact nuclei exclude FITC-dextran [F], whereas
Triton-washed nuclei do not [G]. Bar, 2 μm.

Figure 2. Density substitution of G1 nuclei in-
ubated in yeast nuclear extracts. (A) About 10^7
GA-59 nuclei isolated from cells arrested in
G1 with α-factor were incubated under standard
conditions in a GA-59 S-phase extract with
[α-32p]dATP and 20 μM BrdUTP replacing dTTP
(see Materials and Methods) in the presence (●)
or absence (©) of aphidicolin. DNA was sepa-
rated on a CsC1 gradient, the refractory index
was measured for each fraction and the incorpor-
ated [α-32p]dATP was quantified on a Packard
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Triton-washed nuclei do not [G]. Bar, 2 μm.

bars). In parallel, the fraction of nuclei incorporat-
ing label was determined [Fig. 3D, lightly shaded bars]. In summary, nuclei isolated from cells arrested in G1 replicate ef-
ficiently [65% of input nuclei contain foci], whereas
nuclei from metaphase cells do not (<3%), suggest-
ing that post-S-phase chromosomes can not rereplicate in
our S-phase extracts.

To confirm that the absence of replication in these
nuclei represents a postreplicative control, and not an
irreversible cdc16-specific defect, nuclei were isolated
from cells traversing G2/M synchronously after an α-factor
block/release step [Fig. 3E, lane 3], from cells arrested
in late G2 by either the cdc28-1N mutation [Fig. 3E, lane
4; Surana et al. 1991] or the addition of nocodazole [Fig.
3E, lane 5]. As for cdc16-1 M-phase nuclei [Fig. 3E, lane
6], in all cases the total nucleotide incorporation in these
nuclei is ~60% lower than that in G1- or S-phase nuclei
(α-factor block; Fig. 3E, lane 2, or block-release; Fig. 3E,
lane 1). When analyzed, the G2/M nuclei also fail to
reveal replication foci [e.g., Fig. 3C]. The difference in
nucleotide incorporation between G1- and M-phase nu-
uclei is less pronounced when quantified by [α-32p]dATP
incorporation for the reasons outlined above; the aphidi-
colin-sensitive background incorporation into sheared
DNA is not visualized when replication is scored by
DIG-dUTP fluorescence microscopy. In addition to G2-
and M-phase nuclei, nuclei isolated from noncycling
cells, obtained after a 5-day incubation of cells on glyco-
el-containing media, also reveal negligible amounts of
deoxynucleotide incorporation on introduction into an
S-phase extract, consistent with the absence of the pre-
replication complex at ARS1 in these nuclei [Diffley et
al. 1994; H. Renault and P. Pasero, unpubl.].

Initiation of DNA replication occurs in S-phase
extracts in vitro

In the above experiments, G1-phase nuclei were isolated
from MATa cells that had been synchronized at Start by
the yeast pheromone, α-factor. Nuclear isolation re-
quires that the cell wall be degraded by incubation with
glycolytic enzymes in an osmotically buffered yeast me-
dria for 15 min at 25°C. Because α-factor and its cell-surface receptor are sensitive to degradation, some cells may progress past Start during this treatment, resulting in an ill-defined population of G1 spheroplasts situated between Start and the beginning of S-phase. We can con-
Figure 4. Nuclei prepared from cells blocked in late G1-phase with the cdc7 mutation or in the absence of cyclins are able to initiate DNA replication in an S-phase extract. (A) cdc7-1 cells were arrested in late G1 using the double block procedure described by Brewer and Fangman (1987). Half of the culture was immediately spheroplasted at 37°C for late G1-phase nuclei, whereas the rest of the cells were released into S-phase before spheroplasting. (B) Two-dimensional gel analysis of the 2µ circle from spheroplasts blocked at the CDC7 execution point. Electrophoresis, transfer, and hybridization were carried out as described in Materials and Methods after digestion of genomic DNA with ApaI. (C) Two-dimensional gel analysis of the 2µ circle from spheroplasts in S-phase. The arrowhead points to the replication intermediates. (D) Cdc7-arrested nuclei were incubated in a wild-type S-phase extract at 23°C, and DIG-dUTP incorporation (green) is superimposed on the DNA stain (red). Bar, 1 µm. (E) K3130 cells [cln1::hisG, cln2::del, cln3::GAL::CLN3] were grown on YP/Raff/Gal to the density of 10^7/ml and were blocked in G1, either by transferring the culture to YP/Glu or to YP/Raff until all the cells presented the dumbbell phenotype. Cells blocked on YP/Glu were spheroplasted in the presence of glucose to prepare G1 nuclei, whereas cells blocked on YP/Raff were released into S-phase with the addition of 2% galactose and were spheroplasted when >80% of the cells showed small buds. K3130 G1-phase [F] and S-phase [G] nuclei were assayed for in vitro DNA replication (90 min, 25°C) in a wild-type S-phase extract in the presence of DIG-dUTP, which is detected as described above. The insets show a low magnification image of identical nuclei incubated in the absence of the S-phase extract. [H] The incorporation of DIG-dUTP in G1 [lanes 1,2] or S-phase nuclei [lanes 3,4] in the presence [lanes 1,3] or the absence [lanes 2,4] of S-phase extract was performed as described above. Darkly shaded bars indicate the ratio of FITC::EtBr, whereas lightly shaded bars show the fraction of nuclei containing replication loci. [I] Time-course of total DNA synthesis determined by incorporation of [α-32P]dATP in G1- and S-phase nuclei incubated under standard conditions in a wild-type S-phase extract.

Elongation of engaged polymerases in the absence of extract is inefficient

When S-phase nuclei are incubated with nucleotides and the ATP-regenerating system, but without the S-phase extract [Fig. 4, G, inset, and H, lane 4], we detect a fairly
low level of incorporated DIG-dUTP, which is roughly five times the background level in G1-phase nuclei without extract (Fig. 4, F, inset, and H, lane 2). Moreover, although the proportion of nuclei that have foci is roughly the same for G1- and S-phase nuclei in the presence of S-phase extract [Fig. 4F–H], the net incorporation measured by either the dUTP:DNA ratio (darkly shaded bars, Fig. 4H), or by [α-32P]dATP incorporation [Fig. 4I], is higher in the S-phase nuclei after a 3-hr incubation. Because much of the nucleotide incorporation in S-phase nuclei is likely to be attributable to DNA synthesis by engaged polymerases (elongation), it appears that, in addition to initiation, the elongation rates and/or extent of replication are stimulated by S-phase factors. We can rule out that the extracts themselves nick or partially degrade DNA to stimulate nonreplicative DNA synthesis, because supercoiled, open circular and linear DNAs are stable in S-phase extracts for at least 180 min at 30°C [D. Braguglia, P. Pasero, B. Duncker, P. Heus, and S. Gasser, in prep.].

A 6-DMAP-sensitive component is supplied by the S-phase extract

The induction of replication in G1-phase nuclei is entirely dependent on a protein component of the S-phase extract, because heat-denaturation of its proteins (Fig. 5A, lane 4), or substitution with a G1-phase extract (Fig. 5A, lane 3) reduces semiconservative DNA replication by >75%, as judged by integration of the heavy-light peaks after density gradient sedimentation. Similarly, omission of the extract eliminates the appearance of replication foci (Figs. 4F and 5B, lane 4). We also present evidence that the initiation-promoting factor in the extract is sensitive to 6-dimethylaminopurine (6-DMAP), a purine analog that inhibits a variety of protein kinases, including cdc2-like kinases (Meijer and Pondaven 1988). Incubation of the S-phase extract with 6-DMAP, reduces the DIG-dUTP incorporation in G1-phase nuclei by 3.5-fold (Fig. 5B, lane 2). This is consistent with its ability to inhibit the initiation, but not elongation, of DNA replication in Xenopus egg extracts (Blow 1993).

The initiation-stimulating activity of the extract does not depend on an active pol-primase complex, because an extract prepared from the prl1-1 strain, which is temperature sensitive for pol-primase activity (Francesconi et al. 1991) promotes replication in G1-phase nuclei at both permissive (25°C) and restrictive (37°C) temperatures, at levels comparable with wild-type extracts [Fig. 5C, lanes 1,2; E, darkly shaded bars]. The temperature-sensitivity of the primase activity in these extracts is confirmed by the fact that both label incorporation [Fig. 5D, lanes 1,2; E, lightly shaded bars] and the appearance of replication intermediates in supercoiled plasmid, drop significantly at restrictive temperature, which is not the case in wild-type nuclear extracts [D. Braguglia, P. Pasero, B. Duncker, P. Heus, and S. Gasser, in prep.].

DNA synthesis initiates at replication origins in isolated yeast nuclei

Is initiation origin-specific in vitro? The neutral/neutral two-dimensional gel technique of Brewer and Fangman (1987) enables us to analyze initiation on multicopy plasmids carried by the isolated nuclei. We first examine...
initiation at the ARS of the endogenous 2μ circle in nuclei from α-factor-arrested cells, and later extend the analysis to a replicating plasmid in nuclei of a cdc7-arrested culture, as detection of a single-copy origin would require 20-40× more nuclei than that used in a standard reaction.

Initiation in the 2μ circle is a sensitive marker for entry into S-phase: Its origin fires early (10 min after release from a cdc7 block; Brewer and Fangman 1987), intermediates remain detectable into mid-S-phase, and both the regulation and chromatin structure reflect that of chromosomal origins (see Diffley et al. 1994). To rule out that cells escape the α-factor block during spheroplasting, we looked for the presence of 2μ plasmid replication intermediates by two-dimensional gel analysis in spheroplasts formed after the standard α-factor block (<5% buds; Fig. 6B, labeled as 2). No replicative forms were detected (Fig. 6C, labeled 2). In contrast, replication intermediates are abundant in spheroplasts from cells with a high budding index (Fig. 6B,C, labeled 3), and are detectable in those from a random population of cells (Fig. 6B,C, labeled 1) in which <15% of cells are in early S-phase. The complex pattern of replication intermediates observed at the 2μ plasmid in vivo reflects origin-specific initiation within two recombination isomers (forms A and B, in Fig. 6D). We observe some breakage of the bubble arc during DNA isolation, enhancing the Y-arcs. A very similar pattern, consistent with initiation from the 2μ origin, is observed when G1 nuclei, isolated from the spheroplasts shown in Figure 6C, panel 2, are incubated in S-phase extracts (Fig. 6E).

The centered bubble arc characteristic of localized initiation (arc B, Fig. 6D), is more readily seen in a plasmid that does not recombine like the 2μ circle. To this end,
A tight G\textsubscript{1}/S block to replication has never been shown vitrō. Moss 1996), suggesting that the termination of DNA replication in the S-phase extract (Fig. 6G), we conclude that replication initiates at the origin during incubation in vitro. Because we can detect no replication intermediates in the pRS424 plasmid in G\textsubscript{1} nuclei before incubation in the S-phase extract (Fig. 6G), we conclude that replication initiates at the origin during incubation in vitro.

Replication of yeast nuclei in vitro is ORC-dependent

For temperature-sensitive mutations in budding yeast ORC subunits, although it is expected that ORC is essential for the initiation event (Bell et al. 1993; Micklem et al. 1993; Liang et al. 1995). Our in vitro assay allows us to examine whether ORC is required for initiation itself, or for the establishment of an initiation-competent complex, within which it is no longer essential. To test this, orc2-1 mutant cells were grown and synchronized in G\textsubscript{1} by α-factor arrest at permissive temperature, and both G\textsubscript{1}-phase nuclei and S-phase nuclear extracts were prepared from cells synchronized as described in Figure 6A.

The incubation of the G\textsubscript{1}-phase orc2-1 nuclei in orc2-1 S-phase extracts at permissive temperature reveals the incorporation of DIG-dUTP in well-defined foci in most of the nuclei (Fig. 7C, orc2/orc2 23°C). Quantitation of these results gives a fluorescein::EtBr ratio of 0.7, with over 65% of the nuclei containing replication foci (Fig. 7D, lane 1). At restrictive temperature, in the same extracts and nuclei, foci are observed in less than 10% of the cells (Fig. 7C, orc2/orc2 37°C), indicating that inactivation of Orc2p in late G\textsubscript{1} nuclei results in a sixfold drop in both overall DIG–dUTP incorporation and in the

**Figure 7.** Initiation of DNA replication is ORC-dependent in vitro. [A] The synchronization in G\textsubscript{1} of orc2-1 cells is for 120 min in the presence of α-factor at 23°C. [B] Proteins from a total yeast extract (GA-59; lane 1) or from orc2-1 nuclei after incubation for 45 min at permissive (23°C, lane 2) or restrictive (37°C, lane 3) temperature were denatured and probed by Western blot using an anti-Orc2p antibody (gift of J. Diffley, Imperial Cancer Research Fund, Herts, UK) to show the absence of degradation of the ORC2 protein. (C) Nuclei isolated from orc2-1 or wild-type (wt) cells synchronized in G\textsubscript{1} were assayed for in vitro DNA replication in an orc2-1 or a wild-type S-phase extract for 90 min at 23°C or 37°C in the presence of DIG–dUTP. [orc2/orc2] orc2 nuclei in orc2 extract. (wt/orc2) Wild-type nuclei in orc2 extract. [orc2/wt] orc2 nuclei in wild-type extract. DIG–dUTP (green) and EtBr (red) staining are as described above. Bar, 1 μm. [D] Quantitative analysis of DIG–dUTP incorporation in orc2-1 G\textsubscript{1} nuclei incubated for 90 min at 23°C in an orc2-1 S-phase extract (lane 1); for 90 min at 37°C in an orc2-1 S-phase extract (lane 2); in an orc2-1 S-phase extract for 15 min at 37°C (lane 3), then 75 min at 23°C with DIG–dUTP. (Lane 4) Wild-type nuclei incubated for 90 min at 37°C in an orc2-1 S-phase extract. The incorporation of DIG–dUTP (dark gray bars) and the proportion of replicating nuclei (light gray bars) were determined as indicated in Materials and Methods. [E] Quantitation of semiconservative replication in 10° wild-type (lanes 1,2) and orc2 nuclei (lanes 3,4) incubated for 3 hr in an orc2 extract at 23°C (lanes 1,3) or 37°C (lanes 2,4). The reaction and separation on CsCl gradients were performed as described in Materials and Methods. The moles of semiconservatively synthesized DNA was calculated from the [α-32P]dATP recovered in the heavy–light peak.
number of nuclei containing replication foci [Fig. 7D, lane 2]. We show next that a shift of the isolated G1-phase orc2-1 nuclei to 37°C for as little as 15 min, followed by a return to permissive temperature, is sufficient to eliminate the appearance of replication foci (quantitation in Fig. 7D, lane 3), although wild-type nuclei replicate efficiently in orc2 S-phase extracts even when incubated continuously at 37°C [Fig. 7C, wt/orc2, and D, lane 4]. The lack of replication in G1 orc2-1 nuclei at restrictive temperature was confirmed by BrdUTP substitution and quantitation of the heavy-light peak on CsCl gradients [Fig. 7E, cf. lane 2, wild type, with lane 4, orc2-1]. This confirms that foci are not simply disorganized at restrictive temperature, but that replication itself is blocked. Western blots for Orc2p on a whole-cell extract of GA-59 cells [wild type, Fig. 7B, lane 1] and on equal amounts of protein from orc2-1 nuclei incubated at either 23°C or 37°C [Fig. 7B, lanes 2 and 3], rule out that Orc2p is degraded at 37°C in the temperature-sensitive strain. Orc2p is also readily detectable on Western blots of both wild-type and orc2 S-phase extracts prepared at permissive temperature.

**ORC-deficient nuclei are not complemented by wild-type extracts**

If ORC also serves an essential role in G1-phase for establishing a replication competent origin complex, we predict that the orc2-1 deficiency cannot be complemented by ORC components found in a wild-type S-phase extract. To test this, orc2-1 G1-phase nuclei were incubated in wild-type S-phase extracts at either 23°C or 37°C, and replication was monitored by both isotope incorporation and the appearance of DIG-dUTP-containing foci. As observed in the orc2-1 extracts, the replication foci appear normal at permissive temperature, but little nucleotide is incorporated at 37°C [Fig. 7C]. On the other hand, wild-type nuclei incubated in an orc2-1 extract incorporate DIG-dUTP with high efficiency at both permissive and restrictive temperatures [Fig. 7C], in an aphidicolin-sensitive manner [data not shown]. Therefore, the orc2-1 deficiency is manifest in the substrate nuclei, and cannot be complemented in *trans* with the wild-type ORC present in S-phase extracts.

**Discussion**

We have established a reliable DNA replication system from yeast supporting efficient semiconservative DNA synthesis in vitro. This replication is sensitive to the cell-cycle controls that ensure that the genome is replicated once and only once per cell cycle, because neither G2-M, nor noncycling G0-phase nuclei replicate in the S-phase extract. Moreover, replication initiates in an origin-specific and ORC-dependent fashion in vitro. The application of this assay to the many cell cycle- and DNA replication-specific mutants in yeast should allow a detailed biochemical characterization of the G1-to-S phase transition and its relation to the initiation of DNA replication.

**Replication occurs in distinct foci within yeast nuclei**

A large fraction of the DNA synthesis that we observe in vitro is semiconservative as judged by density substitution [Fig. 2]. Quantitation of incorporated nucleotides indicates that between 20 and 30 ng of DNA is synthesized in the 90-min reaction representing 20%-30% of the input DNA [Table 1]. These values include both replicative and nonreplicative events within high-molecular-weight DNA. To avoid any signal from the repair of damage in contaminating free DNA, we visualize DNA replication through the incorporation of derivatized deoxyribonucleotides into the chromosomes of intact nuclei, and follow the appearance of foci by confocal microscopy [Fig. 1]. We show that yeast nuclear replication, like that of vertebrate nuclei [Nakamura et al. 1986], occurs at distinct subnuclear foci containing clusters of replication forks, and that their appearance correlates with the recovery of a heavy-light peak on density gradient centrifugation. The incorporation detected in this manner can be quantified in a relative fashion, as units of nucleotide-bound fluorescence relative to the EtBr signal of the entire genome. Calculating the number of replicating nuclei in a given population indicates that 60%-70% of the nuclei isolated from either G1- or S-phase spheroplasts, replicate in an S-phase extract, indicating a high efficiency of in vitro initiation.

**Initiation in vitro is origin specific and ORC-dependent**

Origins of replication are well characterized in yeast [for review, see Campbell and Newton 1991]. This has allowed us to demonstrate that the initiation of replication in vitro is origin-specific, by mapping bubble arcs on both multicopy plasmids and the endogenous 2μ circle [Fig. 6]. This initiation activity is compromised at restrictive temperature in the conditional orc2-1 mutant [Fig. 7]. In contrast, we observed that initiation on supercoiled plasmids introduced into these same S-phase extracts occurred randomly and in an ARS- and ORC-independent manner [D. Braguglia, P. Pasero, B. Duncker, P. Heus, and S. Gasser, in prep.; data not shown]. This suggests either that subnuclear organization aids in origin function, or that the necessary preinitiation complexes cannot form on plasmid DNA in S-phase extracts. This is consistent with observations in the *Xenopus* system, where replication of naked DNA occurs with little or no sequence specificity [Hyrien and Méchali 1992; Gilbert et al. 1995], whereas "preorganized" Chinese hamster ovary (CHO) cell nuclei introduced into *Xenopus* extracts initiate preferentially at a putative dihydrofolate reductase [DHFR] origin [Gilbert et al. 1995; Wu and Gilbert 1996].

Interestingly, ORC has an essential role in the de novo formation of replication centers in *Xenopus* egg extracts, although this organization does not fully mimic that in...
somatic cells. Orc2p associates with chromatin in the first stages of nuclear formation in egg extracts [Carpenter et al. 1996], and is thought to be required for the recruitment of Cdc6p and MCMs, both essential for initiation of DNA replication in vitro [Coleman et al. 1996; Romanowski et al. 1996]. Elsewhere we show that ORC and Cdc6p are associated with an insoluble nuclear scaffold fraction in G1, and S-phase yeast nuclei [P. Pasero and S.M. Gasser, in prep.], consistent with a role for ORC in the formation of replication centers. The inactivation of preformed replication complexes by a shift to restrictive temperature in orc2-1 nuclei, indicates that ORC is not only required for the assembly step, but also for initiation itself.

Cis- and trans-acting factors are required for the initiation of DNA replication in vitro

The “licensing factor” model, essentially based on observations in the Xenopus system, proposes that replication competence is attributable to the presence of a positive factor bound to chromatin in G1, which is essential for the initiation of replication. The factors are subsequently inactivated during replication and remain inactive until the transit of mitosis [Blow and Laskey 1988]. Several reviews have pointed out that this licensing event in budding yeast could reflect the association of Cdc6p, Dbf4p, or MCM proteins to the origin-bound ORC, which can occur only after anaphase [for review, see Muzi-Falconi et al. 1996; Donovan and Diffley 1996; Kearsley et al. 1996]. Consistently, we show here that S-phase nuclei, cdc7-arrested, cln-deficient, or α-factor-blocked G1 nuclei can replicate in S-phase extract, whereas G2- or M-phase nuclei cannot. Initiation in G1-phase nuclei is lost at the restrictive temperature in a conditional orc2-1 mutant. This shows clearly that ORC is required for initiation of DNA replication, resolving the ambiguity remaining from in vivo temperature shift experiments, that is, initiation events were retarded but not blocked fully until 3 hr after the temperature shift [Liang et al. 1995], and temperature-sensitive cells arrested at restrictive temperature only slowly, with nearly a 2N DNA content [Bell et al. 1993; Micklem et al. 1993].

The demonstration that cln-deficient nuclei are able to initiate DNA replication in an S-phase extract [Fig. 4], suggests that a functional prereplication complex (pRC) is formed before activation of the G1 cyclin/Cdk complexes. This suggests that neither the second burst of Cdc6p synthesis that occurs in late G1 [Piatti et al. 1995] nor other events promoted by G1 cyclins, are essential G1 events for DNA replication. In other words, even if there are post-Start events that prepare origins for initiation, these can apparently occur in the presence of S-phase activities, and are therefore not obligatorily G1-specific events. These results are fully consistent with genetic studies showing that Clnb/Cdk1 kinase can promote S-phase in the triple cln disruption background (Schwob and Nasmyth 1993).

Candidates for SPF activity

As demonstrated in Xenopus and a mammalian replication system [Krude et al. 1997], the formation of a pRC is insufficient to promote replication, and initiation must be catalyzed by the protein kinase-containing S-phase promoting factor [SPF; for review, see Romanowski and Madine 1996]. In our yeast system, the initiation of DNA replication in G1-phase nuclei requires an S-phase extract, which contains a 6-DMPA-sensitive activity required for promoting initiation. Replication is not only reduced in the presence of the inhibitor, but also in heat-inactivated S-phase extracts, and in extracts obtained from cells blocked with pheromone in G1 [Figs. 2 and 5]. The positive signal for replication may in fact require multiple events, such as the activation of Clb5/Cdk1 and Cdc7/Dbf4 protein kinases, and the inactivation of p40SIC1, a specific inhibitor of the Clb/Cdk complex [Jackson et al. 1993; Schwob and Nasmyth 1993; Schwob et al. 1994]. We do not know whether p40SIC1 is degraded by addition of the S-phase extract, or whether the S-phase kinase level simply overwhelms it. The enhanced levels of elongation observed in S-phase nuclei may reflect their lower levels of this kinase inhibitor, as it has been demonstrated that the mammalian Cdk inhibitor p21 represses pol6 activity through interaction with proliferating cell nuclear antigen [PCNA] [Waga et al. 1994; Podust et al. 1995].

Interestingly, we observed that an extract from mitotically blocked cdc16-1 mutant cells is sufficient to stimulate replication in both G1- and S-phase nuclei, suggesting that the Clb/Cdk1 complexes present in mitosis can also trigger initiation [data not shown]. This is consistent with in vivo observations showing that any of the six B-type cyclins can promote the G1/S transition [for review, see Nasmyth 1996]. Because mitotic extracts have been reported to be deficient for Cdc7 [Jackson et al. 1993], the essential Cdc7 kinase activity may be provided in cis by the G1-phase nuclei, and perhaps activated by Clb/Cdk1 in trans. Further analysis of extracts and nuclei from a range of kinase and cyclin mutants will determine whether Cdc7 and Cdc28 operate on the same, or on parallel pathways [see Jackson et al. 1993; Yoon et al. 1993; Hardy and Pautz 1996].

In yeast, Cdk1 [i.e., Cdc28 kinase] has a dual role in the control of DNA replication as it is involved in triggering initiation and simultaneously appears to inhibit the reestablishment of pRCs [for review, see Nasmyth 1996]. We observe no replication foci when G2- and M-phase nuclei are added to S-phase extracts, suggesting that either pRC assembly factors are missing, or that pRC formation is prevented by the S-phase extract. Because the orc2-1 mutation is not dominant in vivo and does not impede the binding of wild-type ORC in vitro [Bell et al. 1993], our inability to complement the orc2-1 deficiency with a wild-type S-phase extract, is consistent with the notion that pRCs are unable to form attributable to the S-phase Cdk1 activity. Our yeast-based replication system should now enable us to “reprogram” postreplicative nuclei in vitro, by inactivating the Clb/Cdk1 ki-
nases, and sequentially adding back components of the
genetically-defined complexes required for the initiation of
eukaryotic DNA synthesis.

Materials and Methods

Yeast strains

The yeast strain Saccharomyces cerevisiae GA-59 [MATa, leu2, trp1, ural3-52, prb1-1122, pep4::URA3, prc1-407, gal2], lacking three vacuolar proteases, was used as our standard wild type. Other strains include GA-266 [MATa, pri1-1, ural3-52, ino1, can1-1, pep4::URA3], provided by P. Plevani [University of Milan, Italy]; GA-161 [MATa, cdc28-1N, ural3, his7], originally from L. Hartwell [University of Washington, Seattle]; GA-85 [MATa, his6, bar1, trp1-289, leu2-2112, ural3-52, cdc7-1], formerly RM 14-3A, from B. Brewer and W. Fangman [University of Washington, Seattle]; K3130 [MATa, ade2-1, ade3, trp1-1, can1-100, leu2-3-112, his3-11,15, ural3, GAL, cln1::hisG, cln2::del, cln5::GAL, CLN3 [URA3], CLA1, (SD1)], provided by E. Schwob [IGM, Montpellier, France]; GA-361 [MATa, cdc28-1N, ade2-1, ural3-1, his3-11, trp1-1, len3-2,112, can1-100] and GA-462 [MATa, orc2-1, ade2-1, ural3-1, his3-11, trp1-1, leu2-3,112, can1-100, pep4::URA3], which resulted from crossing the cdc28-1N and orc2-1 temperature-sensitive strains into a W303 background.

The replication assay works in all strain backgrounds tested to date. The pep4 yeast assay were tested regularly for absence of carboxyypeptidase Y activity [Jones 1977]. To test whether mitochondrial DNA influences our assay, the strains GA-59 and GA-462 were rendered rho0. Yeast media were as described in Rose et al. [1990] and contained 2% glucose [Glu or D], raffinose [Raf], or galactose [Gal], as indicated 1-1% yeast extract and 2% Bacto-peptone (Difco).

Synchronization, isolation, and extraction of yeast nuclei

Yeast cells were grown in YPD at 30°C to a density of 0.5 to 1 x 107 cells/ml. Temperature-sensitive strains were grown at 25°C [23°C for orc2-1 and cdc7-1] and shifted to 37°C for arrest. After harvesting and washing with centrifugation at 2000g for 5 min at room temperature, the cells were resuspended in 1/4 of the initial volume in YPD at pH 5.0. α-Factor was added to an optimal concentration [1.5 x 10-7 M for the GA-59 strain]. Cells were incubated at 30°C [or at permissive temperature] -generation time until no small buds were visible, indicating a block at the 211 origin.

For S-phase extracts, cells were washed and resuspended in the same volume of YPD prewarmed to growth temperature. Release from the block was checked microscopically, and the cells were harvested as soon as small buds appeared. Spheroplasting of G1- or S-phase cells, isolation of crude nuclei, and extraction with [NH4]2SO4 for the replication extract are described in Verdier et al. [1990]. Both Orc2 and Cdc6p, as well as the replication polymerases are extracted efficiently from nuclei with the detergents and high concentrations of ammonium sulfate used for preparing nuclear extracts [D. Brauglia, P. Pasero, B. Duncker, P. Heus, and S. Gasser, in prep.]. Purification of β-I,3 glucanase was carried out according to Shen et al. [1991]. For G1 extracts, α factor was present during spheroplasting.

The isolation of nuclei for in vitro replication used yeast cells synchronized and spheroplasted as for nuclear extract preparation [Verdier et al. 1990]. Spheroplasts were carefully suspended at 0.2 g/ml in 5 ml of an ice-cold breakage-buffer (buffer 0.25x A, which contains 20 mM Tris-HCl, at pH 7.4, 20 mM KC1, 2 mM EDTA-KOH, 0.05 mM spermine, 0.125 mM spermidine, 300 ng/ml of benzamidine, 1 µg/ml of pepstatin, 0.5 µg/ml of leupeptin, 1% Trasylol (aprotinin, Bayer), 0.5 mM PMSF, 1% thiodiglycol). Usually 1 gram of spheroplasts was immediately layered over 45 ml of a solution containing 10% sucrose in breakage buffer, and nuclei were separated by centrifugation at 2500g for 20 min at 4°C in a Sorvall RC600 centrifuge. The nuclei, which sediment at the middle of the gradient, were collected and diluted by addition of 1 vol of 0.25x A, 0.5 mM PMSF. Sucrose-purified nuclei were recovered by two rounds of centrifugation [5000g; 10 min at 4°C] and resuspended in a minimal volume of glycerol buffer (60% glycerol, 0.25x A, 0.2 mM PMSF) and stored at -20°C. Yield (generally 5 OD260/gram of spheroplast) was estimated by optical density in 1% SDS [1 OD260 - 1010 nuclei].

The integrity of the nuclear membrane was checked using >150-kD FITC dextran as described by Peters [1983]. Permeabilized nuclei were prepared by incubation for 15 min in 0.1% Triton X-100 in 0.25x A, 0.5 mM PMSF at 4°C.

In vitro replication reaction

The 25-µl standard reaction mixture contained 2.5 µl of 10x replication buffer (120 mM HEPES-NaOH at pH 7.6, 48 mM MgCl2, 3 mM EDTA-NaOH at pH 7.6, 6 mM DTT), 107 nuclei [corresponding to 100 ng of genomic DNA], an energy regeneration system of 40 mM creatine phosphate and 0.125 mg/ml of creatine kinase, 20 µM of each dCTP, dGTP, dTTP, 8 µM dATP, 5 µCi/µl [α-32P]dATP, 20 µM of each ATP, CTP, GTP, UTP, and 60 µg of protein extract. Where indicated, aphidicolin, dissolved in dimethylsulfoxide (DMSO), was added to a final concentration of 0.5 mg/ml. The replication reaction was stopped after 90 min at 25°C (or as indicated) with final concentrations of 0.3 M Na-acetate at pH 5.2, 0.1% SDS, 5 mM EDTA, 100 µg/ml of Proteinase K, and was incubated for 60 min further at 37°C. The DNA was phenol extracted, precipitated, washed with 1 ml of 70% ethanol, dried, and resuspended in 20 µl of distilled water. All other methods were performed according to standard protocols [Sambrook et al. 1989]. Replication of plasmid DNA in the soluble extract was carried out as described for nuclei, except that 300 ng of pH4ARS DNA was used as template [D. Brauglia, P. Pasero, B. Duncker, P. Heus, and S. Gasser, in prep.]. Quantitation of [α-32P]dATP incorporation in the high-molecular-weight genomic DNA was done on dried gels by PhosphorImager scanning using serial dilutions of the [α-32P]dATP-labeling mixture to convert the arbitrary units of PhosphorImager readings into moles of dATP incorporated. The internal pool of dATP in the nuclei was shown by isotope dilution to vary from 8 to 15 µM. For immunofluorescence and confocal microscopy, biotin-16–DUTP [Boehringer Mannheim], biotin-7–DUTP [BRL], and digoxigenin-11–DUTP [Boehringer Mannheim] were used for labeling. The best results were obtained with 20 µM DIG-DUTP, as determined by titration.

Two-dimensional gel electrophoresis and gradient centrifugation

Two-dimensional gel electrophoresis was carried out on genomic DNA [4 µg DNA] or on nuclei replicated in vitro [1-2 µg], as described by Brewer and Fangman [1987]. Gels were transferred to nitrocellulose and hybridized with the 981−bp HpaI–Xbal fragment of Yep13 [Fig. 6E] or the 2.6-kb Scal–EcoRV fragment of pRS424 [Figs. 4B, C, and 6C], both containing the 2α ARS. Replication at the pRS424 plasmid was detected using the 1.87-kb EcoRV–Scal fragment of pRS424, that does not contain the 2α origin.

BrdUTP substitution in G1 nuclei was for 180 min at 25°C in an S-phase extract with 200 µM BrdUTP and all other additions as indicated above. Following phenol/chloroform extraction and the removal of unincorporated label using Nuc–Trap col-
ORC-dependent initiation of DNA replication in vitro

Orcs [Stratagene], the genomic DNA was digested with EcoRI, and mixed with a CsCl solution to a final density of 1.7176 grams/ml at 25°C (n = 1.4010). The gradient was generated at 35,000 rpm in a fixed angle Ti27 rotor [Sorvall] for 40 hr at 20°C. Fractions of 30 μl were collected from the bottom of the gradient, and [α-32P]dATP was counted in a Packard liquid scintillation counter. Gradients usually span densities from 1.6742 grams/ml (n = 1.3970) to 1.7936 grams/ml (n = 1.4080), whereas the unsubstituted runs at n = 1.4000 to 1.4010. The sedimentation of the light-light and heavy-light DNA was standardized by elongating a primed M13 template with Sequenase [Boehringer Mannheim] incorporating either BrdUTP or dTTP. Quantitation of semiconservative replication was done by integrating the peak of heavy-light DNA and converting the counts per minute into moles of dATP incorporated, as described above.

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