Requirement of Cyclin/Cdk2 and Protein Phosphatase 1 Activity for Chromatin Assembly Factor 1-dependent Chromatin Assembly during DNA Synthesis*

The influence of reversible protein phosphorylation on nucleosome assembly during DNA replication was analyzed in extracts from human cells. Inhibitor studies and add-back experiments indicated requirements of cyclin A/Cdk2, cyclin E/Cdk2, and protein phosphatase type 1 (PP1) activities for nucleosome assembly during DNA synthesis by chromatin assembly factor 1 (CAF-1). The p60 subunit of CAF-1 is a molecular target for reversible phosphorylation by cyclin/Cdk complexes and PP1 during nucleosome assembly and DNA synthesis in vitro. Purified p60 can be directly phosphorylated by purified cyclin A/Cdk2, cyclin E/Cdk2, and cyclin B1/Cdk1, but not by cyclin D/Cdk4 complexes in vitro. Cyclin B1/Cdk1 triggers hyperphosphorylation of p60 in the presence of additional cytosolic factors. CAF-1 containing hyperphosphorylated p60 prepared from mitotic cells is inactive in nucleosome assembly and becomes activated by dephosphorylation in vitro. These data provide functional evidence for a requirement of the cell cycle machinery for nucleosome assembly by CAF-1 during DNA replication.

The replication of eukaryotic chromosomes is integrated into the regulation of the cell division cycle (1). During S phase, replication of the genomic DNA is tightly coupled to an assembly of the nascent DNA into chromatin (2–5). Chromatin assembly not only allows packaging of the genomic DNA into the nucleus but it is also involved in the regulation of essential DNA transactions such as gene expression, recombination, and repair. The fundamental repeated unit of chromatin structure is the nucleosome core particle, which contains 146 base pairs of DNA wrapped in 1.75 left-handed superhelical turns around a core of two copies of each core histone protein H2A, H2B, H3, and H4 (6). Synthesis of histone proteins occurs during S phase and is coupled to ongoing DNA replication (7). The assembly of new nucleosomes during DNA replication can be analyzed in cytosolic extracts from human somatic cells. Double stranded circular DNA containing the simian virus 40 (SV40) replication origin is able to replicate in a human cytosolic extract under the control of the virally encoded initiator protein and DNA helicase, T antigen (8). The cytosolic extract also catalyzes complementary DNA strand synthesis in the absence of any viral factors on single-stranded DNA templates (9). Nucleosome assembly on replicating DNA molecules in these systems depends on the addition of the nuclear chromatin assembly factor 1 (CAF-1) to the reaction (9, 10). CAF-1 mediates deposition of histone H3/H4 tetramers on the DNA from newly synthesized H3 and H4, which are present in the cytosolic extract (11). In a second step independently of CAF-1, the nucleosome core particles are completed by association of two H2A/H2B dimers to a H3/H4 tetramer (11).

CAF-1 was purified from human cell nuclei as a trimeric protein complex of the subunits p150, p60, and p48 (10), or as a larger complex termed chromatin assembly complex (CAC), also containing newly synthesized histones H3 and H4 (12). The two larger subunits p150 and p60 directly interact with each other and both are essential for nucleosome assembly during DNA replication (13). Immunoprecipitation experiments demonstrated a direct interaction of the p150 subunit with newly synthesized and modified histones H3 and H4 (13). The small subunit p48 binds to free, but not to nucleosomal histone H4 in the absence of the p150 and p60 subunits (12, 14). These histone-binding properties support a role for CAF-1 as a chaperone (15), targeting free histones to replicating DNA for an assembly into nucleosomes. CAF-1 also mediates nucleosome assembly during DNA repair synthesis following UV-induced damage of the DNA templates (16–18). The chaperone model of CAF-1 is supported by the non-cooperative and stoichiometric nucleosome assembly mechanism during complementary DNA strand synthesis in vitro (9).

CAF-1 is a prime candidate for nucleosome assembly during S phase in somatic cells because of its substrate specificity for replicating DNA templates (9, 10). However, nucleosome assembly by CAF-1 can be experimentally uncoupled from ongoing DNA replication by adding CAF-1 to an in vitro reaction after DNA strand synthesis has been inhibited by aphidicolin, suggesting that newly replicated DNA is marked for subsequent nucleosome assembly by CAF-1 (19). This marking depends on a replication factor, proliferating cell nuclear antigen (PCNA) (20). During DNA replication, PCNA is reversibly loaded onto the DNA by replication factor C, forming a topologically closed ring structure around the duplex DNA strand (reviewed in Refs. 21–23). Unloading of PCNA from replicated templates by replication factor C prevents subsequent nucleo-

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‡ Present address: Dept. of Biology, University of Konstanz, D-78464 Konstanz, Germany.
§ A Royal Society university research fellow. To whom correspondence should be addressed. E-mail: tk1@mole.bio.cam.ac.uk.

1 The abbreviations used are: CAF-1, chromatin assembly factor 1; PCNA, proliferating cell nuclear antigen; GST, glutathione S-transferase; PP1, protein phosphatase type 1; PP2A, protein phosphatase type 2A; Cdk, cyclin-dependent, serine/threonine protein kinase; CAC, chromatin assembly complex.
some assembly by CAF-1, and antibodies against PCNA inhibit CAF-1-mediated chromatin assembly, suggesting that the continued presence of PCNA on replicated templates is required for CAF-1 activity (20). These results established a molecular link between the replication fork and the nucleosome assembly machinery of CAF-1 via PCNA. However, successful coupling of these two processes required additional, as yet unknown factors present in the unfractonated cytosolic extract (20). We are addressing the identity of accessory factors required for nucleosome assembly by CAF-1 during DNA replication using an independent approach. During S phase, the two large subunits of CAF-1 co-localize with the intranuclear sites of DNA replication (24). The p60 subunit of CAF-1 changes its phosphorylation state at key regulatory transitions during the cell cycle. Coincident with a recruitment to replication foci at the G1 to S phase transition, p60 becomes partially dephosphorylated (25). In mitosis, p60 becomes hyperphosphorylated, coinciding with loss of nucleosome activity of CAF-1 and its displacement from chromatin (25). These data suggest an involvement of reversible phosphorylation by cell cycle-specific protein kinases and protein phosphatases in regulating CAF-1 activity during the cell division cycle.

Key players of cell cycle control in somatic mammalian cells are cyclins and cyclin-dependent, serine/threonine protein kinases (Cdks) (reviewed in Refs. 26–33). The cyclin subunits contribute to timing, substrate specificity, intracellular localization, and binding to other regulatory proteins of the catalytic Cdk subunits. Following mitogen stimulation, a cascade of Cdk activities is required for timely triggering initiation of nuclear DNA replication at the onset of S phase. A sequential synthesis of the regulatory D-, E-, and A-type cyclins results in sequential binding to, and activation of, the appropriate Cdk subunits. During G1 phase progression, D-type cyclins associate with Cdk4/6 and their activity is involved in expression of S phase-specific genes. Initiation of and progression through S phase is under control of cyclin E/Cdk2 and cyclin A/Cdk2 activity. Following successful replication, condensation of interphase chromatin into mitotic chromosomes and their segregation into the two daughter cells is controlled by B-type cyclins complexed to Cdk1. Phosphorylation by active Cdns is reversible, and phosphates on serine and threonine residues can be removed by protein phosphatase types 1 and 2 (32, 34).

In this paper, we investigate the functional requirement of reversible protein phosphorylation for nucleosome assembly by CAF-1 during DNA replication in human cell extracts. Specific inhibitors for cyclin-dependent kinases and protein phosphatases inhibited nucleosome assembly by CAF-1. These results establish a requirement for reversible protein phosphorylation by G1/S phase-specific Cdns and protein phosphatase type 1 (PP1) for CAF-1 activity in vitro. Furthermore, the p60 subunit of CAF-1 is reversibly phosphorylated by cyclin/Cdns and PP1 activities during the nucleosome assembly reaction in vitro. These data provide a novel molecular link between the control of chromatin assembly during DNA synthesis and the cell cycle machinery.

MATERIALS AND METHODS

Cell Culture and Extraction Preparation—HeLa-S3 cells were cultured as exponentially growing subconfluent monolayers on 145-mm plates in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.), supplemented with 10% fetal calf serum (Life Technologies, Inc.), 10 units/ml penicillin (Sigma), and 0.1 mg/ml streptomycin (Sigma). Cytosolic extracts and CAF-1 were prepared as detailed previously (9, 25).

DNA Synthesis and Nucleosome Assembly Reactions—Complementary DNA strand synthesis and nucleosome assembly reactions were essentially performed as described (9), with the following modifications. Standard reactions contained cytosolic extract (150 μg of protein) from asynchronously proliferating cells, unless indicated otherwise, and 30 ng of single-stranded M13mp18 DNA as template. Semiconservative DNA replication of double-stranded DNA was performed under identical conditions, but using 15 ng of double-stranded M1301 DNA containing the SV40 control region as template (35) and 5 μg of purified SV40 large T antigen as initiator protein (gift of C. Gruss, University of Konstanz, Konstanz, Germany) (9). CAF-1 was isolated from interphase nuclei (9), or from mitotic chromatin (25), and was added at 6 μg of total protein where indicated.

Stock solutions of roscovitine (50 mM), olomoucine (50 mM), okadak acid (50 μM), and protein phosphatase inhibitor 2 (45 μM; all Calbiochem) were dissolved in Me2SO. Sodium vanadate was dissolved in distilled H2O at 100 mM. The first 90 amino acids from human p21 protein comprising the cyclin-binding motif and the Cdk inhibitory motif were obtained as purified GST fusion protein expressed in Escherichia coli (pG21N-GST; gift of N. Furuno, N. den Elzen, and J. Pines, Wellcome/CRC Institute, University of Cambridge, Cambridge, United Kingdom (Ref. 36)). As control, purified GST was also used (gift of J. Pines). These compounds were added to the reactions at the final concentrations indicated in the figures, and control reactions contained equivalent volumes of the respective solvents only.

Reactions containing protein phosphatase λ (New England Biolabs) were supplemented with 20 μM MnCl2. Preincubations in the absence of template DNA to allow dephosphorylation of phosphoproteins were performed at 30 °C for 60 min. Single-stranded M13mp18 DNA was then added to start complementary DNA strand synthesis and mini-chromosomes assembly. Reactions were transferred to 37 °C for 120 min.

Nucleosome assembly in the absence of DNA synthesis was performed on double-stranded M13mp18 DNA in the presence of cytosolic extract, a buffered mix of ribo- and deoxyribonucleoside triphosphates, an energy-regenerating system, and purified core histones as described (35). All reactions were mixed on ice and started by transferring to 37 °C. Standard reaction time was 120 min.

Processing of the Reaction Products—Replisome reactions were stopped by the addition of 50 μl of 2× stop mix (2% sarkosyl, 0.2% SDS, 20 mM EDTA) and extracted in phenol-chloroform. DNA was ethanol-precipitated, dissolved in TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8), and loaded onto 0.75% agarose gels. Gel electrophoresis was performed at room temperature in 1× TBE buffer (45 mM Tris borate, 0.5 mM EDTA, pH 8.4) at 3–4 V/cm. Two-dimensional gel electrophoresis (37) was performed with 0.45–0.55 μM chlorquine in the second dimension as detailed previously (9). DNA was visualized either by autoradiography of the dried gel or by ethidium bromide staining after RNase A digestion, as indicated in the figure legends.

Micrococcal nuclease (MNase) digestions were performed by adjusting replisome reactions after 120 min to 3 mM CaCl2 on ice. MNase (Roche) was added at the specified amounts, and the reactions were transferred to 37 °C for 15 min. Reactions were stopped by addition of 12.5 μl of 5× stop buffer (2.5% sarkosyl, 100 mM EDTA), and the amount of nuclease-resistant DNA was quantitated by trichloroacetic acid precipitation and scintillation counting. For product analysis, nuclease-resistant DNA was purified from the stopped reactions and analyzed on 1.5% agarose gels.

Protein Kinase Assays—Recombinant cyclin/Cdk complexes purified from S9 cells infected with recombinant baculovirus expression vectors were gifts of M. Jackman, Dawn Coverley, and J. Pines (Wellcome/CRC Institute, University of Cambridge, Cambridge, United Kingdom (Ref. 38)) and E. Laue and W. Zhang (Department of Biochemistry, University of Cambridge). For in vitro phosphorylation of the p80 subunit of CAF-1, recombinant p60 protein was purified from E. coli C41 (BL21) (25). Purified p60 was adjusted to kinase buffer (50 mM Tris, 1 mM dithiothreitol, 5 mM ATP, 150 mM NaCl, and 10 mM MgCl2) using PD-10 column chromatography (Amersham Pharmacia Biotech). Phosphorylation reactions were performed in kinase buffer containing purified p60 (4 μg of protein), 0.1 μg of protein of the recombinant cyclin/Cdk complexes, and 10 μCi of [γ-32P]ATP (Amersham Pharmacia Biotech) for 60 min at 30 °C. Samples were stopped and electrophoresed on a 12% denaturing polyacrylamide gel, and proteins were stained by Coomassie Blue. Phosphorylated proteins were detected by autoradiography of the dried gel. Analysis of p60 phosphorylation by immunoblotting using antibody pAb1 was performed as detailed previously (25).

RESULTS

Inhibition of Cyclin-dependent Protein Kinase Activity Partially Inhibits Replication-dependent Nucleosome Assembly in Human Cell Extracts—Single-stranded circular DNA is converted into double-stranded DNA upon incubation in cytosol extract from human cells, supplemented with ribo- and de-
Nucleosome Assembly during DNA Synthesis

To directly test an involvement of Cdks we added recombinant human cyclin/Cdk complexes to reactions containing both CAF-1 and roscovitine and asked whether cyclin/Cdk complexes could negate the inhibition of CAF-1 activity by roscovitine. Clearly, addition of cyclin A/Cdk2 or cyclin E/Cdk2 fully negated the inhibition of CAF-1-dependent nucleosome assembly by roscovitine (Fig. 1D). In control experiments, cyclin B1/Cdk1 or cyclin D2/Cdk6 did not negate this inhibition (data not shown). These results strongly suggest that cyclin A/Cdk2 and/or E/Cdk2 activity is required for nucleosome activity by CAF-1.

We sought to confirm independently a requirement of cyclin/ Cdk2 for CAF-1 activity during DNA synthesis by using a protein comprising the N-terminal 90 amino acids from human p21 protein fused to GST (p21N-GST; Ref. 36). The N terminus of p21 binds to and inhibits the kinase activity of cyclin A/Cdk2, cyclin E/Cdk2, and cyclin D/Cdk4, but not cyclin B1/Cdk1 (42–45). In contrast, the C terminus binds to PCNA and inhibits DNA replication by an independent mechanism (46, 48–
Addition of purified p21N-GST protein clearly inhibited nucleosome assembly by CAF-1 during complementary DNA strand synthesis by 50% (Fig. 2A), whereas the GST control protein did not inhibit nucleosome assembly (Fig. 2B).

Taken together, these inhibitor and add-back experiments demonstrate that the protein kinase activity of cyclin/Cdk2 complexes is required for efficient nucleosome assembly by CAF-1 during complementary DNA strand synthesis. However, about half of the nucleosome assembly activity in this system appears to be resistant to the protein kinase inhibitors and therefore not to depend on protein kinase activity. We asked next whether inhibiting the reverse reaction, namely protein dephosphorylation also influences nucleosome assembly during DNA synthesis.

Inhibition of Serine/Threonine Protein Phosphatase Activity Partially Inhibits Replication-dependent Nucleosome Assembly in Human Extracts—Addition of increasing amounts of sodium vanadate neither inhibited complementary DNA strand synthesis nor nucleosome assembly (Fig. 3A), indicating that protein dephosphorylation at tyrosine residues is not essential in this system. In contrast, addition of the inhibitor of the serine/threonine protein phosphatase types 1 (PP1) and 2A (PP2A), okadaic acid, reduced the amount of superhelical form I DNA with a compensating gain of relaxed topoisomers (Fig. 3B). The number of nucleosomes assembled by CAF-1 during complementary DNA strand synthesis in the presence of okadaic acid was determined by two-dimensional gel electrophoresis (Fig. 3C). Addition of 0.5 μM okadaic acid reduced the average number of nucleosomes assembled by CAF-1 to about 60% (Fig. 3D). To discriminate between PP1 and PP2A, we added increasing amounts of the PP1-specific inhibitor, I-2 (34, 49, 50). I-2 clearly inhibited the supercoiling of double-stranded reaction products (Fig. 3B), suggesting that PP1 activity, rather than PP2A activity, is required for efficient nucleosome assembly by CAF-1 during DNA synthesis. In support of this conclusion, addition of recombinant PP1 to assembly reactions negated the inhibition by okadaic acid (Fig. 3D).

Nucleosome Assembly by CAF-1 during Semiconservative DNA Replication also Depends on Cyclin-dependent Kinase and Protein Phosphatase Activities—So far, CAF-1 activity was analyzed during complementary DNA strand synthesis using single-stranded DNA templates. To assess whether the dependence of CAF-1 activity on cyclin/Cdk and PP1 activities also exists for nucleosome assembly by CAF-1 during semiconservative DNA replication of double-stranded DNA, we performed similar experiments using the SV40 DNA replication system (Fig. 4). Replication depends on cytosolic extract, double-stranded DNA containing the SV40 origin and the addition of the initiator protein and DNA helicase T antigen (8). However, the interaction of T antigen with cytosolic DNA polymerase α/primase also depends on reversible serine/threonine phosphorylation (51, 52). We therefore tested first the influence of roscovitine and okadaic acid on SV40 DNA replication (Fig. 4A). Both compounds inhibited DNA replication strongly, as expected, but in the presence of excess T antigen, some covalently closed circular mature DNA replication products were still formed in the presence of 0.5 mM roscovitine or 1 μM okadaic acid (Fig. 4A). Interestingly, formation of replication intermediates was less sensitive to these compounds (Fig. 4A). In the presence of CAF-1, efficient nucleosome assembly was strongly inhibited by roscovitine and by okadaic acid (Fig. 4B), demonstrating that CAF-1 activity during semiconservative
Nucleosome Assembly during DNA Synthesis

Figure 4. Nucleosome assembly by CAF-1 during semiconservative replication of double-stranded DNA containing the SV40 origin also depends on cyclin-Cdk and PP1 activity. A, sensitivity of SV40 T antigen and origin-dependent DNA replication to roscovitine and okadaic acid. Double-stranded M1301 DNA containing the SV40 control region (35) was replicated in the presence of the indicated amounts of roscovitine (ros) and okadaic acid (OA). Replication products were analyzed by gel electrophoresis and autoradiography. Positions of DNA forms II and III and of intermediates of semiconservative and bidirectional replication containing α and φ structures (RD) are indicated. B, nucleosome assembly by CAF-1 during semiconservative replication of double-stranded DNA is inhibited by roscovitine and okadaic acid. DNA replication reactions were performed in the absence of CAF-1 (reaction 1), in the presence of CAF-1 and 0.5 mM roscovitine (reaction 2) or 1 μM okadaic acid (reaction 3), and in the presence of CAF-1 without inhibitors (reaction 4). Purified DNA replication product reactions were analyzed by two-dimensional gel electrophoresis and autoradiography as detailed for Fig. 1B. The gel was overexposed to reveal the topoisomers of reactions 2 and 3.

Figure 5. Nucleosome assembly during DNA synthesis is inhibited by roscovitine and okadaic acid. A, calibration curves for degradation of chromatin by micrococcal nuclease (MNase). Complementary DNA strand synthesis reactions were supplemented with either saturating amounts of CAF-1 (6 μg; 100% CAF-1, squares), semisaturating amounts of CAF-1 (3 μg; 50% CAF-1, circles), or no CAF-1 (0% CAF-1, triangles). Reaction products were digested with 0, 1, or 5 units of MNase at 37 °C for 15 min. Nucleosome-resistant DNA was precipitated with trichloroacetic acid and quantitated by scintillation counting. B, quantitation of chromatin assembled in the presence of roscovitine and okadaic acid. Assembly reactions in the presence of saturating amounts of CAF-1 were supplemented with Me2SO as control (squares), 0.5 mM roscovitine (circles), 0.5 μM okadaic acid (open triangles), and both roscovitine and okadaic acid (open diamonds). Reaction products were digested and quantitated as described in panel A. Mean values of three to five independent experiments are shown for each panel. C, analysis of nucleosome-resistant DNA by agarose gel electrophoresis. Assembly reactions were performed as in panel B, and reaction products were digested with 0, 0.2, 1, and 5 units of MNase at 37 °C for 15 min. Nucleosome-resistant DNA was purified by ethanol precipitation, separated on a 1.5% agarose gel, and visualized by autoradiography. Positions of uncut DNA forms I, II, and III and of nucleosome-resistant DNA fragments of mono-, di-, tri-, and tetranucleosomal length are indicated on the left, top, and bottom, respectively.

Taken together, these results suggest that protein phosphorylation by S phase-specific cyclin/Cdk2 complexes and its reverse reaction, protein dephosphorylation by PP1, are both required for an efficient nucleosome assembly by CAF-1 during DNA replication. However, some nucleosome assembly activity appears to be resistant to either of the two kinds of inhibitors. It is thus possible that both types of partial inhibition act independently of each other, or act in an additive way, or cancel each other out. Therefore, we analyzed the influence of both inhibitors together on nucleosome assembly during DNA synthesis.

Inhibition of Both Protein Kinase and Phosphatase Activity Prevents Nucleosome Assembly by CAF-1—Addition of both 0.5 mM roscovitine and 0.5 μM okadaic acid to DNA synthesis-dependent nucleosome assembly reactions resulted predominately in the formation of linear form III and open circular form II double-stranded DNA synthesis products (data not shown). These unligated DNA synthesis products thus do not allow analysis of nucleosome assembly by the supercoiling assay. Therefore, we analyzed nucleosome assembly in the presence and absence of these inhibitors by MNase digestions of the double-stranded DNA reaction products (Fig. 5).

CAF-1 assembles nucleosomes during complementary DNA strand synthesis in human cell extracts in a stoichiometric manner (9). As reference, we first calibrated the resistance of chromatin assembled to various nucleosomal densities to a digestion with MNase (Fig. 5A). Minichromosomes assembled during complementary DNA strand synthesis in the presence of saturating amounts of CAF-1 were 76% resistant to a 15 min digestion with 5 units of MNase (Fig. 5A, squares), compared with an efficient degradation of reaction products in the absence of CAF-1 to 40% (Fig. 5A, triangles). Reaction products assembled in the presence of halfmaximal amounts of CAF-1 contain half the complement of nucleosomes (9), and they are degraded by MNase to an intermediate value of 55% (Fig. 5A, circles).

Minichromosomes assembled in the presence of either 0.5 mM roscovitine or 0.5 μM okadaic acid were resistant at intermediate levels of 62% and 69% to degradation with MNase (Fig. 5B, circles and triangles, respectively). These data suggest that only about half the maximal number of nucleosomes are assembled in the presence of either 0.5 mM roscovitine or 0.5 μM okadaic acid, as compared with the control in the absence of inhibitors (Fig. 5B, squares). These observations are entirely consistent with, and independently confirm the supercoiling data of Figs. 1 and 3.

Reaction products assembled in the presence of both inhibitors were efficiently degraded by MNase to 49% (Fig. 5B, diamonds), suggesting that only very few nucleosomes are assembled during this reaction. These data establish that the inhibitory effects of cyclin/Cdk and protein phosphatase inhibitors act in an additive way on chromatin assembly during DNA synthesis.

These quantitative data were supported by the analysis of the nucleosome-resistant DNA products by agarose gel electrophoresis (Fig. 5C). Reaction products assembled in the absence of either inhibitor showed nucleosome-resistant DNA fragments of mono- and spaced oligonucleosomal length (Fig. 5C, control). Addition of either roscovitine or okadaic acid resulted in an
increased nuclease sensitivity of the reaction products, apparent in the shorter average fragment length in the presence of 0.2 unit of MNase and the presence of smaller amounts of predominantly mononucleosomal fragments at higher concentrations of MNase (Fig. 5C). In the presence of both inhibitors, roscovitine and okadaic acid, reaction products were efficiently degraded, and only trace amounts of mononucleosomal DNA fragments were observed.

Taken together, these results show that nucleosome assembly by CAF-1 during complementary DNA strand synthesis is strongly inhibited, if not prevented, when both protein kinase and phosphatase activities are inhibited. This observation could be explained either by a general requirement of these activities for an assembly of nucleosomes in human cell extracts or by a specific requirement for the DNA synthesis-dependent nucleosome assembly pathway mediated by CAF-1. To distinguish between these possibilities, we asked whether replication-independent nucleosome assembly on double-stranded DNA in human cytosolic extracts is also inhibited by these inhibitors.

**Nucleosome Assembly in the Absence of DNA Synthesis Is Not Inhibited by Roscovitine or Okadaic Acid**—Nucleosomes can be efficiently assembled in human cytosolic extracts on double-stranded DNA in the absence of DNA replication and CAF-1 by a different pathway, which relies on purified core histones and a cytosolic assembly factor (35, 53). Addition of core histones purified from human interphase nuclei into reactions containing circular double-stranded DNA substrates and cytosolic extract induced formation of supercoiled form I DNA in a dose-dependent manner (Fig. 6A; cf. Refs. 35 and 53). Importantly, addition of either roscovitine or okadaic acid, or a combination of both, did not inhibit this nucleosome assembly pathway at any concentrations of core histones (Fig. 6A). We furthermore analyzed possible subtle changes in the efficiency of this nucleosome assembly by gel electrophoresis in the presence of chloroquine (Fig. 6B). As control, addition of either or both compounds did not significantly affect the relaxation of supercoiled DNA by cytosolic topoisomerase (Fig. 6B, *core histones*). Upon addition of a 1:1 mass ratio of core histones to the DNA, nucleosome assembly as measured by constrained DNA supercoiling was also not affected by either, or both, of roscovitine and okadaic acid (Fig. 6B, *+core histones*). These results demonstrate that protein kinase and phosphatase activities are not required for nucleosome assembly in human cell extracts *per se*, but they are specifically required for the assembly pathway mediated by CAF-1 during DNA synthesis.

In intact human cells, previous correlational studies described phosphorylation changes of the p60 subunit upon recruitment of CAF-1 to intranuclear sites of DNA replication and repair (17, 25). We therefore investigated in human cell extracts whether CAF-1 is a target for reversible protein phosphorylation during complementary DNA strand synthesis and nucleosome assembly.

**CAF-1 Is Targeted by Phosphorylation and Dephosphorylation during DNA Synthesis-dependent Nucleosome Assembly**—The phosphorylation state of p60 was analyzed by Western blotting (Fig. 7A), using antibody pAb1, which detects the phosphorylated isoforms of p60 (25). The p60 subunit of CAF-1 prepared from interphase nuclei consists of two distinct forms, a phosphorylated form (c) and an intermediately phosphorylated form (b), whereas in mitosis, p60 appears as hyperphosphorylated form (a) (Ref. 25, and see Fig. 7A).

Addition of increasing amounts of roscovitine to nucleosome assembly reactions during complementary DNA strand synthesis resulted in a partial, dose-dependent dephosphorylation of the p60 subunit (Fig. 7A). Conversely, addition of increasing amounts of okadaic acid resulted in a partial, dose-dependent hyperphosphorylation of the p60 subunit (Fig. 7A). Adding both inhibitors together did not result in either dephosphorylation or hyperphosphorylation of p60, but resulted predominantly in the intermediately phosphorylated form (b) of p60 (Fig. 7A). In conjunction with the results of the previous sections, these data show that CAF-1 is targeted by ongoing reversible protein phosphorylation, which is required for the nucleosome assembly activity of CAF-1 during complementary DNA strand synthesis. We asked next whether p60 can be directly phosphorylated by purified cyclin-dependent kinases.

Recombinant p60 was purified from transformed bacteria (25) and used as *in vitro* substrate for phosphorylation by recombinant human cyclin/Cdk complexes, prepared from baculovirus-infected insect cells (38). Analysis of the amino acid sequence of p60 (13) revealed two Cdk phosphorylation motifs fitting the consensus sequence (T/S)P or (T/S)P (Ref. 35 and 53). Importantly, p60 was phosphorylated by recombinant Cdk complexes only when treated with a dephosphorylated form (b) of Cdk2 (Fig. 7B). As control, addition of either or both inhibitors (r/a/o). Marker was supercoiled form I DNA (lane 1) and DNA relaxed by endogenous topoisomerases in the extract in the presence of chloroquine (Fig. 6B). As control, addition of either or both compounds did not significantly affect the relaxation of supercoiled DNA by cytosolic topoisomerase (Fig. 6B, *core histones*). Upon addition of a 1:1 mass ratio of core histones to the DNA, nucleosome assembly as measured by constrained DNA supercoiling was also not affected by either, or both, of roscovitine and okadaic acid (Fig. 6B, *+core histones*). These results demonstrate that protein kinase and phosphatase activities are not required for nucleosome assembly in human cell extracts *per se*, but they are specifically required for the assembly pathway mediated by CAF-1 during DNA synthesis.
mediated direct phosphorylation of p60 (Fig. 7B). Furthermore, mitotic cyclin B1/Cdk1 also phosphorylated p60 (Fig. 7B). In contrast, cyclin D1/Cdk4 as a representative of the cyclin D-Cdk4/6 protein kinase family did not phosphorylate p60 (Fig. 7B). We obtained similar results when CAF-1 prepared from human cell nuclei was used as substrate (data not shown). Therefore, S and M phase-specific cyclin/Cdk complexes can directly phosphorylate p60 in vitro.

In this assay, phosphorylation of recombinant p60 by cyclin/Cdkks did not result in formation of the hyperphosphorylated form (a) of p60 (Fig. 7B), which is observed when nucleosome assembly in human cell extracts is inhibited by okadaic acid (Fig. 7A), or when CAF-1 is prepared from mitotic cells (Ref. 25; cf. Fig. 7A). We therefore asked whether this mobility shift is dependent on additional factors present in the cytosolic extract. Indeed, addition of recombinant cyclin B1/Cdk1, but not of cyclin A/Cdk2, to DNA synthesis reactions resulted in the electrophoretic mobility shift characteristic for hyperphosphorylated p60 (Fig. 7C), suggesting that cyclin B1/Cdk1 is required in conjunction with other factors or modifications to induce hyperphosphorylation of p60. As mitotic CAF-1, containing a hyperphosphorylated p60 subunit, is inactive in supporting nucleosome assembly in vitro (25), we finally asked whether inactive hyperphosphorylated mitotic CAF-1 can be functionally activated in human extracts by dephosphorylation.

Reconstitution of Nucleosome Assembly Activity of Hyperphosphorylated Mitotic CAF-1 by Dephosphorylation—CAF-1 was prepared from mitotic chromatin and used in nucleosome assembly reactions during complementary DNA strand synthesis in human G1 phase cytosolic extracts. As shown before (25), p60 of mitotic CAF-1 is hyperphosphorylated (Fig. 8A) and does not support efficient nucleosome assembly in vitro (Fig. 8B, lane C). Treatment with increasing amounts of λ phosphatase led to a dephosphorylation of the p60 subunit of CAF-1 in the cytosolic extract (Fig. 8A). Even in the absence of λ phosphatase, a limited dephosphorylation of p60 occurred by endogenous phosphatase activity in the extract, which is activated by the addition of 20 μM MnCl2 (Fig. 8A, reaction 1). We then added single-stranded template DNA to these reactions to start complementary DNA strand synthesis and nucleosome assembly. The extent of p60 phosphorylation was not significantly altered during this subsequent incubation (Fig. 8A). Analysis of DNA reaction products demonstrated that dephosphorylation of CAF-1 during preincubation resulted in the formation of supercoiled form I DNA products (Fig. 8B). These data demonstrate that dephosphorylation of inactive hyperphosphorylated CAF-1 causes the activation of its nucleosome assembly activity during DNA synthesis, thus adding further evidence for the requirement of reversible phosphorylation for nucleosome assembly by CAF-1 during DNA synthesis.

**DISCUSSION**

The major conclusion from this work is that nucleosome assembly by CAF-1 during DNA synthesis in human cell extracts depends on reversible protein phosphorylation involving G1/S phase-specific cyclin-dependent protein kinase 2 and type 1 protein phosphatase. The p60 subunit of CAF-1 changes its phosphorylation state during nucleosome assembly in human cytosolic cell extract and becomes dephosphorylated in the presence of Cdk inhibitors and hyperphosphorylated when PP1 is inhibited. Purified p60 can also be directly phosphorylated by purified cyclin/Cdk kinases in vitro, suggesting that CAF-1 is a molecular target for reversible phosphorylation. We cannot exclude an involvement of additional kinase and phosphatase activities during reversible phosphorylation of CAF-1 in cell extracts during nucleosome assembly. However, a defined experimental system that uses purified components for replication-dependent nucleosome assembly by CAF-1 has not been reported to date to identify all the essential molecules during this reaction. Therefore, our results provide the first functional evidence for a requirement of the main players of the cell cycle machinery for CAF-1 activity during DNA synthesis.

**Requirement of Reversible Protein Phosphorylation for Nucleosome Assembly by CAF-1**—We studied the requirement of reversible protein phosphorylation for nucleosome assembly by CAF-1 during DNA synthesis in human cell extracts by using natural compounds, or their synthetic derivatives, as inhibitors. Roscovitine and the related compound olomoucine specifically inhibit purified Cdkks 1, 2, and 5 complexed to their cognate cyclin partners in vitro by competing with ATP in a linear fashion at its binding site on the kinases (39, 40, 54). We found that a 0.5 mM concentration of roscovitine inhibited CAF-1 activity, whereas increased concentrations also inhibited DNA replication. In supplementation experiments, we
Nucleosome Assembly during DNA Synthesis

In mitosis, the p60 subunit of CAF-1 is hyperphosphorylated and CAF-1 prepared from mitotic cells does not support nucleosome assembly by CAF-1 during DNA replication in vitro.

Okadaic acid is a specific inhibitor of the protein phosphatases PP1 and PP2A, which have broad and overlapping substrate specificities, including serine and threonine residues phosphorylated by Cdkas (34, 50, 56). Inhibition of CAF-1 activity by okadaic acid functionally demonstrates a role of either PP1 or PP2A for nucleosome assembly by CAF-1 during DNA synthesis. The PP1-specific peptide inhibitor I-2, which does not inhibit PP2A (50), also inhibited CAF-1 activity, indicating that PP1, rather than PP2A, is the type of protein phosphatase required for CAF-1 activity during DNA synthesis. Furthermore, in supplementation experiments, the inhibition of CAF-1 activity by okadaic acid was entirely relieved by addition of the purified recombinant catalytic subunit of human PP1. Taken together, these experiments established a requirement of PP1 activity for nucleosome assembly by CAF-1 during DNA replication in vitro.

Changes of CAF-1 Phosphorylation during the Cell Cycle in Vivo—Our experiments established functional requirements of cyclin/Cdk complexes and PP1 for nucleosome assembly by CAF-1 during DNA replication in human cell extracts. Furthermore, the phosphorylation state of the p60 subunit changes accordingly in the presence of the inhibitors used (Fig. 7A) and p60 can be directly phosphorylated by purified cyclin/Cdk complexes (Fig. 7B). These experiments demonstrate that CAF-1 is a target for ongoing reversible protein phosphorylation in the cell extracts used for nucleosome assembly and DNA synthesis in vitro. However, these data cannot rule out an indirect involvement of additional kinases and phosphatases, which could also be required for reversibly phosphorylating p60 during these assembly reactions in vitro.

Roles of cyclin/Cdk complexes and PP1 for regulating CAF-1 activity during chromosome dynamics in the cell cycle in vivo are supported by their respective intracellular localizations in human and other mammalian somatic cells. During S phase, CAF-1 co-localizes with intranuclear replication foci (24). Cyclins A, E, and Cdk2 are nuclear proteins in S phase (57, 58), and cyclin A and Cdk2 have been shown to co-localize with replication foci during S phase (59). These observations support a physiological role of these proteins in chromosome replication, and one further function of these cyclin/Cdk complexes may be the phosphorylation of CAF-1.

The intracellular localization of PP1 during the cell cycle also supports a physiological role in chromosome replication. The catalytic subunit of PP1 is predominantly cytosolic in mammalian G1 phase cells and accumulates in nuclei during S and G2 phases (60). Analysis of the three different subtypes of PP1 catalytic subunit showed nuclear localization of all subtypes during interphase and a specific association of PP1α with chromatin (61), suggesting that this subtype is a likely candidate for regulating chromatin assembly during DNA replication in S phase nuclei. Therefore, cyclin A/Cdk2, cyclin E/Cdk2, and PP1, which are required for efficient nucleosome assembly by CAF-1 during DNA replication in vitro (this paper), are at the nuclear location of DNA replication and nucleosome assembly during S phase in vivo.

In mitosis, the p60 subunit of CAF-1 is hyperphosphorylated and CAF-1 prepared from mitotic cells does not support nucleosome assembly in vitro (25). Mitotic cyclin B1/Cdk1 can hyperphosphorylate p60 in vitro, and its protein kinase activity is not involved in targeting CAF-1 to DNA templates undergoing replication (20) and repair (18).

Taken together, these experiments established that the G1/S phase-specific cyclin/Cdk2 complexes, which are required for initiating DNA replication in isolated human cell nuclei (38, 55), are also required for nucleosome assembly by CAF-1 during DNA replication in vitro.

This conclusion is supported by the sensitivity of CAF-1 activity to inhibition by the amino terminus of the natural human Cdk inhibitor p21. p21 directly binds to and significantly inhibits the kinase activity of cyclin A/Cdk2, cyclin E/Cdk2 (and cyclin D/Cdk4), but not cyclin B1/Cdk1 (45). The amino terminus of p21 contains the binding motifs for cyclins and Cdkks (42–44). Microinjection experiments demonstrated an inhibition by p21N of cyclin A/Cdk2 but not of cyclin B1/Cdk1 in human cells at a concentration similar to the one used in our in vitro studies (36). We deliberately used p21N rather than the entire p21 protein in order to exclude inhibitory reactions through interaction of the C terminus of p21 with PCNA (42, 48). PCNA has independently been shown to be a factor demonstrated that the addition of recombinant cyclin A/Cdk2 and E/Cdk2 complexes negated the inhibitory effect of 0.5 mM roscovitine on CAF-1 activity. Addition of cyclin B1/Cdk1 or D/Cdk6 did not. These experiments strongly suggest that the kinase activity of Cdk2, when complexed to either cyclin A or E, is required for nucleosome assembly by CAF-1.

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required for nucleosome assembly by CAF-1 (this paper). Cyclin B1 enters the nucleus at the beginning of mitosis and associates with condensed chromosomes in prophase and metaphase (57), consistent with a role in hyperphosphorylating p60, histone H1 and other mitotic substrates. A functional role for the cyclin B1/Cdk1-dependent hyperphosphorylation of p60 in reversibly inactivating nucleosome assembly activity of CAF-1 in mitosis was supported by our observation that dephosphorylation of inactive mitotic CAF-1 in vitro restores its nucleosome assembly activity (Fig. 8). The p60 subunit of CAF-1 appears fully dephosphorylated in the presence of excess λ phosphatase, and nucleosome assembly by CAF-1 is activated. (Note that this experiment does not contradict our inhibitor studies showing that an inhibition of Cdk activity results in dephosphorylation of p60 and an inhibition of nucleosome assembly by CAF-1. In the presence of excess of λ phosphatase the endogenous Cdk activity in the extract is not inhibited, allowing reversible phosphorylation to continue with the equilibrium of p60 phosphorylation being shifted toward the dephosphorylated form.)

PP1 is bound to condensed chromosomes throughout mitosis (60, 61), and its phosphatase activity is required for the metaphase to anaphase transition and exit from mitosis (60), coincident with cyclin B1 destruction (57). In human cells, PP1 activity has also been found as histone H1 phosphatase, which is required for chromatin decondensation during exit from mitosis (62). During the M to G1 phase transition, the hyperphosphorylation of p60 is also removed (25), consistent with a possible role for PP1 in dephosphorylating p60 and other substrates upon exit from mitosis.

**Models for a Requirement of Reversible Phosphorylation for CAF-1 Activity**—We observed that inhibiting either directions of reversible protein phosphorylation, namely phosphorylation by cyclin/Cdk complexes and dephosphorylation by PP1 partially inhibits nucleosome assembly by CAF-1 (Figs. 1–5). These apparently opposing observations can be explained by different working models.

In a simplistic model, only phosphorylation by Cdk2 would be directly required for nucleosome assembly by CAF-1. In this model, inhibition of PP1 by okadaic acid would allow accumulation of hyperphosphorylated CAF-1 generated by small amounts of cyclin B1/Cdk1 kinase activity present in the extracts from asynchronous cells. Indeed, hyperphosphorylated p60 is observed in the presence of okadaic acid in the reaction (Fig. 7A), and CAF-1 containing hyperphosphorylated p60 does not promote efficient nucleosome assembly (Figs. 3, 7A, and 8). These data are consistent with our previous observation that CAF-1 containing hyperphosphorylated p60 is inactive when isolated from mitotic cells (Fig. 8 and Ref. 25). However, this model cannot easily explain the significant residual CAF-1 activity in the presence of okadaic acid or roscovitine/p21N. It is also difficult to accommodate into this working model the additive inhibitory effect on CAF-1 activity when both protein phosphorylation and dephosphorylation are inhibited at the same time (Fig. 5).

Instead, several mechanistic working models involving reaction cycles could explain our observations (Fig. 9). CAF-1 is a histone chaperone and assembles nucleosomes in a non-cooperative and stoichiometric fashion during DNA synthesis in human cell extracts (9, 13, 15, 17). All three subunits of CAF-1 are histone-binding proteins (12, 13, 63). In the first working model, reversible binding of histones to CAF-1 may depend on the phosphorylation status of CAF-1. The binding of newly synthesized histones to CAF-1 forming CAC (Fig. 9, reaction 1) and the release of histones onto double-stranded DNA to form a nucleosome (Fig. 9, reaction 3) may require a cycle of reversible phosphorylation and dephosphorylation.

In the second working model, the reversible association of CAF-1 with replicating DNA, involving interaction with replication fork-associated protein PCNA, could depend on reversible phosphorylation. CAF-1 is present at replication foci in S phase nuclei (24), and it is recruited to the replication fork involving an association of p150 with PCNA (18, 20). The association of CAC with the replicating DNA to form an assembly intermediate (Fig. 9, reaction 2), and the release of CAF-1 from this intermediate to allow formation of a mature new nucleosome (Fig. 9, reaction 4) may require a cycle of reversible phosphorylation and dephosphorylation.

These two working models involving reaction cycles would also allow reconciliation of the partial nature of the inhibition of nucleosome assembly by CAF-1 by inhibiting Cdk2 and PP1 activities. The starting material of CAF-1 used for nucleosome assembly in this study consists of a mixture of unphosphorylated and phosphorylated p60 (Fig. 7). Conversion of one form of p60 into the other during nucleosome assembly in vitro, while preventing the reverse reaction by inhibitors, would therefore still be possible for about half of the p60 present in the reaction. This inhibitor-resistant conversion could be responsible for the residual CAF-1 activity observed in the presence of either inhibitor alone, but not in the presence of both inhibitors together.

We cannot currently discern whether dephosphorylated or phosphorylated CAF-1 is at the start of any of those reversible phosphorylation cycles. However, during the cell division cycle in vivo, phosphorylated CAF-1 is associated with chromatin during G2 phase and the occurrence of a dephosphorylated form of p60 is characteristic for S phase (25). During repair of UV-induced DNA damage in human G2 phase cell nuclei, the phosphorylated form of p60 is found in a detergent-resistant chromatin fraction, whereas in non-irradiated G2 phase cells, p60 is predominantly dephosphorylated in this fraction (17). These results may suggest that recruitment of CAF-1 to sites of DNA synthesis may first require phosphorylation, and dissociation would then depend on dephosphorylation.

![FIG. 9. Pathways of CAF-1-mediated nucleosome assembly during DNA replication that may be regulated by reversible protein phosphorylation. 1, association of CAF-1 with newly synthesized histones forming the chromatin assembly complex CAC. 2, association of CAC with replicating DNA to form an assembly intermediate, involving interaction of p150 with replication fork-associated protein PCNA. 3, formation of a newly assembled nucleosome from the assembly intermediate. 4, detachment of CAF-1 from the intermediate. For more details, see references (2, 5, 15). Reversible pathways during the CAF-1 reaction cycle that could be regulated by reversible phosphorylation, involving the p60 subunit, are the association with and the dissociation from replicated DNA (reactions 2 and 4 along the vertical; open arrows), or the association with and the dissociation from histones (reactions 1 and 3 shown along the horizontal; solid arrows).](image-url)
Future experiments using purified components with defined phosphorylation status will be therefore required to discriminate between these scenarios on a molecular level. To date, no experimental system exists that mediates nucleosome assembly by CAF-1 during DNA synthesis using purified components. However, the identification of two novel factors reported here, namely cyclin/Cdk2 and protein phosphatase type 1, which are required for efficient nucleosome assembly by CAF-1 during DNA synthesis in unfractionated human cell extracts will help future attempts to establish such defined cell-free systems from purified components.

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