Cell Cycle Regulation of Human CDC6 Protein

INTRACELLULAR LOCALIZATION, INTERACTION WITH THE HUMAN MCM COMPLEX, AND CDC2 KINASE-MEDIATED HYPERPHOSPHORYLATION

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The binding of mammalian MCM complexes to chromatin is cell cycle-regulated and under CDC2 kinase negative control. Here, we investigated the properties of mammalian CDC6 protein, a candidate regulator of MCM. The levels of CDC6 were relatively constant during the HeLa cell cycle. In asynchronous cells, CDC6 was mainly detected in the nuclei with immunostaining, but some CDC6 was not extractable with nonionic detergent. In contrast to the chromatin-bound MCM, this fraction of CDC6 was resistant to DNase I treatment, suggesting that it binds to the detergent- and nuclease-resistant nuclear structure. In S phase cells, CDC6 became detectable in the cytoplasm with immunostaining; however, the level of the bound CDC6 was unchanged. In G2/M phase cells, the level of the bound CDC6 was still maintained, which was hyperphosphorylated by CDC2 kinase. These data suggest that some CDC6 protein is associated with the specific nuclear structure throughout the cell cycle and that major binding sites on chromatin differ between MCM and CDC6. However, co-immunoprecipitation assays with chemical cross-linking indicated that a small part of the chromatin-bound MCM is present close to the bound CDC6.

In the budding yeast, DNA replication initiates at definitive replication origins, autonomously replicating sequences (ARSs), and an origin recognition complex (ORC), consisting of six protein subunits, binds directly to ARSs (1). Genomic footprint analyses of isolated nuclei have suggested that the ORC remains bound to ARSs throughout the cell cycle but that additional proteins associate around the ARSs in the G1 phase, forming a prereplication complex (2). CDC6 protein and the six MCM proteins, which physically associate with one another (3–6), are good candidates for the components of the prereplication complex. Its formation is dependent on CDC6, the levels of which drop dramatically as cells enter S phase (7–9), and MCM proteins are loaded on prereplication chromatin in an ORC/CDC6-dependent manner and displaced from postreplication chromatin (10–13). Recently, it was suggested on the basis of immunoprecipitation of cross-linked chromatin that CDC6 and MCMs may associate with ARSs in the G1 phase and that MCMs might move with replication forks after initiation (11, 13).

In the DNA replication system with Xenopus egg extracts, although replication occurs sequence-nonspecifically (14), the homologues of the yeast initiation proteins are similarly loaded onto prereplication chromatin; first, Xenopus ORC binds, then Xenopus CDC6 Xenopus ORC-dependently binds, and finally Xenopus MCM complexes are recruited (15, 16). It seems likely that yeast prereplication complex-like complexes are organized on prereplication chromatin. So far no physical interactions have been detected among Xenopus ORC, Xenopus CDC6, and the Xenopus MCM complex (15, 16). Chromatin-bound Xenopus MCMs are dissociated as replication proceeds (17–19), and Xenopus CDC6 appears to associate with nonchromatin region in M phase nuclei (15).

In both systems and also in mammalian somatic cells, assembly of the prereplication complex and subsequent reassembly after authentic replication seems to be prohibited by the S phase and mitotic CDKs (8, 11, 20–24). There are several possible mechanisms for the CDK-directed inhibition. One is the phosphorylation of CDC6 by the CDK, e.g. in fission yeast, phosphorylation of Cdc18, a homologue of CDC6, by the CDK leads to its rapid degradation, and overexpression of its stable form or CDK phosphorylation site-mutant form drives cells into rereplication (21, 25, 26). Another possible mechanism is the CDK-mediated phosphorylation of MCM complexes (22, 24, 27).

In mammalian somatic cells, DNA replication appears to initiate from specific regions, differing from the egg extract system, although the specific cis-acting elements have not been defined (28). Recently, mammalian homologues of the initiation proteins have been identified, including the mammalian MCM protein family (29–32). MCM proteins appear essential for DNA replication in mammalian cells, and their binding to chromatin is cell cycle-regulated (30–33). It is assumed that they also play regulatory roles in mammalian cell DNA replication, as in the yeast and Xenopus egg systems, although specific differences should also exist. Previously, we found that the six human MCMs (hMCMs) are present as heterohexameric complexes throughout the cell cycle, whether bound to chromatin or not (24, 34). We also found that CDC2 kinase phosphorylates MCM2 and MCM4 in the complexes and plays a crucial role in inhibiting reloading of the MCM complexes at G1/M (24). Recently a human CDC6 (hCDC6) protein was identified that would be expected to be an important factor for regulation of MCM-chromatin interactions and DNA replication (35–37). In the present study, we prepared antibodies against the CDC6 protein and performed a characterization.
Cell Culture and Synchronization—HeLa cells were grown in Dulbecco's modified Eagle's medium with 5% fetal calf serum and synchronized at G2/M by treatment with 50 ng/ml nocodazole for 16–18 h. Cells arrested in early S phase were obtained by 2.5 mM hydroxyurea treatment for 18–20 h. G1 cells were obtained as described previously (24). Raji cells were grown in RPMI 1640 with 10% fetal calf serum. FT210 cells (25) were maintained at 32 °C in RPMI 1640 containing 25 mM Hepes and 10% fetal calf serum. For G2/M synchronization, they were treated with 50 ng/ml nocodazole for 18 h at either 32 or 39 °C.

Production of Antibodies—hCDC6 and hORC1 cDNA were kindly provided by Dr. K. Ohtani (40). A glutathione S-transferase (GST) fusion protein containing amino acids 1–326 of hCDC6 was produced in Escherichia coli, isolated as inclusion bodies, and purified by SDS-polyacrylamide gel electrophoresis (PAGE) (32). Rabbits were injected with approximately 200 μg of the purified protein mixed with RIBI adjuvant system R-730 (RIBI Immunocochem Research). Anti-hCDC6 antibodies were affinity-purified from the antiserum with GST-hCDC6 coupled-Sepharose 4B (Amersham Pharmacia Biotech) and then passed through a GST-Sepharose column to remove anti-GST antibodies.

Preparation of Cell Extracts—HeLa cells cultured in 100-mm plates or FT210 or Raji cells in 20 ml of culture were lysed for 10 min on ice with 1 ml of ice-cold modified CSK buffer containing 0.1% Triton X-100 and 0.1 mM ATP (0.1%TX-100mCSK; Refs. 24 and 34). Multiple protease inhibitors (Protease Inhibitor Mixture for Mammalian Cell Extracts, Sigma; 25 μM l-mercaptoethanol, 200 μM Na3VO4, and 20 mM NaF were also added to the buffer. The cells were then subjected to centrifugation to obtain the extractable fractions. The extracted nuclei were either directly added to SDS sample buffer or digested with 1000 units/ml DNase I (10 units/μl, Roche Molecular Biochemicals) in 0.1%TX-100mCSK containing 1 mM ATP and the multiple protease inhibitors at 25 °C for 30 min (24, 34).

Immunoprecipitation—For immunoprecipitation, antibodies were cross-linked to protein A-Sepharose beads. HeLa cells cultured in 100-mm plates were lysed for 10 min on ice with 1 ml RIPA buffer (32) containing 1% Triton X-100, 10 μg/ml aprotinin, 200 μM Na3VO4, and 20 mM NaF. Separate aliquots were diluted with NET gel buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 1 mM EDTA) containing 0.5% bovine serum albumin, mixed with the beads, and rocked for 1 h. The beads were then washed with NET gel buffer, and the immunoprecipitates were boiled in SDS sample buffer without β-mercaptoethanol. The supernatants were collected, added to β-mercaptoethanol, reboiled, and subjected to 10% (10:0.1 acrylamide:bis-acrylamide) SDS-PAGE.

Cross-linked nuclear fraction was prepared as follows. Triton X-100-extracted nuclei prepared as above were washed once with cross-linking buffer (phosphate-buffered saline, pH 8.0, 1 mM MgCl2, 100 mM sucrose, 0.01% Triton X-100) by low speed centrifugation and resuspended in the buffer. Dithiothreitol was added to a final concentration of 100 μg/ml, and the samples were rocked for 10 min at 4 °C. The reaction was quenched with Tris-glycine buffer, and then the nuclei were washed with cross-link buffer, added to RIPA buffer containing 1% SDS, diluted with NET gel buffer, and sonicated. The washing buffer for the immunoprecipitation was NET gel buffer containing 0.05% SDS, 0.25% sodium deoxycholate, and 0.5% Triton X-100.

Immunoblotting—Whole cell lysates were prepared by directly adding SDS sample buffer to cell pellets. In vitro translated hCDC6 and hORC1 proteins were obtained from their cDNAs with a coupled transcription-translation system (Promega). Multiple protease inhibitors were included in the reaction. Immunoblotting was performed as described previously (24, 34), and protein blots were incubated with purified anti-hCDC6 at 1 μg/ml.

Immunofluorescence Analysis—Cells grown in culture chambers (Nunc) were fixed for 10 min with 3.7% formaldehyde and then permeabilized with methanol at −20 °C for 2 min. The samples were then incubated with phosphate-buffered saline with 10% normal goat serum and then with anti-hCDC6 antibodies (2.5 μg/ml) for 1 h. After washing, the samples were reacted with 20 μg/ml fluorescein isothiocyanate-conjugated second antibodies (Zymed Laboratories Inc.) for 1 h, followed by 0.1 μg/ml diamidophenylindole to stain DNA.

λ-Phosphatase Treatment of the Extracted Nuclei and CDC2 Kinase Treatment of the Immunoprecipitates—Triton X-100-extracted nuclei were resuspended in phosphatase buffer (24) supplemented with 0.01% Triton X-100 and the multiple protease inhibitors and incubated with λ-phosphatase (New England Biolabs) for 20 min at 30 °C in the presence or absence of 400 μM Na3VO4. Immunoprecipitates were resuspended in 30 μl of kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 2 mM MnCl2, 1 mM dithiothreitol, 1 mM ATP, 25 mM NaF, 1 mM β-glycerophosphate, 1 μM calyculin A, 0.5 mM phenylmethylsulfonyl fluoride) and incubated with CDC2/cyclin B purified from FM3A cells (41) for 60 min at 30 °C. The reactions were stopped by addition of 2× SDS sample buffer and processed for SDS-PAGE.

RESULTS AND DISCUSSION

Specificity of Anti-hCDC6 Polyclonal Antibodies—With Western blotting, anti-hCDC6 antibodies could detect the bacterially produced hCDC6 at levels as low as 100 pg (data not shown). The antibodies recognized in vitro translated hCDC6 protein but not in vitro translated hORC1 protein (Fig. 1A). A protein migrating at approximately 62 kDa was detected in whole cell lysates from HeLa cells, the mobility of which on SDS-PAGE was concordant with that of the in vitro translated hCDC6 protein (Fig. 1A). The antibodies also could immunoprecipitate the 62-kDa protein (Fig. 1B). Together, we concluded that the 62-kDa protein is hCDC6.

We note that hCDC6 protein is unstable in vitro. We have used 0.1%TX-100mCSK buffer to fractionate cells (24, 34). In the buffer supplemented with 1 mM phenylmethylsulfonyl fluoride and 10 μg/ml aprotinin, proteins such as hMMCs and proliferating cell nuclear antigen are relatively stable (34). In contrast, hCDC6 was readily degraded; even when held on ice, most was degraded after 10 min (data not shown). Therefore, to inhibit proteolysis as much as possible, we should add sufficient amounts of multiple protease inhibitors to the extraction buffer.

Levels of hCDC6 Protein Are Relatively Constant during the Cell Cycle—To investigate whether the levels of hCDC6 protein change during the cell cycle, we prepared whole cell lysates from asynchronous (where 60–70% cells are in G1 phase), early S phase (hydroxyurea-treated), and G2/M phase (nocodazole-treated) HeLa cells and analyzed them by immunoblotting. A 2–3-fold increase was observed in the S phase cells, and the protein from G2/M cells resolved into a doublet on SDS-PAGE (Fig. 2). The levels of hMCM7 were unchanged among the lysates (Fig. 2), in agreement with our previous results (32). There is some discrepancy among two previous reports and our present findings. Williams et al. (35) reported nuclear hCDC6 protein levels to be constant during the HeLa cell cycle while fluctuating in W138 cells, and Sabat et al. (36) experienced no remarkable change in either HeLa or NIH3T3 cell cycles. The reason for the difference is currently unknown. At least, our observed increase in the S phase hCDC6 protein levels is in line with the reported enhanced expression of its mRNA in S phase (35, 36). However, compared with yeast case, in which CDC6/Cdc18 protein is rapidly degraded through the S phase-CDK

![Figure 1. Characterization of anti-hCDC6 antibodies. A. in vitro translated (JVT) hORC1 and hCDC6 and the whole cell lysate from approximately 1 × 10^6 HeLa cells were immunoblotted with anti-hCDC6 antibodies. B. total cell lysates prepared from HeLa cells with RIP buffer were immunoprecipitated with a control rabbit immunoglobulin or anti-hCDC6 antibody beads. The immunoprecipitates (IP) and the lysates (input) were then immunoblotted with the antibodies.](image-url)
phosphorylation (9, 21), the cell cycle variation of CDC6 protein levels in mammalian cells appears to be limited, similar to Xenopus CDC6 in the Xenopus egg extract system (15). Although it remains unclear whether Xenopus CDC6 is phosphorylated by CDKs, our data suggest that hCDC6 may be phosphorylated by CDC2 kinase in the G2/M phase (see below). However, this does not appear to lead to dramatic reduction in hCDC6 protein levels.

**Subcellular Localization of hCDC6 Protein during the Cell Cycle Assessed by Immunostaining**—To investigate the subcellular localization of hCDC6 protein, immunofluorescence staining was applied to asynchronous HeLa cells. Approximately 60–70% of the cells showed dominant nuclear staining with some cytoplasmic positivity, whereas remaining interphase cells demonstrated rather strong cytoplasmic staining (data not shown). Taking into account the previous finding that exogenously transfected hCDC6 protein is present in nuclei in G1 but in cytoplasm in S phase cells (36), we investigated hCDC6 localization in synchronized HeLa cells. In the G1 phase cells, dominant nuclear staining with some cytoplasmic staining was observed (Fig. 3, panel 1). However, in most of the early S phase cells, strong cytoplasmic staining was observed (Fig. 3, panel 5), which remained also in G2 phase cells (Fig. 3, panel 9, left two cells). In mitotic cells, homogeneous staining was obtained (Fig. 3, panel 9). Although the significance of the cytoplasmic staining in G1 cells is currently unclear, the changing pattern of major localization of endogenous hCDC6 protein during the cell cycle is in agreement with previous findings for transfected hCDC6 (36).

**hCDC6 Protein Is Associated with Detergent- and Nuclease-resistant Nuclear Structure throughout the Cell Cycle**—We next investigated subcellular localization of hCDC6 protein with cell fractionation. First, cells were treated with 0.1%TX-100mCSK, which extracts not only cytoplasmic but also nuclear proteins not tightly bound to nuclear structures, and second, the extracted nuclei were digested with DNase I to remove the bulk of chromatin (24, 34). As we showed previously (32, 34), in asynchronous HeLa cells, about two-thirds of the hMCM7 protein was extracted with the buffer, and most of the rest was released by DNase I digestion (Fig. 4A). The DNase I treatment also liberated about two-thirds of core histones and DNA from the extracted nuclei (data not shown, but see our previous paper; Ref. 34). The same fractions were then examined with anti-hCDC6 antibodies. About two-thirds of the hCDC6 protein was detected in the extractable fraction, and the rest were detected in the extracted nuclei. However, in contrast to the chromatin-bound hMCMs, most of the nucleus-bound hCDC6 was resistant to DNase I treatment (Fig. 4A). Similar results were also obtained with the restriction enzyme HaeIII and other human cell lines (data not shown). We could not obtain positive hCDC6 immunostaining in the extracted nuclei (data...
not shown). The bound form hCDC6 may be inaccessible to our antibodies.

We next examined early S phase HeLa cells with the fractionation and found essentially the same profile as with asynchronous cells (Fig. 4B). The insoluble hCDC6 was still present in G2/M phase HeLa, where little hMCMs bind to chromatin (Fig. 4C). These data, in combination with the immunostaining, suggest the following: 1) in the G1 phase, hCDC6 is present mainly in nuclei, and a part of it is associated with the detergent- and nuclease-resistant nuclear structure; 2) in the S phase, hCDC6, probably present in the nucleoplasm in G1, is translocated to the cytoplasm, but the bound form is still present; and 3) it remains bound to the nuclear structure or condensed chromosomes in the G2/M phase.

In our nuclear preparation, about 30% of the total chromatin still remains (34). Therefore, it is currently unknown whether the structure to which hCDC6 binds is nuclear matrix or nuclease-resistant specific chromatin regions such as heterochromatin. Because the detergent-insoluble hCDC6 is extractable with the buffer containing 0.5 M NaCl (data not shown), it is difficult with classical salt extraction to further strip the remnant chromatin from our nuclear preparation while preserving hCDC6 binding. This issue should be addressed in the future.

Saha et al. (36) suggest that hCDC6 protein may be selectively eliminated from nuclei in the S phase, and thereby DNA replication may be regulated. However, our data suggest that this is not necessarily the case. On the other hand, it is currently unclear whether the majority of the bound hCDC6 is actually associated with chromatin or not. Given the conservation of CDC6 function, the hCDC6 may bind to prereplication chromatin in G1. However, it might dissociate from chromatin in S phase, as suggested for the Xenopus egg system (42).

**hCDC6-hMCM Complex Interaction**—The data described above also suggest that major binding sites on chromatin differ between hMCMs and hCDC6. This is also supported by the observation that significant co-immunoprecipitation between hCDC6 and hMCM complex was undetectable in the solubilized chromatin fraction as well as in the Triton X-100-extractable fraction and that the levels of the bound hMCM7 were at least several times those of bound hCDC6 (data not shown). hCDC6 protein is suggested to physically interact with hORC1 (36), and interestingly, it was found with the cell fractionation that almost all hORC1 protein may be associated with the detergent- and nuclease-resistant nuclear structure throughout the cell cycle. It has been suggested that also in Xenopus eggs and yeast MCMs may not be tethered to chromatin via ORC and CDC6 (10, 42). Rather these latter might act as loaders for MCMs, as does replication factor C for proliferating cell nuclear antigen (43). Recently, hORC5 was shown to tightly bind to nucleus (44). It has also been shown that hORC1, 2, 4, and 5 can form a complex (44–46).

Taking the available information into consideration, we propose a hypothetical model as shown in Fig. 5 for the state of prereplication chromatin in human cell nuclei. In this model, hORC and hCDC6 are associated with chromatin on the detergent- and nuclease-resistant nuclear structure. The hMCM complexes are loaded, presumably by hORC/hCDC6, mainly onto the chromatin regions that are not associated with the nuclear structure. Given the analogy with the yeast system (11, 13), however, it is possible that at least a small proportion of the hMCM complexes is loaded onto chromatin regions near hCDC6-binding sites. To address this point, the extracted nuclei from asynchronous HeLa cells were chemically cross-linked with DSP, then solubilized in 1% SDS buffer with sonication, and subjected to immunoprecipitation (Fig. 6A). Because hMCM hexameric complexes are disrupted with SDS, anti-hMCM7 antibodies could not co-precipitate hMCM3 protein without cross-linking. However, with cross-linking, hMCM3 became efficiently co-precipitated with anti-hMCM7 antibodies as expected. Under such conditions, small but significant amounts of hMCM3 and 7 were co-precipitated with anti-hCDC6 antibodies. On the other hand, anti-hMCM7 antibodies could hardly co-precipitate hCDC6, possibly because of the existence of excess hMCM complexes that are not associated with hCDC6. Lamin B, one of the most abundant proteins in nuclei, was not co-precipitated in all of the cases tested. We further repeated the analysis with Epstein-Barr virus-infected Raji cells (Fig. 6B). In this case, reciprocal co-precipitation of hCDC6 with anti-hMCM7 antibodies as well as co-precipitation of hMCM7 with anti-hCDC6 was found. These data suggest that the notion described above may be correct.

Our model is not necessarily similar to that for yeast and also has many problems. We have no information as to what chromatin region is associated with human initiator proteins. What is the significance of the association of the initiator proteins with the specific nuclear structure? In Xenopus egg extracts, specific nuclear structures and DNA sequences may not necessarily be required for replication (14). In this regard, during the process of differentiation, more complex regulation of nuclear functions would be expected to have evolved, requiring a more complex network of nuclear structures. It also remains unclear whether the chromatin-bound hMCM complexes spread over the entire chromatin or are clustered within regions near hCDC6. One possible way to address these points would be by immunoprecipitation assay for cross-linked chromatin.

**Hyper phosphorylation of the Nuclear Structure-associated hCDC6 Protein in the G2/M Phase and Involvement of CDC2**

Y. Tatsumi, T. Tsurimoto, K. Shirahige, H. Yoshikawa, and C. Obuse, submitted for publication.
Kinase—To assess the nature of the slow migrating form observed for bound hCDC6 in the G2/M phase (Figs. 2 and 4C), Triton X-100-extracted nuclei from G2/M phase HeLa cells were treated with λ-phosphatase. This resulted in conversion to the normally migrating form, a change that was inhibited by vanadate, an inhibitor for λ-phosphatase (Fig. 7A). These findings indicate that the bound hCDC6 is hyperphosphorylated at G2/M. CDC2 kinase is maximally active in late G2/M phase, can phosphorylate mammalian MCM2 and 4, and plays a crucial role in inhibiting reloading of mammalian MCM complexes (24). As shown in Fig. 7B, we found that purified CDC2/cyclin B can phosphorylate in vitro the hCDC6 immunoprecipitated from S phase HeLa cells and promote a shift in its mobility.

We next examined its in vivo role using murine CDC2 kinase temperature-sensitive mutant FT210 cells (24, 38, 39). Our antibodies could detect murine CDC6 protein, but most of it was distributed in the the detergent- and nuclelease-resistant nuclear fraction in asynchronous FT210 cells (data not shown, but see below). The reason for the difference between the FT210 cells and tested human cells including HeLa, in which a significant amount of CDC6 is Triton-soluble, is currently unknown. The FT210 cells were incubated with nocodazole for 16 h at a permissive or a nonpermissive temperature for synchronization at the G2/M phase, and whole cell lysates, Triton X-100-extractable fractions, and nuclear pellets were prepared. Immunoblotting with a monoclonal antibody 4A4 specific for CDC2-phosphorylated vimentin confirmed the CDC2 inactivation at a nonpermissive temperature (data not shown) as described previously (24). The samples were then immunoblotted with anti-hCDC6 antibodies, showing the presence of slow migrating hyperphosphorylated murine CDC6 in the G2/M phase FT210 cells cultured at a permissive temperature and its conversion to the normally migrating form at a nonpermissive temperature (Fig. 7C). We have repeated the experiment and consistently obtained the same result.

Together, the data indicate that the hyperphosphorylation of the nuclear structure-bound CDC6 at G2/M phase is under the control of CDC2 kinase. Given that the bound form is active for regulation of DNA replication, a bound form-specific phosphorylation seems reasonable. Although the details are currently unknown, the function of CDC2 kinase to suppress the reloading of mammalian MCM complexes during G2/M phase might be mediated by the phosphorylation of CDC6 as well as phosphorylation of MCM complexes by CDC2 kinase (24). During preparing this manuscript, three related papers for mammalian CDC6 were published. Petersen et al. (47) reported that the cytoplasmic relocation of mammalian CDC6 in S phase may be regulated by phosphorylation by CDK2/cyclin A. However, because localization of CDC6 was only determined by immunostaining, the nucleus-bound form CDC6 was not well characterized in the study. On the other hand, Stoeber et al. (48) demonstrated that similar to the case in FT210 cells described above, almost all CDC6 is distributed in Triton-insoluble fraction in murine NIH3T3 cells, although it was not shown whether it is associated with chromatin or not. Williams et al. (49) also reported that in S phase human fibroblasts, CDC6 shows nuclear but not cytoplasmic staining. The exact regulation of mammalian CDC6 protein during the cell cycle, and its biological significance will require further study for complete understanding.

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REFERENCES

1. Bell, S. P., and Stillman, B. (1992) Nature **357**, 128–134

2. Diffley, J. F. X., Cocker, J. H., Dowell, S. J., and Rowley, A. (1994) Cell **78**, 303–316

3. Yan, H., Merchant, A. M., and Tye, B. K. (1993) Genes Dev. **7**, 2149–2160

4. Hennessy, K. M., Lee, A., Chen, E., and Botstein, D. (1991) Genes Dev. **5**, 958–969

5. Dalten, S., and Whitbread, L. (1995) Proc. Natl. Acad. Sci. U. S. A. **92**, 2514–2518

6. Lei, M., Kawasaki, Y., and Tye, B. K. (1996) Proc. Natl. Acad. Sci. U. S. A. **93**, 601–609

7. Lei, M., Kawasaki, Y., and Tye, B. K. (1996) Proc. Natl. Acad. Sci. U. S. A. **93**, 5375–5386

8. Hennessy, K. M., Lee, A., Chen, E., and Botstein, D. (1991) Genes Dev. **5**, 397–495

9. Piatti, S., Lengauer, C., and Nasmyth, K. (1998) EMBO J. **17**, 17095–17101

10. Donovan, S., Harwood, J., Drury, L. S., and Diffley, J. F. X. (1997) Proc. Natl. Acad. Sci. U. S. A. **94**, 958–969

11. Tanaka, T., Knapp, D., and Nasmyth, K. (1997) EMBO J. **16**, 5081–5090

12. Liang, C., and Stillman, B. (1997) Science **275**, 27247–27251

13. Aparicio, O. M., Weinstein, D. M., and Bell, S. P. (1997) Cell **88**, 418–422

14. Walter, J. Sun, L., and Newport, J. (1998) Mol. Cell Biol. **18**, 396–410

15. Coleman, T. R., Carpenter, P. B., and Dunphy, W. G. (1996) Cell **87**, 287–296

16. Rowles, A., Chung, J. P. J., Brown, L., Howell, M., Egan, I. V., and Blow, J. J. (1996) Cell **87**, 287–296

17. Kubota, Y., Mimura, S., Nishimoto, S., Takisawa, H., and Nojima, H. (1995) Cell **81**, 601–609

18. Chong, J. P. J., Mahbubani, H. M., Khoa, C.-Y., and Blow, J. J. (1995) Nature **375**, 418–421

19. Madine, M. A., Khoa, C.-Y., Mills, A. D., and Laskey, R. A. (1995) Nature **375**, 421–424

20. Hayles, J., Fisher, D., Woollard, A., and Nurse, P. (1994) Cell **78**, 813–822

21. Jallepalli, P. V., Brown, G. W., Muni-Falconii, M., Tien, D., and Kelly, T. J. (1997) Genes Dev. **11**, 2767–2779

22. Hendrickson, M., Madine, M., Dalten, S., and Gautier, J. (1996) Proc. Natl. Acad. Sci. U. S. A. **93**, 12223–12228

23. Hua, X. H., Yan, H., and Newport, J. (1997) J. Cell Biol. **137**, 183–192

24. Fujita, M., Yamada, C., Tsurumi, T., Hanaoka, F., Matsuoka, K., and Inagaki, M. (1998) J. Biol. Chem. **273**, 17095–17101

25. Kelly, T. J., Martin, G. S., Pardue, S. L., Stephen, R. J., Russo, A., and Nurse, P. (1993) Cell **74**, 371–382

26. Nishitani, H., and Nurse, P. (1995) Cell **83**, 397–495

27. Coue, M., Kerceay, S. E., and Mechal, M. (1996) EMBO J. **15**, 1085–1097

28. DePamphilis, M. L. (1996) in DNA Replication in Eukaryotic Cells (DePamphilis, M. L., ed) pp. 45–86. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

29. Thommes, P., Pett, R., Schray, B., Burkhart, R., Barnes, M., Kennedy, C., Brown, N. C., and Knippers, R. (1992) Nucleic Acids Res. **20**, 1069–1074

30. Kimura, H., Nozaki, N., and Sugimoto, K. (1994) EMBO J. **13**, 4311–4320

31. Todorov, I. T., Attaran, A., and Karseay, S. E. (1995) J. Cell Biol. **129**, 1433–1445

32. Fujita, M., Kiyono, T., Hayashi, Y., and Ishibashi, M. (1996) J. Biol. Chem. **271**, 4349–4354

33. Fujita, M., Kiyono, T., Hayashi, Y., and Ishibashi, M. (1996) Biochem. Biophys. Res. Commun. **219**, 664–670

34. Fujita, M., Kiyono, T., Hayashi, Y., and Ishibashi, M. (1997) J. Biol. Chem. **272**, 10928–10935

35. Williams, R. S., Shohe, R. V., and Stillman, B. (1997) Proc. Natl. Acad. Sci. U. S. A. **94**, 142–147

36. Saha, P., Chen, J., Thome, K. C., Lawlis, S. J., Hou, Z., Hendreics, M., Parvin, J. D., and Dutta, A. (1998) Mol. Cell Biol. **18**, 2758–2767

37. Yan, Z., DeGregori, J., Shohet, R., Leine, G., Stillman, B., Nevins, J. R., and Williams, R. S. (1998) Proc. Natl. Acad. Sci. U. S. A. **95**, 3603–3608

38. Mineo, C., Murakami, Y., Ishimi, Y., Hanaoka, F., and Yamada, M. (1986) Exp. Cell Res. **175**, 53–627

39. Thang, J. P. H., Wright, P. S., Hamaguchi, J. R., Lee, M. G., Norbury, C. J., Nurse, P., and Bradbury, E. M. (1990) Cell **63**, 313–324

40. Tho¨mmes, P., Fett, R., Schray, B., Burkhart, R., Barnes, M., Kennedy, C., Yatani, R., and Inagaki, M. (1992) J. Biol. Chem. **267**, 20937–20942

41. Williams, R. S., Shohe, R. V., and Stillman, B. (1997) Proc. Natl. Acad. Sci. U. S. A. **94**, 12223–12228

42. Hua, X. H., and Newport, J. (1998) J. Cell Biol. **140**, 271–281

43. Perkins, G., and Diffley, J. F. X. (1998) Mol. Cell **2**, 23–32

44. Quintana, D. G., Thome, K. C., Hou, Z., Ligon, A. H., Morton, C. C., and Dutta, A. (1998) J. Biol. Chem. **273**, 27157–27165

45. Quintana, D. G., Hou, Z., Thome, K. C., Hendricks, M., Saha, P., and Dutta, A. (1997) J. Biol. Chem. **272**, 28247–28251

46. Gavin, K. A., Hidaka, M., and Stillman, B. (1995) Science **269**, 1667–1671

47. Petersen, B. O., Lukas, J., Sørensen, C. S., Barr, K., and Helin, K. (1999) EMBO J. **18**, 396–410

48. Stoeber, K., Mills, A. D., Kubota, Y., Krude, T., Romanowski, P., Marheineke, K., Laskey, R. A., and Williams, G. H. (1998) EMBO J. **17**, 7219–7229

49. Williams, G. H., Romanowski, P., Morris, L., Madine, M., Mills, A. D., Stoeber, K., Marr, J., Laskey, R. A., and Coleman, N. (1998) Proc. Natl. Acad. Sci. U. S. A. **95**, 14932–14937