

Nucleosome Assembly Activity and Intracellular Localization of Human CAF-1 Changes during the Cell Division Cycle*

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We characterized changes of nucleosome assembly activity, intracellular localization, and reversible phosphorylation of the human chromatin assembly factor CAF-1 during the somatic cell division cycle. HeLa cells were synchronized in the G₁, S, G₂, and M phases of the cell cycle. All three subunits of human CAF-1 (p150, p60, and p48) are present during the entire cell cycle. In interphase, p150 and p60 are bound to the nucleus, but they predominantly dissociate from chromatin during mitosis. During S phase, p150 and p60 are concentrated at sites of intranuclear DNA replication. Only a fraction of total p48 is associated with p150 and p60, and the majority is present in other high molecular weight complexes. The other nucleosome assembly protein, NAP-1, is predominantly cytosolic throughout the cell cycle. Human CAF-1 efficiently mediates nucleosome assembly during complementary DNA strand synthesis in G₁, S, and G₂ phase cytosolic extracts. Active CAF-1 can be isolated as a 6.5 S complex from G₁, S, and G₂ phase nuclei. In contrast, CAF-1 isolated from mitotic cytosol does not support nucleosome assembly during DNA synthesis. In mitosis, the p60 subunit of inactive CAF-1 is hyperphosphorylated, whereas active CAF-1 in interphase contains hypophosphorylated and/or phosphorylated forms of p60.

The genomic DNA of a eukaryotic cell is assembled into chromatin. The repeated structural subunit is the nucleosome core particle, in which 145 base pairs of DNA are wrapped in 1.75 superhelical turns around a central core histone octamer, consisting of two copies of each histone H2A, H2B, H3, and H4 (1). Higher order structures can be formed by association of nucleosome core arrays on the genomic DNA with linker histones and non-histone chromatin proteins (reviewed in Ref. 2). This assembly not only serves as a means to compact the genomic DNA within the confines of the nucleus but also participates in the regulation of gene expression and, perhaps, DNA replication and repair (for reviews see Refs. 3–7).

In proliferating cells, the bulk of chromatin is assembled following DNA replication during the S phase of the cell division cycle. Histone proteins are synthesized in S phase and incorporated into nucleosomes on newly replicated DNA (8–10). Newly synthesized histones differ from bulk histones in

their posttranslational modification. Newly synthesized histones H3 and H4 are acetylated on specific lysine residues before incorporation into nucleosomes (3, 11–13). Following nucleosome assembly, these sites become deacetylated by nuclear histone deacetylases (14).

De novo assembly of nucleosomes on replicating DNA can be mediated by chromatin assembly factors. Human chromatin assembly factor 1 (CAF-1) was purified from proliferating cell nuclei due to its ability to assemble nucleosomes on DNA templates replicating in cell-free extracts (15) (reviewed in Refs. 16 and 17). It mediates the first step of nucleosome assembly, depositing an H3/H4 tetramer onto replicating DNA (18). In human cells, CAF-1 is complexed to newly synthesized and acetylated histones H3 and H4 (12). H2A/H2B dimers are added to the nucleosome precursor in a second step, which is independent of ongoing DNA replication (18) and might involve another nucleosome assembly factor, nucleosome assembly protein 1 (NAP-1)¹ (19, 20). The assembly of new nucleosomes by CAF-1 seems to be an evolutionarily conserved mechanism in eukaryotes, and homologues of CAF-1 have been identified in *Drosophila* (21–23) and yeast (24).

Purified human CAF-1 contains three subunits, termed p150, p60, and p48. The p150 and p60 subunits are both essential for replication-dependent nucleosome assembly in human cell extracts and physically interact with each other (25). These two larger subunits of human CAF-1 also co-localize with the sites of nuclear DNA replication in S phase cells (26). These observations suggest that CAF-1 may be a physiologically relevant factor for nucleosome assembly during DNA replication *in vivo*. In yeast cells, however, mutants lacking the p150 and p60 homologues are viable, indicating that alternative nucleosome assembly pathways exist. However, these mutants are deficient in telomeric silencing, implying an involvement of CAF-1 in chromatin assembly *in vivo* (24, 27). The smallest subunit p48 was shown to be a member of a multiprotein family involved in histone binding (12, 13), and it is identical with protein RbAp48 in humans (28, 29). In addition, p48 is also a subunit of the human histone deacetylase HDAC1 (or HD1 (14)) and of a homologous protein in *Drosophila* (23). Furthermore, in yeast, a homologous protein, Hat2p, is a noncatalytic subunit of a cytoplasmic histone H4 acetyltransferase (30) and, in human cells, the closely related protein RbAp46 is a histone binding and noncatalytic subunit of the HAT1 histone acetyltransferase (13). These data suggest that the smallest subunit of CAF-1 is present in many multisubunit protein complexes involving histone metabolism (31).

The nucleosome assembly activity of CAF-1 is not restricted to DNA replication. It has been shown recently that CAF-1 is

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¹ The abbreviations used are: NAP-1, nucleosome assembly protein 1; TE, 10 mM Tris-Cl, 1 mM EDTA, pH 7.5; TBE, 45 mM Tris-borate, 0.5 mM EDTA, pH 8.4; MNase, micrococcal nuclease; pAb, polyclonal antibody; mAb, monoclonal antibody.

also able to mediate *de novo* assembly of nucleosomes on UV-irradiated DNA plasmids undergoing DNA excision repair synthesis *in vitro* (32). In yeast, mutations in the two larger subunits of CAF-1 show enhanced UV-sensitivity, supporting an involvement of CAF-1 in DNA repair *in vivo* (24). It appears that CAF-1 might be generally recruited to any DNA molecule undergoing DNA synthesis, be it DNA replication or repair.

These findings warrant an analysis of CAF-1 during the cell division cycle. If CAF-1 is to participate in DNA repair *in vivo*, we reasoned that it should also be present in an active form in the nonreplicative phases of the cell division cycle. We therefore analyzed the intracellular localization and nucleosome assembly activity of all human CAF-1 subunits during the cell cycle. CAF-1 is present throughout the entire cell cycle, and, during S phase, it is concentrated at sites of intranuclear DNA replication. Importantly, active human CAF-1 is not restricted to S phase nuclei and can be isolated in active form from G₁, S, and G₂ phase nuclei. CAF-1 predominantly dissociates from chromatin in mitosis and becomes inactive in nucleosome assembly. Hyperphosphorylation of the p60 subunit of CAF-1 during mitosis correlates with chromatin displacement and inactivation, suggesting that nucleosome assembly activity might be regulated by reversible phosphorylation.

EXPERIMENTAL PROCEDURES

Cell Culture and Synchronization—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 unit/ml penicillin (Sigma), and 0.1 mg/ml streptomycin (Sigma).

Synchronization of cells was performed as described (33). Cell cycle synchronization was monitored by flow cytometry of isolated interphase nuclei or of mitotic cells. Mitotic cells were fixed in 70% ethanol and washed in PBS before staining with propidium iodide (1 ml of solution of 5 μ g/ml propidium iodide in PBS containing 0.4% Triton X-100). Isolated interphase nuclei were stained without fixation. Samples were analyzed by FACScan (Becton Dickinson) using the Lysis II-software. Data are presented as histograms showing relative DNA content (*x* axis) and cell number (*y* axis).

Preparation of Cell-free Extracts—Cytosolic extracts of synchronized HeLa cells were prepared by hypotonic treatment of the cells, followed by Dounce homogenization and centrifugation at 20,000 $\times g$ as described previously (34). Cytosolic extracts contained about 9–11 μ g/ μ l protein, determined with the Bio-Rad protein assay using bovine serum albumin as a standard.

Nuclear extracts were prepared by resuspending pelleted nuclei from the preparation of cytosolic extracts in 20 mM K-HEPES (pH 7.8), 0.4 M NaCl, 5 mM potassium acetate, 0.5 mM MgCl₂, and 0.5 mM dithiothreitol at 1.5×10^6 nuclei/ml (33). Proteins bound to nuclei were extracted with constant agitation for 60 min at 4 °C. Residual nuclear material was pelleted for 30 min at 14,000 rpm in an Eppendorf 5415C centrifuge. The supernatant was freshly prepared for each experiment. For an extraction of chromosomal proteins from mitotic cells, condensed chromatin from nocodazole-arrested cells was used in place of interphase nuclei. These extracts contained 4–7 μ g/ μ l protein.

For fractionation by glycerol gradient centrifugation (34), 300–400 μ l (1.2–2 mg of protein) of extract were directly applied to a linear 5-ml glycerol gradient (15–30%) in gradient buffer (100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.01% Nonidet P40; 25 mM Tris-Cl, pH 7.5). Centrifugation was performed in a Beckman SW55 rotor at 45,000 rpm for 20 h at 4 °C. The gradient was fractionated and stored at –20 °C.

DNA Synthesis Reaction—Reactions were essentially performed as described (34), with the following modifications: standard reactions contained 30 mM NaCl, cytosolic extract (150 μ g of protein for G₁, G₂, and M phase and 200 μ g of protein for S phase extracts), the buffered nucleotide mixture, including an ATP-regenerating system and 25 ng of M13mp18-single-stranded DNA (34). Where indicated, 4–10 μ l (6 μ g of total protein) of glycerol gradient fractions were added before the nucleotide mixture. In control reactions, identical volumes of gradient buffer (15% glycerol) were used. Reactions were mixed on ice and started by transferring to 37 °C. Standard reaction time was 120 min.

For reactions monitoring DNA supercoiling in the absence of DNA synthesis, 50 ng of double-stranded M13mp18 DNA were used as substrate in the absence of radioactive dATP. Four identical reactions were

pooled to yield sufficient DNA for visualization by ethidium bromide.

Processing of the Reaction Products—Replication reactions were stopped by the addition of 50 μ l of 2 \times stop mixture (2% sarcosyl, 0.2% SDS, 20 mM EDTA) and extracted in phenol-chloroform. DNA was ethanol precipitated, dissolved in TE buffer, and loaded onto 0.75% agarose gels. Gel electrophoresis was performed at room temperature in 0.5 \times TBE buffer at 4 V/cm. DNA was visualized either by autoradiography of the dried gel or by ethidium bromide staining after RNaseA digestion, as indicated in the figure legends.

For micrococcal nuclease (MNase) digestions, the products of *in vitro* DNA synthesis reactions were treated with the indicated amount of MNase (Boehringer Mannheim): four identical assays were pooled, adjusted to 2 mM CaCl₂, subsequently digested with MNase at room temperature for 15 min, and processed as described (35).

Generation of Polyclonal Antibody pAb1, Directed against the Recombinant p60 Subunit of CAF-1—CAF-1 p60 encoding cDNA was isolated by reverse transcription of total HeLa RNA and PCR using primers matching the 5' and 3' coding region of the published sequence (25). The PCR product was cloned in the pET23a vector (Novagen). *Escherichia coli* C41(BL21) (36) was transformed with pETp60 and overexpressed p60 protein was extracted from inclusion bodies with 8 M urea. The protein was further purified to >90% homogeneity by Ni²⁺-NTA chromatography (Quiagen). Rabbits were immunized with the purified recombinant p60 to give anti-p60 antisera. Antibodies against p60 were affinity-purified from the antisera by column chromatography with a p60 resin, which was prepared by coupling purified recombinant p60 to Sulfolink Gel (Pierce). The antibodies were eluted from the resin with 100 mM glycine-HCl (pH 2.0). The resulting solution was neutralized with ¼ volume of 1 M Tris-HCl (pH 8.0) and then precipitated with ammonium sulfate. The pellet was resuspended in sodium phosphate buffer (pH 8.0) containing 10% glycerol.

Immunoprecipitations—Purified antibody was cross-linked to Sepharose A beads (Amersham Pharmacia Biotech) using dimethyl-pimelimidate as described (37). Precleared S phase nuclear extract (100 μ g of protein) was incubated with cross-linked antibodies for 1 h at 4 °C and washed 5 times with 25 mM Tris-Cl, pH 7.4, 135 mM NaCl, 3 mM KCl, 0.1% Nonidet P-40. Bound proteins were eluted with SDS-polyacrylamide gel electrophoresis sample buffer without β -mercaptoethanol.

SDS-Polyacrylamide Gel Electrophoresis, Western Blot Analysis, and Immunofluorescence—Protein samples were subjected to electrophoresis on denaturing 8% polyacrylamide gels (38). Proteins were transferred to polyvinylidene difluoride membranes (Immobilon P, Millipore) and analyzed by Western blotting, using rabbit polyclonal antibodies (pAb1) or mouse monoclonal antibodies (mAb60–53 and mAb1–150 (39); mAb11G10 (29)). The proteins were detected with ECL (Amersham Pharmacia Biotech) in conjunction with horseradish peroxidase-coupled secondary antibodies. Dephosphorylation reactions were carried out with λ phosphatase (New England Biolabs) according to the manufacturer's manual. 10 units of enzyme per μ l of the reaction were used.

Confocal Immunofluorescent Microscopy—The analysis of isolated HeLa cell nuclei by confocal laser scanning immunofluorescence microscopy was performed exactly as detailed previously (26, 33, 40). Primary antibodies used are specified in the figure legends. Secondary antibodies were fluorescein-conjugated anti-rabbit IgG and Texas Red-conjugated anti-mouse IgG (both Amersham Pharmacia Biotech).

RESULTS

Synchronization of HeLa Cells—HeLa cells were synchronized in the G₁, S, G₂, and M phases of the the somatic cell cycle. Flow cytometry of isolated G₁, S, and G₂ phase nuclei and of entire mitotic cells confirmed successful synchronization (Fig. 1). Preparations of S phase cells were enriched in early S phase, and preparations of G₂ phase cells contained up to 30% late S phase contaminants (Fig. 1) as consequences of the synchronization procedures. These synchronized cell populations were used as sources for preparation of cytosolic extracts and for the isolation of CAF-1.

Cytosolic Extracts from G₁, S, and G₂ Phase Cells Support Complementary DNA Strand Synthesis and Nucleosome Assembly by Human CAF-1—CAF-1 assembles nucleosomes on DNA templates undergoing DNA synthesis (15, 22, 25, 32, 34). As a prerequisite for studying DNA synthesis-dependent nucleosome assembly *in vitro*, we first asked whether cytosolic extracts from synchronized cells are able to support DNA syn-

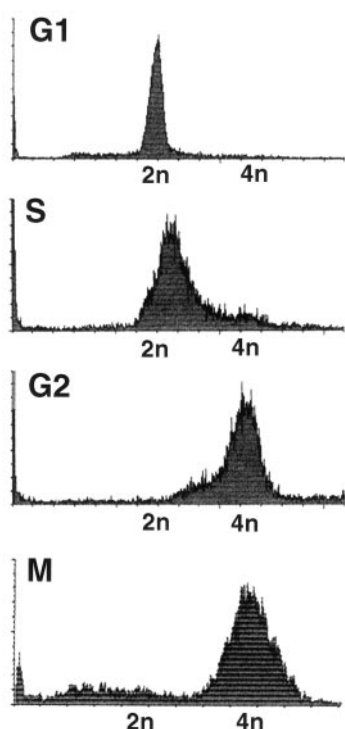


FIG. 1. **Synchronization of human cells.** HeLa cells were synchronized in all phases of the cell cycle as detailed under "Experimental Procedures." Isolated nuclei of G_1 , S, and G_2 phase cells and entire M phase cells were analyzed by flow cytometry. The nuclear DNA content of unreplicated ($2n$) G_1 phase and fully replicated ($4n$) G_2 /M phase cells is indicated.

thesis. Single-stranded circular DNA was used as template for complementary DNA strand synthesis in cytosolic extracts, supplemented with deoxy- and ribonucleoside triphosphates and radioactive dATP as a tracer (34). After deproteinization, replicated double-stranded DNA was detected by agarose gel electrophoresis and autoradiography. Double-stranded DNA reaction products comprise (i) fully replicated and covalently closed molecules migrating as relaxed or supercoiled topoisomers, (ii) relaxed open circular form II DNA products still containing single-stranded gaps, and (iii) linear form III DNA products due to a limited nucleolytic cleavage of the single-stranded templates during the reaction (34).

Cytosolic extracts from G_1 , S, and G_2 phases supported efficient complementary DNA strand synthesis, but mitotic extracts did not (Fig. 2A). In G_1 and S phase extracts, the covalently closed double-stranded DNA reaction products were predominantly relaxed or contained only a few supercoils. Surprisingly, in G_2 phase extract, these reaction products were predominantly supercoiled, suggesting nucleosome assembly during the reaction. Supercoiling during DNA synthesis in cytosolic extracts depends on DNA synthesis because double-stranded DNA templates incubated under identical conditions were efficiently relaxed by endogenous topoisomerases (Fig. 2B).

We next isolated human CAF-1 from proliferating cell nuclei and analyzed its nucleosome assembly activity during complementary DNA strand synthesis in cytosolic extracts from synchronized cells (Fig. 3). CAF-1 mediated efficient supercoiling of the covalently closed double-stranded circular DNA reaction products in G_1 , S, and G_2 phase extracts (Fig. 3A). Two-dimensional gel electrophoresis confirmed the formation of negative superhelices in these reactions, indicating an efficient nucleosome assembly mediated by CAF-1 (data not shown, but see Ref. 34). In the absence of DNA synthesis, double-stranded

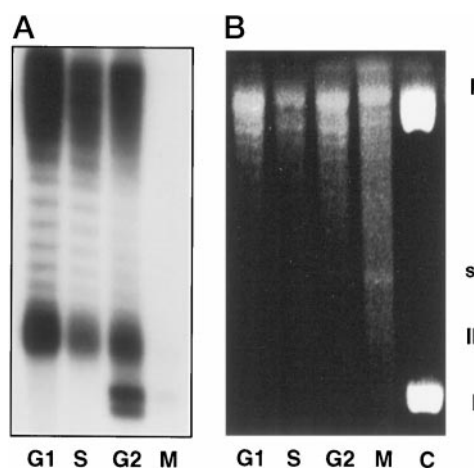


FIG. 2. **Complementary DNA strand synthesis and DNA supercoiling occurs in cytosolic extracts from G_1 , S, and G_2 phase cells.** A, complementary DNA strand synthesis and DNA supercoiling. Single-stranded circular M13mp DNA was incubated in cytosolic extracts from HeLa cells synchronized as indicated. Radiolabeled DNA reaction products were purified, separated on a 0.75% agarose gel, and visualized by autoradiography. B, lack of DNA supercoiling of double-stranded DNA in these extracts in the absence of DNA synthesis. Double-stranded M13mp18 DNA was incubated under conditions identical those in A. DNA reaction products were purified, analyzed on a parallel gel, and visualized by staining with ethidium bromide. The positions of DNA forms I (superhelical), II (relaxed or nicked circular), and III (unit length linear) and of single-stranded DNA (ss) are indicated. The control lane (C) contains superhelical and nicked circular double-stranded M13mp18 DNA.

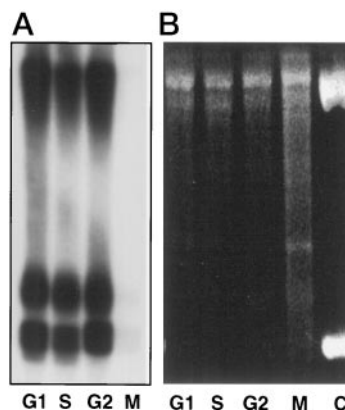


FIG. 3. **CAF-1 mediates nucleosome assembly during DNA synthesis in cytosolic extracts from G_1 , S, and G_2 phase cells.** A, CAF-1 was isolated from asynchronously proliferating cells and added to complementary DNA strand synthesis reactions using cytosolic extracts from synchronized cells. Reaction products were analyzed as detailed in the legend to Fig. 2A. B, lack of DNA supercoiling by CAF-1 in the absence of DNA synthesis in these reactions. Reaction products were analyzed as detailed in the legend to Fig. 2B.

DNA was relaxed and not supercoiled under otherwise identical conditions (Fig. 3B), indicating that nucleosome assembly mediated by CAF-1 depends on DNA synthesis in G_1 , S, and G_2 phase cytosolic extracts.

We next analyzed the assembled minichromosomes by MNase digestions (Fig. 4). MNase cleaves chromatin preferentially in the linker DNA between adjacent nucleosomes, resulting in nuclease-resistant DNA fragments of mono- and oligonucleosomal length. The nuclease cleavage pattern of minichromosomes confirmed nucleosome assembly by CAF-1 in G_1 , S, and G_2 phase cytosolic extracts (Fig. 4). We observed patterns of oligonucleosomes in G_1 and G_2 phase extracts that are similar to the patterns assembled by CAF-1 in extracts from asynchronously proliferating cells (15, 18, 34). However,

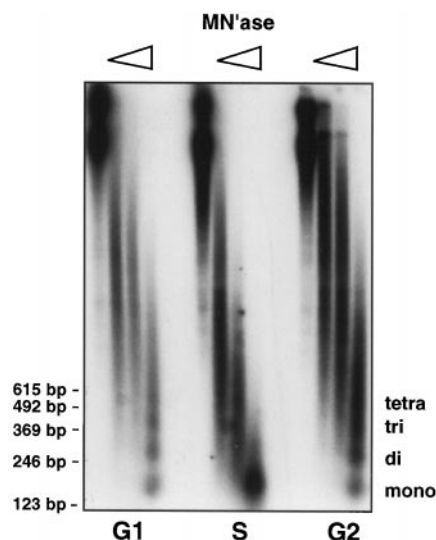


FIG. 4. MNase digestion of minichromosomes assembled by CAF-1 during complementary DNA strand synthesis reactions in cytosolic extracts from G_1 , S, and G_2 phase cells. Reactions were digested with 0, 2, 5, and 10 units of MNase and the nuclease-resistant DNA was purified, separated on a 1.5% DNA agarose gel, and visualized by autoradiography. Positions of DNA size markers are indicated on the left, and positions of mono- and oligomeric nucleosomal DNA fragments on the right are indicated on the right.

we were not able to resolve more than three or four nucleosomes (Fig. 4), indicating an assembly of arrays of irregularly spaced nucleosomes in these extracts. On minichromosomes assembled in S phase extract, we were only able to resolve mononucleosomes containing ~ 145 base pairs of DNA as reaction products (Fig. 4), indicating an assembly of irregularly or randomly spaced nucleosomes.

We conclude that CAF-1 supports an efficient assembly of nucleosomes in cytosolic extracts from G_1 , S, and G_2 phase cells in a DNA synthesis-dependent manner. Interestingly, neither the ability to support DNA synthesis nor the ability to allow CAF-1 mediated nucleosome assembly during DNA synthesis is restricted to S phase cytosolic extracts, but it occurs also in G_1 and G_2 phase extracts.

In the next set of experiments, we investigated whether active CAF-1 itself is restricted to S phase nuclei or whether it can also be detected in and isolated from cells synchronized in the other phases of the cell cycle.

Phosphorylated Forms of the p60 Subunit of Human CAF-1 Can Be Identified by a Polyclonal Antibody—To detect the p60 subunit of human CAF-1, we raised a polyclonal rabbit antiserum against the bacterially expressed product of a full-length cDNA (see under "Experimental Procedures") and purified monospecific anti-p60 antibodies, termed pAb1. In Western blots of HeLa cell nuclear extracts pAb1 detected multiple bands of p60 (Fig. 5, lane 2), whereas the monoclonal antibody mAb60–53 (39) detected only one major band (Fig. 5, lane 1). Purified human CAF-1 contains at least three p60 polypeptides (15), reminiscent of the bands detected by pAb1, and it is phosphorylated *in vivo* (39). We therefore tested whether the different forms of p60 are due to phosphorylation. Treatment of isolated CAF-1 by λ phosphatase, followed by Western blotting with pAb1, resulted in a single band of faster mobility (Fig. 5, lane 3). Inhibition of phosphatase by pyrophosphate resulted in the detection of the full set of p60 bands by pAb1 (Fig. 5, lane 4). We conclude that phosphorylated p60 can be detected by pAb1 as the slower migrating forms in Western blots.

CAF-1 Is Present during the Entire Cell Cycle—The p150 and p60 subunits of CAF-1 were originally shown by immunohisto-

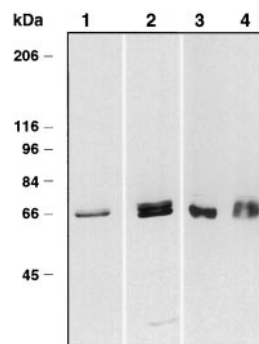


FIG. 5. Polyclonal antibody pAb1 detects differently phosphorylated isoforms of the CAF-1 subunit p60. Nuclear extracts of asynchronously proliferating HeLa cells were separated on 8% SDS-polyacrylamide gels, blotted and probed with control monoclonal antibody mAb60–53 (39) (lane 1) and with pAb1 (lanes 2–4). The hypophosphorylated isoform of p60 was identified by treatment of isolated CAF-1 (34) with λ phosphatase (lane 3). As the control, λ phosphatase was inhibited by the addition of 1 mM sodium pyrophosphate (lane 4).

chemistry to be nuclear proteins in asynchronously proliferating cells (39). When analyzed by confocal immunofluorescence microscopy using monoclonal antibodies, they co-localized with sites of DNA replication in isolated S phase nuclei (26). We therefore re-investigated the intranuclear localization of CAF-1 using polyclonal antibody pAb1.

Intranuclear sites of CAF-1 were first analyzed by confocal immunofluorescence microscopy (Fig. 6). The p60 subunit (Fig. 6A) directly co-localized with p150 (Fig. 6B), confirming that these two subunits are exclusively located at the same intranuclear sites. These sites further coincide with replication foci in S phase nuclei (Fig. 6, C and D). We did not detect any significant p150 and p60 signal above background fluorescence in G_1 or G_2 phase nuclei (data not shown; cf. Ref. 26), indicating that in proliferating cells CAF-1 is clearly detected by immunofluorescence microscopy only at replication foci in S phase nuclei. We did not detect a significant p48 signal in isolated HeLa nuclei by immunofluorescence microscopy with antibody mAb11G10 (data not shown).

We therefore analyzed the localization of all three subunits of CAF-1 during the cell cycle by fractionation of synchronized cells into extracts from interphase nuclei or mitotic chromatin and cytosol, followed by Western blotting (Fig. 7). The two large subunits p150 and p60 are clearly detected in nuclear extracts from G_1 and G_2 phase cells, in addition to S phase cells (Fig. 7A). Only subthreshold amounts of both subunits are detected in G_1 and S phase cytosolic extracts (Fig. 7B). However, in G_2 phase cytosolic extracts, small amounts of p150 and trace amounts of p60 can be detected (Fig. 7B and data not shown), possibly accounting for the increased supercoiling activity of these extracts during complementary DNA strand synthesis (cf. Fig. 2). In contrast, mitotic cells contain significant amounts of cytosolic p150 and p60 (Fig. 7B), but some p150 and p60 are also present in the extract from mitotic chromatin (Fig. 7A). The small subunit p48 was present in the cytosol during the entire cell cycle and in extracts from interphase nuclei and from mitotic chromatin. Therefore, all three subunits of CAF-1 are present throughout the entire cell cycle, and p150 and p60 were detected by immunofluorescence microscopy at the intranuclear sites of DNA replication during S phase.

Phosphorylation of p60 Changes at the G_1 -to-S Phase Transition and in Mitosis—The phosphorylation pattern of p60 changed during the cell cycle (Fig. 7). In G_1 phase, nuclear bound p60 migrated as a slower form corresponding to the upper and middle band in the p60 triplet and is therefore predominantly phosphorylated. In S and G_2 phase, some p60

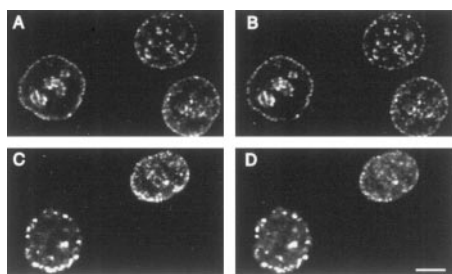


FIG. 6. **The p60 subunit of CAF-1 co-localizes with p150 and replication foci in S phase nuclei *in vivo*.** In isolated S phase nuclei, p60 was detected by pAb1 (A) and p150 by mAb150-1 (39) (B) using confocal immunofluorescence microscopy. HeLa cells were pulse-labeled with bromodeoxyuridine for 15 min, p60 was detected by pAb1 (C), and sites of DNA replication in the same S phase nuclei were visualized by anti-bromodeoxyuridine antibodies (D). Scale bar, 5 μ m.

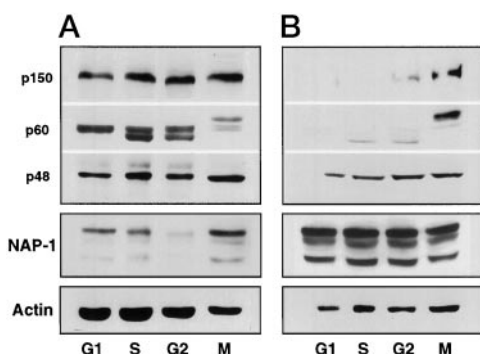


FIG. 7. **Intracellular distribution of the nucleosome assembly factors CAF-1 and NAP-1 during the cell division cycle.** A, extracts from G₁, S, and G₂ phase nuclei and from mitotic chromatin. B, cytosolic extracts from the same synchronized cells containing soluble nuclear and cytoplasmic proteins. Extracts were separated on 8% SDS-polyacrylamide gels, blotted, and sequentially probed with the CAF-1 specific antibodies mAb150-1 (specific for p150), pAb1 (p60), and mAb11G10 (specific for p48 (29)), the NAP-1 specific monoclonal antibody 4A8 (19), and, as a loading control, an actin-specific antibody (Sigma). A total mass of 40 μ g of protein was analyzed per lane.

also migrated as the faster form of hypophosphorylated p60 in addition to the slower migrating phosphorylated form. In mitosis, cytosolic and chromatin-bound p60 were detected as a doublet of even slower migrating bands than in interphase, consistent with a hyperphosphorylated state. Phosphatase-treatment of mitotic CAF-1 resulted in the fast migrating form (data not shown), confirming hyperphosphorylation of p60 in mitosis. This hyperphosphorylation coincides with a displacement of p60 into the cytosol (Fig. 7). In summary, reversible changes of p60 phosphorylation occur at key regulatory steps of the cell cycle, namely at the G₁ to S transition and during mitosis.

NAP-1 Is Predominantly Cytosolic during the Cell Cycle—We also analyzed the intracellular localization of the other human nucleosome assembly factor, NAP-1 (19) (Fig. 7). In *Drosophila*, NAP-1 was shown to be nuclear in S phase and to become cytoplasmic in G₂ phase (20). A similar behavior in human cells might be an explanation for the increased supercoiling activity during DNA synthesis observed in cytosolic G₂ phase extracts (*cf.* Fig. 2). However, in human cells, NAP-1 is predominantly cytosolic throughout the cell cycle (Fig. 7B), but small and variable amounts were also detected in nuclear extracts (Fig. 7A). Therefore, nucleosome assembly reactions using unfractionated cytosolic extracts from asynchronously proliferating or synchronized HeLa cells all contain NAP-1.

All Three Subunits of CAF-1 Are Complexed with Each Other, but p60 and p48 Also Exist in Different Complexes during the Cell Cycle—We next analyzed the composition of the

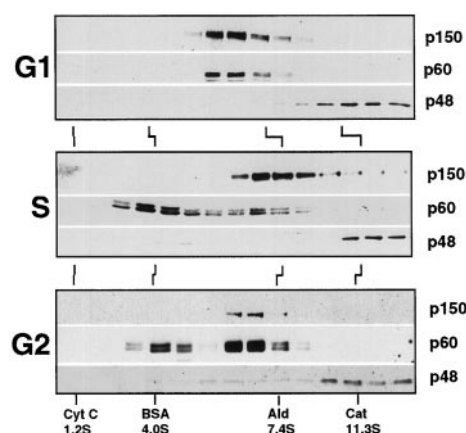


FIG. 8. **Sedimentation of CAF-1 subunits present in nuclear extracts from G₁, S, and G₂ phase.** Nuclear extracts were separated on linear 15–30% glycerol gradients and fractions were analyzed for the presence of the CAF-1 subunits by Western blotting as detailed in the legend to Fig. 7. The signal intensities of the different blots do not reflect the relative protein levels. Sedimentation of marker proteins were analyzed in parallel gradients, and their sedimentation coefficients are indicated at the bottom. Cyt C, cytochrome C; BSA, bovine serum albumin; Ald, aldolase; Cat, catalase.

multisubunit complexes of CAF-1 isolated from nuclear extracts of cells synchronized at all stages of the cell cycle. The individual subunits were detected by Western blotting of fractionated glycerol gradients (Fig. 8).

In G₁ phase, the two large subunits, p150 and p60, were exclusively complexed with each other and sedimented at about 6.5 S. In contrast, the majority of p48 did not co-sediment with p150/p60 but sedimented at about 12 S at the bottom of the gradient. Overexposure of the blot showed that only minor amounts of p48 co-sedimented with p150 and p60 (data not shown). Similar sedimentation properties were observed for CAF-1 prepared from asynchronously proliferating cells (12, 15, 34).

In S and in G₂ phases, nuclear CAF-1 complexes had different properties (Fig. 8). The sedimentation properties of the p150 and p48 subunits appear to be the same as in G₁ phase. Overexposure confirmed the presence of small amounts of p48 sedimenting at 6.5 S (data not shown). However, only a fraction of p60 co-sedimented with the other subunits at 6.5 S, whereas the remainder now sedimented near the top of the gradient at about 3 S. This subpopulation of p60 sediments at a position consistent with monomeric p60 and is not detectably complexed to either p150 or p48. The existence of these two subpopulations was confirmed by gel filtration (data not shown). To test whether co-sedimentation of the three subunits reflects complex formation with each other, we immunoprecipitated p60 from an S phase nuclear extract using pAb1 (Fig. 9) and analyzed the presence of all three subunits by Western blotting using monoclonal antibodies. All three subunits were immunoprecipitated by pAb1 (Fig. 9, lane 3), confirming that p60 is complexed with p150 and with at least a fraction of p48 in S phase.

The 6.5 S Complex of CAF-1 Prepared from G₁, S, and G₂ Phase Cells Supports Nucleosome Assembly—We next characterized the nucleosome assembly activities during complementary DNA strand synthesis of the isolated glycerol gradient fractions (Fig. 10). The 3 S form of monomeric p60 found in S and G₂ phase (Fig. 10, lanes 1) and the 12 S complex containing p48 (Fig. 10, lanes 4) did not support significant nucleosome assembly. In contrast, the 6.5 S fractions prepared from G₁, S, and G₂ phase cells supported efficient nucleosome assembly in this system (Fig. 10, lanes 3). In fact, when all fractions of the gradients were analyzed, the peaks of nucleosome assembly

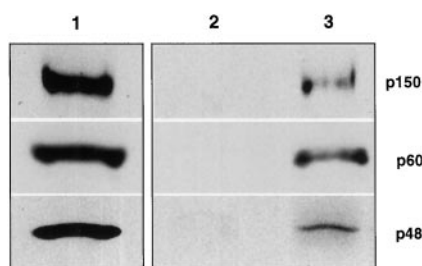


FIG. 9. All three subunits of CAF-1 immunoprecipitate with anti-p60 antibodies. CAF-1 was immunoprecipitated with pAb1 from an S phase nuclear extract as specified under "Experimental Procedures." The input nuclear extract (lane 1) and immunoprecipitations using a control polyclonal antibody against human MCM5 protein (lane 2) and pAb1 (lane 3) were analyzed by Western blotting. p150 was detected by mAb150-1, p60 by mAb60-53, and p48 by mAb11G10.

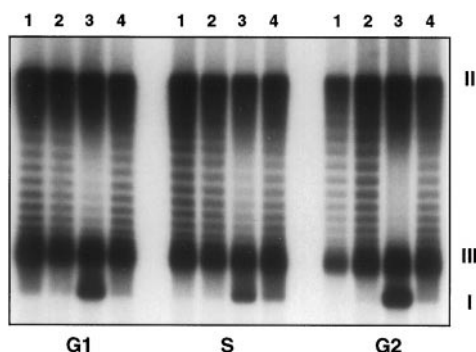


FIG. 10. Nucleosome assembly activity co-sediments with the complexed subunits of CAF-1 but not with the uncomplexed individual subunits in G_1 , S, and G_2 phase nuclear extracts. Nucleosome assembly activity was analyzed in complementary DNA strand synthesis reactions supplemented with gradient buffer (lanes 1), or with the 4 S fractions (lanes 2), the 6.5 S fractions (lanes 3) or the 12 S fractions (lanes 4) of the glycerol gradients shown in Fig. 8. DNA synthesis reactions were carried out in G_1 phase cytosolic extracts, and the products were visualized by gel electrophoresis and autoradiography.

activity overlapped with the peaks of p150 and p60 proteins seen in Fig. 10 (data not shown).

We conclude that active CAF-1 can be isolated as a 6.5 S complex from G_1 , S, and G_2 phase nuclei, and the nucleosome assembly activity of this complex does not correlate with a partial hypophosphorylation of p60 and the occurrence of the 3 S form of p60 in S and G_2 phase.

CAF-1 from Mitotic Cells Does Not Support Efficient Nucleosome Assembly—In mitosis, all three subunits exist predominantly in a cytosolic form, but some CAF-1 is still bound to chromatin (Fig. 7). Finally, we analyzed the sedimentation properties and nucleosome assembly activity during complementary DNA strand synthesis of these forms (Fig. 11). In the chromatin-bound form of mitotic CAF-1, p150 co-sediments with the majority of p60 at 6.5 S (Fig. 11A), but a small proportion of p60 also sediments at 3 S, as seen in S and G_2 phase (cf. Fig. 8). The majority of p48 sediments at about 3 S, apparently overlapping with the small subpopulation of monomeric p60 (Fig. 11A). As shown in Fig. 7, mitotic p60 is hyperphosphorylated, and it was seen as a dimer of slow migrating hyperphosphorylated bands in the complex with p150 (Fig. 11A). A very similar sedimentation pattern was detected for the cytosolic form of mitotic CAF-1 (Fig. 11A).

We detected only inefficient nucleosome assembly activity of the 6.5 S form of chromatin-bound mitotic CAF-1 and, importantly, no nucleosome assembly activity of cytosolic CAF-1 at all (Fig. 11B).

We conclude that CAF-1 becomes inactivated in mitosis and

that this inactivation correlates with a hyperphosphorylation of p60 and a displacement of p60 and p150 from chromatin.

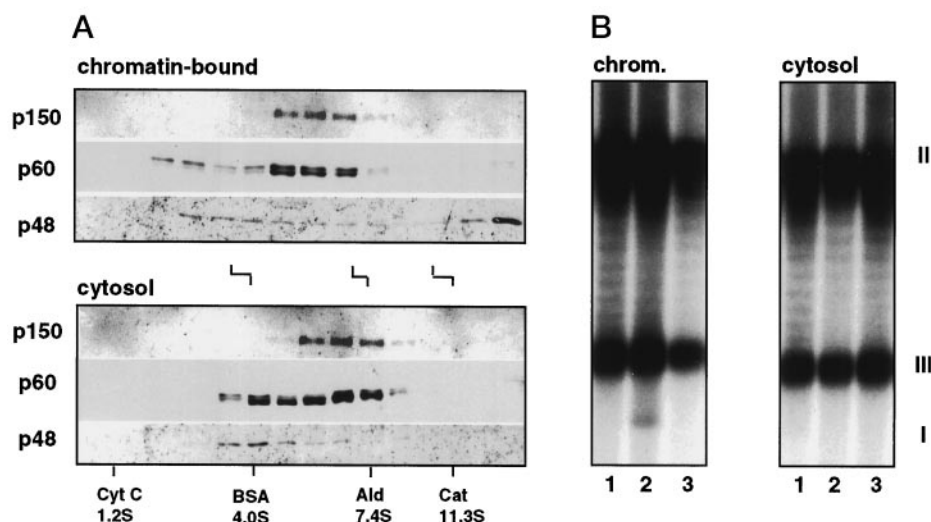
DISCUSSION

In this study, we described changes in intracellular localization and DNA synthesis-dependent nucleosome assembly activity of human CAF-1 during the somatic cell division cycle. CAF-1 protein is present during the entire cell cycle, but active CAF-1 can be isolated only from G_1 , S, and G_2 phase nuclei, and not from cells in mitosis. Inactivation in mitosis coincides with hyperphosphorylation of the p60 subunit and a displacement from chromatin. These data have implications for the involvement of CAF-1 in both DNA replication and DNA repair.

As a prerequisite for nucleosome assembly during DNA synthesis, we first tested cytosolic extracts from different stages of the cell cycle for supporting complementary DNA strand synthesis. Interestingly, all interphase extracts supported efficient DNA synthesis. This is different from the requirements for replication of double-stranded SV40 DNA in extracts from synchronized human cells. SV40 replication is restricted to S phase cytosol or to G_1 phase cytosol supplemented with cyclin A/Cdc2 or cyclinB/Cdc2 protein kinases (41). Our data indicate that replication proteins required for priming, elongating, and ligating complementary DNA strands are present and active throughout interphase and are not inactivated in G_1 and G_2 phases. The capability of G_1 and G_2 phase extracts to synthesize complementary DNA strands and to mediate nucleosome assembly by CAF-1 may indicate involvement of replication and chromatin assembly proteins in DNA synthesis other than S phase-specific DNA replication. In fact, this is consistent with the enzymology of DNA excision repair synthesis, which, unlike nuclear DNA replication, is not restricted to S phase. In human cell-free DNA repair systems, the gap-filling DNA synthesis observed after lesion recognition and excision is carried out by enzymes also involved in DNA replication (42). We reason that the ability of G_1 and G_2 phase cytosolic extracts to carry out complementary DNA strand synthesis and mediate nucleosome assembly by CAF-1 could reflect the potential of a cell for DNA excision repair synthesis outside S phase. Consistent with this scenario, a role for human CAF-1 in nucleosome assembly during DNA repair *in vitro* has been described (32); furthermore, yeast mutants lacking CAF-1 show enhanced UV sensitivity (24, 27).

Even though histone synthesis is induced during S phase, a background synthesis of basal histone variants occurs outside S phase (10). Efficient nucleosome assembly in G_1 and G_2 phase could therefore be achieved by using these basal histone variants by human CAF-1, as found in extracts from asynchronously proliferating cells (18). However, we noted a minor difference between S phase and G_1 or G_2 phase cytosolic extracts, because nucleosomes assembled by CAF-1 in S phase cytosol were irregularly spaced, and the linkers were more accessible to nuclease than those assembled in G_1 or G_2 phase cytosol. Control experiments using increased concentrations of DNA templates demonstrated that histones were not limiting for an assembly of the DNA templates undergoing DNA synthesis into minichromosomes under standard conditions (data not shown). It is therefore possible that factors involved in postreplicative chromatin spacing and remodeling such as human homologues of *Drosophila* CHRAC (43) or ACF (44) are present in lower concentrations in S phase cytosol than in G_1 or G_2 , accounting for the comparatively lower degree of nucleosome spacing. In any case, nucleosomes assembled during complementary DNA strand synthesis in human cell extracts were generally less regularly spaced than on minichromosomes assembled in *Xenopus* or *Drosophila* extracts on double-stranded DNA in the absence of replication (2, 6, 43, 44, 45), possibly

FIG. 11. Chromatin-bound and cytosolic CAF-1 isolated from mitotic cells is inactivated in nucleosome assembly during complementary DNA strand synthesis. A, extracts from isolated mitotic chromosomes and mitotic cytosol were analyzed for the presence of CAF-1 subunits by glycerol gradient centrifugation and Western blotting as described in the legend to Fig. 8. B, nucleosome assembly of mitotic CAF-1 was analyzed in complementary DNA strand synthesis reactions supplemented with the 4 S fractions (lanes 1), the 6.5 S fractions (lanes 2), or the 12 S fractions (lanes 3) of the glycerol gradients shown in A. DNA synthesis reactions were carried out in G₁ phase cytosolic extracts, and the products were visualized by gel electrophoresis and autoradiography.



reflecting the different mechanisms of assembly in these systems. However, full nucleosomes protecting ~145 base pairs of DNA were assembled in human G₁, S, and G₂ phase extracts, consistent with the presence of NAP-1 in these extracts. NAP-1 allows efficient deposition of histones H2A and H2B (20) to the precursor particle containing H3 and H4 assembled during DNA synthesis by CAF-1 (18).

Active CAF-1 was isolated by salt extraction not only from S phase nuclei but also from G₁ and G₂ phase nuclei, and inactive CAF-1 is present in mitosis. The amino acid sequences of the p150 and p60 subunits of human CAF-1 (25) contain PEST box motifs thought to be involved in cell cycle specific degradation of proteins (46, 47). As CAF-1 was detected by immunofluorescence microscopy only in S phase nuclei (26), a cell cycle-specific degradation of CAF-1 following S phase could be considered as an attractive possibility to regulate replication-dependent nucleosome assembly during the cell cycle. The data presented here are not consistent with this scenario but provide evidence that CAF-1 is not regulated during the cell cycle by complete degradation and *de novo* synthesis. However, we cannot exclude the possibility of a concomitant degradation of old CAF-1 and a synthesis of new CAF-1.

A small proportion of the p48 subunit of CAF-1 is associated with the active 6.5 S form of CAF-1 during interphase, whereas the vast majority is present in other complexes of higher molecular weight. Similar sedimentation has been reported for CAF-1 isolated from nuclei of asynchronously proliferating cells (12). It is most likely that other p48-containing protein complexes, such as transcriptional co-activators and repressors containing histone acetylases and deacetylases (14, 23, 30, 48, 49) are also present in the high molecular weight fractions. In fact, we detected human HDAC1 and Sin3 polypeptides in these fractions co-sedimenting with p48 (data not shown).

We found posttranslational modifications of CAF-1 at key transitions of the cell cycle. At the G₁ to S transition, a fraction of the p60 subunit of CAF-1 becomes hypophosphorylated, and this coincides with the occurrence of an apparently monomeric form of p60 in addition to the complexed form. The physiological relevance of this free p60 is unclear at the moment because it does not contain nucleosome assembly activity on its own. It could be due to newly synthesized p60 not yet complexed to p150, or it could reflect a different binding affinity of p60 to the other subunits in S phase, resulting in a partial dissociation upon salt extraction. This form persists through G₂ until mitosis, but we cannot rule out the possibility that a reversion of p60 to a phosphorylated and a fully complexed form as in G₁ phase occurs in G₂ phase, because preparations of G₂ phase

nuclei contain up to 30% of late S phase contaminants as an unavoidable consequence of the synchronization procedure (*cf.* Fig. 1). In any case, this hypophosphorylation of p60 does not correlate with an apparent change in nucleosome assembly activity, because CAF-1 containing either phosphorylated p60 in G₁, S, and G₂ phase or hypophosphorylated p60 in S and G₂ phase efficiently supports nucleosome assembly. This conclusion was confirmed by using λ phosphatase-treated CAF-1 in complementary DNA strand synthesis without observing an apparent loss of nucleosome assembly (data not shown). However, hypophosphorylated p60 in S phase coincides with a change in subnuclear localization, resulting in a concentration at replication foci. Because the G₂ phase preparations contain up to 30% of late S phase contaminants, we cannot exclude the interesting possibility that hypophosphorylation of p60 is specific for S phase, possibly reflecting a recruitment of CAF-1 to replication foci.

In mitosis, the p60 subunit becomes hyperphosphorylated, and a significant fraction of CAF-1 is detached from condensed chromatin. This cytosolic CAF-1 from mitotic cells does not support nucleosome assembly during complementary DNA strand synthesis. It is therefore possible that nucleosome assembly by CAF-1 is negatively regulated by mitosis-specific hyperphosphorylation. The amino acid sequence of p60 (25) reveals several phosphorylation consensus motifs for cyclin-dependent protein kinases, providing candidate sites for such reversible phosphorylation by high cyclin-dependent protein kinase levels in mitosis. Analysis of p60 mutants lacking phosphorylation sites could elucidate roles for cyclin-dependent protein kinase phosphorylation of CAF-1 in inactivation of nucleosome assembly activity and in regulating mitotic displacement. A precedent for phosphorylation-accompanied displacement from chromatin into a soluble form and inactivation has been described for the minichromosome maintenance (MCM) replication protein complex in S phase (40, 50–53). Hypophosphorylated MCMs bind to chromatin in late G₁ phase as part of the pathway licensing chromatin to initiate DNA replication, and they are displaced from their sites during DNA replication in S phase. Soluble MCMs are hyperphosphorylated and do not rebind to chromatin, reflecting an inactivation of their licensing activity.

In conclusion, recruiting the nucleosome assembly activity of CAF-1 to sites of DNA synthesis in the G₁, S, and G₂ phases allows maintenance of chromatin organization during repair and replication of genomic DNA in the cell cycle.

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