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Identification of a Rapidly Formed Nonnucleosomal Histone-DNA Intermediate that Is Converted into Chromatin by ACF

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SUMMARY

Chromatin assembly involves the combined action of histone chaperones and ATP-dependent motor proteins. Here, we investigate the mechanism of nucleosome assembly with a purified chromatin assembly system containing the histone chaperone NAP1 and the ATP-dependent motor protein ACF. These studies revealed the rapid formation of a stable nonnucleosomal histone-DNA intermediate that is converted into canonical nucleosomes by ACF. The histone-DNA intermediate does not supercoil DNA like a canonical nucleosome, but has a nucleosome-like appearance by atomic force microscopy. This intermediate contains all four core histones, lacks NAP1, and is formed by the initial deposition of histones H3-H4. Conversion of the intermediate into histone H1-containing chromatin results in increased resistance to micrococcal nuclease digestion. These findings suggest that the histone-DNA intermediate corresponds to nascent nucleosome-like structures, such as those observed at DNA replication forks. Related complexes might be formed during other chromatin-directed processes such as transcription, DNA repair, and histone exchange.

INTRODUCTION

Nucleosome assembly is required for the duplication of eukaryotic chromosomes, as well as for the packaging of DNA into chromatin upon transcription and DNA repair. Nucleosomes are assembled by the combined action of ATP-driven motor proteins, such as ACF (ATP-utilizing chromatin assembly and remodeling factor), RSF (remodeling and spacing factor), and CHD1 (chromo-ATPase/helicase-DNA-binding protein 1) (for reviews, see Haushalter and Kadonaga, 2003; Kadam and Emerson, 2002; Lusser and Kadonaga, 2004), and histone chaperones, such as NAP1 (nucleosome assembly protein 1), CAF1 (chromatin assembly factor 1), Asf1 (antisilencing function 1), FACT (facilitates chromatin transcription), nucleoplasmin, and HIRA (histone regulatory protein A), that deliver the histones to the sites of chromatin assembly (for reviews, see Campos and Reinberg, 2010; Corpet and Almouzni, 2009; Das et al., 2010; Ransom et al., 2010; Avvakumov et al., 2011).

The assembly of periodic arrays of nucleosomes was observed to be an ATP-dependent process by Worcel and colleagues (see, for example, Glikin et al., 1984; Ruberti and Worcel, 1986). This effect was originally studied in crude extracts derived from *Xenopus* oocytes (Glikin et al., 1984) or *Drosophila* embryos (Becker and Wu, 1992; Kamakaka et al., 1993). Then, the individual components that mediate the ATP-dependent assembly of chromatin were fractionated, purified, and cloned, and it was found that a purified and defined system comprising ACF, NAP1, core histones, ATP, and relaxed DNA is able to mediate the assembly of periodic arrays of nucleosomes (Ito et al., 1997, 1999).

Although the efficient assembly of canonical nucleosomes on extended relaxed DNA is dependent upon a motor protein such as ACF (see, for example, Ito et al., 1997, 1999; Loyola et al., 2001; Fyodorov and Kadonaga, 2002a; Lusser et al., 2005), there are conditions in which nucleosomes can be formed at physiological salt concentrations in the absence of an ATPase. First, with short DNA fragments typically ranging from 146 bp to 207 bp, NAP1 and histones can yield mononucleosomes (see, for example, Mazurkiewicz et al., 2006; Andrews et al., 2010). In such cases, the free ends of the short DNA fragments are able to wrap around the histone octamers to form mononucleosomes. Second, with longer DNA substrates, such as plasmid DNA, the efficiency of spontaneous nucleosome formation in the absence of a motor protein is significantly enhanced by negative supercoiling (see, for example, Pfaffle and Jackson, 1990; Nakagawa et al., 2001). In such cases, the formation of nucleosomes is driven forward by the release of DNA supercoiling energy, which is relieved by the wrapping of the DNA around the histone octamer. In the eukaryotic nucleus, however, the bulk of the DNA is relaxed (Sinden et al., 1980; Giaever and Wang, 1988). Hence, we have focused on the mechanism of ACF-dependent assembly of extended nucleosome arrays with relaxed DNA substrates.

In this work, we studied the early steps in the formation of nucleosomes. We sought, in particular, to address the apparent paradox that nucleosome-like structures appear to form immediately (within seconds) upon passage of DNA replication forks (see, for example, McKnight and Miller, 1977; McKnight et al., 1978; Sogo et al., 1986), whereas canonical "mature"

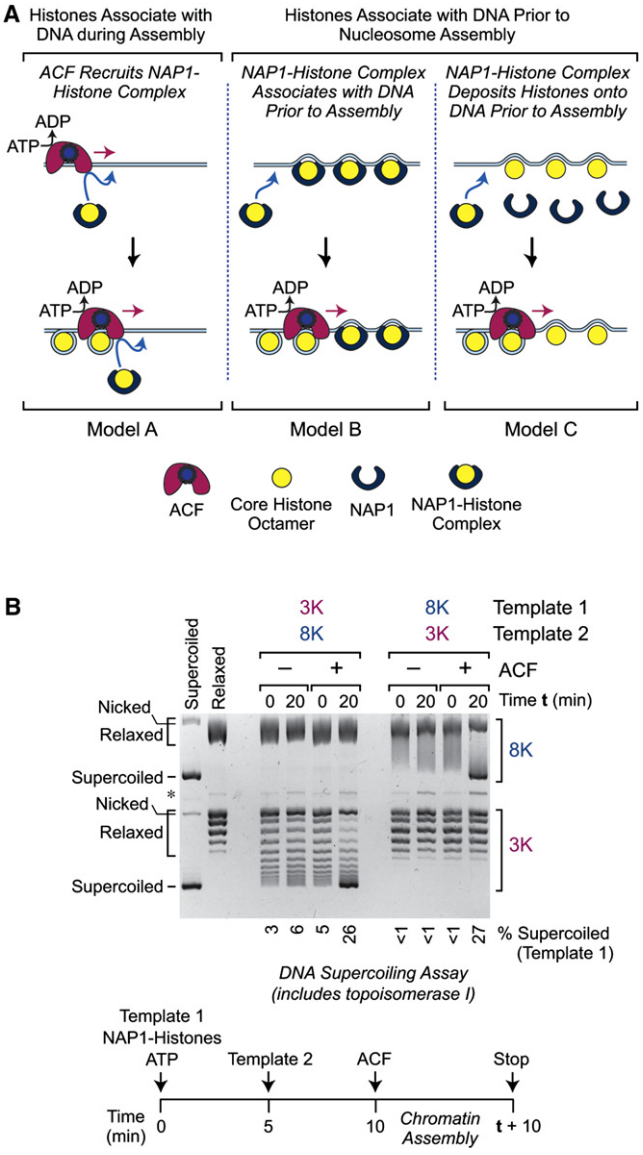

Figure 1. Core Histones Associate with the DNA Template prior to ACF-Catalyzed Nucleosome Assembly

(A) Three models for the mechanism of chromatin assembly. In model A, core histone deposition and nucleosome formation occur in a single step, whereas in models B and C, the histones are initially deposited onto the DNA to give a non-nucleosomal intermediate that is subsequently converted into canonical ("mature") chromatin by ACF.

(B) Core histones stably associate with DNA prior to chromatin assembly by ACF. Template association experiments were performed with equivalent masses of two different circular DNA templates of approximately 3 kbp (3K) and 8 kbp (8K). Core histones (amount sufficient for complete assembly of one DNA template) were initially incubated with template 1 (either 3K or 8K). After 5 min, template 2 (either 8K or 3K) was added to the mixture, which was then allowed to incubate for another 5 min (to allow potential exchange of histones from template 1 to template 2) prior to the addition of ACF. Purified topoisomerase I was included in the reactions, and the formation of nucleosomes was monitored by the DNA supercoiling assay. The 3K and 8K templates were resolved by agarose gel electrophoresis. For each reaction, the percent supercoiling of template 1 DNA [(supercoiled DNA/total DNA) × 100] is indicated. Due to the higher resolution of the different supercoiled species with the 3K plasmid than the 8K plasmid, the quantitation of percent supercoiling is likely to be more accurate with the 3K plasmid than the 8K plasmid. As references, supercoiled and relaxed DNAs were also included. The positions of relaxed, supercoiled, and nicked DNA species are shown. The asterisk indicates a contaminant in the preparation of the 3K plasmid.

See also Figure S1.

nucleosomes, as characterized by nuclease digestion and sedimentation properties, are more slowly generated after approximately 10 to 20 min (see, for example, Seale, 1975, 1976; Levy and Jakob, 1978; Worcel et al., 1978; Schlaeger and Knippers, 1979; Klempnauer et al., 1980; Jackson and Chalkley, 1981). To this end, we examined the initial steps in the formation of chromatin with the purified chromatin assembly system that comprises ACF, NAP1, histones, ATP, and relaxed DNA. We investigated the mechanisms by which the histones could be deposited onto the DNA and converted into nucleosomes. These studies revealed a nonnucleosomal histone-DNA complex that is rapidly formed within 15 s in the absence of ACF. These intermediate species, which resemble nucleosomes by atomic force (scanning probe) microscopy, can be slowly converted into canonical chromatin by ACF. It is likely that this nonnucleosomal intermediate in the chromatin assembly pathway corresponds to the rapidly formed nucleosome-like species that was observed at DNA replication forks.

RESULTS AND DISCUSSION

A Stable Nonnucleosomal Complex Is Formed prior to Nucleosome Assembly

In our analysis of the initial steps in chromatin assembly, we examined when the histones become associated with the DNA template (Figure 1). Specifically, we sought to distinguish between model A, wherein histone deposition occurs concurrently with nucleosome formation, and models B and C, in which histone deposition precedes the formation of canonical nucleosomes (Figure 1A). To this end, we carried out template association experiments in which NAP1-histone complexes are first incubated with DNA template #1 and subsequently challenged with DNA template #2 prior to the addition of ACF (see reaction scheme at bottom of Figure 1B). If the histones stably associate with template #1 (as in models B and C), then there would be preferential chromatin assembly on template #1 relative to template #2. Alternatively, if the histones do not stably associate with the DNA template prior to ACF-mediated chromatin assembly (as in model A), then there would be roughly equivalent levels of assembly on templates #1 and #2.

We monitored the extent of chromatin assembly by using the DNA supercoiling assay, which is based on the observation that the wrapping of DNA around the core histone octamer results in a change in the linking number of approximately −1 (Germond et al., 1975; Simpson et al., 1985). Thus, in this assay, the formation of nucleosomes is detected by the supercoiling of relaxed closed circular DNA templates in the presence of topoisomerase I. As controls, we confirmed that the chromatin assembly reactions are dependent upon NAP1 and ATP (Figure S1A available online).

We performed the template association experiments with equivalent masses of two DNA templates of approximately

3 kbp ("3K") and 8 kbp ("8K") that can be resolved by agarose gel electrophoresis (Figure 1B). These experiments revealed essentially exclusive assembly of chromatin onto template #1 relative to template #2 (Figure 1B). Template association by the histones was observed whether the 3K plasmid or the 8K plasmid was used as template #1. Thus, prior to the formation of nucleosomes by ACF, the histones associate with template #1 as a nonnucleosomal histone-DNA complex that does not induce DNA supercoiling. This property suggests that DNA in the nonnucleosomal histone-DNA complex is not fully wrapped around the core histone octamer.

As observed previously (Ito et al., 1999; Fyodorov and Kadonaga, 2002a; Lusser et al., 2005), a small amount of DNA supercoiling is observed with NAP1 and histones in the absence of a motor protein (Figure 1B). The extent of the ACF-independent DNA supercoiling is typically about 20%–25% of that obtained with ACF. Given that NAP1 can spontaneously form nucleosomes on supercoiled DNA (see, for example, Nakagawa et al., 2001), the ACF-independent partial supercoiling of the relaxed DNA is likely to be due to the spontaneous folding of a fraction (~20%–25%) of the deposited histones into canonical nucleosomes. It is important to note, however, that the amount of DNA supercoiling in the absence of ACF does not increase significantly upon extended incubation for up to 90 min (Figure S1B) (Ito et al., 1999; Fyodorov and Kadonaga, 2002a; Lusser et al., 2005). Hence, the efficient assembly of chromatin onto relaxed DNA with purified recombinant NAP1 requires an ATP-utilizing motor protein such as ACF or CHD1.

ACF comprises the Acf1 polypeptide along with the ISWI ATPase. The ISWI polypeptide alone is able to catalyze the assembly of chromatin, although with a lower efficiency than ACF (see, for example, Ito et al., 1999; Fyodorov and Kadonaga, 2002b). To assess whether the Acf1 subunit of ACF is required for conversion of the histone-DNA complex into chromatin, we performed template association experiments with purified ISWI protein. As shown in Figure S1C, we observed that ISWI protein alone is able to convert the nonnucleosomal complex into chromatin. Hence, this activity can be carried out by the core ISWI subunit of ACF.

To complement the DNA supercoiling data, we characterized the histone-DNA complexes by partial micrococcal nuclease digestion analysis (Noll and Kornberg, 1977). As shown in Figure S1D, digestion of the histone-DNA complexes formed in the absence of ACF yields a diffuse heterogeneous mixture of DNA fragments, which is in contrast to the distinct bands of DNA that are obtained upon micrococcal nuclease digestion of ACF-assembled chromatin. Notably, the digestion products obtained from the histone-DNA complexes resemble the micrococcal nuclease digestion products of nuclei from HTC cells that were pulse-labeled with [^{3}H]thymidine for 2 min (Smith et al., 1984). In both cases, there is a broad range of DNA fragments with some detectable bands that may derive from mono- and dinucleosomes. The absence of distinct bands derived from periodically spaced oligonucleosomes is unlike the pattern seen in bulk chromatin or chromatin that is assembled in the presence of ACF (Figure S1D). Hence, these findings, combined with the DNA supercoiling data (Figure 1B), suggest that the nonnucleosomal complexes are distinct from chromatin.

Formation of the Nonnucleosomal Complex and Its Conversion into Nucleosomes

To understand the properties of the nonnucleosomal complex, we investigated its stability as well as the rates of its formation and ACF-catalyzed conversion into canonical nucleosomes. To analyze its stability, we formed the complex on template #1 and then incubated it with template #2 for time periods ranging from 5 min to 120 min prior to the addition of ACF (Figure 2A). We found that the histones remain stably associated with template #1 for at least 120 min, at which time there is no detectable exchange of histones from template #1 to template #2. A slight decrease in the amount of supercoiled DNA can be seen at the 60 min and 120 min time points, but this effect appears to be due to increased nicking of the DNA rather than instability of the complexes. Then, to examine the rate of formation of the histone-DNA complexes, we combined NAP1-histones with template #1 and subsequently added template #2 at time points ranging from 15 s to 5 min (Figure 2B). These experiments revealed that formation of the template-associated complexes is complete within 15 s. Hence, the nonnucleosomal histone-DNA complexes are fully formed within 15 s and remain associated with the template for at least 120 min.

To test whether the nonnucleosomal complex has the kinetic properties of an intermediate in the assembly of chromatin, we measured the rate of conversion of the histone-DNA complexes into chromatin. If the nonnucleosomal complex is an intermediate in the chromatin assembly process, then the rate at which it is converted into chromatin would be greater than or equal to the rate of the overall chromatin assembly reaction, because an intermediate in a multistep reaction must be converted into the product at least as rapidly as the overall reaction. By carrying out standard one-template chromatin assembly reactions, we compared the rate of the overall chromatin assembly reaction (in which ACF was combined with NAP1-histones prior to the addition of DNA) with the rate of conversion of histone-DNA complexes into chromatin by ACF (Figure 2C). These experiments revealed that the rate of conversion of the histone-DNA complexes into chromatin is comparable to that of the overall reaction.

Therefore, the nonnucleosomal histone-DNA complex satisfies the two fundamental criteria for an intermediate in the chromatin assembly process—first, the intermediate can be converted into chromatin, and second, the rate of conversion of the intermediate into chromatin is comparable to the rate of the overall chromatin assembly reaction. Combined with the observation that the intermediate is formed within 15 s, the data lead to a model for chromatin assembly in which there is rapid ACF-independent formation of the non-nucleosomal histone-DNA intermediate followed by a rate-limiting ACF-mediated conversion of the intermediate into canonical nucleosomes.

The Nonnucleosomal Intermediate Lacks NAP1

Next, we sought to determine whether the nonnucleosomal intermediate contains NAP1, which would enable us to distinguish between models B and C in Figure 1A. To address this issue, we formed the intermediate with NAP1-histones and template DNA, and depleted the His_6-tagged NAP1 from solution by incubation with Ni-NTA agarose followed by pelleting of the

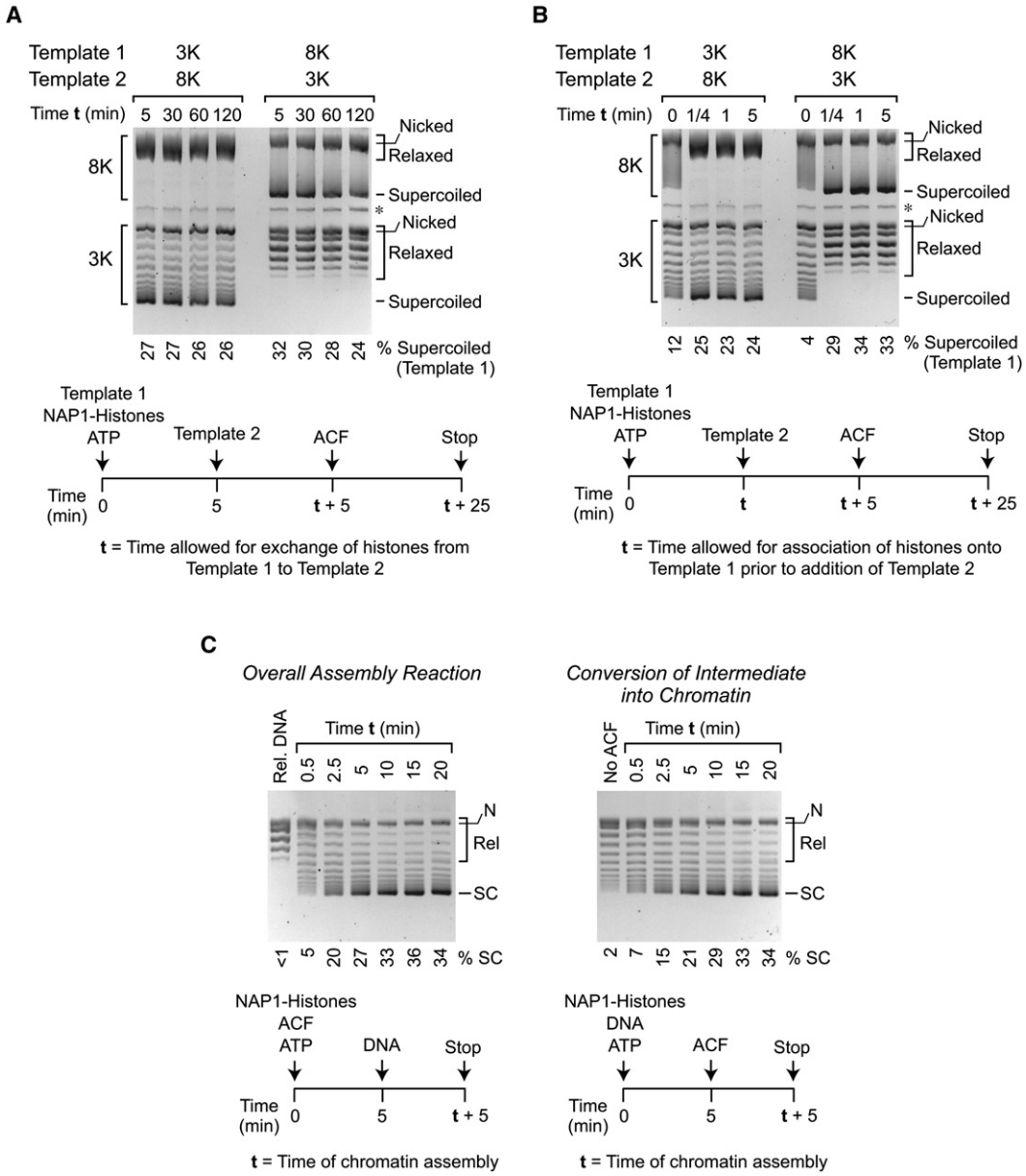

Figure 2. Dynamics of the Formation of the Nonnucleosomal Histone-DNA Intermediate and Its Conversion to Canonical Nucleosomes by ACF

(A) The nonnucleosomal histone-DNA complexes remain associated with the DNA template for at least 120 min. Template association experiments were performed as in Figure 1B, except that the time of incubation of the template 1-histone complex with template 2 (to allow potential exchange of histones from template 1 to template 2 prior to the addition of ACF) was varied as indicated.

(B) The nonnucleosomal histone-DNA complexes are formed within 15 s. Template association experiments were performed as in Figure 1B, except that the time of addition of template 2 to the template 1 + NAP1-histone mixture was varied as specified.

(C) The nonnucleosomal histone-DNA complexes can be converted into chromatin by ACF at a rate that is comparable to that of the overall ACF-catalyzed chromatin assembly reaction. Standard (one-template) chromatin assembly reactions were performed in parallel under conditions that compared the rate of overall chromatin assembly with the rate of conversion of the non-nucleosomal histone-DNA complexes into chromatin by ACF. Purified topoisomerase I was included in the reactions, and the formation of nucleosomes was monitored by the DNA supercoiling assay.

agarose beads (Figure 3A). As a reference, we carried out parallel depletions with NAP1-histones in the absence of DNA. The resulting supernatant and pellet fractions were analyzed by western blot for NAP1 and histones (H2A-H2B) and by agarose gel electrophoresis and ethidium bromide staining for DNA. In the absence of DNA, the NAP1 and histones are in the pellet, and are not detectable in the supernatant. However, in the presence of DNA, most of the histone-DNA complex is in the supernatant, whereas essentially all of the NAP1 and a small amount of histones and DNA are in the pellet (Figure 3A). In addition, the

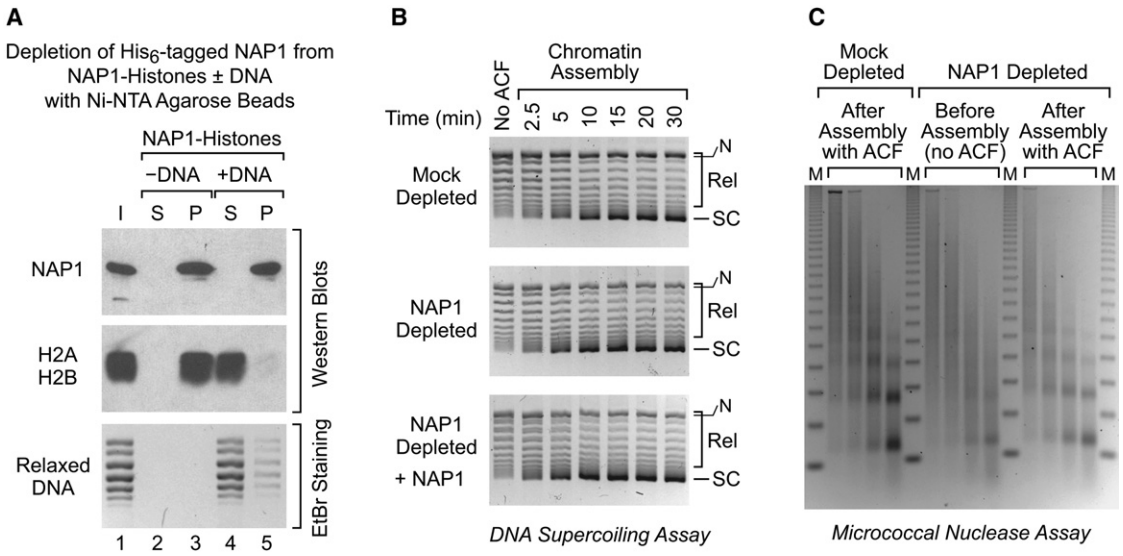

Figure 3. The Nonnucleosomal Histone-DNA Intermediate Does Not Contain NAP1

(A) NAP1 is not associated with the nonnucleosomal histone-DNA complexes. Nonnucleosomal histone-DNA complexes were formed by incubation of NAP1-histones and relaxed template DNA, and then incubated with Ni-NTA agarose beads (QIAGEN) to which the His_6-tagged NAP1 can bind (lanes 4 and 5). The beads were pelleted, and the resulting supernatant (S) and pellet (P) fractions were analyzed by western blot with antibodies against NAP1 and histones H2A-H2B. In addition, the DNA content of the supernatant and pellet was analyzed by agarose gel electrophoresis and staining with ethidium bromide. As a reference, NAP1-histone complexes in the absence of DNA were analyzed in parallel (lanes 2 and 3). Samples of the input (I) NAP1, histones, and DNA were also included (lane 1).

(B) The NAP1-depleted intermediate can be converted into chromatin by ACF at a rate that is comparable to that of the overall chromatin assembly reaction. Upon depletion of NAP1 in (A), the non-nucleosomal histone-DNA complexes in the supernatant (S) fraction were incubated with ACF for the indicated reaction times. Chromatin assembly was monitored by DNA supercoiling analysis in the presence of purified topoisomerase I. Assembly reactions were performed in parallel with NAP1-depleted samples (middle), NAP1-depleted samples that were subsequently supplemented with purified NAP1 (equivalent amount of NAP1 as depleted; bottom), and mock-depleted samples (top). The % supercoiling [(supercoiled DNA/total DNA) x 100] versus time for each series of reactions is shown in Figure S3. The positions of relaxed (Rel), supercoiled (SC), and nicked (N) DNA are indicated.

(C) The NAP1-depleted histone-DNA complexes can be converted into periodic nucleosome arrays by ACF. NAP1-depleted histone-DNA complexes were incubated with ACF and ATP under standard chromatin assembly conditions. Samples that were obtained either before or after incubation with ACF were subjected to partial micrococcal nuclease digestion analysis. As a reference, mock-depleted histone-DNA complexes that were incubated with ACF were analyzed in parallel.

See also Figure S2.

relative amounts of the four core histones in the histone-DNA complex in the supernatant (Figure 3A, lane 4) are the same as those in the input sample (Figure 3A, lane 1), as assessed by SDS-polyacrylamide gel electrophoresis and silver staining (Figure S2A). These results show that most of the non-nucleosomal histone-DNA complex is not associated with NAP1.

We then tested whether the NAP1-depleted histone-DNA complexes can be assembled into chromatin by ACF. These experiments revealed that ACF can catalyze the conversion of the NAP1-deficient histone-DNA complexes into chromatin at a rate that is comparable to that seen with mock-depleted complexes (Figure 3B and Figure S2B) as well as the overall chromatin assembly reaction (Figure 2C). Moreover, the addition of purified NAP1 to the NAP1-depleted histone-DNA complexes does not affect the rate or efficiency of chromatin assembly by ACF (Figure 3B and Figure S2B). These findings indicate that the non-nucleosomal intermediate lacks NAP1, and are therefore consistent with model C in Figure 1A.

We further examined whether the NAP1-depleted histone-DNA complexes yield periodic nucleosome arrays upon incubation with ACF. To clarify this point, we incubated the NAP1-depleted complexes with ACF under standard chromatin assembly conditions and then subjected the reaction products to micrococcal nuclease digestion analysis. These experiments revealed that the NAP1-depleted complexes are converted into canonical periodic nucleosome arrays by ACF (Figure 3C). Hence, the NAP1-depleted complexes satisfy the criteria for an intermediate, as they yield chromatin at a rate that is comparable to the overall reaction.

Analysis of Chromatin Assembly with Histone H1

Chromatin in metazoans contains approximately one molecule of histone H1 per nucleosome (see, for example, Bates and Thomas, 1981; Happel and Doenecke, 2009). We therefore analyzed the assembly of chromatin in the presence of purified histone H1. To examine the effect of H1 on the formation of the histone-DNA intermediate, we carried out template association experiments in the presence of one molecule of histone H1 per core histone octamer. As shown in Figure 4A, we observed template association during chromatin assembly in the presence of H1. Thus, the histone-DNA intermediate is formed and converted into chromatin by ACF in the presence or absence of histone H1.

Then, we compared the properties of naked DNA, the histone-DNA intermediate (DNA, core histones, NAP1), chromatin lacking

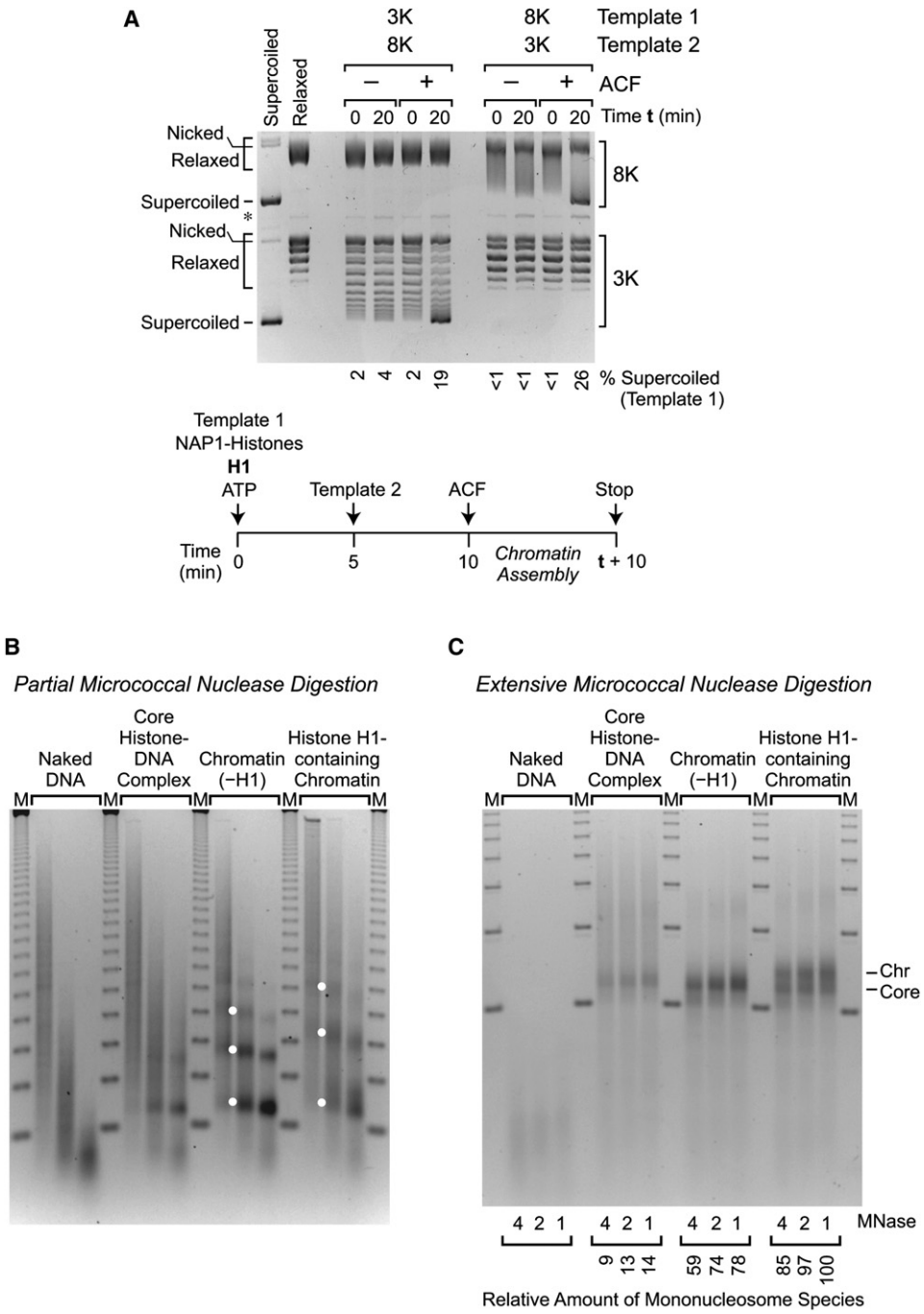

Figure 4. Analysis of Chromatin Assembled with Histone H1

(A) Template association during chromatin assembly occurs in the presence of histone H1. Chromatin assembly reactions were performed as in Figure 1B, except that histone H1 was included at approximately one molecule of H1 per core histone octamer. The asterisk denotes a contaminant in the preparation of the 3K plasmid.

(B) Partial micrococcal nuclease digestion analysis of chromatin at different stages of assembly. Naked DNA (DNA only), core histone-DNA complexes (DNA, NAP1, core histones), chromatin lacking H1 (DNA, NAP1, core histones, ACF, ATP), and histone H1-containing chromatin (DNA, NAP1, core histones, H1, ACF, ATP) were subjected to micrococcal nuclease digestion analysis. Each sample was treated with three different concentrations (increasing from left to right) of micrococcal nuclease. The three concentrations of micrococcal nuclease used with the different samples were identical. The DNA size markers are the 123 bp ladder (Invitrogen). The white dots denote positions of DNA fragments derived from mono-, di-, and trinucleosomes.

(C) Extensive micrococcal nuclease digestion analysis of chromatin at different stages of assembly. Samples that are similar to those in (B) were extensively digested with micrococcal nuclease for 1 min. The relative concentrations of micrococcal nuclease are indicated. The DNA size markers are the 123 bp ladder. The positions of DNA fragments derived from core particles ("Core") and chromatosomes ("Chr") are denoted. The relative amounts of total mononucleosome species (core particles and chromatosomes) are indicated.

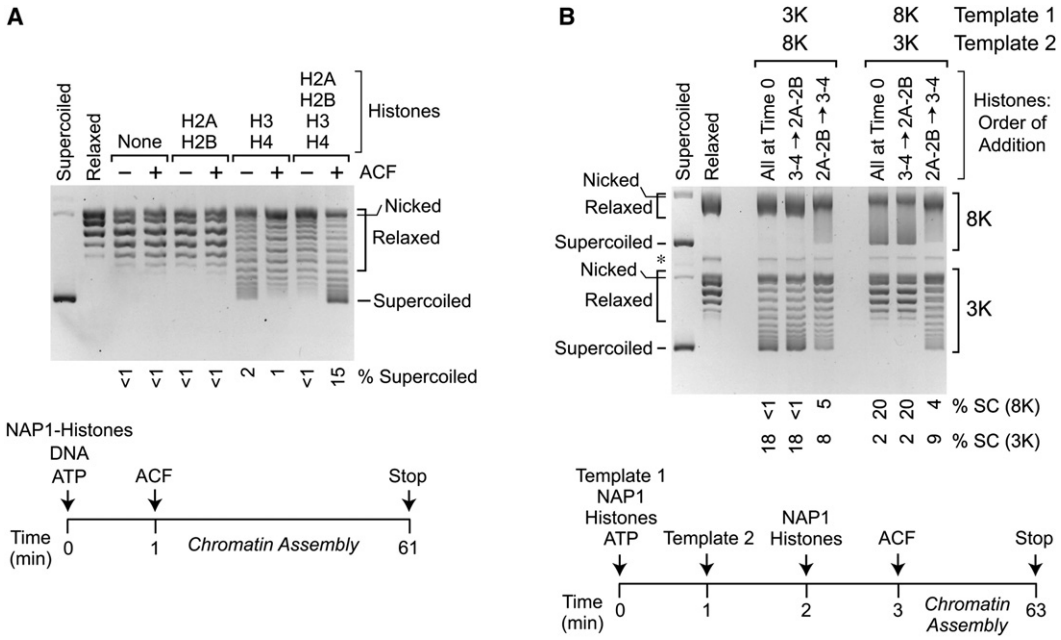

Figure 5. The Deposition of Histones H3-H4, but Not H2A-H2B, Is Sufficient for Template Association

(A) Chromatin assembly reactions with H2A-H2B alone, H3-H4 alone, or all four core histones. Chromatin assembly reactions were performed, as outlined in the scheme at the bottom of the figure, in the absence or presence of ACF with equimolar amounts of the indicated histones. The reaction products were analyzed by using the DNA supercoiling assay.

(B) Order-of-addition reactions reveal that H3-H4, but not H2A-H2B, is sufficient for template association. Template association reactions were carried out, as outlined in the scheme at the bottom of the figure, by modification of the basic procedure used in Figure 1B. Histones (H2A-H2B, H3-H4) were added either at 0 min (prior to the addition of template 2) or at 2 min (after the addition of template 2). For each reaction, the order of addition of H3-H4 and H2A-H2B is indicated. The percent supercoiling (%SC) is given for both the 3K and 8K templates.

See also Figure S3.

H1 (DNA, core histones, NAP1, ACF), and H1-containing chromatin (DNA, core histones, NAP1, ACF, H1), which represent key stages in the assembly of chromatin. To examine the structure of mono- and oligonucleosomal species, we carried out partial micrococcal nuclease digestion analysis (Figure 4B). As noted above, digestion of the histone-DNA intermediate yields a diffuse heterogeneous mixture of DNA fragments, which are similar to those seen upon micrococcal nuclease digestion of newly synthesized DNA (Smith et al., 1984). Upon the addition of ACF, periodic arrays of nucleosomes are generated. We also found that the H1-containing chromatin is more nuclease resistant than the H1-deficient chromatin as well as the histone-DNA intermediate, which are, in turn, more resistant than naked DNA. In addition, we observed an increase in the nucleosome repeat length upon addition of histone H1. The increased repeat length and decreased micrococcal nuclease sensitivity of H1-containing chromatin relative to H1-deficient chromatin are similar to the properties of chromatin associated with bulk DNA relative to newly synthesized DNA in nuclei (see, for example, Levy and Jakob, 1978; Schlaeger and Knippers, 1979).

Next, to investigate the nature and amount of mononucleosome species, we performed an extensive micrococcal nuclease "limit" digestion of DNA, histone-DNA complexes, and chromatin lacking or containing H1 (Figure 4C). First, with the chromatin samples, we saw ~147 bp DNA species (derived from core particles) in the absence of H1 and a mixture of ~166 bp and ~147 bp DNA fragments (derived from chromatosomes [H1-containing mononucleosomes; see Simpson (1978)] and core particles) in the presence of H1. Second, with the histone-DNA complexes, we observed only about 15%–20% of the amount of the ~147 bp DNA species (which roughly corresponds to the low level of ACF-independent nucleosome formation [see, for example, Figure 1B]) that was obtained with the corresponding H1-deficient chromatin. Third, with the naked DNA, the DNA was extensively digested and ~147 bp DNA species were not seen. Hence, the results of the "limit" digests reveal that the histone-DNA complexes are much more sensitive to micrococcal nuclease digestion than nucleosomes.

Template Association Involves the Initial Deposition of Histones H3 and H4

Chromatin assembly in cells appears to involve the initial deposition of histones H3 and H4 prior to the incorporation of histones H2A and H2B (see, for example, Worcel et al., 1978; Crémisi and Yaniv, 1980). We therefore examined template association by the histones with equimolar amounts of purified preparations of histones H3-H4 and H2A-H2B (Figure S3). First, we carried out standard chromatin assembly reactions with H2A-H2B alone, H3-H4 alone, and all four core histones (Figure 5A). With the DNA supercoiling assay, there was no detectable supercoiling with H2A-H2B alone and a small amount of negative DNA supercoiling with H3-H4 alone. (The negative supercoiling by

H3-H4 alone was confirmed by agarose gel electrophoresis of the DNA species in the absence versus the presence of chloroquine [data not shown].) These findings are consistent with results obtained in previous studies on the deposition of H3-H4 onto DNA (see, for example, Camerini-Otero and Felsenfeld, 1977; Bina-Stein and Simpson, 1977; Peterson et al., 2007). In addition, upon addition of ACF to H3-H4-DNA complexes, we observed a reproducible decrease in DNA supercoiling, which may be due to some disruption or reorganization of H3-H4-DNA species by ACF. With regard to the present study, however, the main point of this experiment is that chromatin assembly can be achieved with the purified preparations of H2A-H2B and H3-H4 and that the properties of H2A-H2B alone and H3-H4 alone are in accord with those observed in previous work.

We then examined the specific roles of H2A-H2B and H3-H4 in template association. To this end, we carried out template association experiments in which H2A-H2B were added prior to H3-H4, and vice versa (Figure 5B). When histones H3-H4 were combined with template #1 and then template #2 and histones H2A-H2B were added sequentially, we observed chromatin assembly specifically on template #1 relative to template #2. In contrast, when H2A-H2B were combined with template #1 and then template #2 and H3-H4 were added sequentially, chromatin assembly occurred on both template #1 and template #2. Therefore, H3-H4, but not H2A-H2B, are sufficient for the formation of a template associated complex. These findings indicate that the histone-DNA intermediate is formed by the initial deposition of histones H3 and H4 onto the DNA, and are consistent with studies in cells in which the deposition of H3-H4 was seen to precede the incorporation of H2A-H2B into chromatin.

Visualization of the Nonnucleosomal Intermediate

To gain a better understanding of the nature of the nonnucleosomal complex, we imaged the intermediate and chromatin by atomic force microscopy. In these experiments, we performed template association reactions with the 3K plasmid as template #1 and the 8K plasmid as template #2 in the absence or presence of ACF. As shown in Figure 6A, both the nonnucleosomal intermediate and chromatin exhibit the characteristic ''beads-on-a-string'' morphology that has been observed in the imaging of chromatin by electron microscopy and atomic force microscopy (see, for example, McKnight and Miller, 1977; McKnight et al., 1978; Bustamante et al., 1997). Notably, the histones in both the nonnucleosomal intermediate and chromatin samples are associated only with the smaller ~3 kbp template #1. Figure 6B shows magnified images of the histone-DNA complexes and nucleosomes. The apparent areas (mean ± standard deviation) of individual histone-DNA particles and nucleosomes were observed to be 113 ± 69 nm^2 (n = 338) and 99 ± 55 nm^2 (n = 558), respectively. The rather large values for the standard deviation are partly attributable to heterogeneity in orientations of individual particles deposited on the surface, overlapping of some particles, and variations in AFM probe sharpness (see, for example, Bustamante et al., 1997). The distributions are shown in Figure 6C. Additional images of the histone-DNA complexes and chromatin are shown in Figure S4. To within the obtained resolution, the morphology and size of the nonnucleosomal histone-DNA particles are similar to those of canonical nucleosomes.

Histone Deposition and Chromatin Maturation

We have identified a non-nucleosomal histone-DNA intermediate that is formed during the assembly of chromatin by ACF. These histone-DNA complexes do not supercoil DNA like a canonical nucleosome (Figures 1B), lack NAP1 (Figure 3), are more sensitive to micrococcal nuclease digestion than nucleosomes (Figure 4), can be formed in the presence of histone H1 (Figure 4), are formed by the initial deposition of H3-H4 (Figure 5), resemble nucleosomes by atomic force microscopy analysis (Figure 6), and can be converted into nucleosomes by ACF (Figure 3). The rapid rate of formation of the nonnucleosomal intermediate corresponds to the timing of the appearance of similar bead-like structures that were observed on newly synthesized DNA at replication forks in *Drosophila* embryos (McKnight and Miller, 1977; McKnight et al., 1978), as well as of the nucleosome-like species that were deduced by the inhibition of psoralen crosslinking of DNA at SV40 DNA replication forks (Sogo et al., 1986). In addition, the rate of the ACF-catalyzed conversion of the nonnucleosomal intermediate into canonical chromatin correlates with the timing of chromatin ''maturation'' (see, for example, Seale, 1975, 1976; Levy and Jakob, 1978; Worcel et al., 1978; Schlaeger and Knippers, 1979; Klempnauer et al., 1980; Jackson and Chalkley, 1981). Hence, these results, combined with the studies of the processivity of chromatin assembly by ACF and CHD1 (Fyodorov and Kadonaga, 2002a; Lusser et al., 2005), lead to a model for nucleosome assembly in which there is rapid formation of nonnucleosomal histone-DNA complexes and subsequent conversion of this ''nascent'' chromatin into ''mature'' canonical chromatin by the processive action of a motor protein (Figure 7).

Given the existence of multiple histone chaperones, there are probably multiple mechanisms by which the nonnucleosomal intermediate (or analogous species) can be generated. For example, in studies of DNA replication-coupled chromatin assembly by CAF1, Smith and Stillman (1991) observed a chromatin assembly intermediate that, like the histone-DNA complex described in this study, does not induce DNA supercoiling. Although the CAF1-H3-H4-DNA intermediate differs in content from the non-nucleosomal complex described in this work, both species may share a related function and interaction of H3-H4 with the DNA.

We envision the rapidly formed histone-DNA intermediate to be a species that resembles an ''open'' nucleosome in which the DNA is not fully wound around the histones. Based on the observation that H3 and H4 are sufficient for template association (Figure 5B), the primary contacts are likely to be with histones H3 and H4. Then, the complete wrapping of the DNA around the histone octamer to give ''mature'' chromatin would be achieved by the ATP-dependent motor activity of ACF. It is also useful to consider that species that are related to the intermediate that we describe in this study could be generated during processes such as transcription, DNA repair, and histone exchange. Hence, these findings may be relevant to a broad range of phenomena in the nucleus.

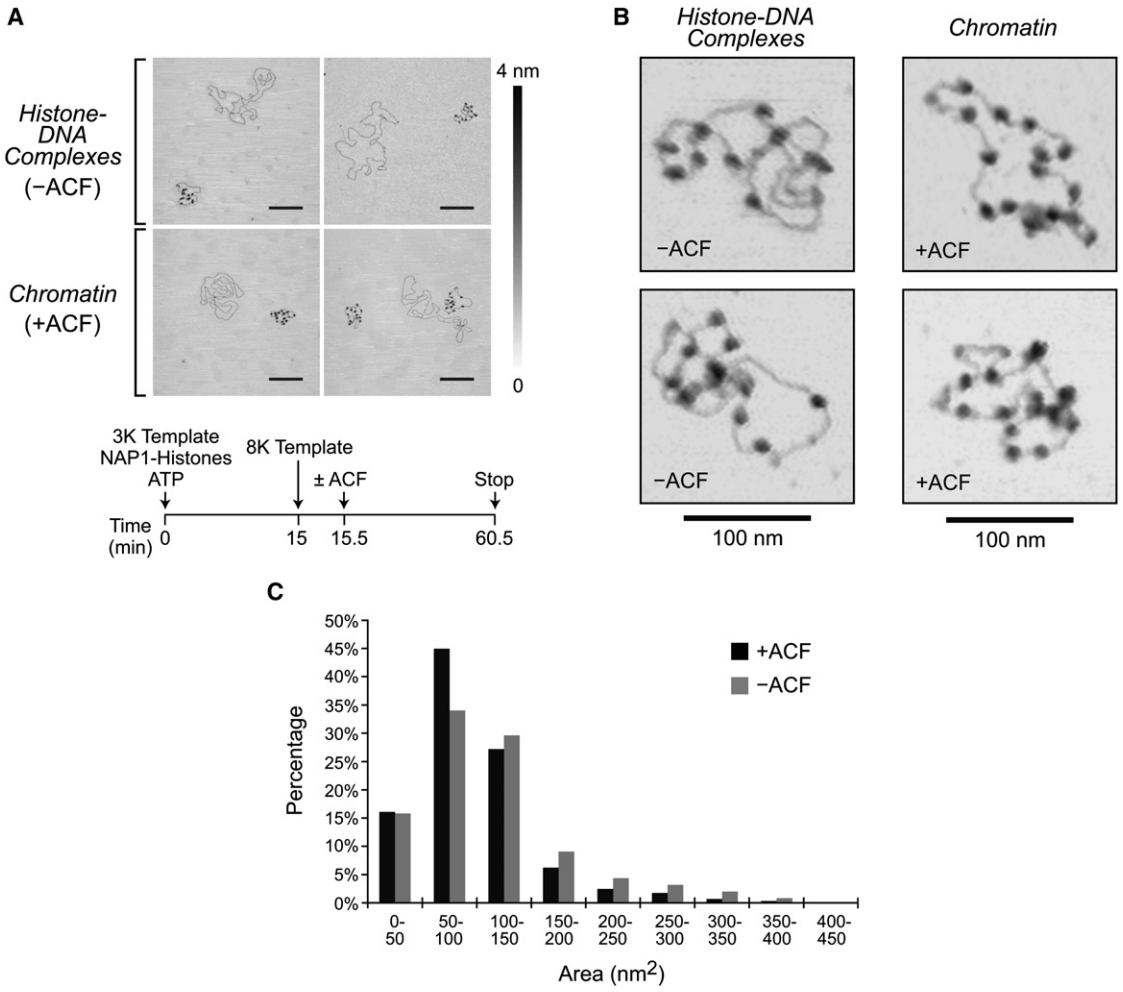

Figure 6. Visualization of the Nonnucleosomal Histone-DNA Complexes by Atomic Force Microscopy

(A) Template association experiments were performed, as depicted in the diagram, in the absence or presence of ACF. The resulting samples were immobilized on mica and then analyzed by atomic force microscopy. Representative images are shown. The sample field for each image is 1 μm × 1 μm, and the height scale is from 0 to 4 nm, as shown on the right. The scale bars represent 200 nm.

(B) Magnified images of histone-DNA complexes (−ACF) and nucleosomes (+ACF).

(C) Distribution of apparent areas of individual histone-DNA intermediates (−ACF; n = 338) and nucleosomes (+ACF; n = 558).

See also Figure S4.

EXPERIMENTAL PROCEDURES

Chromatin Assembly

Chromatin assembly reactions were performed as described by Fyodorov and Kadonaga (2003). All reactions contained core histones (0.35 μg), NAP1 (1.4 μg), ACF (6 nM), ATP (3 mM), topoisomerase I (1 nM), and an ATP regeneration system (3 mM phosphoenolpyruvate, 20 U/ml pyruvate kinase) in a final volume of 70 μl. The buffer composition of the final reaction mixture was as follows: 15 mM K-HEPES (pH 7.6), 3 mM Tris, 100 mM KCl, 5 mM NaCl, 5.5 mM $MgCl_2$, 0.1 mM EDTA, 6.6% (v/v) glycerol, 1% (w/v) polyvinyl alcohol (average MW 10,000), 1% (w/v) polyethylene glycol 8000, 20 μg/ml bovine serum albumin, and 20 μg/ml human insulin. Standard (one template) reactions contained one relaxed circular DNA plasmid (0.294 μg, pGIE-0, which is the "3K" template), whereas template association (two template) reactions contained two different relaxed circular DNA plasmids (0.294 μg each; pGIE-0 and pJH187, which is the "8K" template). The reaction products were analyzed by DNA supercoiling and micrococcal nuclease digestion assays (Fyodorov and Kadonaga, 2003). The percent supercoiling [(amount of supercoiled DNA/amount of total DNA species) × 100] in the DNA supercoiling assays was quantitated with ImageQuantTL (GE Healthcare). It is important to note that nicked DNA that is packaged into chromatin is included in the "amount of total DNA species" but not in the "amount of supercoiling DNA." Therefore, "percent supercoiling" reflects the amount of closed circular plasmid DNA that is packaged into chromatin and does not include the nicked DNA that is packaged into chromatin. In addition, due to the higher resolution of the different supercoiled species with the 3K plasmid than the 8K plasmid, the quantitation of percent supercoiling is likely to be more accurate with the 3K plasmid than the 8K plasmid.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at doi:10.1016/j.molcel.2011.07.017.

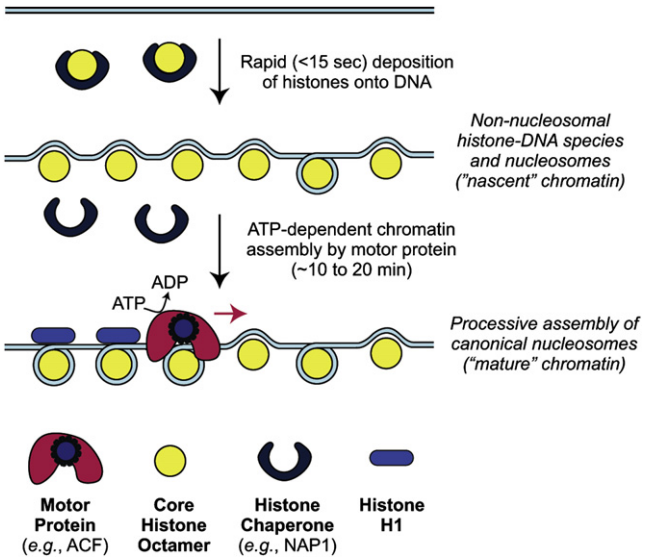

Figure 7. A New Working Model for the Steps in Chromatin Assembly

In the assembly of chromatin, a non-nucleosomal histone-DNA intermediate is rapidly formed in a process that does not require ACF. Unlike a canonical nucleosome, this intermediate does not significantly supercoil DNA. The intermediate is also more sensitive to micrococcal nuclease digestion than a canonical nucleosome. The formation of the intermediate appears to involve the initial deposition of histones H3-H4 followed by H2A-H2B. ACF is able to convert the intermediate into a canonical nucleosome in an ATP-dependent process at a rate that is comparable to that of the overall chromatin assembly reaction. By atomic force microscopy, the nonnucleosomal intermediate resembles a nucleosome. Thus, the intermediate may correspond to "nascent" chromatin that is formed immediately behind a DNA replication fork. This nascent chromatin is then converted by ACF into "mature" chromatin containing histone H1.

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