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Human CAF-1-dependent nucleosome assembly in a defined system

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Abbreviations: ASF1, anti-silencing function 1; CAF-1, chromatin assembly factor 1; PCNA, proliferating cell nuclear antigen; RFC, replication factor C; NAP1L1, nucleosome assembly protein 1L1; ccDNA, covalently closed DNA; scDNA, supercoiled DNA; ssDNA, single-stranded DNA; DTT, dithiothreitol

Replication-coupled nucleosome assembly is a critical step in packaging newly synthesized DNA into chromatin. Previous studies have defined the importance of the histone chaperones CAF-1 and ASF1A, the replicative clamp PCNA, and the clamp loader RFC for the assembly of nucleosomes during DNA replication. Despite significant progress in the field, replication-coupled nucleosome assembly is not well understood. One of the complications in elucidating the mechanisms of replication-coupled nucleosome assembly is the lack of a defined system that faithfully recapitulates this important biological process in vitro. We describe here a defined system that assembles nucleosomal arrays in a manner dependent on the presence of CAF-1, ASF1A-H3-H4, H2A-H2B, PCNA, RFC, NAP1L1, ATP, and strand breaks. The loss of CAF-1 p48 subunit causes a strong defect in packaging DNA into nucleosomes by this system. We also show that the defined system forms nucleosomes on nascent DNA synthesized by the replicative polymerase δ. Thus, the developed system reproduces several key features of replication-coupled nucleosome assembly.

Introduction

Chromatin is a dynamic and complex DNA–protein structure that tightly controls access of diverse factors to the packaged DNA. The key unit of chromatin is the nucleosome, which is comprised of a histone octamer wrapped around with a 146 bp DNA in 1.65 left-handed superhelical turns.[1,2] A canonical histone octamer contains 2 copies each of H2A, H2B, H3, and H4. Nucleosomes are assembled and disassembled during diverse nuclear processes that include transcription, replication, and repair. Assembly and disassembly of nucleosomes are promoted by histone chaperones, a specialized class of proteins that bind histones with high affinity.[3,4] The bulk of nucleosome assembly takes place during DNA replication and transcription.[3-7] The assembly of a nucleosome consists of the loading of an $(H3\text{-}H4)_2$ tetramer that is followed by the addition of 2 H2A-H2B dimers. Histone chaperones play important roles in deposition of both $(H3\text{-}H4)_2$ tetramers and H2A-H2B dimers. The current evidence indicates that histone chaperones facilitate nucleosome assembly by transporting histones from cytoplasm to the sites of their deposition in the nucleus and by suppressing non-productive interactions between the core histones and DNA.[8]

Eukaryotic DNA replication is tightly coordinated with the disassembly of nucleosomes in front of advancing replication forks, histone synthesis and transport, and rapid packaging of the nascent strands into nucleosomes.[9] Replication-coupled nucleosome assembly has the complicated task of generating approximately 30 million nucleosomes per replication per human cell. Several conserved proteins have been implicated in the assembly of nucleosomes during DNA replication.[3,4] The major factor that orchestrates replication-coupled nucleosome assembly is the heterotrimeric CAF-1.[10,11] Human CAF-1 is an essential histone H3-H4 chaperone composed of p150, p60, and p48 subunits.[12-14] An siRNA-directed depletion of the p150 subunit slows down DNA replication and results in an accumulation of HeLa cells in early and mid S phase.[11] Silencing of the p60 subunit triggers apoptosis in proliferating cells.[15] The CAF-1 p60 subunit is often overexpressed in different cancers, and its overexpression is associated with poor clinical outcome.[16] Unlike human CAF-1, yeast Caf1 is not an essential protein.[17] Nevertheless, deletion of any of the 3 Caf1 genes (*CAC1*, *CAC2*, or *CAC3*) increases the rate of gross chromosomal rearrangements.[18] Yeast mutants lacking Caf1 display longer Okazaki fragments, suggesting that this histone chaperone plays a role in lagging-strand synthesis.[19] In addition, the lack of Caf1 sensitizes the yeast cells to UV irradiation.[17] CAF-1 directly interacts with the PCNA clamp.[20,21]

PCNA is an essential homotrimeric protein required for numerous DNA replication and repair transactions.[22,23] PCNA is loaded on the 3′ ends of the primer/template junctions by the

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clamp loader RFC in a reaction requiring the energy of ATP hydrolysis.[24,25] RFC can also load PCNA on a DNA lacking a strand break, but the efficiency of this reaction is much lower compared with that observed in the reactions with a nicked or gapped DNA.[26] Because strand breaks (nicks and gaps) are present at replication forks, they are believed to be the natural substrates for loading PCNA by RFC. During DNA replication, the loaded PCNA increases the processivity of replicative DNA polymerases.[22] This function of PCNA is required for DNA replication and the survival of eukaryotic cells. The PCNA-CAF-1 interaction couples nucleosome assembly to DNA replication. Consistent with the role of the loaded PCNA in replication-coupled nucleosome assembly, we have recently shown that RFC is required for CAF-1- and PCNA-dependent histone H3-H4 deposition in a defined system.[27]

Human cells contain 3 histone H3 variants, referred to as H3 or H3.1, H3.2, and H3.3. Of these, H3 is the major histone variant. H3 and H3.2 are replication-dependent variants that are synthesized and incorporated into chromatin in S phase.[28,29] The histone variant H3.3 is produced throughout the cell cycle and replaces H3 and H3.2 during transcription, marking actively transcribed genes.[29,30] A mass-spectrometric analysis of histone deposition complexes isolated from human cells indicates that CAF-1 forms a complex with H3-H4, whereas the transcription-coupled histone chaperone HIRA associates with H3.3-H4.[31]

ASF1 histone chaperones assist the action of CAF-1 at replication forks.[32,33] ASF1 proteins form heterotrimeric complexes with newly synthesized H3-H4 dimers in cytoplasm and transport them to the sites of nucleosome assembly at the nucleus. Furthermore, the histone chaperones of the ASF1 family bind parental H3-H4 dimers generating the heterotrimeric complexes.[34,35] ASF1-H3-H4 complex was originally purified from *Drosophila* embryo extract as a factor that stimulated in vitro SV40 replication-coupled nucleosome assembly at a low concentration of CAF-1.[32] Human cells have 2 isoforms of ASF1, ASF1A and ASF1B.[33] Amino acid sequences of human ASF1A and ASF1B are 71% identical to each other, with the majority of the sequence differences being within their C-terminal parts.[33] Both ASF1A and ASF1B are involved in replication-coupled chromatin assembly.[36] The expression of ASF1B, but not ASF1A, drops down about 5-fold upon the exit of cells from the cell cycle,[37] suggesting that the action of ASF1B-H3-H4 in replication-coupled assembly may be different from that of ASF1A-H3-H4. Depletion of ASF1 proteins in human, chicken, and fly cells results in accumulation of the cells in S phase and decreased DNA replication, eventually leading to cell death.[38-40] The globular domain of Asf1 has a fold found in immunoglobulins.[41] In the crystal structures of Asf1-H3-H4 and ASF1A-H3-H4 complexes, the histone chaperone has extensive contacts with the core of the H3-H4 dimer in a way that physically blocks the formation of $(H3\text{-}H4)_2$ tetramer.[35,42]

Mammalian cells possess 5 members of NAP1 family histone chaperones.[43] Of these 5, 3 are exclusively expressed in the nervous system, and the other 2 referred to as NAP1L1 and NAP1L4 are ubiquitous. NAP1 histone chaperones bind to both H2A-H2B and H3-H4 histones. The mechanism of nucleosome assembly by yeast Nap1 is well understood.[8] The major function of Nap1 in the assembly of nucleosomes in vitro is to suppress nonspecific contacts between H2A-H2B dimer and DNA. Consistently, yeast strains lacking *NAP1* accumulate histone H2A-H2B-DNA complexes that deregulate gene transcription.[8] It remains unclear whether H2A-H2B-DNA complexes detected in *nap1*Δ strains are produced during DNA replication, transcription, or both processes. A support for a role of NAP1 in DNA replication comes from the observations that in *Drosophila* embryos, NAP1 is localized to nuclei in S phase and in the cytoplasm in G_2.[44] Analysis of *S. pombe nap1* cells further corroborates the idea that NAP1 has a role in DNA replication.[45] Because DNA replication requires the presence of ongoing nucleosome assembly, these observations suggest that NAP1 might participate in replication-coupled nucleosome assembly.

Previous studies have mainly investigated replication-coupled nucleosome assembly in vivo and in cell-free extracts in vitro.[3,4,6,7] Replication-coupled nucleosome assembly has not been demonstrated in a defined system. Here, we describe a defined human system that assembles nucleosomal arrays in a CAF-1-, ASF1A-H3-H4-, H2A-H2B-, PCNA-, RFC-, NAP1L1-, ATP-, and strand break-dependent manner. Therefore, our results indicate that the presence of CAF-1, ASF1A, PCNA, RFC, and NAP1L1 is sufficient to drive the nick-dependent formation of nucleosomes in a defined system.

Results

Purification of human ASF1A-H3-H4

We initiated this work with the aim of developing a defined human system that would recapitulate some aspects of replication-coupled chromatin assembly. Human ASF1A-H3-H4 plays a key role in this process.[3,4] A previous study was able to isolate a fragment of human ASF1A, lacking the last 31 amino acid residues, in the complex with *X. laevis* histone H3-H4 dimer.[35] However, the complex consisting of full-sized human ASF1A and H3-H4 dimer has not yet been isolated. In order to prepare full-sized human ASF1A-H3-H4, we constructed a plasmid that permits co-expression of the 3 proteins in *E. coli*. The presence of soluble ASF1A, H3, and H4 in the *E. coli* crude extracts was confirmed by western blot analyses with the specific antibodies (data not shown). Next, we purified the recombinant human ASF1A-H3-H4. The final preparation that we obtained contained 3 proteins that run in SDS gels with mobilities expected of human ASF1A (23.0 kDa), H3 (15.4 kDa), and H4 (11.4 kDa) (**Fig. 1A**). Furthermore, antibodies against human ASF1A/ASF1B recognized the top band, whereas antibodies against human histones H3 and H4 recognized the middle and bottom bands, respectively (**Fig. 1B**). Based on these results, we concluded that we prepared a recombinant human ASF1A-H3-H4 in a highly purified form.

Purified yeast Caf1 and Asf1-H3-H4 interact with one another in solution.[46] Furthermore, human ASF1A and ASF1B pull down CAF-1 from cell-free extracts.[36] Nevertheless, an interaction between human CAF-1 and ASF1A-H3-H4 has not been established. We analyzed whether the recombinant ASF1A-H3-H4

(**Fig. 1A**) interacts with CAF-1 using pull-down assays. Nearly homogeneous FLAG-tagged CAF-1 was tethered to α-FLAG beads and unbound CAF-1 was removed by washing. The beads were then incubated with the purified ASF1A-H3-H4. At the end of incubation, the beads were extensively washed, and the bound proteins were eluted and analyzed by western blots with the α-ASF1 and α-H3 antibodies. We found that the eluted fraction contained both ASF1A and histone H3 (**Fig. 1C**). These results established that the recombinant human ASF1A-H3-H4 interacts with human CAF-1.

High concentrations of *Drosophila* ASF1-H3-H4 and yeast Asf1-H3-H4, but not Asf1, promote packaging of relaxed ccDNA into nucleosomes in reactions containing human cell-free extracts.[32,47] This activity is consistent with the role of Asf1 in replication-independent chromatin assembly.[48] In such a reaction, the formation of one nucleosome produces approximately one negative supercoil in the substrate ccDNA. To determine if the purified ASF1A-H3-H4 has a similar activity, we analyzed this protein in the supercoiling assays. We found that the addition of the purified ASF1A-H3-H4 to HeLa cytosolic extracts stimulates negative supercoiling of the substrate DNA (**Fig. 1D**). These results established that the recombinant human ASF1A-H3-H4 is able to promote nucleosome assembly occurring on a relaxed ccDNA in cytosolic extracts.

Assembly of DNA into nucleosomes in defined reactions

Strands breaks (nicks and gaps) are natural intermediates of leading- and lagging-strand syntheses at replication forks and are necessary for loading PCNA by RFC in vitro.[25,26] Having purified ASF1A-H3-H4, we sought to define a minimal set of human factors that would be proficient in packaging DNA into nucleosomes in a strand break-dependent manner. In these experiments, we utilized a DNA substrate that contained 12 strand breaks distributed evenly in the complementary strand of phage MR59 DNA (**Fig. 2A and B**). (Analysis of this DNA by ligation with T4 DNA ligase showed that at least 60% of the strand breaks are nicks [data not shown].) The formation of the characteristic mono- and di-nucleosomal DNA fragments that resisted micrococcal nuclease cleavage was used to score assembly of nucleosomes in this series of experiments (**Figs. 2–7**). Previous studies have implicated ASF1A-H3-H4, CAF-1, PCNA, and RFC in replication-coupled nucleosome assembly.[10,13,20,27,32] We analyzed whether the substrate DNA could be assembled into nucleosomes in the presence of nearly homogeneous ASF1A-H3-H4, H2A-H2B, CAF-1, PCNA, and RFC (**Fig. 2C**). We observed that the majority of products in these 5-protein reactions were non-nucleosomal complexes that were apparently generated between the core histones and DNA (**Fig. 2C**, lane 7). Yeast Nap1 eliminates non-nucleosomal DNA-histone complexes in vitro and in vivo.[8] Furthermore, NAP1 may have a role in replication-coupled nucleosome assembly in vivo.[8,44,45] Based on this, we studied if human NAP1, NAP1L1, is proficient in promoting nucleosome assembly in the presence of ASF1A-H3-H4, H2A-H2B, CAF-1, PCNA, and RFC. Consistent with the previous study,[8] we found that NAP1L1 suppressed the formation of the non-nucleosomal complexes and supported nucleosome assembly in the presence of the other 5 proteins (**Fig. 2C**, compare DNA products in lanes 2 and 7). Quantification of the data demonstrated that nucleosome assembly in this reaction displayed only a very modest dependence on RFC or PCNA (**Fig. 2C**). Nevertheless, our results showed that the observed nucleosome assembly depends on the major replicative histone chaperone CAF-1 (**Fig. 2C and D**).

Replication-coupled nucleosome assembly in cell-free extracts and in vivo requires the presence of loaded PCNA.[20,21] To determine whether the dependence of nucleosome assembly on PCNA and RFC in a defined system might be increased, we performed the 6-protein reactions in the presence of competitor DNA. Under these conditions, the omission of PCNA or RFC decreased nucleosome assembly by 3- and 5-fold, respectively (**Fig. 3A and B**). Thus, nucleosome assembly in the 6-protein reaction that included competitor DNA largely depends on

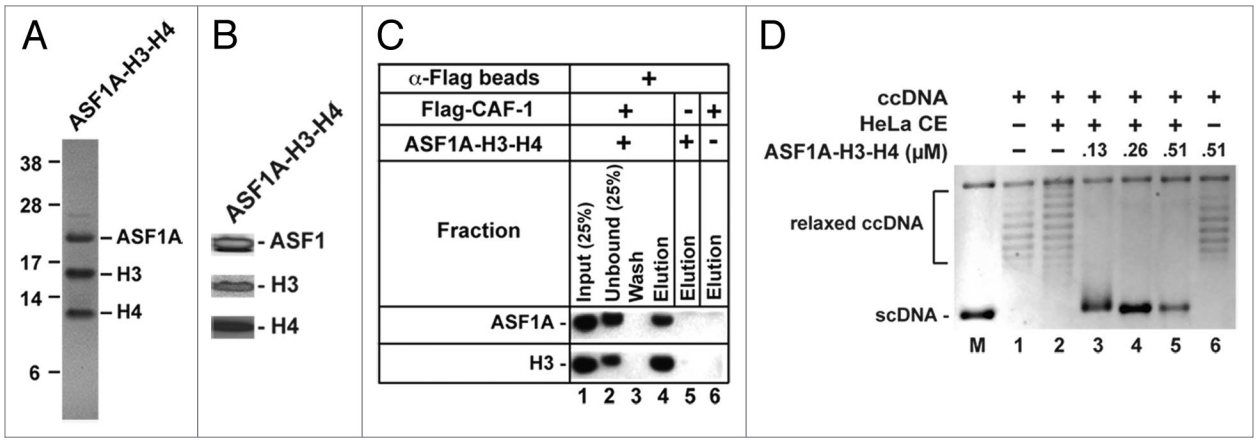


Figure 1. Isolation of the recombinant human ASF1A-H3-H4 complex. ASF1A-H3-H4 was purified from *E. coil* cells and analyzed as described in "Materials and Methods". (**A**) Human ASF1A-H3-H4 (2.6 μg) obtained at the final purification step was separated in an SDS gel and visualized by staining with Coomassie brilliant blue R-250. (**B**) The purified ASF1A-H3-H4 separated in an SDS gel was transferred on a PVDF membrane and probed with antibodies against human ASF1A/ASF1B (top panel), H3 (middle panel), or H4 (bottom panel). (**C**) Detection of interactions between purified human ASF1A-H3-H4 and CAF-1. (**D**) Involvement of the purified ASF1A-H3-H4 complex in packaging of relaxed MR59 ccDNA into nucleosomes in HeLa cytosolic extracts (HeLa CE). The reactions were performed in the presence of the indicated components. M, negatively supercoiled DNA of MR59 was used as the standard.

PCNA and RFC. Next, we carried out a detailed analysis of the 6-protein nucleosome assembly reaction occurring in the presence of competitor DNA (**Fig. 3A and B**). We found that the circular DNA with 12 strand breaks supported about 4 times more nucleosome assembly than relaxed ccDNA (**Fig. 3B**). The circular DNA with a single-strand break was assembled into nucleosomes less efficiently than the DNA with 12 strand breaks (**Fig. 3B**). As expected, no nucleosome assembly was detected when ASF1A-H3-H4 or H2A-H2B was omitted from the defined reaction (**Fig. 3A and B**). Importantly, the lack of the replicative histone chaperone CAF-1 caused a ~19-fold decrease in nucleosome assembly (**Fig. 3A and B**). Wild-type CAF-1 present at the standard concentration of 15 nM in the 6-protein reaction supported about 6 times more nucleosome assembly than 15 nM CAF-1Δp48 (**Fig. 3B**). The assembly of nucleosomes observed in the presence of 50 nM CAF-1Δp48 was about 40% that in the reaction containing wild-type CAF-1. These results indicated that the p48 subunit is necessary for the action of CAF-1 in the nick-directed formation of nucleosomes. The omission of ATP, required for PCNA loading by RFC, strongly impaired the formation of nucleosomes by ~11-fold (**Fig. 3B**). The lack of both RFC and PCNA decreased nucleosome assembly by 6-fold (**Fig. 3B**). NAP1L1 stimulated nucleosome assembly 2-fold (**Fig. 3A and B**). Interestingly, the omission of NAP1L1 in the reactions containing competitor DNA (**Fig. 3A**, lane 7) did not cause the formation of non-nucleosomal complexes seen in the reactions lacking competitor DNA (**Fig. 2C**, lane 7). This result suggested that competitor DNA, like NAP1L1, is able to prevent the formation of non-specific complexes between the core histone and the substrate DNA. Together, these results defined a minimal human system that assembles nucleosomes in a CAF-1-, ASF1A-H3-H4-, H2A-H2B-, PCNA-, RFC-, NAP1L1-, ATP-, and strand break-dependent manner.

To further characterize the defined nucleosome assembly system, we titrated the substrate DNA, H2A-H2B, ASF1A-H3-H4, PCNA, NAP1L1, CAF-1, and RFC into reactions containing the other components at the standard concentrations (**Fig. 4A–G**). We found that increasing concentration of the 12 strand break-containing DNA substrate from 0.08 nM to 1.2 nM enhanced nucleosome assembly in a nearly linear manner (**Fig. 4A**). Furthermore, the results of our titration experiments (**Fig. 4A–G**) indicated that each of the 6 nucleosome assembly

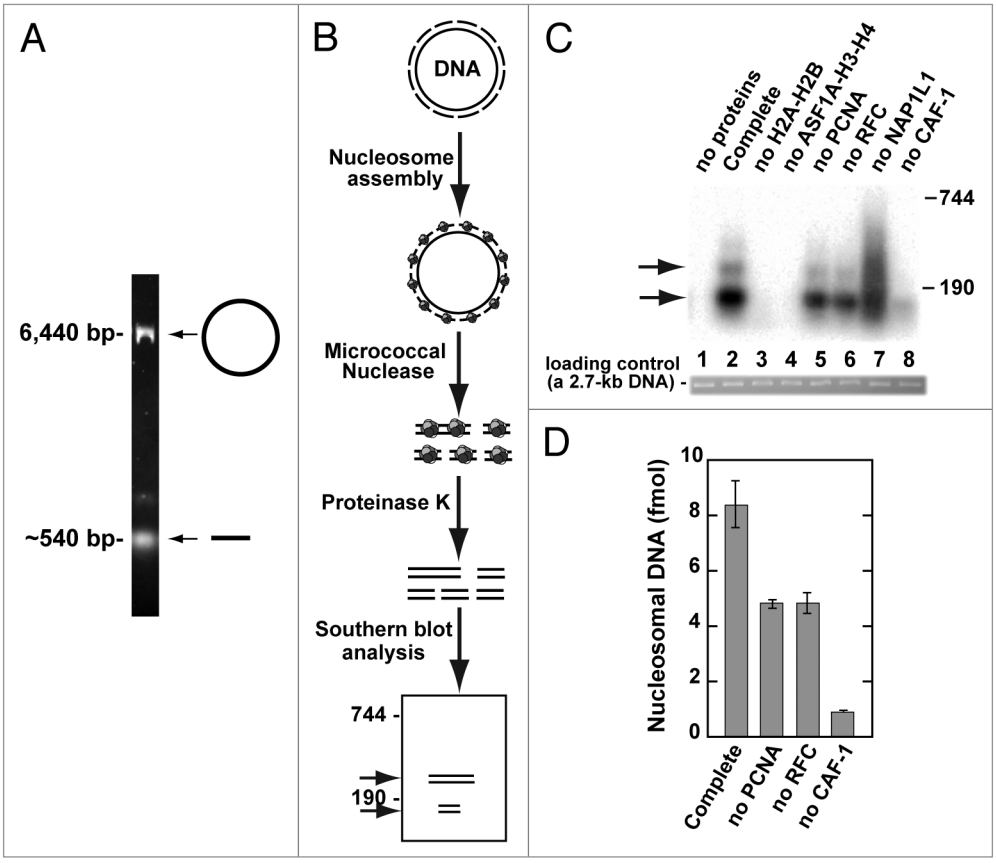


Figure 2. Assembly of nucleosomes in defined reactions lacking competitor DNA. (**A**) Circular DNA with 12-strand breaks used as the substrate in the nucleosome assembly reactions shown in **Figures 2–6**. The DNA substrate was obtained as described in "Materials and Methods". After the final purification step, DNA was separated in a denaturing agarose gel in 40 mM NaOH and 2 mM EDTA and visualized by staining with ethidium bromide. The positions of the circular and discontinuous strands are indicated. (**B**) Outline of the defined nucleosome assembly reactions and their analysis. (**C and D**) The complete nucleosome assembly reactions contained CAF-1, ASF1A-H3-H4, H2A-H2B, PCNA, RFC, NAP1L1, ATP, and the circular DNA with 12 strand breaks. The indicated reactions were performed and analyzed as described in "Materials and Methods", except that the reactions lacked competitor DNA and were incubated for 4 min. The arrows in (**C**) show locations of mono- and di-nucleosomal DNAs. The size of mononucleosomal DNAs is ~160–170 bp. The data in (**D**) were obtained by quantification of images including the one shown in (**C**) and are the means ± 1 SD, n = 4.

factors was present at a concentration that did not limit nucleosome assembly in the defined system. During replication the nucleosome assembly machinery packages newly synthesized DNA into nucleosomal arrays. We tested whether the defined system could form nucleosomal arrays. As shown in **Figure 4H** (lanes 1 and 2), the limited cleavage of the products of the 6-protein reactions with micrococcal nuclease revealed the presence of tetranucleosomal arrays. Thus, the defined nucleosome assembly system is proficient in the formation of nucleosomal arrays.

Histone chaperone FACT plays a critical role in transcription elongation.[49] Furthermore, yeast strains containing mutations in the *POB3* gene of FACT (Pob3-Spt16 heterodimer) progress slowly through S phase, indicating that this histone chaperone is required for normal DNA replication.[50] However, the function of FACT at replication forks remains unknown. It has been suggested that FACT may function as a histone H2A-H2B chaperone at replication forks.[4] Therefore we analyzed whether human FACT (SSRP1-SPT16 heterodimer) might promote nucleosome assembly in the defined reactions containing no NAP1L1. The results of our omission and titration experiments suggested that FACT weakly stimulates nucleosome assembly in the reactions that lacked NAP1L1 (**Fig. 5A–C**). We also analyzed nucleosome assembly in defined reactions that contained both NAP1L1 and FACT. The results of these experiments suggested that FACT did not have a significant effect on nucleosome assembly reactions containing NAP1L1 (**Fig. 5D**). Collectively, our observations supported a conclusion that FACT promotes the defined nucleosome assembly by CAF-1, ASF1A-H3-H4, H2AB-H2B, PCNA, and RFC, but the effect of FACT is weaker than that of NAP1L1.

Next, we compared the strand break-dependent nucleosome assembly occurring in the presence of CAF-1, ASF1A-H3-H4, H2AB-H2B, PCNA, RFC, and NAP1L1 (**Fig. 3A**) with that in HeLa cytosolic extracts. As expected from previous studies,[27,51] nucleosomes in HeLa cytosolic extracts were assembled onto the DNA with 12 strand breaks in a CAF-1-dependent manner (**Fig. 6A and B**). Furthermore, we found that the 12 strand break-containing DNA was assembled into nucleosomes in the extract with about 6 times higher efficiency than the relaxed ccDNA (**Fig. 6B**). We than compared the rates of nucleosome assembly in the minimal and extract systems. To enable efficient nucleosome assembly in the HeLa cytosolic extract, we used 220 μg of the extract and supplemented it with purified CAF-1 and H2A-H2B.[10,52] As shown in **Figure 6B**, nucleosomes were rapidly formed in both systems, but the rate of nucleosome assembly in the defined reaction was almost 2 times slower than that in the cytosolic reaction. The measurements of concentrations of the proteins in the cytosolic extracts by semi-quantitative western blots indicated that the amounts of several proteins in the defined reactions were higher compared with those in the cytosolic reactions (**Fig. 6C**). These results suggested that the 2-fold difference between the rates of nucleosome assembly in the defined and cytosolic extract reactions is probably due to the absence of one or several factors in the former reaction.

Replication-coupled nucleosome assembly packages the nascent duplexes into nucleosomes behind replication forks.[3,4] One of the principal DNA polymerases involved in human nuclear replication is the 4-subunit Pol δ.[53] We investigated whether the minimal system could package nascent DNA synthesized by

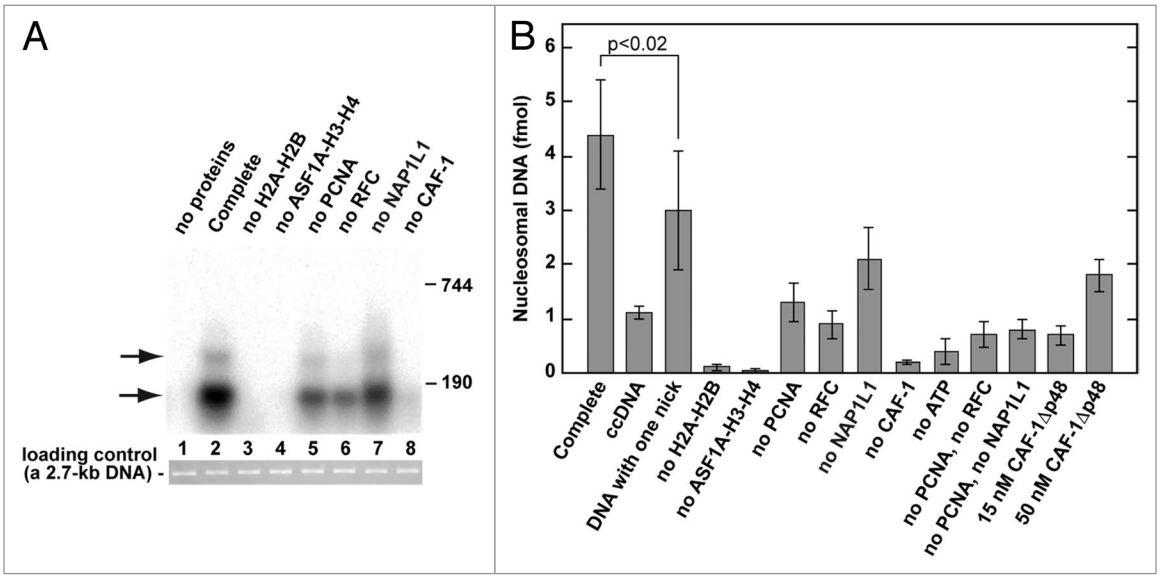


Figure 3. Assembly of nucleosomes in defined reactions containing human histone chaperones and replication factors. The defined reactions that package DNA into nucleosomes were performed and analyzed as detailed in "Materials and Methods" and described below. The complete reactions contained CAF-1, ASF1A-H3-H4, H2A-H2B, PCNA, RFC, NAP1L1, ATP, the circular DNA with 12 strand breaks, and competitor DNA. (**A and B**) Effects of the indicated omissions or replacements on the yield of nucleosomal DNA in the defined reactions. The bars labeled ccDNA and DNA with one nick indicate the amounts of nucleosomal DNA produced in the complete reactions containing relaxed MR59 ccDNA and MR59 DNA with a single nick, respectively, instead of the substrate MR59 DNA with 12-strand breaks. The bars labeled 15 nM CAF-1Δp48 and 50 nM CAF-1Δp48 in (**B**) show the amounts of nucleosomal DNA formed in the complete reactions in which 15 nM CAF-1 was replaced with 15 nM CAF-1Δp48 and 50 nM CAF-1Δp48, respectively. The data presented in (**B**) were obtained by quantification of images including the one shown in (**A**) and represent the means ± 1 SD, n ≥ 4.

human Pol δ. A 12-primed 6.4 kb circular ssDNA was used as the substrate in these experiments (**Fig. 7**). Previous studies determined that in addition to the four dNTPs the polymerase activity of Pol δ depends on PCNA, RFC, and the ssDNA-binding protein RPA,[54-56] Therefore, we supplemented the minimal 6-protein system, already containing PCNA and RFC, with Pol δ, RPA, and the 4 dNTPs. Consistent with the results of the previous studies,[54-56] we observed that DNA synthesis in this system required the presence of the 4-subunit DNA polymerase δ, RFC, PCNA, and the 4 dNTPs, and was strongly stimulated by RPA (**Fig. 7A and B**). Histone H2A-H2B dimer weakly stimulated DNA synthesis, while ASF1A-H3-H4, CAF-1, and NAP1L1 had no effect on the synthesis of DNA in the 8-protein system. Importantly, the nascent DNA was packaged into nucleosomes under these conditions (**Fig. 7C and D**). The observed nucleosome assembly required the presence of CAF-1, ASF1A-H3-H4, and H2A-H2B and was strongly stimulated by NAP1L1 (**Fig. 7C and D**). We concluded that the minimal system consisting of CAF-1, ASF1A-H3-H4, H2A-H2B, RFC, PCNA, and NAP1L1 is proficient in the assembly of nucleosomes onto nascent DNA synthesized by a replicative DNA polymerase.

Discussion

The discovery that chromatin assembly is coupled to DNA replication and the development of the in vitro assay that permits monitoring chromatin assembly during SV40 replication[57] led to the identification of CAF-1, the loaded PCNA, and ASF1-H3-H4 as the key components of replication-coupled chromatin assembly.[10,13,14,20,32] Because RFC loads PCNA onto DNA,[24-26] the clamp loader should be necessary for replication-coupled chromatin assembly. Consistent with this assumption, we recently found that RFC is required for CAF-1- and PCNA-dependent histone H3-H4 deposition onto a nicked DNA in vitro.[27] Furthermore, indirect evidence supports the view that NAP1 and FACT may participate in replication-coupled chromatin assembly.[44,45,50] Despite the significant progress in uncovering the key and candidate players in replication-coupled chromatin assembly, our

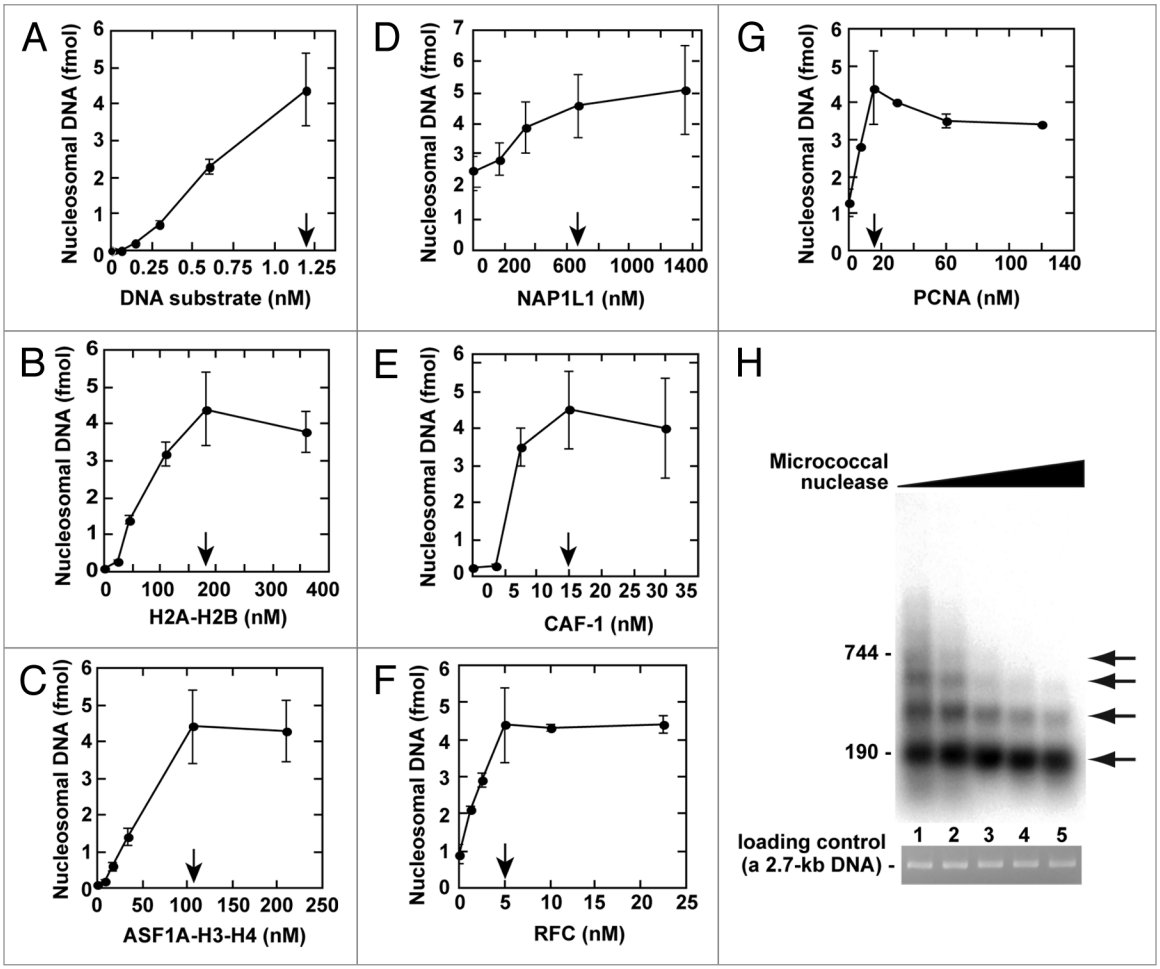


Figure 4. Characterization of nucleosome assembly in a defined system. The indicated reactions were performed and analyzed as described in **Figure 3**. (**A**–**G**) Nucleosome assembly as a function of concentration of the circular DNA with 12-strand breaks (**A**), H2A-H2B (**B**), ASF1A-H3-H4 (**C**), NAP1L1 (**D**), CAF-1 (**E**), RFC (**F**), and PCNA (**G**). The data were obtained by quantifying the products of nucleosome assembly reactions in which concentration of the indicated component varied as shown, while all the other components were present at standard concentrations indicated by the arrows. (**H**) Identification of nucleosomal arrays in the 6-protein reactions. The products of the 6-protein reactions were cleaved with 1 U (lane 1), 2 U (lane 2), 5 U (lane 3), 10 U (lane 4), or 20 U of micrococcal nuclease. The arrows indicate the positions of mono-, di-, tri-, and tetra-nucleosomal fragments.

understanding of the mechanisms of this process remains rather limited.

Analysis of a molecular process in defined systems provides an important approach for both elucidating the mechanisms of the process and complementing the results of the genetic and cell biology studies. In this work, we took advantage of the results of the previous studies to investigate whether nearly homogenous human CAF-1, ASF1A-H3-H4, H2A-H2B, PCNA, RFC, NAP1L1, and FACT are sufficient to form nucleosomes on DNA carrying strand breaks. We show here that a circular DNA containing 12 strand breaks is assembled into nucleosomal arrays in a reaction dependent on the presence of CAF-1, ASF1A-H3-H4, H2-H2B, PCNA, RFC, NAP1L1, ATP, and strand breaks (**Figs. 3 and 4**). In contrast, a circular DNA lacking a strand break is much less efficient in supporting nucleosome assembly (**Fig. 3B**). Because a circular DNA is a poor substrate for PCNA loading by RFC,[26] the latter observation (**Fig. 3B**) highlights the importance of the loaded PCNA for nucleosome assembly in the defined system. Collectively, our results support the view that CAF-1, ASF1A, PCNA, and RFC are the core components of replication-coupled chromatin assembly.[10,13,14,20,27,32]

Studies of human replication-coupled chromatin assembly in the past were hampered by the unavailability of purified full-sized human ASF1A(B)-H3-H4.[58] We developed a procedure that yields a recombinant human ASF1A-H3-H4 in a highly purified form (**Fig. 1A**). The recombinant protein was active in supporting the assembly of nucleosomal arrays onto both relaxed ccDNA in HeLa cytosolic extract and the circular DNA with 12-strand breaks in the defined reactions (**Figs. 1D and 4H**). We anticipate that this protein will be instrumental for understanding the mechanisms of replication-coupled chromatin assembly in human system.

In the 6-protein system, NAP1L1 prevents the non-productive interactions between DNA and the core histones (**Fig. 2C**, lane 7). This activity of NAP1L1 preserves both DNA and the histones for their incorporation into nucleosomes and probably is the basis of the ability of the protein to stimulate the defined nucleosome assembly. Our finding that NAP1L1 promotes the nick-dependent nucleosome assembly is in accord with the previous studies that suggested that NAP1 might have a role in replication-coupled chromatin assembly.[44,45] Human cells contain 2 ubiquitous NAP1 homologs, NAP1L1 and NAP1L4,[43] that display 68% identity to each other. Given the level of identity, it is likely that NAP1L4 can act as NAP1L1 in the defined reaction. We also found that FACT was able to replace NAP1L1 in the defined reactions, though the effect of FACT is weaker than that of NAP1L1 (**Fig. 5D**). It is possible that our defined system misses one or more factors that make FACT act more effectively in the strand break-dependent nucleosome assembly. Together, our in vitro results support the view that NAP1L1, NAP1L4, and FACT may participate in replication-coupled chromatin assembly in vivo.[4,44,45,50] However, in vivo evidence that the human NAP1 proteins and FACT are involved in replication-coupled nucleosome assembly is still missing.

Based on the results described previously and here, we envision that nucleosome assembly occurring in the defined 6-protein system proceeds via several distinct steps. Initially, the interactions between ASF1A-H3-H4 and CAF-1 produce CAF-1-$(H3\text{-}H4)_2$ complex.[59,60] Concurrently, RFC loads PCNA

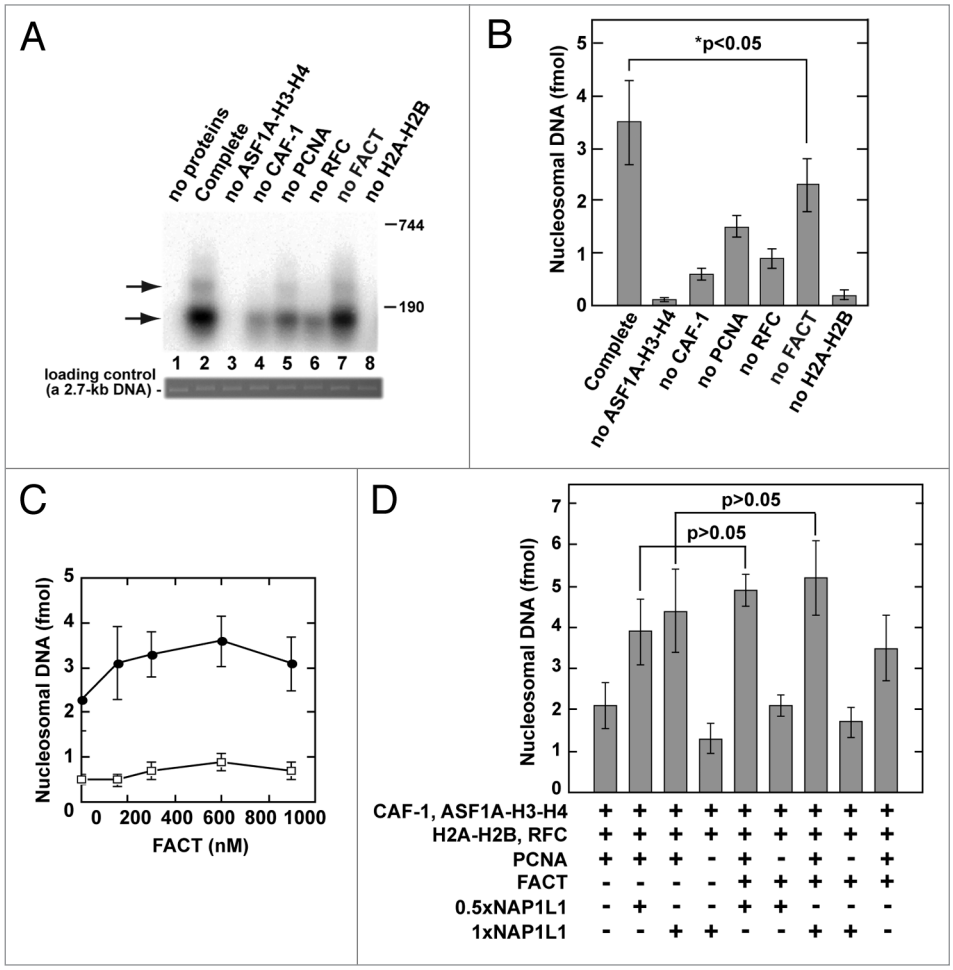


Figure 5. Analysis of nucleosome assembly in defined reactions containing the histone chaperone FACT. (**A and B**) The indicated nucleosome assembly reactions were carried and analyzed as described in **Figure 3**, but NAP1L1 was replaced with 450 nM FACT. The complete reactions contained CAF-1, ASF1A-H3-H4, H2A-H2B, PCNA, RFC, FACT, ATP, the circular DNA carrying 12 strand breaks and competitor DNA. (**C**) Nucleosome assembly as a function of FACT concentration. The nucleosome assembly reactions containing (●) or lacking (□) PCNA and RFC were performed as detailed above, but the concentration of FACT differed as shown. (**D**) Nucleosome assembly in defined reactions containing both NAP1L1 and FACT.

onto a nick or a gap in an ATP- and strand break-dependent manner[61] and NAP1L1 prevents H2A-H2B dimer from forming a non-productive complex with DNA.[8] Next, the loaded PCNA tethers CAF-1-$(H3\text{-}H4)_2$ complex to DNA.[20,21] The tethered CAF-1 then deposits H3-H4 tetramer onto DNA.[12,27] Finally, 2 H2A-H2B dimers join the deposited tetramer, forming a nucleosome.

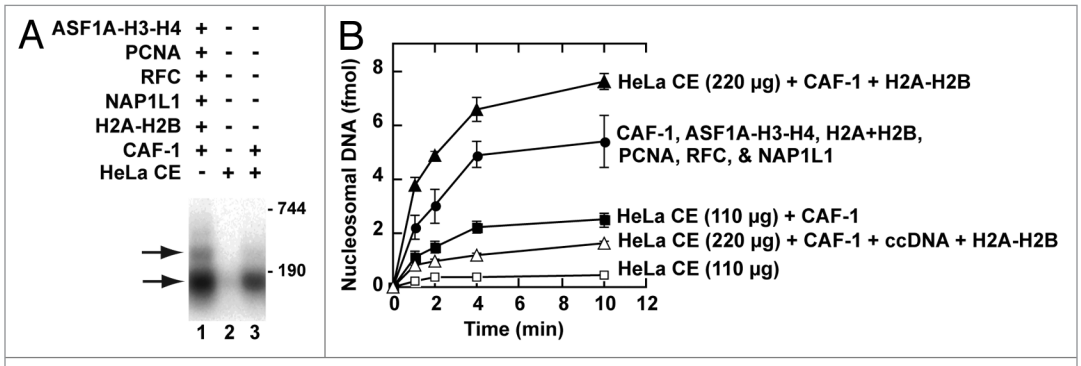
Replication-coupled nucleosome assembly rapidly packages nascent DNA into nucleosomes.[9,62] During DNA replication, human Pol δ is involved in lagging-strand synthesis.[63,64] We have demonstrated that DNA synthesized by the Pol δ holoenzyme is assembled into nucleosomes in the defined 8-protein system (**Fig. 7**). Thus, the presence of nucleosome assembly factors does not inhibit the activity of Pol δ holoenzyme. The substrate that we used in these experiments resembles the template for the lagging-strand synthesis (**Fig. 7A**). A recent work has demonstrated that *S. cerevisiae* replication-coupled nucleosome assembly in vivo is intrinsically coupled to the lagging-strand synthesis producing nucleosomes that carry at their midpoints the 5′ and 3′ ends of Okazaki fragments.[19] It will be interesting to determine whether our defined system can package DNA, such that its strand breaks are at the midpoints of nucleosomes.

Despite the fact that concentrations of several protein factors in the defined system are higher than those in the HeLa cytosolic extract system (**Fig. 6C**), the rate of the strand break-dependent nucleosome assembly in the former was almost twice lower than that in the latter (**Fig. 6B**). The simplest interpretation of these results is that the defined system lacks one or several factors that participate in strand break-dependent nucleosome assembly in the cytosolic extracts. Notably, our defined system does not contain any ATP-dependent chromatin remodeler. Recent studies have demonstrated that chromatin remodelers Isw2, Ino80, and ACF1-ISWI are involved in DNA replication.[65-67] Therefore, it is possible that these chromatin remodelers play roles in assembly of nucleosomes during DNA replication. Isw2, Ino80, and ACF1-ISWI may contribute to replication-coupled nucleosome assembly by moving the newly assembled nucleosomes to correct positions.[44] A recent in vitro study suggested that a nonnucleosomal complex is an intermediate in nucleosome assembly, and that these intermediates are converted into nucleosomal arrays by a chromatin remodeling enzyme.[68] Though our defined system lacks a chromatin remodeler and is proficient in the formation of nucleosomal arrays (**Figs. 3 and 4**), it is possible that the presence of a chromatin remodeler will promote nucleosome assembly in the defined system.

Materials and Methods

Cell extracts, recombinant proteins, DNA substrates, and oligonucleotides

HeLa S3 cells were cultured in suspension and HeLa cytosolic extracts were prepared according to the standard procedures.[27,69]



C

Protein	Amount in the defined reaction, fmol	Amount in the reaction with HeLa CE (220 μg), fmol
CAF-1	600	600
ASF1A/ASF1B	4200	800
H3-H4	4200	2400
H2A-H2B	7200	7200 or 880*
NAP1L1	27000	1200
FACT	0	1100
RFC	200	90
PCNA	600	900

Figure 6. Comparison of assembly of nucleosomes in defined and cytosolic-extract reactions. (**A** and **B**) The indicated nucleosome assembly reactions were performed and analyzed as described in "Materials and Methods" and below. The composition of the complete reaction is the same as in **Figure 3**. (**A**) Visualization of nucleosomal DNAs accumulated in the complete reaction (lane 1) and in the reactions containing 110 μg HeLa CE (lane 2) or 110 μg HeLa CE supplemented with 15 nM CAF-1 (lane 3) in 10 min of the incubation. (**B**) Time course of the formation of nucleosomes in the complete reaction (●) and in the HeLa CE reactions that included 220 μg of the extract supplemented with purified CAF-1 and H2A-H2B (▲), 110 μg of the extract supplemented with purified CAF-1 (■), 220 μg of the extract supplemented with purified CAF-1 and H2A-H2B and the relaxed ccDNA instead of the circular DNA with 12-strand breaks (△), or 110 μg of the extract (□). (**C**) Amounts of the nucleosome assembly proteins present in the defined reaction and the reaction containing 220 μg HeLa CE supplemented with 15 nM purified CAF-1. *, amounts of H2A-H2B present in the reactions supplemented or not with purified H2A-H2B. The amounts of the proteins in the HeLa CE were determined in western blots using the corresponding purified proteins as standards and antibodies described in "Materials and Methods".

Recombinant human DNA polymerase δ, PCNA, RFC, RPA, CAF-1, CAF-1Δp48, NAP1L1, FACT, and HeLa histone H2A-H2B were obtained in nearly homogenous forms according to previously described procedures.[27,49,70,71] In order to obtain recombinant human ASF1A-H3-H4 complex, we constructed a plasmid, a derivative of pET-11a, containing the histone *H3* and *H4* ORFs and the *ASF1A* ORF fused in frame at its 3′ end with the his_6-encoding sequence. In this plasmid, pET11a-ASF1A-His_6-H3-H4, transcription of each of the 3 human genes is under control of a separate T7 promoter, *lac* operator, and T7 terminator. The presence of the desired sequences in the constructed plasmid was verified by DNA sequencing. To express ASF1A-H3-H4 complex, pET11a-ASF1A-His_6-H3-H4 plasmid was transformed into *E.coli* BLR(DE3) competent cells. The fresh transformants were immediately inoculated into a 4.5 L media (12 g/l of Bacto-Tryptone, 42 g/l of yeast extract, 0.4% [v/v] glycerol, 50 mM K-phosphate, pH 7.2, and 0.5% casamino acid) and grown at 37 °C until OD_{600} = 0.7. At that point, the temperature was switched to 15 °C, and IPTG was added to the final concentration of 0.5 mM. After a 16 h incubation at the indicated conditions, the cells were harvested and lysed by sonication in the presence of lysozyme. The supernatant was clarified by centrifugation, and the recombinant ASF1A-H3-H4 complex was purified by chromatography on Ni-beads (Affimetrix). The His_6-tagged protein eluted from the Ni-beads was then purified by chromatography on a 1 ml MonoS column. Nearly homogenous ASF1A-H3-H4 (**Fig. 1A**) eluted from the MonoS column at 700 mM KCl. The peak fractions were pooled, aliquoted, frozen in liquid N_2, and stored at –80 °C.

The circular DNA carrying a single-strand break and ccDNA were made as previously described.[72] To prepare a 12-primed circular ssDNA, 12 20-nt oligonucleotides were annealed to the 6.44 kb phage MR59 ssDNA[73] producing a circular DNA that has 12 primers equally separated by ~517 nt gaps. To obtain the circular DNA with 12 strand breaks, the complementary strand of the 12-primed DNA was synthesized by T4 DNA polymerase (gp43) in the presence of the T4 clamp loader (gp44/62), the T4 replication clamp (gp45), the 4 dNTPs, and ATP. After phenol/chloroform extraction and ethanol precipitation, the reaction products were treated with the lambda exonuclease (NEB) to remove linear dsDNA contaminants. The DNA molecules carrying 12 strand breaks were separated from ssDNA impurities by chromatography on BND-cellulose (Sigma) as previously described.[74]

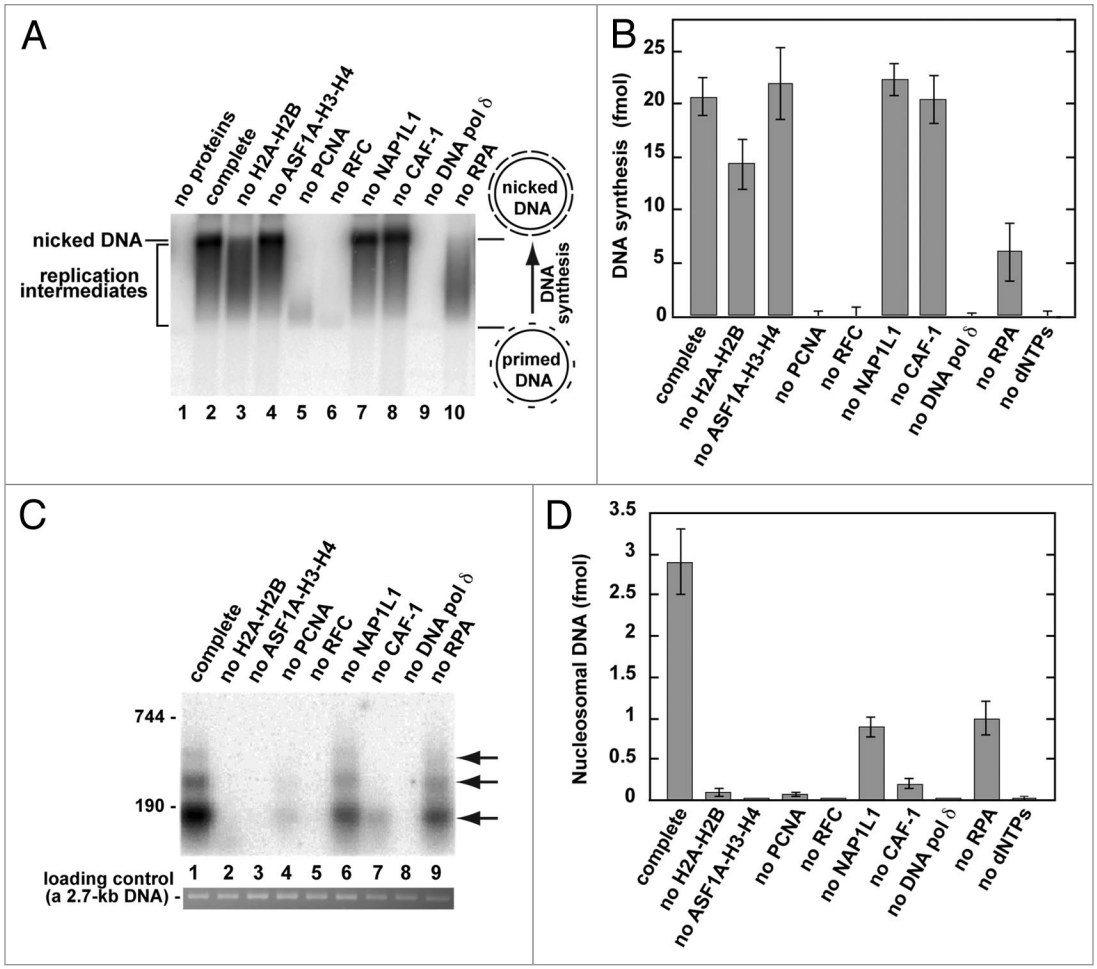


Figure 7. Packaging of nascent DNA into nucleosomes in a purified system. The coupled DNA synthesis and nucleosome assembly reactions were performed and analyzed as described in "Materials and Methods" and below. Visualization of DNA synthesis (**A and B**) and nucleosome assembly (**C and D**) in the indicated reactions. The complete reactions contained DNA polymerase δ, RPA, CAF-1, ASF1A-H3-H4, H2A-H2B, RFC, PCNA, NAP1L1, 12-primed DNA, competitor DNA, and the 4 dNTPs. The arrows show the positions of mono-, di-, and tri-nucleosomal DNA.

The sequences of all oligonucleotides used in this work are available upon request.

Pool-down assay, antibodies, and detection of immunological complexes

To analyze if there is an interaction between human CAF-1 and ASF1A-H3-H4, the purified CAF-1 (2 μg) was bound to 10-μl anti-FLAG M2 agarose beads (Sigma) in a buffer containing 20 mM HEPES, pH 7.4, 0.1 mM EDTA, 0.01% (v/v) NP40, 12.5% (v/v) glycerol, 243 mM NaCl, 0.05 mg/ml BSA, 1 mM DTT, 0.1 mM PMSF at 4 °C for 1 h. After extensive washing, the CAF-1-containing beads were incubated with the purified ASF1A-H3-H4 (0.5 μg) in a 50-μl binding buffer (20 mM HEPES, pH 7.4, 0.1 mM EDTA, 0.01% [v/v] NP40, 20% [v/v] glycerol, 92 mM KCl, 28 mM NaCl, 9 μg/ml BSA, 0.1 mM DTT, and 0.2 mM PMSF) at 4 °C for 1 h. The beads were extensively washed with the binding buffer and bound proteins were eluted from the beads by incubating in a 40 μl buffer (10 mM MOPS-NaOH, pH 7.0, 1% SDS, 1% β-merkaptoethanol, 25% [v/v] glycerol, and 5 mM EDTA) at 70 °C for 10 min. The input, wash, and eluted fractions were analyzed in western blot analyses with antibodies against ASF1A/B and histone H3.

When indicated, the described experiments involved α-ASF1A/ASF1B antibodies (Santa Cruz Biotechnology, sc-53171), α-H2B antibodies (Millipore, 07–371), α-H3 antibodies (Santa Cruz Biotechnology, sc-8654), α-H4 antibodies (Millipore, 07–108), α-NAP1L1 antibodies (Abcam, ab21630), α-PCNA antibodies (Santa Cruz Biotechnology, sc-7907), α-RFC-p140 antibodies (Santa Cruz Biotechnology, sc-20993), and α-FACT-SPT16 antibodies (Santa Cruz Biotechnology, sc-7907). Antigen-antibody complexes during western blot analyses were detected using the ECL plus detection reagents (GE HealthCare).

Assembly of relaxed covalently closed DNA into nucleosomes

To analyze ASF1A-H3-H4-dependent assembly of relaxed covalently closed DNA (ccDNA) into nucleosomes in HeLa cytosolic extracts, an assay based on a previously published method[47] was used. Forty μl reactions contained 20 mM Hepes-NaOH (pH 7.4), 90 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, 2 mM ATP, 2 mM DTT, 0.2 mg/ml BSA, 0.5% (v/v) glycerol, 0.6 nM (0.1 μg) of ccDNA,[72] 90 μg HeLa cytosolic extract, and the indicated concentrations of the purified ASF1A-H3-H4. The reactions were incubated at 37 °C for 60 min and then terminated by the addition of a 30 μl stop mixture (0.35% SDS, 0.4 M NaCl, 0.17 mg/ml Proteinase K, 0.33 mg/ml glycogen, and 13 mM EDTA) and incubated at 50 °C for 15 min. DNA products were extracted with phenol/chloroform, precipitated with isopropanol, washed with 75% ethanol, dissolved in 1xTE buffer (pH 7.5) containing 0.02 mg/ml RNase A, and separated in a native 0.8% agarose gel in 1xTAE buffer. The DNA products were visualized by the ethidium bromide staining.

Assembly of nucleosomes onto DNA carrying strand breaks

Unless specified otherwise, a nucleosome assembly reaction was performed in a 40 μl mixture containing 20 mM HEPES-NaOH, pH 7.4, 150 mM KCl, 5 mM $MgCl_2$, 2 mM ATP, 2 mM DTT, 0.2 mg/ml BSA, 2% (v/v) glycerol, 1.2 nM DNA with 12 strand breaks, and 5.7 nM competitor DNA (supercoiled pUC19). (The competitor DNA shares no sequence homology with the substrate DNA.) A complete defined nucleosome assembly reaction also included 15 nM CAF-1, 105 nM ASF1A-H3-H4, 180 nM H2A-H2B dimer, 5 nM RFC, 15 nM PCNA, and 675 nM NAP1L1. A cytosolic extract nucleosome assembly reaction contained 110 μg or 220 μg HeLa cytosolic extract supplemented or not with 15 nM CAF-1. When indicated, the cytosolic reactions with 220 μg HeLa cytosolic extract were supplemented with both 15 nM CAF-1 and 155 nM H2A-H2B. (The addition of 155 nM H2A-H2B produces the same final concentration [180 nM] of the histone dimer in the cytosolic reaction as in the complete defined reaction.)

After incubation at 37 °C for 10 min or the indicated time, a defined or cytosolic nucleosome assembly reaction was supplemented with 20 U micrococcal nuclease (Worthington) and $CaCl_2$ to the final concentration of 2.5 mM and then incubated at room temperature (21–23 °C) for 10 min. Micrococcal nuclease cleavage was terminated by the addition of a 9 μl stop mixture (0.47% SDS, 66 mM EDTA, 1 mg/ml Proteinase K, 38% glycerol, and 18 ng *Hind*III-cleaved pUC18 DNA [a gel loading control]), followed by incubation of the reaction at 50 °C for 15 min. Proteinase K was inactivated by the addition of 2 μl 16 mM PMSF. The deproteinized DNA products were separated in native 1.7% agarose gels in 1xTAE buffer, transferred onto nylone membranes, and analyzed by Southern hybridizations with a ^{32}P-labeled probe as previously described.[72] The images were visualized by phosphorimaging, and the data were quantified. Where indicated, the obtained data were analyzed by unpaired *t* test.

The probe was an equimolar mixture of 36 different 5′-^{32}P-labeled oligonucleotides that were labeled to about the same specific activity. Each of the 36 oligonucleotides is complementary to a unique 23–24 nt sequence in the complementary strand of the 12-nicked substrate DNA. The oligonucleotides were designed such that after annealing they are evenly distributed along the length of the complementary strand.

Coupled DNA synthesis and nucleosome assembly in a defined system

The coupled DNA-synthesis and nucleosome assembly reactions were carried similarly to the defined nucleosome assembly reactions. The complete 40 μl reactions contained 20 mM HEPES-NaOH, pH 7.4, 150 mM KCl, 5 mM $MgCl_2$, 2 mM ATP, 0.2 mM dGTP, 0.2 mM dATP, 0.2 mM dTTP, 0.2 mM dCTP, 2 mM DTT, 0.2 mg/ml BSA, 2% (v/v) glycerol, 15 nM CAF-1, 105 nM ASF1A-H3-H4, 5 nM RFC, 15 nM PCNA, 675 nM NAP1L1, 180 nM H2A-H2B dimer, 9 nM DNA polymerase δ, 100 nM RPA, 1.2 nM 12-primed DNA, and 5.7 nM competitor DNA (supercoiled pUC19), and was incubated at 37 °C for 10 min. To score DNA synthesis, 25 μl of a reaction were mixed with a 15 μl stop mixture (0.47% [w/v] SDS, 66 mM EDTA, 1 mg/ml Proteinase K, 38% [v/v] glycerol, and 30 ng *Hind*III-cleaved pUC18 DNA [a gel loading control]) at the end of the incubation. DNAs of the reactions were separated in 0.8% native agarose gels and transferred on nylon membranes, and the newly synthesized DNAs were visualized by Southern blot hybridizations with a ^{32}P-labeled probe. The probe was prepared by cleaving MR59 ssDNA with restriction endonucleases *Hin*fI

(NEB) and *Hae*I (NEB), followed by dephosphorylation of the 5′ ends with the antarctic phosphatase (NEB) and 5′-^{32}P-labeling of the ssDNA fragments with T4 polynucleotide kinase (NEB). Nucleosome assembly in the coupled reactions was scored by Southern hybridization as described above for the strand break-dependent reactions.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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