UNIVERSITY OF CALIFORNIA, SAN DIEGO

The Study of Non-Nucleosomal Chromatin Particles and the Development of a Simple and Versatile System for the ATP-Dependent Assembly of Chromatin

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by

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DEDICATION

To my family

Tran, Hung, and Quang Khuong

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ABSTRACT OF THE DISSERTATION

The Study of Non-Nucleosomal Chromatin Particles and the Development of a Simple and Versatile System for the ATP-Dependent Assembly of Chromatin

by

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Chromatin is a nucleoprotein complex that is responsible for both the organization of the genome as well as the regulation of DNA-utilizing processes. The basic repeating unit of chromatin is the nucleosome, which consists of approximately 147 bp of DNA wrapped around a core histone octamer. Non-nucleosomal chromatin particles also exist and are of interest due to their localization at regions of active chromatin. They are thought to prime chromatin for the initiation of DNA-utilizing processes, such as replication, repair, and transcription. For this dissertation, I developed methods to investigate two non-nucleosomal chromatin particles, the H3C110 particle and the prenucleosome, in cells using thiol-specific reagents and antibodies targeting acetylated H3K56, respectively. In addition, the formation of the prenucleosome was further investigated using a previously published *in vitro* chromatin assembly system with histone chaperone NLP in lieu of NAP1. The investigation into the H3C110 particles and prenucleosomes *in vivo* were ultimately discontinued due to a lack of enrichment of active chromatin. However, further *in vitro* work with the prenucleosome revealed that prenucleosomes can be formed with either NAP1 or NLP. This finding ultimately led to the development of a simplified and versatile *in vitro* chromatin assembly system. In this system, NLP has been substituted for NAP-1 and ATPase ISWI for ACF. Both proteins are synthesized in bacteria and easily purified making the system simpler to set up. In addition, this system can assemble chromatin with a variety of histones and DNA and can also incorporate histone H1 and nonhistone chromosomal protein HMGN2 into the chromatin. The simplicity and versatility of this system will allow a broader range of scientists to assemble customized chromatin for structural and functional analysis

CHAPTER 1:

Introduction

Introduction to non-nucleosomal chromatin particles

In eukaryotic cells, chromatin is a nucleoprotein complex that is responsible for organizing the entire genome, often over a meter in length, into a nucleus with a diameter of several microns. It is also responsible for regulating DNA-utilizing processes such as replication, transcription, repair, and recombination (Chen and Li, 2010; Campos and Reinberg, 2009; Paranjape *et al*. 1994).

The basic repeating unit of chromatin is the nucleosome. In metazoans, the nucleosome core consists of approximately 147 base pairs of DNA that is wrapped around a core histone octamer in a left handed superhelix. The core octamer comprises of two copies each of histones H2A, H2B, H3, and H4, with two H2A-H2B heterodimers flanking an H3-H4 tetramer (Luger *et al*. 1997). The nucleosomes are connected by linker DNA forming a 10 nm fiber structure (also referred to as "beads on a string"). It is important to note that this structure only exists in water. In physiological salt, the 10 nm fiber condenses into a 30 nm structure with the help of linker histone H1, a histone that binds to linker DNA.

Chromatin is diverse and dynamic and thus contains not only canonical nucleosomes, but also non-nucleosomal particles that may play a large part in active chromatin. Nucleosomes have been extensively studied and are well understood, whereas much about non-nucleosomal particles is still unknown. These species are likely to be biologically important as many of them are found in regions of active chromatin such as promoters and enhancers (Jin *et al*., 2009; Allegra *et al.* 1987; Chen-Cleland *et al.* 1993; Henikoff *et al*. 2011; Ishii *et al*. 2015; Rhee *et al*. 2014). These particles have been

described by various investigators in different contexts; however, it is unclear how these species relate to one another. Study of these particles may shed light on their importance in the regulation of DNA-utilizing processes.

Salt-labile nucleosomes

Salt-labile nucleosomes are defined as nucleosomes that are unusually unstable to increases in salt concentration. Typically, nucleosome core particles (NCPs) exposed to increasing salt concentrations will lose first the two H2A/H2B dimers and at higher salt concentrations, the H3/H4 tetramers will follow (Burton *et al*. 1978). Salt-labile nucleosomes dissociate at much lower concentrations. Studies have suggested that this may be due to the presence of histone variants H2A.Z and H3.3 (Jin and Felsenfeld, 2007; Henikoff *et al*. 2007), but the data are controversial (Park *et al*. 2004; Thakar *et al*. 2009). In one study, NCPs containing H2A.Z/H3.3 were destabilized by an increase in salt concentration (Jin and Felsenfeld, 2007, This data was further supported by a genome-wide profiling of salt fractions performed by Henikoff and colleagues (Henikoff *et al*. 2007). In contrast, other studies suggest that the presence of H2A.Z slightly stabilized the octamer (Park *et al*. 2004) or that there were no difference in stability between NCPs containing H2A.Z/H3.1 and those containing H2A.Z/H3.3 during the salt increase (Thakar *et al*. 2009). Despite the controversial data, salt-labile nucleosomes remain of interest due to their instability to salt increases and possible role in priming regions for activation.

Fragile nucleosomes

Micrococcal nuclease (MNase) digestion is a method commonly used to isolate oligonucleosomes for analyses. However, there are many studies that revealed the presence

of “fragile nucleosomes,” which are nucleosomes that are unusually susceptible to digestion by MNase. These fragile nucleosomes, like salt-labile nucleosomes, have been found at promoters and enhancers of actively transcribed genes (Weiner *et* al. 2010; Xi *et al*. 2011; Ishii *et al*. 2015). Genome mapping in yeast revealed that these nucleosomes are enriched at promoters, whereas nucleosomes more resistant to micrococcal nuclease are in the mid-coding region (Weiner *et al*. 2010). Fragile nucleosomes also appear to have a role in poising genes for activation. In yeast, they are found enriched in a subset of Environmental-Stress-Response (ESR) genes. In optimally growing cells, fragile nucleosomes were found at ESR genes that are upregulated following stress induction; in heat stressed cells, fragile nucleosomes are instead found at ESR genes that are upregulated in optimal growth conditions (Xi *et al*. 2011). Furthermore, upon activation, fragile nucleosomes are more likely to be evicted rather than stay a fragile nucleosome or be converted into a mature canonical nucleosome (Xi *et al*. 2011). This eviction converts the site into a nucleosome free region and makes it more primed for DNA-utilizing processes to occur.

Because of their sensitivity to MNase and the common use of MNase to extensively digest and analyze chromatin, it is likely that fragile nucleosomes were under-represented or even missing in previous analyses of various chromatin properties. MPE-seq (methidiumpropyl-EDTA sequencing), a new method for the genome-wide characterization of chromatin, was developed to better differentiate between MNase sensitive and MNase resistant nucleosomes (Ishii *et al*. 2015). The use of MPE can detect subnucleosomal particles (101-140 bp DNA containing histones) immediately upstream of

the transcription start site, whereas high amounts of MNase cannot. Only a low amount of MNase can detect these particles (Ishii *et al*. 2015) suggesting that the subnucleosomal particles may be related to fragile nucleosomes observed previously.

The stability of nucleosomes is of interest due to their suspected role in regulating DNA-utilizing processes. Processes like replication, transcription, and DNA repair require nucleosome eviction, sliding, or disassembly; therefore, it would be ideal for nucleosomes to be primed for removal. Studies of salt-labile nucleosomes and fragile nucleosomes suggest that their presence at promoters may be to prime the region for activation.

Hg-bound nucleosomes

Hg-bound nucleosomes, originally discovered in rats, are non-nucleosomal chromatin particles that can bind to Hg(II)-resin (Chen and Allfrey, 1987, Chen *et al*. 1991; Chen-Cleland *et al*. 1993). Referred to as lexosomes by Allfrey, these nucleosomes are enriched at transcriptionally poised and active promoters, contain all four core histones, and have an exposed histone H3 cysteine 110 (H3C110) thiol group allowing Hg(II) affinity resin to bind to it (Allegra *et al*. 1987; Chen and Allfrey, 1987). Particles lacking this exposed thiol group at H3C110 do not bind. The specificity of this interaction was demonstrated by the analysis of this phenomenon in yeast *S. cerevisiae*. In wild type yeast, histone H3 alanine 110 (H3A110) was mutated to a cysteine to give H3C110 particles. Nucleosomes at transcriptionally poised and active promoters in wild type yeast (H3A110) did not bind to Hg(II) resin, whereas nucleosomes at transcriptionally poised and active promoters in the mutated yeast did bind (Chen *et al*. 1991; Chen-Cleland *et al*. 1993).

Both Hg-bound nucleosomes and prenucleosomes, which are discussed next, differ from canonical nucleosomes in structure. In addition to an exposed thiol group in Hg(II) bound nucleosomes, structural analysis revealed that these nucleosomes underwent a conformational change that is consistent with particle unfolding (Bazett-Jones *et al*. 1996). This structural difference may allow them to be more accessible to factors such as chemical reagents, transcription factors, and histone modifying enzymes.

Prenucleosomes

Prenucleosomes were originally discovered as a precursor to nucleosomes in the assembly of chromatin (Torigoe *et al*. 2011). Studies revealed that prenucleosomes contain all four core histones and are formed by the deposition of histones onto DNA by histone chaperone *Drosophila* NAP-1 (dNAP-1), and can be converted into mature canonical nucleosomes by ATP-dependent chromatin assembly motor protein such as ACF and CHD1. Prenucleosomes are formed within 15 seconds, lack dNAP-1, and are stably associated with the first DNA template they encounter for at least 2 hours.

When viewed by atomic force microscopy, prenucleosomes and nucleosomes both exhibit the same characteristic "beads-on-a-string" appearance (Torigoe *et al*. 2011). Although this characteristic makes it impossible to differentiate between the two species by atomic force microscopy, this identical morphology resolved a paradox from the 1970s. At that time, it was observed that nucleosome-like structures formed within seconds after DNA replication (McKnight and Miller, 1977). However, other findings demonstrated that mature canonical nucleosomes do not form until 10 to 20 minutes after replication (Worcel

et al. 1978). The discovery of the prenucleosomes and their formation within seconds can now explain this disagreement.

Prenucleosomes differ from nucleosomes in various ways. Prenucleosomes do not supercoil DNA like a nucleosome does and also associate with 80 bp of DNA, whereas nucleosomes associate with 147 bp of DNA (Torigoe *et al*. 2011; Fei *et al*. 2015). Prenucleosomes are also suspectible to acetylation at H3K56 by p300 relative to nucleosomes (Fei *et al*. 2015). Despite prenucleosomes being discovered in an *in vitro* context, investigating their characteristics will allow us to study prenucleosome-like structures in cells. A few studies have already indicated the presence of prenucleosome-like particles in cells (Ishii *et al*. 2015; Brown *et al*. 2013). Further studies will only aid in our understanding of their roles in chromatin

Summary

Although not fully understood, non-nucleosomal chromatin particles appear to play an important role in chromatin regulation. Those previously described *in vivo* are found at regions of active genes and are less stable than mature canonical nucleosomes, suggesting that they are primed for removal by other factors. This would ensure that processes occur rapidly in response to cellular changes. It would be interesting to study how structurally and functionally different they are from canonical nucleosomes. Do they contain histone variants? Are they enriched for specific epigenetic modifications? What factors are bound to them and where are they localized along the genome? For the second chapter of this dissertation, I sought to isolate Hg-bound nucleosomes in *Drosophila* by replicating experiments done in murine fibroblasts and comparing them to non-nucleosomal chromatin

particles previously described. I also sought to isolate acetylated H3K56 particles in both *Drosophila* and humans and determine their relationship to prenucleosomes. Unfortunately, however, I was not successful in these endeavors, and I will discuss the possible reasons why the experiments did not work as well as possible future directions.

For the third chapter of this dissertation, I sought to determine if other histone chaperones could form prenucleosomes or if prenucleosome formation was solely a property of *Drosophila* NAP-1 (nucleosome assembly protein-1). My work demonstrates that prenucleosome formation could occur with histone chaperone *Drosophila* NLP (nucleoplasmin-like protein) and that prenucleosomes formed by dNLP exhibited the same characteristics as those formed by dNAP-1.

Introduction to chromatin assembly methods

The natural state of the genome in the eukaryotic nucleus is in the form of chromatin and because chromatin is responsible for the regulation of many DNA-utilizing processes, it is ideal to study these processes in the context of chromatin. In the eukaryotic cell, chromatin assembly is an ATP-dependent process that is mediated by histone chaperones and an ATP-dependent chromatin assembly motor protein. Histone chaperones like NAP-1, CAF-1 (chromatin assembly factor-1), Np (nucleoplasmin), and NLP are responsible for the deposition of the histones onto DNA. A $(H3\text{-}H4)_2$ tetramer is first deposited onto DNA forming a tetrasome. Two H2A-H2B dimers are then deposited onto the tetrasome forming the octamer (see review Das *et al*. 2010). Chromatin assembly motor proteins like ACF (ATP-utilizing chromatin assembly and remodeling factor), CHD1 (chromo-ATPase/helicase-*DNA* binding domain-1), and RSF (remodeling and spacing factor) are

then responsible for wrapping the DNA around the histone-DNA complex and forming the mature canonical nucleosome.

There are a few methods to reconstitute chromatin *in vitro*, and each has its own advantages and disadvantages. The most commonly used method is the salt dialysis method and although this method produces pure chromatin (containing only histones and DNA), the nucleosomes are randomly distributed and do not resemble bulk native chromatin. The use of positioning sequences with the salt dialysis method produces regularly spaced nucleosomes (Chao *et al*. 1979, Simpson *et al*. 1983, Shrader *et al*. 1989, Lowary and Widom, 1989), but introduces the use of artificial sequences, which isn't ideal when studying DNA-utilizing processes.

Crude cell extracts are used to produce periodic nucleosome arrays on natural sequences, which resemble bulk chromatin (Becker *et al*. 1994; Sessa and Ruberti, 1990); however, complications can arise during future analyses from contaminants in the extract. To decrease possible contamination, a completely purified in vitro chromatin assembly system was developed using histone chaperone *Drosophila* nucleosome assembly protein 1 (dNAP-1), ATP-utilizing chromatin assembly and remodeling factor (ACF), and native *Drosophila* core histones (Fyodorov and Kadonaga, 2003). This system generates periodic nucleosome arrays on natural sequences with purified factors and has been used in several different applications (Torigoe *et al.* 2011; Allemand *et al*. 2016; Theisen *et al*. 2013; Torigoe *et al*. 2013; Emelyanov *et al*. 2012, Rattner *et al*. 2009, Sharma and Nyborg, 2008). However, the complexity of the expression and purification of the factors prevent many labs from using this system.

Histone chaperones

Originally called "molecular chaperones," histone chaperones were first characterized as proteins that prevented the formation of DNA-protein aggregates under physiological conditions (Laskey *et al*. 1978). There are several histone chaperones and they differ in sequence and structure (see selected reviews Philpott *et al*. 2000; Akey and Luger, 2003; Hammond *et* al. 2017), but share the same role in mediating histone-DNA and histone-histone interactions to help promote nucleosome formation. Some chaperones preferentially bind H2A-H2B dimers like NAP-1 and Np (Ishimi *et al*. 1987; Laskey *et al*. 1978; Earnshaw *et al*. 1980; Dilworth *et al*. 1987), whereas other chaperones preferentially bind H3-H4 tetramers like N1/N2, CAF-1,and ASF1 (Philpott *et al*. 2000; Kleinschmidt *et al*. 1986; Smith and Stillman, 1991; Tyler et al. 1999). Some chaperones specifically bind S-phase dependent histones like nucleoplasmin and N1/N2 (Kleinschmidt *et al*. 1985, Dilworth *et al*. 1987), whereas other chaperones bind histone variants like FACT and HIR-A (Heo *et al*. 2008; Ray-Gallet *et al*. 2002; Tagami *et al*. 2004). Some chaperones are specific for DNA-utilizing processes. For instance, histone chaperones ASF1 and CAF-1 bind to newly synthesized histones H3 and H4 (Verreault *et al*. 1996; Tyler *et al*. 1999) and are localized at DNA replication forks (Krude, 1995; Schulz and Tyler, 2006) suggesting a role for them in the deposition of histones after replication. Studies show that many histone chaperones have redundant functions, whereas others have more specialized roles. Select histone chaperones and their roles in chromatin regulation will be discussed below.

Nucleoplasmin, the most abundant protein in the *Xenopus* oocyte nucleus, was the first identified histone chaperone and is often considered the archetype of histone chaperones (Laskey *et al*. 1978; Dingwall and Laskey, 1990). Nucleoplasmin was originally discovered as a thermostable protein that co-purifies with chromatin assembly activity (Laskey *et al*. 1978). It has a pentameric structure (Earnshaw *et* al. 1980; Dingwall *et al.* 1982) and long polyglutamic acid tracts, which make it an ideal candidate for histones to bind to, specifically H2A/H2B (Laskey *et al*. 1978; Kleinschmidt *et al*. 1985; 1990; Dilworth *et al*. 1987). Nucleoplasmin also appears to have a more specialized role in eggs and early embryos; it decondenses sperm chromatin upon fertilization by replacing the sperm nuclear basic proteins (X and Y) found in chromatin with H2A-H2B dimers (Philpott and Leno, 1992; Leno *et al*. 1996). It also promotes transcription factor binding to nucleosomes by depleting H2A-H2B dimers upon the binding of multiple Gal4-AH subunits (Chen *et al*. 1994).

Since the discovery of nucleoplasmin, several other histone chaperones have been identified (Figure 1.1) including N1/N2, NLP (nucleoplasmin-like protein), and NAP-1 (nucleosome assembly protein-1); many of them share characteristics similar to nucleoplasmin. For instance, like nucleoplasmin, the aforementioned chaperones all contain polyacidic tracts (Kleinschmidt *et al.* 1986; Namboodiri *et al*. 2003; Ito *et al*. 1996a), which may aid in the binding of histones. N1/N2 also has a specialized role in eggs and early embryos (Kleinschmidt *et al*. 1986), but differs from nucleoplasmin in that it binds to $(H3\text{-}H4)_2$ tetramers *in vivo* (Kleinschmidt *et al.* 1982; 1985; Dilworth *et al*. 1987). This characteristic complements nucleoplasmin's ability to bind to H2A-H2B dimers and

both chaperones are shown to function together in the cell to assemble nucleosomes (Dilworth *et al*. 1987; Zucker and Worcel, 1990). *Drosophila* NLP (dNLP) also known as CRP1 and p22 resembles nucleoplasmin in both sequence (31%) and function (Kawasaki et al. 1994; Crevel et al. 1997; Ito *et al*. 1996b). It is pentameric in structure and is thermostable (Namboodiri *et al*. 2003). It differs from both nucleoplasmin and *Drosophila* NAP-1 (dNAP-1) in that it cannot decondense sperm chromatin and does not stably associate with histones in *Drosophila* embryos (Ito *et al*. 1996b). It has, however been shown to bind to all four core histones *in vitro* and aids in the assembly of chromatin (Ito *et al*. 1996b).

NAP-1 was the first identified chaperone in mammalian cells (hNAP-1; Ishimi *et al*. 1984). Since then, homologues of NAP-1 have been identified in several other organisms including yeast (yNAP-1; Ishimi and Kikuchi, 1991), *Drosophila* (dNAP-1; Ito *et al*. 1996a), and amphibians (Steer *et al*. 2003). Like nucleoplasmin, all NAP-1 homologues preferentially bind H2A-H2B dimers relative to (H3-H4)2 tetramers (Ishimi *et al*. 1987; Ito *et al*. 1996a). They are also able to substitute for one another in the assembly of chromatin *in vitro* (Ito *et al*. 1996a). dNAP-1 decondenses sperm chromatin (Ito *et al*. 1996a) and there are several studies implicating NAP-1 in transcription (Walter *et al*. 1995; Zlatanova *et al*. 2007).

ATP-dependent chromatin assembly factors

Chromatin assembly (the assembly of periodic nucleosome arrays) was first observed to be an ATP-dependent process in *Xenopus* oocyte extract by Worcel and colleagues (Glikin *et* al. 1984). Further characterization of the histone chaperones revealed

that they do not hydrolyze ATP, which suggested the presence of ATP-utilizing assembly factors, which were yet to be discovered. This led to the discovery of the first ATP-dependent chromatin assembly factor, ACF (ATP-utilizing chromatin assembly and remodeling factor) in *Drosophila* embryo extract (Ito et al. 1997). ATP-dependent chromatin assembly factors are responsible for the conversion of histone-DNA complexes into nucleosomes and for also positioning them into periodic arrays.

Since the discovery of ACF, several other chromatin assembly factors including CHRAC (chromatin accessibility complex), RSF (remodeling and spacing factor), and CHD1 (chromo-ATPase/helicase-DNA-binding protein 1) have been identified and characterized (Figure 1.2) (Varga-Weisz et al., 1997; LeRoy *et al*. 2000; Loyola et al., 2001; Loyola et al., 2003; Lusser *et al*. 2005; Konev *et al*. 2007). These assembly factors all belong to the SNF2-family of proteins, which is characterized by a conserved ATPase domain and includes all known ATP-dependent chromatin remodeling factors (see select reviews: Clapier and Cairns, 2009; Tyagi *et al*. 2016). CHD1 acts as a monomer, whereas ACF, CHRAC, and RSF are in complexes containing the nucleosome stimulated ATPase ISWI (imitation switch) (Lusser *et al*. 2005; Ito *et* al. 1997; Varga-Weisz *et al*. 1997; LeRoy *et al*. 1988). Like other members of the SNF2-family, these chromatin assembly factors are also able to remodel nucleosomes (Ito *et al*. 1997; Eberharter *et al*. 2001; LeRoy *et al*. 2000; Lusser *et al*. 2005; LeRoy *et al.* 1998); however, not all chromatin remodelers can assemble chromatin.

ISWI, the ATPase found in three chromatin assembly factors, is also the catalytic subunit for many chromatin remodeling factors (see select reviews Längst and Becker,

2001; Corona and Tamkun, 2004). Although it has never been isolated alone in a native source following a functional assay, it has been shown to function both as a chromatin assembly and remodeling factor on its own (Corona *et al*. 1999). It is able to facilitate binding of transcription factors to chromatin (Corona *et al*. 1999). ISWI is known to exhibit characteristics similar to the ISWI-containing complexes.

ACF, the first characterized ATP-utilizing assembly factor, was found to assemble periodic arrays of nucleosomes with the help of histone chaperone dNAP-1 (Ito *et al*. 1997; LeRoy *et al*. 2000). It consists of polypeptide Acf1 and ATPase ISWI, which synergistically function to efficiently assemble chromatin (Ito *et al*. 1999). RSF, another assembly factor, was originally discovered in humans by its nucleosome remodeling activity, which allowed the RNA polymerase II transcription initiation complex to form on a chromatin template (LeRoy *et al*. 1998). Like ACF, RSF consists of two subunits, polypeptide Rsf-1 and its ISWI homologue hSNF2H. It differs from both ACF and ISWI in that it does not require a histone chaperone to assemble chromatin (Loyola *et al*. 2001). CHD1, an assembly factor that does not contain ISWI, was first discovered as a protein that contained a chromodomain-helicase-DNA-binding domain (Delmas *et al*. 1993). Despite not containing ISWI, CHD1 exhibits characteristics similar to that of ACF. Like ACF, CHD1, mediates the transfer of histones onto DNA in an ATP-dependent manner and assembles nucleosomes in a processive manner. It differs from ACF in that it cannot assemble chromatin with linker histone H1 (Lusser *et al*. 2005).

Not only do these factors assemble chromatin, but many of them have roles in regulating genomic processes. For instance, CHD1 promotes sperm decondensation by

helping incorporate histone variant H3.3 into chromatin (Konev *et al*. 2007). An Acf1-SNF2H (ISWI homologue) complex in humans promotes DNA replication in heterochromatin and both RSF and ACF facilitate transcription on chromatin templates (Collins *et al*. 2002; LeRoy *et al*. 1998; Ito *et al*. 1997; Levenstein and Kadonaga, 2002; LeRoy *et al*. 2000). Interestingly, evidence suggests that many ISWI-containing complexes not only regulates transcription activation but also repression (see review Corona and Tamkun, 2004). In *Drosophila* salivary gland polytene chromosomes, ISWI is found associated at hundreds of euchromatic sites, but its binding pattern does not generally overlap with RNA polymerase II (Deuring *et al*. 2000) suggesting a more general repressive role for ISWI. In addition, Isw2, one of two ISWI homologues in yeast, can counteract activation by sliding nucleosomes to specific positions and displace bound activators (Kassabov *et al*. 2002).

Summary

The regulation of DNA-utilizing processes is complex. It is further complicated by the fact that DNA is in chromatin form. To better understand how DNA-utilizing processes are regulated by chromatin structure, we must develop a simpler method to reconstitute chromatin and that chromatin should resemble bulk chromatin observed in the eukaryotic nuclei. Our previous method of assembling chromatin (Fyorodov and Kadonaga, 2003) generated periodic arrays of nucleosomes on natural sequences, but the complexity in the expression and purification of the factors involved prevented many labs from using the method. In chapter four of this dissertation, I sought to develop a simplified system that would enable a broader range of scientists to assemble chromatin. All the factors involved

in this system are expressed solely in bacteria and are more easily purified relative to the previous system. This new system will allow more scientists the opportunity to generate customized chromatin and study its structure and function due to its short time investment, low expression costs, and high protein yields.

Chaperones	Histones	Key References
NAP-1	H2A-H2B	Ishimi *et al*. 1983; Ishimi *et al*. 1984; Ishimi *et al*. 1987; Ito *et al*. 1996a
Nucleoplasmin	H2A-H2B	Laskey *et al*. 1978; Earnshaw *et al*. 1980; Dilworth *et al*. 1987; Dingwall *et al*. 1982
FACT	H2A.Z-H2B	Heo *et al*. 2008
ASF1	H3-H4	Tyler *et al*. 1999
CAF-1	H3-H4	Smith and Stillman, 1989; Smith and Stillman, 1991
N1/N2	H3-H4	Kleinschmidt *et al*. 1982; 1985; Dilworth *et al*. 1987
Rtt106	H3-H4	Huang *et al*. 2005
HIRA	H3.3-H4	Ray-Gallet *et al*. 2002; Tagami *et al*. 2004

Figure 1.1 A partial list of known histone chaperones and their histone targets

Name	Functions	Subunits	Key References
ACF	Transcriptional regulation DNA replication	Acf1, ISWI	Ito *et al.* 1997; Ito *et al*. 1999; LeRoy *et al*. 2000
CHRAC	DNA replication	Acf1, p14, p16, ISWI	Varga-Weisz *et al*. 1997; Alexiadis *et* al. 1998; Ito *et al*. 1999; Corona *et al*. 2000
RSF	Transcriptional regulation	Rsf-1, ISWI	LeRoy *et al*. 1988; Loyola *et al*. 2001; Loyola *et al*. 2003
CHD1	Sperm decondensation Transcriptional regulation	CHD1	Delmas *et al*. 1993; Lusser *et al*. 2005; Konev *et al*. 2007

Figure 1.2 A partial list of known ATP-dependent chromatin assembly factors

CHAPTER 2:

Methods Used to Study

H3C110 Particles and Acetylated H3K56-Containing Particles

Abstract

Several methods were used to study both the H3C110 particles and acetylated H3K56-containing particles. Affi-Gel 501 and alternative Hg(II) resins were used to isolate H3C110 particles in *Drosophila* embryos, but the project was ultimately discontinued due to a lack of enrichment for active chromatin particles as assessed by monitoring specific protein markers. Antibodies specific against acetylated H3K56 were used to target acetylated H3K56-containing particles in *Drosophila* embryos, S2 cells, as well as HeLa S3 cells, but the project was also terminated due to a lack of enrichment for active chromatin particles. Despite the termination of both projects, many lessons were learned and future directions are discussed.

Introduction

Recent data suggest that non-nucleosomal chromatin particles have an important role in regulating DNA-utilizing processes. Hg(II)-bound nucleosomes, which will be referred to as H3C110 particles, have an exposed H3C110 residue and are found enriched at transcriptionally poised and active promoters, suggesting that they have some role in promoting transcription initiation (Chen and Allfrey, 1987; Allegra *et al*. 1987; Chen *et al*. 1991; Chen-Cleland *et al*. 1993). Further analysis of the particle revealed that it undergoes a conformational change consistent with particle unfolding (Bazett-Jones *et al*. 1996). Not much else is known about H3C110 particles, and it is unclear how they relate to more recently described non-nucleosomal chromatin particles. Investigating the H3C110 particles and its protein composition, associated proteins, and occurrence within the cell

will likely shed light on their possible role in transcription activation as well as give insight into its relationship with other non-nucleosomal chromatin particles.

The prenucleosome is another non-nucleosomal chromatin particle of interest. It was originally discovered in an *in vitro* context and characterization of it revealed a non-nucleosomal chromatin particle that associates with 80 bp of DNA and is susceptible to acetylation at H3 K56, whereas the nucleosome is not (Torigoe *et al*. 2011; Khuong *et al*., 2015). Recent studies have suggested the presence of particles like the prenucleosome in cells (Khuong *et al*. 2015; Ishii *et al*. 2015) and it would be interesting to isolate these particles in cells. As mentioned, prenucleosomes differ from nucleosomes in that they are able to be acetylated at H3 K56. This is interesting because acetylated H3K56 is present in newly-synthesized H3 and is deposited into chromatin during DNA replication (Han *et al*. 2007; Li *et al*. 2008; Masumoto *et al*. 2005). H3K56ac has also been observed at transcriptionally active promoters and enhancers, again suggesting a role in priming transcription activation (Lo *et al*. 2011; Venkatesh *et al*. 2012). Characterization of the H3K56ac particles in cells could possibly give prenucleosomes an *in vivo* context as well as provide insight as to its possible role in DNA-utilizing processes.

Results

Targeting H3C110 in core histones using alternative Hg-affinity resins

Allfrey and colleagues originally used Affi-Gel 501 (Bio-Rad) resin to isolate nucleosomes that were found at transcriptionally active genes (Chen and Allfrey, 1987, Chen *et al*. 1991; Chen-Cleland *et al*. 1993). Unfortunately, the resin has since been

discontinued and attempts to replicate the resin's properties were made. Two separate attempts were made to replicate the resin. Using Bio-Rad's protocol, p-aminophenylmercuric acetate was coupled to Affi-Gel 10 to create a resin that resembled Affi-Gel 501. 4-(hydroxmercuri)benzoic acid sodium salt was also coupled to amino ethyl magnetic agarose beads to create a magnetic Hg(II) based resin that resembled the organomercurial-agarose resin used in Chen-Cleland *et al* 1993. Modifications were also made to both resins to try and optimize H3C110 elution once the particles were bound.

Many attempts were made to bind H3C110 particles. Core histones were added to the resin to determine the specificity of the resin. Approximately 1 µg of core histones were added 50 µL of prepared resin and incubated on a rotary mixer/nutator at room temperature for 1 h. The resin was then washed with buffer A (10 mM K^+ Hepes, pH 7.6 containing 1 mM EDTA, 10% glycerol) with 0.5 M NaCl to remove non-specific binding. The resin was then washed with buffer A containing 8 M urea to denature the core histones followed by elution with buffer A containing 25 mM DTT. Results were inconclusive. With resin that contained irreversible binding sites, elution of the core histones with DTT were not possible. With resin that had its irreversible binding sites blocked, the core histones would non-specifically elute in the salt wash. The use of alternative Hg-affinity resins was discontinued due to this lack of elution or non-specific elution and efforts were directed towards targeting H3C110 particles using other thiol-specific reagents.

Targeting H3C110 in Drosophila embryos using Affi-gel 501

A limited amount of Affi-Gel 501 was available and used as a last attempt to target H3C110 particles in *Drosophila* embryos. 2.5 g previously frozen dechorionated embryos were resuspended in 10 mL E-lysis buffer (25 mM K^+ Hepes, pH 7.6 containing 10 mM NaCl, 3 mM $MgCl_2$, 0.01% NP-40, 5 mM sodium butyrate, and 0.1 mM PMSF) and homogenized with a Wheaton Dounce homogenizer (tight pestle) 20 times. The suspension was subjected to centrifugation (10,000 x g, 10 min, room temperature). The pellet was resuspended in 1 mL buffer B (25 mM K^+ Hepes, pH 7.6 containing 25 mM NaCl, 25 mM KCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM sodium butyrate, and 0.1 mM PMSF) and digested with 1 unit micrococcal nuclease for 10 min. Reactions were stopped with a final concentration of 5 mM EGTA. The supernatant was then added to a 0.5 mL Affi-Gel 501 column (pre-equilibrated with buffer B with 5 mM EDTA). The column was washed with buffer B with 5 mM EDTA until $A260_{nm}$ readings returned to background and then washed with buffer B containing 0.2 M NaCl and 5 mM EDTA until $A260_{nm}$ readings returned to background. Finally, H3C110 particles were eluted with buffer B containing 10 mM DTT and 5 mM EDTA. Preliminary data indicated that two histone-containing peaks were detected (data not shown), but further analysis did not reveal any enrichment for specific factors in the eluate (H2A.v, H1, H3K56ac). It was concluded that Affi-Gel 501 was able to pull down Hg(II)-bound nucleosomes, but that it lacked factors that are characteristic of non-nucleosomal chromatin particles.

Targeting H3C110 particles in Drosophila embryos using thiol-specific reagents

Thiol-specific reagents, such as EZ link™ Maleimide-PEG_2- Biotin and fluorescein-5-maleimide (Thermo Fisher Scientific; #21901BID and #62245) were first used to determine whether the H3C110 residue was accessible. Core histones were successfully labeled with both fluorescein and biotin indicating that these reagents could target the cysteine on histone H3. Treatment of *Drosophila* nuclei with fluorescein yielded low signals; therefore, treatment of nuclei was mainly done with biotin-tagged maleimides. Multiple approaches were attempted to enrich for the H3C110 particles and the project was ultimately discontinued due to lack of enrichment for specific factors in the eluate.

Targeting H3K56ac octamers in Drosophila embryos, S2 cells, and HeLa S3 cells

The prenucleosome is an intermediate to the nucleosome that was originally discovered during the study of chromatin assembly (Torigoe et al 2011). Its existence *in vivo*, however, remains to be investigated. To try and give prenucleosomes an *in vivo* context, I attempted to immunoprecipitate acetylated H3K56 (H3K56ac) particles in *Drosophila* embryos, S2 cells, and HeLa S3 cells and compare them to prenucleosomes, which have been previously demonstrated to be susceptible to acetylation at H3K56 by p300 (Fei et al 2015). The project was ultimately terminated due to either a lack of enrichment of H3K56ac particles and/or a lack of enrichment of specific post translational modifications and proteins.

Discussion and Lessons Learned

Several attempts were made to isolate nucleosomes and specifically target H3C110 particles. Alternative Hg(II)-affinity resins were made to resemble Affi-Gel 501 in hopes of replicating experiments originally performed by Allfrey and colleagues (Chen and Allfrey, 1987, Chen *et al*. 1991; Chen-Cleland *et al*. 1993). Unfortunately, those resins did not appear to have the same histone binding characteristics as Affi-gel 501. Preliminary experiments with Affi-Gel 501 yielded two histone-containing peaks, which resembled those seen in Allfrey's papers, but due to a limited quantity of the resin, further investigation was not conducted.

The use of thiol-specific reagents to target H3C110 particles was a suitable alternative, but ultimately the project was discontinued due to a lack of enrichment for active chromatin particles as assessed by monitoring specific protein markers. This could be due to a few reasons. The particles may not be as abundant in the cell making them difficult to enrich for. Also, even though attempts were made at digesting the nuclei with low amounts of micrococcal nuclease, the nuclei may have still been overdigested resulting in a loss of H3C110 particles. Overdigestion may also have caused proteins associated with the H3C110 particle to dissociate, particularly those that bind to linker DNA. To prevent the loss of H3C110 particles during micrococcal nuclease digestion, the order of biotinylation and digestion were swapped. However, when biotinylation occurred prior to digestion, there was the concern that the maleimide was unable to access H3C110 particles due to crowding. This led to the use of maleimide-PEG_{11}-biotin, which has a longer linker. Unfortunately, results were the same with the longer linker.

There were also issues regarding the purification of nucleosomes. After biotinylation, sucrose gradient sedimentation or size exclusion column chromatography were implemented to remove excess biotin as well as contaminants that were also labeled. The fractionation of the sample diluted the sample, which made the proteins difficult to detect in an SDS-PAGE by simple staining by Coomassie Brilliant Blue R-250. In addition, despite observing a relatively clean sample of core histones by SDS-PAGE analysis, it was clear by detection of Cy3-streptavidin that this was not the case. It is possible that the proteins detected did in fact bind to the H3C110 particles, but it was more likely that these proteins contained thiol groups and were also biotinylated. The use of streptavidin-HRP (horseradish peroxidase) also yielded extremely dirty blots and it is not advisable to use HRP for these blots. The use of ^{32}P-labeled Protein A was substituted in lieu of a secondary antibody and yields much cleaner signals. It is also much more sensitive. A standard method was used to purify the nucleosomes (Figure 2.1), but steps were often rearranged to address concerns previously mentioned.

Similar purification issues arose for the isolation of H3K56ac particles. Both salt and MNase concentrations were varied in an attempt to optimize immunoprecipitations. The lack of enrichment for H3K56ac particles could be due to the low abundance in the cell, particularly in humans (Drogaris *et al.* 2012). In addition to testing our own generated antibodies, commercially made antibodies specific for H3K56ac were also used for the immunoprecipitations. Unfortunately, it has been revealed that many of the commercial H3K56ac antibodies available are non-specific in mammalian cells and fly tissues (Pal *et al*. 2016). H3K9ac, which is 800 fold more abundant in the cell relative to H3K56ac and

shares three identical neighboring residues, is also detected with many of these H3K56ac specific antibodies (Drogaris *et al*. 2012). This could lead to false positive results in IPs and westerns.

Despite the termination of this project, non-nucleosomal chromatin particles are likely to be biologically important and efforts should be continued to study them in cells. Maleimides were primarily used to target the exposed thiol group in H3C110 particles, but it is possible that other thiol-specific reagents like iodoacetamides may be better at targeting the thiol in cells. It could also be that the factors we looked for as an indicator of enrichment of active chromatin were incorrect. Regarding the isolation of H3K56ac particles, it is inadvisable to continue trying to isolate the particles with antibodies. It would, however, be interesting to enrich first for active chromatin using methyl-specific restriction enzymes and perform mass spectrometry on the particles found rather than specifically target a non-nucleosomal chromatin particle.

Materials and Methods

Preparation of alternative Hg-affinity resin by ST

Affi-Gel 10 (Catalog #1536099; Bio-Rad) was reacted with p-aminophenylmercuric acetate in isopropyl alcohol/dimethylformamide using a published protocol in BioRad's product FAQ to form Hg-affinity resin that resembles Affi-Gel 501. 25 mL Affi-Gel 10 was filtered and washed with 100 mL anhydrous isopropanol. 0.375 g of p-aminophenylmercuric acetate was dissolved in 7.5 mL dimethylformamide and mixed with Affi-Gel 10 on a rotary mixer/nutator at room temperature for 4 h. 250 uL of

ethanolamine was added and mixed on a rotary mixer/nutator at room temperature for 1 h to block unreacted succinimide groups. Gel slurry was filtered and washed with 75 mL of dimethylformamide followed by 200 mL anhydrous isopropanol. Gel was resuspended in anhydrous isopropanol and stored at 4°C.

A modified version of the resin where irreversible binding sites from the resin were blocked was prepared as follows: 1.5 mL of the resin was poured into a column and washed with 10 mL ddH_20. The column was then washed with 5 mL 50 mM Tris, pH 8.0. 1 mL 2.5 mM β mercaptoethanol was loaded onto the column five times with a 15 minute wait in between each load. The column was further washed with 5 mL 50 mM Tris, pH 8.0; 5 mL ddH_20; 2 mL 50 mM MES, pH 4.8 twice; 1 mL $HgCl_2$ (in 10 mM MES, pH 4.8) five times with a 10 min wait in between each wash; 2 mL 50 mM MES, pH 4.8 five times; 10 mL ddH20; and 10 mL 50 mM Tris, pH 8.0. The resin was stored in 10 mL 40 mM K+ Hepes, pH 7.8 containing 0.02% NaN3.

Preparation of alternative Hg-affinity resin by GK

Amino ethyl magnetic agarose beads (BioScience bead division) was reacted with 4-(hydroxymercuri) benzoic acid sodium salt to form p-mercuribenzoyl magnetic agarose beads. 4 mL beads was filtered and washed with 15 mL ddH_20 four times. Beads were then washed with 10 mL 150 mM MES, pH 4.8 two times. Beads were left to settle and supernatant was removed. 4 mL 40% dimethylformamide was added followed by 125 mg 4-(hydroxymercuri) benzoic acid sodium salt to give a final concentration of 43 mM. pH was adjusted to 4.8 with 1 N H HCl. 155 mg N-(3-dimethylaminopropyl)-N’ ethyl

carbodiimide HCl was added. pH was checked before slurry was mixed on rotary mixer/nutator at room temperature overnight. Slurry was transferred to a Kontes column (1.5 x 10 cm) and washed with 10 mL ddH_20 three times; 10 mL 100 mM $NaHCO_3$, pH 8.5 two times; 80 mL 100 mM $NaHCO_3$, pH 8.5; and 20 mL 50 mM K^+ Hepes, pH 7.8. Slurry was stored in 40 mM K^+ Hepes, pH 7.8 containing 0.2% NaN_3. A modified version of this resin with the irreversible binding sites blocked was also prepared using a previously described method.

Thiol-specific reagents

The following thiol-specific reagents from Invitrogen were used to target H3C110 particles: fluorescein-5-maleimide (#62245), 5-iodoacetamido-fluorescein (#62246), maleimide-PEG_2-biotin (#21902BID), and maleimide-PEG_{11}-biotin (#21911). Fluorescent reagents were resuspended in F-buffer (50 mM K^+ Hepes, pH 7.6, containing 5 mM $MgCl_2$, 0.1 mM EDTA, 0.01% NP-40, and 5% glycerol) prior to use. Biotin-tagged reagents were resuspended in buffer BIO (30 mM K^+ Hepes, pH 7.0, 4 mM $MgCl_2$, 60 mM KCl, 1 mM $CaCl_2$, 0.01% NP-40, 0.25 mM PMSF, and 0.5 μg/mL leupeptin) with 15 mM NaCl prior to use. Modifications to buffer BIO were made in an attempt to optimize labeling of particles. Varying amounts of $MgCl_2$ (0 to 4 mM), NaCl (10 to 300 mM), and KCl (0 to 60 mM) were tried. All modifications didn't affect the labeling of thiol groups. Reactions were more effected by pH changes.

Preparation of oligonucleosomes from Drosophila embryos

5 g dechorionated embryos were resuspended in 20 mL buffer C110 (30 mM K^+ Hepes, pH 7.0, 3 mM $MgCl_2$, 1 mM $CaCl_2$, 0.01% NP-40, 0.25 mM PMSF, 0.5 μg/mL leupeptin, 10 mM sodium butyrate) containing 25 mM NaCl. Resuspension was homogenized on ice using a Wheaton dounce (tight pestle) before being subjected to centrifugation (Fiberlite F21S-8x50y rotor; 3,000 rpm for 10 min, 4°C). The pellet was then resuspended in 2 mL buffer C110 containing 25 mM NaCl. 2 uL or 10 uL 1000 UN/mL of MNase (experimentally pre-determined) was added to resuspension and incubated for 10 min at 37°C. Reaction was stopped with a final concentration of 12.5 mM EDTA. The mixture was then subjected to centrifugation (microcentrifuge; 2500 rpm, 5 min). The supernatant contains the oligonucleosomes.

Isolation of H3C110 particles in Drosophila embryos

The oligonucleosomes were then treated with 1.5 mM maleimide-biotin to acquire a final concentration of 30 uM and incubated at 22°C for 1.5 h on a rotary mixer/nutator. 1 M β-mercaptoethanol was added to a final concentration of 10 mM and incubated at 22°C for 10 min on a rotary mixer/nutator to quench the reaction. (The labeling of nucleosomes was sometimes done prior to digestion by micrococcal nuclease). Histone-containing particles were then purified by one of two ways. The sample could have been loaded onto a 15-45% sucrose gradient (in TE' containing 15 mM NaCl) and subjected to ultracentrifugation (SW55 rotor; 32k rpm, 16 h, 4°C) or the sample could have been subjected to 1 mL sephadex G-100 column in buffer C110 (without $CaCl_2$) containing 50

mM NaCl. Histone-containing particles were then consolidated and treated with streptavidin beads (Thermo Fisher Scientific; #65305 or #20349) and incubated at 22°C for 1.5 h. The beads were washed with buffer S (30 mM K^+ Hepes, pH 7.0, containing 3 mM $MgCl_2$, 0.01% NP-40) with 500 mM NaCl before eluting with sample buffer. Samples were analyzed by western using streptavidin-HRP antibodies (Jackson Immuno; #016-030-084) or Cy3-Streptavidin (Jackson Immuno; #016-160-084).

Isolation of H3K56ac particles in Drosophila embryos

The preparation of oligonucleosomes from *Drosophila* embryos was performed as previously stated except that buffer C110 should be substituted with buffer K56 (30 mM K^+ Hepes, pH 7.6, containing 3 mM $MgCl_2$, 1 mM $CaCl_2$, 0.05% NP-40, 0.5 μg/mL leupeptin, 0.5 μg/mL aprotinin, 0.5 μg/mL pepstatin A, 1 mM DTT, 0.25 mM PMSF, 1 mM sodium metabisulfite, 1 mM benzamidine, and 10 mM sodium butyrate) with 25 mM NaCl. The oligonucleosomes were then loaded onto a 14 mL sephadex G-100 column (in buffer K56 containing 100 mM NaCl) and then washed with buffer K56 containing 100 mM NaCl. 500 uL fractions were taken and the peak DNA-containing were consolidated for treatment with anti-H3K56ac. Antibodies were incubated with the sample for 5 h at 4°C on a rotary mixer/nutator. Protein A sepharose beads were then added to the sample and incubated overnight at 4°C on a rotary mixer/nutator. The following day, the beads were spun down and washed multiple times with buffer K56 without $MgCl_2$. H3K56ac particles were eluted with sample buffer. Samples were analyzed by western using various

antibodies (HMG-D, HP1, H1, H2A.V, TBP, su(var)3-9, H3K4me1, H3K4me3, H3K27me3, H3K27ac, H3K9me2, H3K56ac) and ^{32}P-labeled Protein A.

Isolation of H3K56ac particles in S2 cells and HeLa S3 cells

The preparation of oligonucleosomes was adapted from Jin and Felsenfeld, 2007. Cells were grown to confluency and subjected to centrifugation (2k rpm, 4°C, 5 min). Cells were then washed with 1X cold PBS and then resuspended in C-lysis buffer (10 mM Tris, pH 7.5 containing 3 mM $MgCl_2$, 10 mM NaCl, 0.4% NP-40). C-lysis buffer and all following buffers were supplemented with 0.25 mM PMSF, 0.5 μg/mL leupeptin, 0.5 μg/mL aprotinin, 0.5 μg/mL pepstatin A, 10 mM sodium butyrate, 1 mM DTT, 1 mM sodium metabisulfite, 1 mM benzamidine, 100 ng/mL FR901228, and 0.5 mM dihydrocoumarin. Cells were incubated on ice for 10 minute before being passed through a 22G needle and subjected to centrifugation (2k rpm, 5 min, 4°C). Supernatant was removed and nuclear pellet was resuspended in C-resupension buffer (10 mM Tris, pH 7.5, containing 15 mM NaCl, 60 mM KCl, 0.15 mM spermine, and 0.15 mM spermidine). MNase (an experimentally pre-determined amount) was added to the nuclei and incubated for 10 min at room temperature. Digestion was stopped by the addition of EGTA to a final concentration of 20 mM and subjected to centrifugation (2300 rpm, 5 min, 4°C).

The oligonucleosomes were then incubated with antibodies for 5 h at 4°C on a rotary mixer/nutator. Protein A sepharose beads were then added to the sample and incubated overnight at 4°C on a rotary mixer/nutator. The following day, the beads were spun down and washed multiple times with C-wash buffer (10 mM Tris, pH 7.5, containing

100 mM NaCl, 1 mM EDTA, and 0.05% NP-40) before being eluted with sample buffer. Samples were analyzed by western using various antibodies and p32-labeled Protein A.

Batch Purification of H3K56ac Antibodies from Serum using Bakerbond

Antibodies specific against H3K56ac were generated using Pocono Rabbit Farm and Laboratory, Inc (PRF&L) in rabbits #31573 and #31576. The serum was either left as is or purified using different methods (Figure 2.2) and then used for immunoprecipitation. Different denaturing and non-denaturing conditions were tested to determine efficacy of elution (Figure 2.3).

For every 7.5 mL of serum, hydrated 1 g of Bakerbond Abx resin (JT Baker Co, Cat #7269) with 12.5 mL 25 mM MES K^+, pH 5.6 for 10 min. The resin was then washed twice with 12.5 mL 25 mM MES K^+, pH 5.6 and left in 0.5 mL 25 mM MES, K^+, pH 5.6 until serum is prepared. Serum was filtered through a syringe filter (0.22 μm) into a SS-34 tube. Then 5.2 mL 4M ammonium sulfate, pH 7.0 (60% saturation) was added dropwise, while being vortexed. The sample was incubated on ice for 30 min and subjected to centrifugation (Fiberlite F21S-8x50y rotor; 10,000 rpm for 10 min, 4°C). The ammonium sulfate pellet was then resuspended in 10 mL 25 mM MES K^+, pH 5.6. The bakerbond slurry was added to the precipitated serum and mixed on a rotary mixer/nutator for 10 min at room temperature. Most of the supernatant was discarded before being transferred to an Econo-Pac chromotagraphy column (Bio-Rad #7321010). The column was washed with 25 mL 25 mM MES K^+, pH 5.6 and the antibodies were eluted with 0.5 M ammonium sulfate (fraction size: 5 mL). Peak protein concentration fractions were pooled and 1.2

mL/mL 4 M ammonium sulfate was added while vortexing. Sample was incubated on ice for 30 min and subjected to centrifugation (Fiberlite F21S-8x50y rotor; 10,000 rpm for 10 min, 4°C). Pellet was resuspended in 5 mL 1X PBS, dialyzed (molecular weight cutoff: 3500 Da) twice against 2 L 1X PBS, containing 35% glycerol for 2 h each, and once overnight at 4°C. Antibodies were stored at -80°C.

Affinity Purification of H3K56ac Antibodies from Serum

Unacetylated H3K56 or acetylated H3K56 peptides were coupled to cyanogen bromide-activated sepharose 4B (GE Healthcare Life Sciences #17-0430-01) and poured into a 1 mL column. Elution was performed using low pH (100 mM glycine, pH 2.5). Antibodies eluted from the sepharose-H3K56ac peptide column were labeled H3K56ac AP (mod) whereas antibodies that flowed through the sepharose-H3K56 peptide column were labeled H3K56ac AP (unmod). A cleaner preparation of the serum was performed by first subjecting the serum to the sepharose-H3K56ac peptide column. The eluate is then loaded onto the sepharose-H3K56 peptide column and the flow through is saved for use (H3K56ac clean).

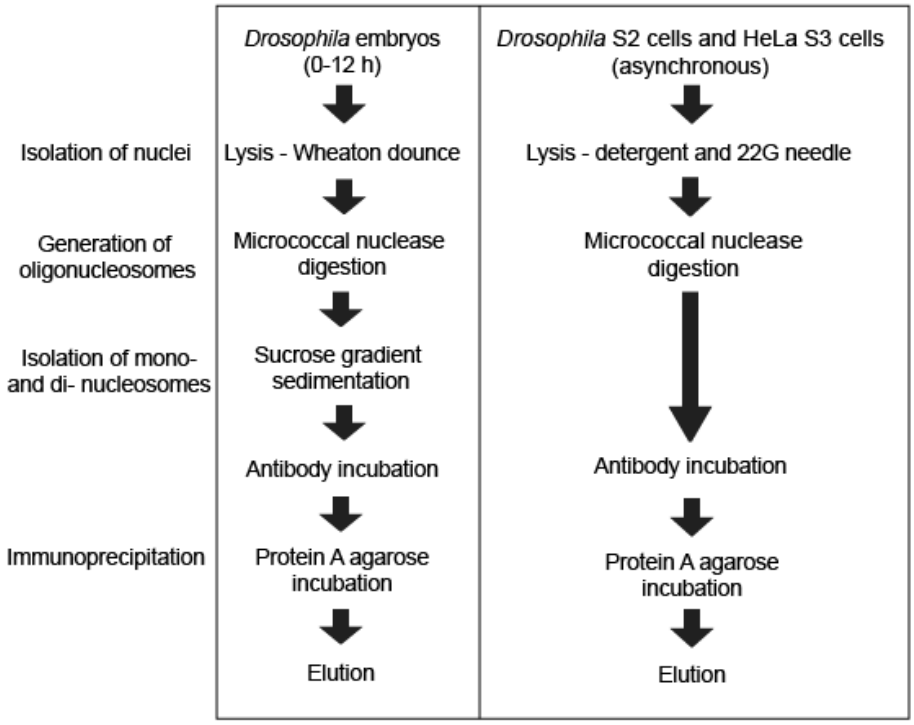

Figure 2.1 Standardized method to isolate oligonucleosomes from *Drosophila* embryos, S2 cells, and HeLa S3 cells

ANTIBODY SAMPLE	METHOD
H3K56AC SERUM	none
H3K56AC BB	Bakerbond purification
H3K56AC AP (MOD)	Affinity purification against H3K56ac peptides
H3K56AC AP (UNMOD)	Affinity purification against H3K56 peptides
H3K56AC CLEAN	Affinity purification against H3K56ac and H3K56 peptides

Figure 2.2 Purification of antibody serum using various methods

Condition	Treatment	Elution efficacy
Denaturing	1X SDS sample buffer followed by boiling for 3 min	+++
Low pH (acid)	100 mM glycine HCl, pH 2.5 for 10 min	+
Salt	30 mM Hepes K^+, pH 7.6 containing 3.5 M $MgCl_2$ for 10 min	++
Polyol/Salt	30 mM Hepes K^+, pH 7.6 containing 1 mM EDTA, 0.5 M ammonium sulfate, 30% ethylene glycol, and 0.05% NP-40 for 20 min	+
Soft	30 mM Hepes K^+, pH 7.6 containing 1 mM EDTA, 0.2% sarkosyl, 3 mM $MgCl_2$, and 0.1% Tween 20 for 10 min	-

Figure 2.3 Denaturing and non-denaturing elution methods and their efficacy

CHAPTER 3:

Prenucleosomes and Active Chromatin

Prenucleosomes and Active Chromatin

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Chromatin consists of nucleosomes as well as nonnucleosomal histone-containing particles. Here we describe the prenucleosome, which is a stable conformational isomer of the nucleosome that associates with ~80 bp DNA. Prenucleosomes are formed rapidly upon the deposition of histones onto DNA and can be converted into canonical nucleosomes by an ATP-driven chromatin assembly factor such as ACF. Different lines of evidence reveal that there are prenucleosome-sized DNA-containing particles with histones in the upstream region of active promoters. Moreover, p300 acetylates histone H3K56 in prenucleosomes but not in nucleosomes, and H3K56 acetylation is found at active promoters and enhancers. These findings therefore suggest that there may be prenucleosomes or prenucleosome-like particles in the upstream region of active promoters. More generally, we postulate that prenucleosomes or prenucleosome-like particles are present at dynamic chromatin, whereas canonical nucleosomes are at static chromatin.

The nucleosome is the basic repeating unit of chromatin in the nuclei of eukaryotic cells. The nucleosome core consists of ~147 bp DNA that is wrapped approximately 1.7 turns around a histone octamer, which contains two copies each of histones H2A, H2B, H3, and H4. Native chromatin in the cell additionally contains ~50 bp of linker DNA between the nucleosome cores as well as other components such as histone H1 and high-mobility group (HMG) proteins.

In addition to the nucleosome, it has been increasingly appreciated that there are nonnucleosomal histone-containing particles in chromatin (Fig. 1A). These noncanonical chromatin particles have been observed at locations, such as active promoter regions, where nucleosomes are disrupted. Biochemical data have also revealed the existence of the prenucleosome, a stable nonnucleosomal histone-containing particle that can be converted into a canonical nucleosome by an ATP-driven motor protein such as ACF (APOBEC1 complementation factor). We postulate that the noncanonical particles reflect dynamic activity in chromatin, whereas canonical nucleosomes occur in relatively static chromatin. Here, we discuss the identification and characterization of the prenucleosome, a conformational isomer of the nucleosome (Torigoe et al. 2011; Fei et al. 2015).

DISCOVERY OF THE PRENUCLEOSOME AS A PRECURSOR TO THE NUCLEOSOME

We initially found the prenucleosome in the course of our studies on chromatin assembly (Torigoe et al. 2011). A simplified depiction of the steps of the chromatin assembly process (Torigoe et al. 2013) is shown in Figure 1B. First, the core histones are synthesized in the cytoplasm and imported into the nucleus in a complex with one of a variety of histone chaperone proteins. In a fast (complete in <15 sec) reaction, the histones are rapidly and spontaneously deposited onto free DNA to give randomly distributed prenucleosomes. Next, in a slower step (complete in ~10–15 min), an ATP-dependent motor protein, such as ACF or Chd1, converts the prenucleosomes to canonical nucleosomes. Then, the randomly distributed nucleosomes are converted into periodic nucleosome arrays by the ATP-dependent motor protein, which can function both as a chromatin assembly factor (for the conversion of prenucleosomes to nucleosomes) and as a chromatin remodeling factor (for the mobilization of nucleosomes into periodic arrays). The organization of the nucleosomes into periodic arrays is likely to be driven by interactions between nucleosomes (such as chromatin folding and compaction) that result in a thermodynamically more stable arrangement with evenly spaced nucleosomes than with randomly distributed nucleosomes.

Prenucleosomes were discovered in the analysis of the deposition of histones onto DNA during chromatin assembly (Torigoe et al. 2011). In these experiments, we used a DNA template association assay (Fig. 1C) as follows: (1) Histone–chaperone complexes are added to Template 1; (2) Template 2 is added; and (3) ATP-dependent nucleosome assembly is mediated by ACF. If stable histone–Template 1 complexes are formed, then nucleosomes would be assembled on Template 1 but not on Template 2. If the histone–Template 1 complex is not stable, then nucleosomes would be formed on both Template 1 and Template 2. With the NAP1 histone chaperone, we observed template association as shown in Figure 1C (Torigoe et al. 2011). Prenucleosomes were identified on the basis of their ability to remain associated with Template 1 before nucleosome assembly by ACF.

We additionally sought to test whether template association can be seen with other core histone chaperones.

[3]These authors contributed equally to this work.

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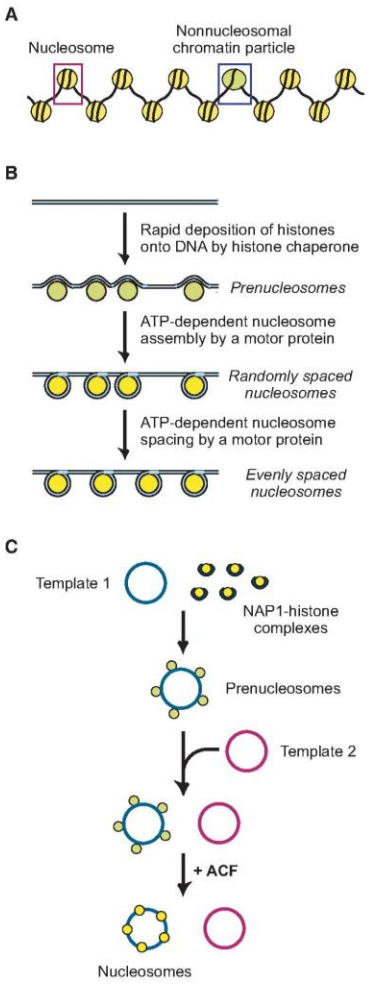

Figure 1. The prenucleosome was initially discovered as a precursor to the nucleosome. (*A*) Chromatin contains nucleosomes as well as nonnucleosomal histone–DNA particles. (*B*) A simple working model for the steps in the assembly of chromatin. This model is based on results from Torigoe et al. (2011, 2013). (*C*) Diagram of the template association assay for chromatin assembly. This assay is described in the text.

To this end, we tested the properties of *Drosophila* nucleoplasmin-like protein, dNLP (Ito et al. 1996). First, we purified dNLP protein (Fig. 2A) and found that it is able to function along with purified ACF in the ATP-dependent assembly of chromatin (Fig. 2B). We then performed template association experiments, as depicted in Figure 1C, and observed the formation of template-associated prenucleosomes with dNLP (Fig. 2C). These experiments therefore show that prenucleosomes can be formed with the dNLP histone chaperone. Hence, the generation of prenucleosomes is not a special property of NAP1 (specifically, *Drosophila* NAP1, as used in Torigoe et al. 2011).

The formation of prenucleosomes and their conversion to nucleosomes can be seen by focusing on the two blue rectangles in Figure 2C (lanes 2 and 4). (As a technical note, in Fig. 2B,C, we monitored chromatin assembly by using the DNA supercoiling assay, in which the formation of nucleosomes is observed by the generation of negative supercoils in the associated circular plasmid DNA [see, e.g., Fyodorov and Kadonaga 2003].) In the absence of ACF (lanes 1 and 2), there is a small increase in DNA supercoiling during the 30-min chromatin assembly reaction, whereas in the presence of ACF (lanes 3 and 4), there is a large increase in supercoiling that is due to the formation of nucleosomes. The key comparison, however, is between the two blue rectangles. In the absence of ACF (lane 2), the histones are associated with Template 1 (the 3K template), but the histone–DNA species (i.e., mostly prenucleosomes) do not substantially supercoil the DNA. In the presence of ACF (lane 4), the histones, which are specifically associated with Template 1, are assembled into nucleosomes by ACF, as seen by the DNA supercoiling. Thus, in the absence of ACF, prenucleosomes (which do not supercoil DNA) are specifically associated with Template 1; then, upon addition of ACF, the prenucleosomes are converted to nucleosomes.

The initial characterization of prenucleosomes revealed the following (Torigoe et al. 2011).

- Prenucleosomes contain all four core histones and lack the NAP1 chaperone.
- Unlike nucleosomes, prenucleosomes do not induce supercoiling of the DNA.
- Prenucleosomes can be converted into nucleosomes by the ACF motor protein.
- The rate of ACF-mediated conversion of prenucleosomes to nucleosomes is comparable to the overall rate of ACF-catalyzed chromatin assembly.
- Prenucleosomes are rapidly formed in the absence of ACF. The formation of prenucleosomes is complete within 15 sec, which is the shortest time point that could be measured in a standard assay. This effect was previously shown with NAP1–histone complexes (Torigoe et al. 2011). We have also seen the rapid formation of prenucleosomes with dNLP–histone complexes (Fig. 3A).
- When a competing free DNA template is added to a prenucleosomal template, the prenucleosomes remain stably associated with the original template for at least 2 h. This property, which was originally seen with NAP1 (Torigoe et al. 2011), is also observed with dNLP as the histone chaperone (Fig. 3B).
- When NAP1–histone complexes are added to relaxed circular DNA, a mixture of ~85% prenucleosomes and 15% nucleosomes is formed. The apparently sponta-

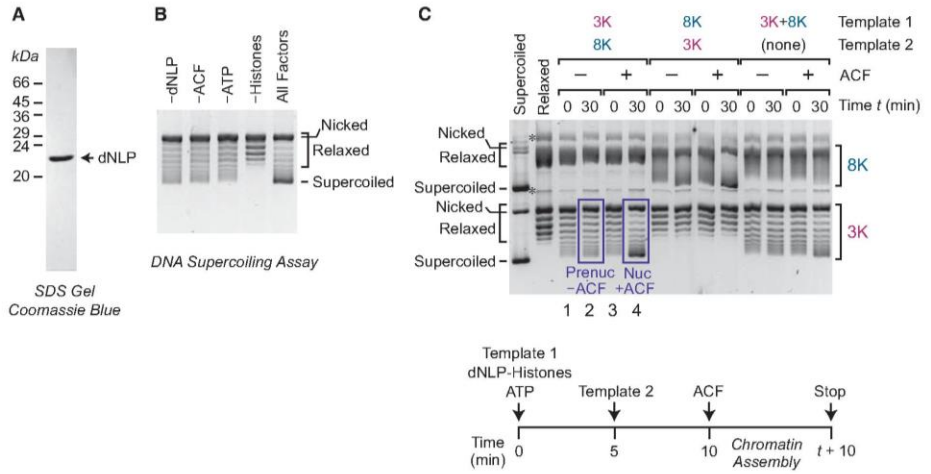

Figure 2. Template association assay with the dNLP core histone chaperone. (*A*) Purification of recombinant dNLP by the method of Ito et al. (1996). (*B*) Chromatin assembly requires dNLP, ACF, core histones, and ATP. Chromatin assembly was performed and monitored by the DNA supercoiling assay, as described in Fyodorov and Kadonaga (2003). (*C*) Template association experiments were performed with equivalent masses of two different relaxed circular DNA templates of ~3 kb (3K) and 8 kb (8K). Core histones (amount sufficient for complete assembly of one DNA template) were initially incubated with Template 1. After 5 min, Template 2 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) before 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. As references, supercoiled and relaxed DNAs are also included. The positions of relaxed, supercoiled, and nicked DNA species are shown. The asterisks indicate contaminants in the plasmid DNA preparations. The blue rectangles that highlight prenucleosomes (Prenuc) and nucleosome (Nuc) are discussed in the text.

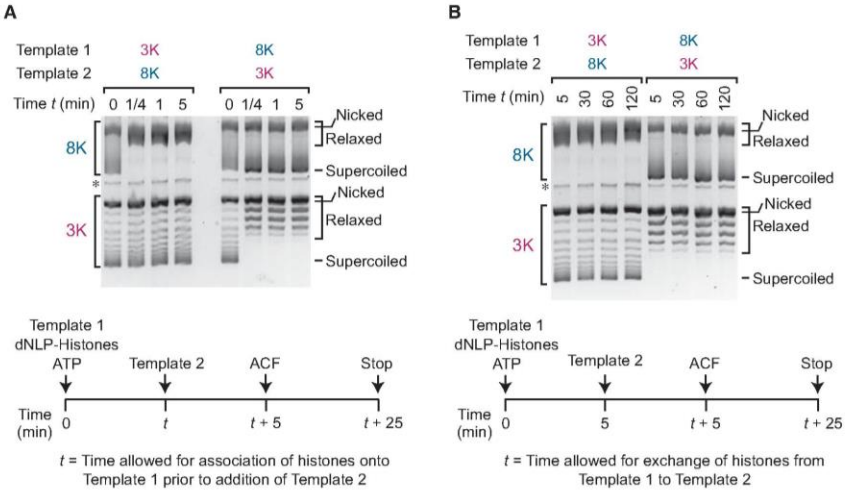

Figure 3. Analysis of the formation and stability of prenucleosomes. (*A*) Prenucleosomes are formed with dNLP–histone complexes and relaxed plasmid DNA within 15 sec. Template association experiments were performed as in Figure 2C, except that the time of addition of Template 2 to the Template 1 + dNLP–histone mixture was varied as specified. (*B*) Prenucleosomes remain associated with the DNA template for at least 120 min. Template association experiments were performed as in Figure 2C, 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 before the addition of ACF) was varied as indicated.

neous formation of a small fraction of nucleosomes is a subject of current investigation. In this regard, it should also be noted that canonical nucleosomes are rapidly and spontaneously formed on negatively supercoiled DNA because the wrapping of the DNA around the histone octamer relieves the superhelical tension (see, e.g., Pfaffle and Jackson 1990; Nakagawa et al. 2001). Thus, negatively supercoiled DNA is "spring loaded" for nucleosome formation. In our studies of ACF-catalyzed chromatin assembly, we include purified eukaryotic topoisomerase I to maintain the DNA in a relaxed state.

- Prenucleosomes can be formed by the initial deposition of histones H3 and H4 onto the DNA followed by the subsequent addition of histones H2A and H2B. Prenucleosomes are not formed by the initial deposition of histones H2A and H2B onto the DNA.
- By atomic force microscopy, prenucleosomes are indistinguishable from nucleosomes.

The discovery of prenucleosomes additionally clarified an early paradox in chromatin assembly. By electron microscopy, it was observed that nucleosome-like structures that contain histones H2A and H3 are formed within seconds upon passage of DNA replication forks (see, e.g., McKnight and Miller 1977; McKnight et al. 1978; Sogo et al. 1986). In contrast, canonical "mature" nucleosomes, as characterized by nuclease digestion and sedimentation properties, were found to require ~10–20 min for assembly in cells (see, e.g., Seale 1975, 1976; Levy and Jakob 1978; Worcel et al. 1978; Schlaeger and Knippers 1979; Klempnauer et al. 1980; Jackson and Chalkley 1981). It was therefore wondered how could nucleosome-like particles be formed within seconds when it was also known that it takes at least 10 min to form a canonical nucleosome (see, e.g., Worcel et al. 1978). It now appears that this paradox can be explained by the rapid formation of prenucleosomes at DNA replication forks. The rapid rate of formation of prenucleosomes in vitro corresponds to the timing of the appearance of similar nucleosome-like structures (which we postulate to be prenucleosomes) at replication forks in vivo. In addition, the rate of the ACF-catalyzed conversion of prenucleosomes into canonical nucleosomes in vitro coincides with the timing of chromatin "maturation" in cells.

Hence, the initial studies revealed that prenucleosomes are rapidly formed nucleosome-like particles that do not supercoil DNA but can be converted into canonical nucleosomes with ACF or Chd1. The term "prenucleosome" was designated because these particles are precursors to nucleosomes (Fig. 1B). Moreover, as shown in Figures 2 and 3, prenucleosomes can be generated with the dNLP histone chaperone as well as with NAP1. These findings collectively suggest that prenucleosomes can be rapidly formed on newly synthesized DNA, such as during DNA replication and repair, and can remain stably associated with the DNA until they are converted into canonical nucleosomes by a motor protein.

PRENUCLEOSOMES ASSOCIATE WITH ~70–80 bp DNA

The next challenge in the study of the prenucleosome was the determination of its composition and structural organization. The first step toward this goal was achieved by the analysis of prenucleosomes by psoralen cross-linking and electron microscopy. In this method, chromatin is treated with psoralen, which cross-links the linker DNA between nucleosomes, and the DNA is visualized by electron microscopy under denaturing conditions (Sogo et al. 1986; Brown et al. 2013). The regions of DNA that are associated with histones appear as single-stranded bubbles in the electron micrographs.

When prenucleosomes and nucleosomes were compared by psoralen cross-linking and electron microscopy, it was found that nucleosomes are bound to ~140–150 bp DNA, as expected, and prenucleosomes are associated with ~70–80 bp DNA (Fig. 4; Fei et al. 2015). The association of less than one turn of DNA around the histones in a prenucleosome is the likely cause of the lack of DNA supercoiling by prenucleosomes, as seen in Torigoe et al. (2011).

The psoralen cross-linking experiments additionally allowed the comparison of the properties of prenucleosomes and nucleosomes in vitro (Fei et al. 2015) with those of chromatin particles at active and repressed promoters in vivo in yeast (Brown et al. 2013). Somewhat strikingly, the in vivo studies revealed a peak of 70–80 bp at the active *PHO5* promoter and a peak of 140–150 bp at the inactive *PHO5* promoter. Direct comparison of the in vitro and in vivo bubble size distributions suggests that active *PHO5* promoter contains prenucleosomes, whereas the inactive *PHO5* promoter contains canonical nucleosomes (Fig. 4). Thus, prenucleosomes may be present at the upstream promoter regions of active genes. This point is discussed in greater detail below.

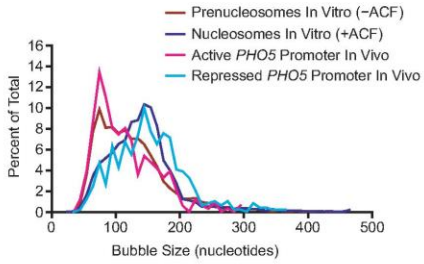

Figure 4. Prenucleosomes generated in vitro as well as noncanonical chromatin particles at the active yeast *PHO5* promoter in vivo both associate with ~70–80 bp DNA. Chromatin particles were analyzed by psoralen cross-linking followed by denaturing electron microscopy, as in Brown et al. (2013). The histone-free DNA is cross-linked by psoralen, and the resulting bubbles represent the locations of prenucleosomes and nucleosomes. The figure shows data from prenucleosomes and nucleosomes in vitro (Fei et al. 2015) and at the active versus repressed *PHO5* promoters in vivo in *Saccharomyces cerevisiae* (Brown et al. 2013). The plots show the distribution of bubble sizes as the average of 10-nt bins. (Reprinted, with permission, from Fei et al. 2015, Cold Spring Harbor Laboratory Press.)

THE PRENUCLEOSOME IS A STABLE CONFORMATIONAL ISOMER OF THE NUCLEOSOME

The observation that prenucleosomes associate with ~70–80 bp DNA led us to develop a monomeric prenucleosome (mono-prenucleosome) system. We found that prenucleosomes are formed with 80 bp DNA fragments and the four core histones under a variety of conditions that include deposition by NAP1 or dNLP as well as salt dialysis methodology (Fig. 5A; Fei et al. 2015). The formation of prenucleosomes by salt dialysis of the four core histones and an 80 bp DNA fragment suggests that the prenucleosome is the thermodynamically most stable arrangement of a histone octamer and 80 bp DNA. Additional experiments, which are described in Fei et al. (2015), further indicated that prenucleosomes contain a histone octamer rather than a histone hexamer (with two copies each of H3 and H4 and one copy each of H2A and H2B).

It was important to test, however, whether monoprenucleosomes that are assembled with 80 bp DNA can be converted into canonical nucleosomes. To address this question, we ligated DNA fragments to the 80 bp mono-prenucleosomal DNA to give mono-prenucleosomes with 85 bp of free flanking DNA on each side (Fig. 5B). We then observed that the resulting species can be assembled into a canonical nucleosome by the ACF motor protein and ATP (Fig. 5B). Thus, the mono-prenucleosomes that are formed with 80 bp DNA are functionally active.

It is intriguing that mono-prenucleosomes with free flanking DNA do not spontaneously fold into nucleosomes. This phenomenon has been observed with mono-prenucleosomes (Fig. 5) as well as with prenucleosomes on relaxed plasmid DNA (Torigoe et al. 2011). Because of the association of only ~80 bp DNA with the histones in a prenucleosome, we hypothesize that the histone octamer in a prenucleosome is slightly expanded or unfolded relative to the octamer in a canonical nucleosome. The mechanism by which ACF can convert prenucleosomes into nucleosomes remains to be determined.

Additional studies revealed that the DNA associated with the prenucleosome is at a location that is analogous to the central 80 bp in a canonical nucleosome (Fei et al. 2015). In addition, micrococcal nuclease (MNase) digestion analysis of prenucleosomes revealed that ~80 bp of DNA are protected from digestion (Fei et al. 2015). The latter observation is consistent with the psoralen cross-linking and electron microscopy data (Fig. 4) and can also be seen at low resolution in Figure 5B.

The assembly and analysis of mono-prenucleosomes has revealed that the prenucleosome is a stable conformational isomer of the nucleosome. It is associated with ~80 bp of DNA and can be converted into a canonical nucleosome by an ATP-driven chromatin assembly factor such as ACF or Chd1. In addition, we found that histone H3K56 can be acetylated by p300 in a prenucleosome but not in a canonical nucleosome (Fei et al. 2015). H3K56 is located at the DNA entry and exit points of the nucleosome. Hence, the accessibility of the p300 acetyltransferase to H3K56 in a prenucleosome is consistent with the experimentally determined central location of the 80 bp of DNA in the prenucleosome. These findings are summarized in Figure 6.

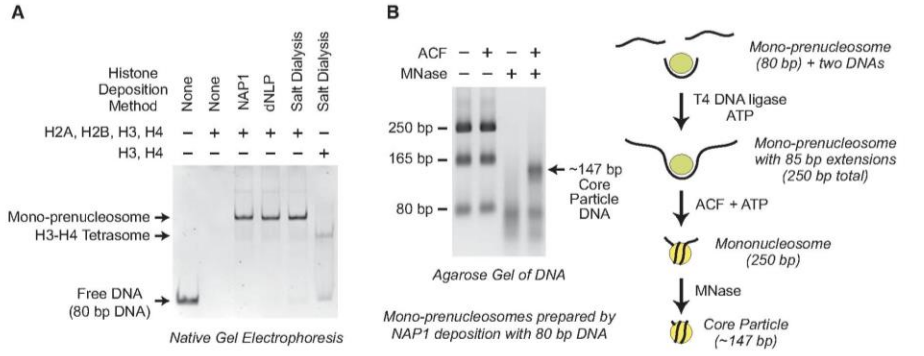

Figure 5. Assembly and analysis of monomeric prenucleosomes (mono-prenucleosomes). (*A*) Mono-prenucleosomes appear to be the thermodynamically most stable arrangement of the four core histones and 80 bp DNA at 50 mM NaCl. Mono-prenucleosomes were formed with the NAP1 or dNLP histone chaperones as well as by salt dialysis of the four core histones with an 80 bp DNA fragment. For comparison, H3-H4 mono-tetrasomes were also generated in parallel by salt dialysis with H3-H4. The histones were used at an octamer equivalent:DNA ratio of 1.0. (*B*) Prenucleosomes can be converted into canonical nucleosomes by ACF. ACF-dependent assembly of mono-prenucleosomes to canonical nucleosomes. Mono-prenucleosomes formed on 80 bp of DNA were ligated to two free 80 bp DNA fragments (each containing a single 5-nt overhang) to give mono-prenucleosomes that are flanked by 85 bp DNA extensions, as illustrated in the diagram. The resulting mono-prenucleosomes were assembled into nucleosomes by ACF. The formation of canonical nucleosomes was assessed by MNase digestion into core particles that contain ~147 bp DNA. The 80 bp and 165 bp DNA fragments are incomplete ligation products. (Reprinted, with permission, from Fei et al. 2015, Cold Spring Harbor Laboratory Press.)

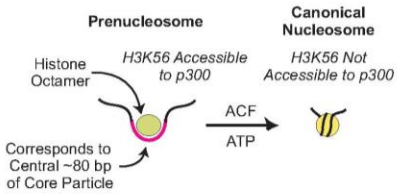

Figure 6. The prenucleosome is a conformational isomer of the nucleosome. Prenucleosomes comprise a core histone octamer and 80 bp DNA at a location that is analogous to that of the central 80 bp of the core particle. H3K56 is accessible to p300 in a prenucleosome but not in a nucleosome. Prenucleosomes can be converted into canonical nucleosomes by a motor protein such as ACF or Chd1. (Reprinted, with permission, from Fei et al. 2015, Cold Spring Harbor Laboratory Press.)

MPE-seq REVEALS PRENUCLEOSOME-LIKE PARTICLES AT THE UPSTREAM REGION OF ACTIVE PROMOTERS

To gain a different perspective on chromatin structure, we used the chemical reagent methidiumpropyl-EDTA-Fe(II) (MPE-Fe(II)) to cleave chromatin in nuclei, and then we analyzed the resulting DNA fragments by genome-wide paired-end sequencing (Ishii et al. 2015). This method, termed MPE-seq, revealed that the upstream promoter regions of active genes in mouse cells contain chromatin particles with subnucleosome-sized DNA fragments.

Because prenucleosomes associate with ~80 bp DNA, we specifically analyzed the properties of chromatin particles with 61–100 bp DNA fragments. We compared the properties of chromatin that was cleaved with MPE-Fe(II) (MPE-seq), the standard concentration of MNase (MNase-seq), or a low concentration of MNase (MNase Low-seq). As shown in Figure 7A (left panels), there is a strong peak of prenucleosome-sized (61–100 bp) DNA fragments at the upstream promoter region by MPE-seq as well as by MNase Low-seq. This location immediately upstream of the transcription start site of active promoters is often termed the "nucleosome-depleted region" (NDR) or "nucleosome-free region" (NFR). Importantly, chromatin immunoprecipitation (ChIP) data indicated that the 61–100 bp DNA particles contain histones H2B and H3 (Fig. 7A, right panels). Heat maps of MPE-seq, MNase-seq, and MNase Low-seq data with 61–100 bp DNA fragments further revealed that intensity of the MPE-seq and MNase Low-seq signals at the NDR increases with promoter activity, as assessed by RNA-seq (Fig. 7B). In addition, the MPE-ChIP-seq and MNase Low-ChIP-seq data with 61–100 bp fragments show that the strength of the H2B and H3 ChIP signals at the NDR increases with promoter activity (Fig. 7C,D). These findings suggest that there are prenucleosome-like particles at the NDRs of active promoters.

It is also notable that 61–100 bp particles are not seen in the NDRs of active genes by standard MNase-seq (Fig. 7B). The MNase sensitivity of these particles is similar to that of "fragile nucleosomes," which have been studied in yeast (see, e.g., Weiner et al. 2010; Xi et al. 2011; Knight et al. 2014; Kubic et al. 2015; also see Henikoff et al. 2011). Hence, there may be a relation between fragile nucleosomes in yeast and the prenucleosome-like particles at the NDRs of mouse cells (Fig. 7).

SUMMARY AND PERSPECTIVES

The prenucleosome is a stable conformational isomer of the nucleosome. It is associated with ~80 bp DNA and can be assembled into a canonical nucleosome by an ATP-driven chromatin assembly factor such as ACF (Fig. 6). Multiple independent lines of evidence suggest that prenucleosomes or prenucleosome-like particles are at active chromatin such as enhancers and promoters (Fig. 8A). First, there is a strong correlation between the size distributions of psoralen cross-linked bubbles in prenucleosomes assembled in vitro and the active yeast *PHO5* promoter in vivo (Fig. 5; Fei et al. 2015). Second, prenucleosome-sized DNA-containing particles with core histones are observed at the NDRs of active promoters in mouse cells (Fig. 7; also see Ishii et al. 2015). Moreover, the occurrence of these particles correlates with the level of promoter activity. Third, prenucleosomes, but not nucleosomes, can be acetylated at H3K56 by p300 (Fei et al. 2015). H3K56 acetylation is associated with chromatin assembly (see, e.g., Masumoto et al. 2005; Han et al. 2007; Chen et al. 2008; Li et al. 2008). In addition, H3K56 is acetylated at active promoters and enhancers in yeast, *Drosophila*, and humans (see, e.g., Lo et al. 2011; Venkatesh et al. 2012; Skalska et al. 2015). H3K56 acetylation in chromatin may be a marker for the presence of prenucleosomes. From a more general perspective, prenucleosomes or prenucleosome-like particles might be found at sites of DNA synthesis and/or chromatin disruption—in other words, at dynamic chromatin (Fig. 8B).

Last, an important question that remains to be resolved is whether prenucleosomes have specific functions at dynamic chromatin. It might be imagined, for instance, that the transcriptional machinery is optimized to function in conjunction with a prenucleosome. In the future, it will be interesting and important to investigate the roles of prenucleosomes at active chromatin.

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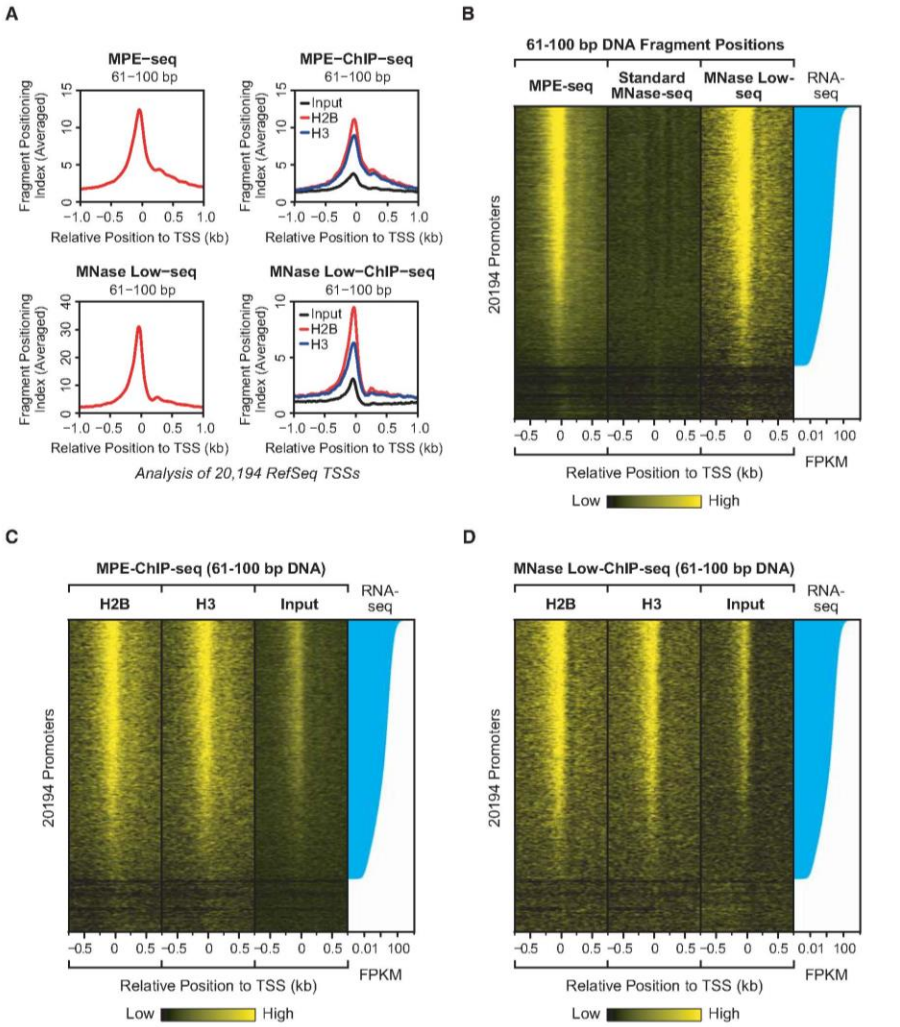

Figure 7. Prenucleosome-sized (61–100 bp) DNA-containing particles with core histones are immediately upstream of transcription start sites of active promoters in mouse J1 embryonic stem cells. The 61–100 bp data from Ishii et al. (2015) were subjected to fragment positioning analysis as described in Ishii et al. (2015). The fragment positioning index depicts the location of the central regions of the DNA fragments, which were identified by paired-end sequencing. Data from the digestion of chromatin with MPE-Fe(II), the standard concentration of MNase, or a low concentration of MNase (MNase Low) are shown. The 20,194 promoters in the heatmaps were ranked according to their transcript levels (FPKM, fragments per kilobase of exon per million fragments mapped) from RNA-seq data. (*A*) Averaged Fragment Positioning Index of chromatin digested with MPE-Fe(II) or MNase Low conditions. The *left* panels show the total 61–100 bp DNA fragments. The *right* panels show the results from ChIP analyses of soluble chromatin fragments. (*B*) Comparison of MPE-seq, standard MNase-seq, and MNase Low-seq data. Heatmaps of the fragment positioning index of 61–100 bp DNA-containing particles are shown. (*C*) Heatmaps of the MPE-ChIP-seq signal of histones H2B and H3 from 61–100 bp DNA-containing particles. (*D*) Heatmaps of the MNase Low-ChIP-seq signal of histones H2B and H3 from 61–100 bp DNA-containing particles.

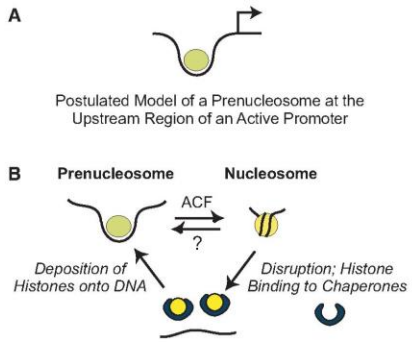

Figure 8. Postulated role of prenucleosomes in active chromatin. (*A*) Prenucleosomes or prenucleosome-related particles may be present in the upstream region of active promoters. (*B*) Model for the productive dynamic interconversion between prenucleosomes and nucleosomes. Prenucleosomes can be formed by the deposition of histones onto DNA and converted into nucleosomes by an ATP-driven motor protein such as ACF or Chd1. Nucleosomes can be disrupted by the action of enzymes such as polymerases as well as some ATP-driven chromatin remodeling factors. The resulting free histones are bound by the chaperones and then re-assembled into prenucleosomes. It is not known whether a canonical nucleosome can be directly converted into a prenucleosome. (Reprinted, with permission, from Fei et al. 2015, Cold Spring Harbor Laboratory Press.)

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CHAPTER 4:

A Simple and Versatile System for the ATP-dependent Assembly of Chromatin

Abstract

Chromatin is the substrate for diverse biological phenomena in the eukaryotic nucleus, and the biochemical analysis of these processes would ideally be carried out with nucleosomal templates. Here we describe a simple, reliable, and versatile method for the ATP-dependent assembly of periodic nucleosome arrays. The minimal chromatin assembly system comprises the *Drosophila* nucleoplasmin-like protein (dNLP) histone chaperone, the imitation switch (ISWI) ATP-driven motor protein, core histones, template DNA, and ATP. The dNLP and ISWI are synthesized in bacteria, and each protein can be purified in a single step by affinity chromatography. We show that the dNLP-ISWI system can be used with different DNA sequences, linear or circular DNA, bulk genomic DNA, recombinant or native *Drosophila* core histones, native human histones, linker histone H1, non-histone chromosomal protein HMGN2, and core histone variants H3.3 and H2A.V. The dNLP-ISWI system should be accessible to a wide range of researchers and enable the assembly of customized chromatin with specifically desired DNA sequences, core histones, and other chromosomal proteins.

Introduction

In the eukaryotic nucleus, DNA is packaged into a nucleoprotein complex known as chromatin (Craig et al. 2014). The chromatin fiber consists of repeating units of nucleosomes, which comprise nucleosome cores (~147 bp of DNA wrapped around a core histone octamer of two copies each of the histones H2A, H2B, H3, and H4) that are connected by about 20 to 50 bp of linker DNA. Chromatin is involved not only in the

compaction and organization of the genome, but it is also the substrate for DNA utilizing processes such as transcription, DNA replication, and DNA repair.

Chromatin is fascinating yet challenging because of its multidimensional nature. In addition to the core histones, chromatin contains the linker histone H1 as well as non-histone chromosomal proteins such as the high mobility group (HMG) proteins. There are also different types of core histones: the canonical S phase-regulated histones as well as S phase-independent histone variants with specialized functions. Furthermore, there are many different covalent modifications of the histones.

To analyze chromatin in vitro, it is essential to reconstitute nucleosomes from DNA and histones (for reviews, see: Stein 1989; Lusser and Kadonaga 2004). For the assembly of periodic arrays of nucleosomes onto natural DNA sequences, there are purified and defined enzyme-driven systems that comprise an ATP-dependent motor protein, such as ACF (ATP-utilizing chromatin assembly and remodeling factor), Chd1 (chromodomain helicase DNA-binding protein 1), or RSF (remodeling and spacing factor), and a core histone chaperone, such as NAP1 (nucleosome assembly protein 1) (for reviews, see: Fyodorov and Kadonaga 2003; Loyola and Reinberg 2003). These enzyme-driven systems have been successfully used for the assembly of chromatin, but they also have some limitations that restrict their more widespread use. For instance, the ACF and RSF motor proteins are synthesized in insect cells by using baculovirus vectors, and it would be easier and less expensive to use bacterially synthesized proteins. On the other hand, Chd1, which can be made in bacteria, is not able to assemble histone H1-chromatin (Lusser et al. 2005).

NAP1 is widely used as a histone chaperone, but bacterially-synthesized NAP1 is often contaminated with a nuclease that can degrade the DNA template (Ito et al. 1996 - MCB).

To circumvent these problems, we have developed and characterized a simple and reliable method for ATP-dependent assembly of periodic arrays of nucleosomes with bacterially-synthesized ISWI (imitation switch) motor protein and dNLP (*Drosophila* nucleoplasmin-like protein) histone chaperone. Importantly, we tested the versatility of this dNLP-ISWI assembly system, and found that it can function with circular or linear DNA templates, human or *Drosophila* core histones, histone H1, an HMGN (high mobility group, nucleosome-binding) protein, and core histone variants. Thus, this system should be useful for the preparation of many different types of customized chromatin for a wide range of specific applications.

Results

ATP-dependent assembly of periodic nucleosome arrays with dNLP and ISWI

In this work, we sought to establish and to characterize a simple, reliable, and versatile system for the ATP-dependent assembly of periodic nucleosome arrays. The minimal chromatin assembly process is mediated by a combination of an ATP-utilizing motor protein, such as ACF, Chd1, or RSF, and a core histone chaperone, such as NAP1 or dNLP. In our simplified system, we chose to use the *Drosophila* ISWI motor protein (Elfring et al. 1994), which is the ATPase subunit of the ACF assembly factor (Ito et al. 1997). ACF comprises ISWI and the Acf1 protein (Ito et al. 1999), and ISWI alone is able to assemble chromatin in the absence or presence of histone H1 in conjunction with the

NAP1 histone chaperone (Ito et al. 1999; Corona et al. 1999; Lusser et al. 2005). Moreover, ISWI can be synthesized in bacteria and purified by one-step affinity chromatography (Corona et al. 1999); thus, the purified protein can be obtained quickly and inexpensively. Hence, ISWI is ideally suited for a simplified assembly system because it could be easily produced in bacteria and could mediate the assembly of chromatin in the presence or absence of histone H1.

For the core histone chaperone, we chose to use the *Drosophila* nucleoplasmin-like protein, dNLP (Ito et al. 1996 - JBC; also known as p22 and CRP1; Kawasaki et al. 1994; Crevel et al. 1997), which was found to function in chromatin assembly in conjunction with purified ACF (Khuong et al. 2015). In the past, we have extensively used dNAP1 that was synthesized in Sf9 insect cells with a baculovirus vector (Fyodorov and Kadonaga 2003). In contrast, we have found that bacterially-synthesized dNAP1 can be contaminated with a bacterial nuclease (Ito et al. 1996 - MCB) and can also lose its activity upon storage at -80˚C. The use of dNLP circumvents these problems. Like *Xenopus* nucleoplasmin, dNLP is a heat-stable protein (Crevel et al. 1997; Namboodiri et al. 2003), and hence, it can be heat-treated under conditions that inactivate contaminating nucleases. In addition, we have found that purified bacterially-synthesized dNLP retains its activity indefinitely upon storage at -80C.

We therefore tested whether purified bacterially-synthesized ISWI can assemble chromatin in conjunction with purified bacterially-synthesized dNLP. To this end, we purified ISWI by a variation of the method of Corona et al. (1999), and we generated and purified an N-terminally His_6-tagged version of dNLP (termed dNLP). Each of the proteins

was purified by a single step of affinity chromatography (Fig. 1*A*). We then carried out chromatin assembly reactions with ISWI and dNLP along with purified recombinant canonical (S phase-regulated) *Drosophila* core histones, relaxed circular plasmid DNA, and ATP. It should also be noted that the reactions additionally contained an ATP regeneration system (pyruvate kinase and phosphoenolpyruvate), purified topoisomerase I to relax DNA superhelical tension, and bovine serum albumin (BSA) as a stabilizing agent.

Chromatin assembly was monitored by the DNA supercoiling and micrococcal nuclease (MNase) digestion assays. During nucleosome assembly, the wrapping of the DNA around the core histone octamer results in a change in the linking number of about -1 (Germond et al. 1975; Simpson et al. 1985). The DNA supercoiling assay monitors the formation of negative supercoiling in the plasmid DNA as nucleosomes are assembled in the presence of topoisomerase I. The extent of nucleosome assembly is revealed by the formation of negative supercoils in the DNA from the deproteinized reaction products. As seen in Fig. 1*B*, we observe the most efficient nucleosome assembly in the complete reaction containing dNLP, ISWI, core histones, and ATP. More detailed analyses of different concentrations of dNLP and ISWI in the chromatin assembly reactions are shown in supplemental Figs. S1 and S2.

The partial MNase digestion assay reveals whether there are extended periodic arrays of nucleosomes in the reconstituted chromatin (Noll and Kornberg 1977). MNase catalyzes the double-stranded cleavage of DNA in the linker region between the nucleosome cores. If the nucleosomes are evenly spaced, then the different DNA fragments derived from the mono- and oligonucleosomes generated by partial MNase cleavage will form a ladder that

correspond to mononucleosomes, dinucleosomes, trinucleosomes, and so on. As seen in Fig. 1*C* (and supplemental Figs. S1 and S2), we observed the most distinct pattern of MNase digestion products ("MNase ladder") with the complete reaction containing dNLP, ISWI, core histones, and ATP. These experiments reveal that purified bacterially-synthesized dNLP, ISWI, and core histones can be efficiently assembled into periodic nucleosome arrays.

The dNLP-ISWI system can assemble chromatin with either human or Drosophila core histones

We next sought to test the versatility of the dNLP-ISWI chromatin assembly system. We initially addressed the following two issues. First, because the dNLP and ISWI are *Drosophila* proteins, it was important to test whether the factors would work with human histones, which would be more widely studied than *Drosophila* histones. Second, bacterially-synthesized recombinant histones are homogeneous polypeptides, whereas native histones consist of S phase-regulated histones and their variants with a variety of different covalent modifications. We therefore tested the ability of the dNLP-ISWI system to assemble chromatin with native core histones. To this end, we purified native core histones from *Drosophila* embryos as well as from human HeLa cells (Fig. 2*A*), and then carried out chromatin assembly reactions with dNLP and ISWI. As shown in Figs. 2*B* and 2*C*, both native and recombinant *Drosophila* core histones as well as native human core histones are efficiently assembled into extended periodic nucleosome arrays with dNLP and ISWI. The core histones are highly conserved among eukaryotes. It is thus likely that

the dNLP-ISWI system will function with core histones from many different organisms. In addition, it is useful to know that the dNLP-ISWI system can be used with native core histones as well as with recombinant core histones, which can be specifically modified as desired.

The dNLP-ISWI system can assemble chromatin onto different DNA templates

We then examined the ability of the dNLP-ISWI system to assemble chromatin onto different DNA templates. First, as shown in Figs. 3*A* and 3*B*, we found that the dNLP-ISWI system is able to assemble chromatin onto relaxed circular DNAs containing sequences from the adenovirus E4 promoter (3 kb), bacteriophage T4 (7 kb), and the *Drosophila Antennapedia* gene (8 kb). Thus, the assembly factors function with both prokaryotic and eukaryotic DNA sequences. Second, we observed that dNLP and ISWI can assemble chromatin efficiently onto linearized plasmid DNA (Fig. 3*C*). Third, we also tested the ability of the dNLP-ISWI system to assemble chromatin with high molecular weight (mostly > 12 kb; supplemental Fig. S3) bulk genomic DNA. These experiments revealed efficient assembly of extended periodic nucleosome arrays with the high molecular weight genomic DNA from calf thymus (Fig. 3*D*). It thus appears that the dNLP-ISWI system can be used for the assembly of chromatin with a wide range of linear and circular DNAs.

Assembly of histone H1-containing chromatin with the dNLP-ISWI system

In metazoa, histone H1 is a major component of chromatin with a stoichiometry that generally ranges from 0.5 to 0.8 molecules per nucleosome (Bates and Thomas 1981; for reviews, see: Woodcock et al. 2006; Happel and Doenecke 2008; Kalashnikova et al. 2016). It is thus important to include histone H1 in the analysis of chromatin structure and function. We therefore tested whether the dNLP-ISWI system could be used to assemble histone H1-containing chromatin. To this end, we purified native histone H1 from *Drosophila* embryos as well as recombinant human histone H1 variant H1.0 (Fig. 4*A*). We then carried out chromatin assembly reactions in the absence or presence of histone H1. Because dNAP1 and ACF can assemble histone H1-containing chromatin (Fyodorov and Kadonaga 2003), we used these factors as a reference. As seen in Figs. 4*B* and 4*C*, chromatin is assembled in the presence of histone H1, and the incorporation of histone H1 into chromatin results in an increase in the nucleosome repeat length, as seen previously in the assembly of histone H1-containing chromatin with crude or purified systems (Rodríguez-Campos et al. 1989; Becker and Wu 1992; Fyodorov and Kadonaga 2003; Lusser et al. 2005). We further analyzed the histone H1-containing chromatin by extensive MNase digestion into mononucleosomes followed by native gel electrophoresis (Fig. 4*D*). In these experiments, we found that the H1-containing chromatin yields chromatosomes (mononucleosomes + H1), as seen previously with chromatin isolated from cells or assembled in vitro with ACF and dNAP1 (Varshavsky et al. 1976, Albright et al. 1980, Lusser et al. 2005). Some differences can be seen between chromatin that is assembled with *Drosophila* H1 relative to human H1.0, and these effects are likely due to different properties of the different H1 molecules. Importantly, histone H1-containing chromatin

that is assembled with dNAP1-ACF is similar to that assembled with dNLP-ISWI. These results indicate that histone H1 is incorporated into chromatin during assembly with the dNLP-ISWI system.

Assembly of HMGN2-containing chromatin with the dNLP-ISWI system

Beyond the core histones and H1, there are other abundant components of chromatin, such as the high mobility group (HMG) proteins (for reviews, see: Reeves 2010; Postnikov and Bustin 2010; Zhu and Hansen 2010). Here, we focus on the HMGN proteins, which are abundant nuclear proteins (Kuehl et al. 1984) that bind with high affinity to two sites on nucleosomes (Sandeen et al. 1980; Mardian et al. 1980; Alfonso et al. 1994). We purified recombinant human HMGN2 (Fig. 4*A*), and then carried out chromatin assembly reactions in the absence or presence of different concentrations of this protein. The DNA supercoiling and partial MNase digestion assays indicated that periodic arrays of nucleosomes are assembled in the presence of HMGN2 (Figs. 5*A* and 5*B*). The incorporation of HMGN2 into chromatin was examined by digestion of the chromatin into mononucleosomes with MNase followed by native gel electrophoresis of the resulting chromatin particles (Fig. 5*C*). These experiments revealed the incorporation of up to two molecules of HMGN2 per nucleosome. Hence, the dNLP-ISWI system can be used for the assembly of periodic nucleosome arrays containing the HMGN proteins.

The dNLP-ISWI system can be used to assemble chromatin containing histone variants H3.3 and H2A.V (Drosophila H2A.X/H2A.Z)

In the study of chromatin structure and function, it is important to analyze the S phase-independent histone variants in addition to the canonical S phase-regulated histones (for reviews, see: Weber and Henikoff 2014; Maze et al. 2014). For instance, nucleosomes containing the histone variants H3.3 and H2A.Z have been found at active promoters, enhancers, and insulator regions in humans (Jin et al. 2009). We therefore investigated whether the dNLP-ISWI system could be used to assemble chromatin containing histone variants. To this end, we synthesized and purified *Drosophila* histone H3.3 and H2A.V (Fig. 6*A*). *Drosophila* H3.3 has the same amino acid sequence as human H3.3, whereas histone H2A.V is a *Drosophila* H2A variant (van Daal et al. 1988) with the combined features of mammalian H2A.X and H2A.Z. By using the dNLP-ISWI system, we performed chromatin assembly reactions with H2A.V and H3.3 along with canonical H2B and H4. DNA supercoiling and partial MNase digestion analyses revealed the formation of evenly-spaced arrays of nucleosomes containing H2A.V and H3.3 (Figs. 6*B* and 6*C*). Thus, the dNLP-ISWI system has considerable promise for the assembly of a wide range of histone substrates into periodic nucleosome arrays.

A minimal dNLP-ISWI chromatin assembly system that lacks an ATP regeneration system, topoisomerase I, and bovine serum albumin

Many investigators may wish to use the simplest possible system for the assembly of chromatin. We therefore examined the effects of the removal of the potentially nonessential protein components in our standard chromatin assembly reactions. First, we tested the omission of the ATP regeneration system (pyruvate kinase and

phosphoenolpyruvate), which converts the ADP that is produced during chromatin assembly back into ATP. As shown in Figs. 7*A* and 7*B*, the absence of the ATP regeneration system has little effect on the efficiency of assembly of periodic nucleosome arrays under our standard reaction conditions. It should be noted, however, that the ATP regeneration system might be essential for efficient chromatin assembly if a higher concentration of the ISWI ATPase were used.

Then, in the absence of the ATP regeneration system, we further investigated the omission of topoisomerase I. Under normal reaction conditions, topoisomerase I serves two functions: first, it is used to relax the negative supercoils in plasmid DNA prior to chromatin assembly; and second, it functions to relax the positive supercoils that are formed as the DNA wraps around the histone octamer during chromatin assembly. The second function is the basis for the DNA supercoiling assay. The first function is relevant to the energy that drives the formation of nucleosomes. In this regard, we prefer to use relaxed DNA for chromatin assembly because negatively supercoiled DNA is "spring loaded" for chromatin assembly, as the formation of nucleosomes relieves the stress of the negative supercoiling. If chromatin is assembled from relaxed DNA in the presence of topoisomerase I, then the energy that drives the formation of nucleosomes is provided only by the ATP-dependent motor protein (in this case, ISWI) without any contribution from superhelical tension in the DNA. This is useful for the study of the mechanism of chromatin assembly. However, if the objective of an experiment is to make chromatin without regard to the specific mechanism, then negatively supercoiled DNA could be used as a substrate for chromatin assembly. Thus, as shown in Fig. 7*C*, we carried out chromatin

assembly reactions with negatively supercoiled plasmid DNA in the absence or presence of topoisomerase I, and observed the assembly of periodic arrays of nucleosomes in the absence of topoisomerase I. [Note: without topoisomerase I, it is not possible to perform the DNA supercoiling assay.] Thus, it should be possible to perform chromatin assembly reactions with the dNLP-ISWI system with negatively supercoiled plasmid DNA in the absence of topoisomerase I. In these reactions, the negative supercoils in the DNA are relieved by the wrapping of the DNA around the histones. It is also important to note that topoisomerase I is not necessary for the assembly of linear DNA, as in Figs. 3*C* and 3*D*.

Lastly, we tested whether bovine serum albumin (BSA) is essential for chromatin assembly with the dNLP-ISWI system. BSA is commonly added to biochemical reactions to minimize adsorption of proteins to plastic as well as to maintain the activity of enzymes in vitro. However, in the absence of the ATP regeneration system and topoisomerase I, we observed that the omission of BSA has no discernable effect on chromatin assembly (Fig. 7*C*). Thus, altogether, it can be seen that the efficient assembly of periodic nucleosome arrays can be achieved with the minimal dNLP-ISWI system, which consists only of dNLP, ISWI, core histones, and ATP, in conjunction with supercoiled or linear DNA. Nevertheless, when possible, we recommend the inclusion of the ATP regeneration system and BSA to provide additional assurance of the consistent success of the reaction. In addition, topoisomerase I would be required if the chromatin is to analyzed by the DNA supercoiling assay.

Discussion

We describe a simple, reliable, and versatile method for the ATP-dependent assembly of periodic nucleosome arrays. The dNLP-ISWI system can be used with different DNA sequences, linear or circular DNA, recombinant or native core histones from *Drosophila* or humans, histone H1, HMGN2, and core histone variants H3.3 and H2A.V (H2A.Z/H2A.X). We have also described a minimal assembly system that does not involve the inclusion of an ATP regeneration system, topoisomerase I, or BSA. The dNLP-ISWI system should be accessible to a wide range of researchers and enable the assembly of customized chromatin with specifically desired DNA sequences, core histones, and other chromosomal proteins.

Because there are many different possible approaches to the use of the dNLP-ISWI system, some specific comments and recommendations are as follows.

• dNLP and ISWI. For all applications, it is necessary to purify dNLP and ISWI.

• Core histones from various organisms. The core histones are highly conserved proteins, and it is likely that the dNLP-ISWI system will work with core histones from many different organisms. For any given organism, if the core histones (especially histones H3 and H4) have a strong similarity to the human histones, it is likely that they will function in the dNLP-ISWI system.

• Recombinant versus native core histones. We have generally found that native core histones yield slightly better (*i.e.*, more evenly spaced) chromatin than recombinant histones (for example, see Fig. 2*C* and Levenstein and Kadonaga 2002). However, native core histones are a mixture of S phase-regulated histones and histone variants, and have a

variety of different covalent modifications. Thus, one key advantage of recombinant core histones is that each individual histone is homogeneous. In addition, a second important advantage of recombinant histones is the ability to use mutant histones, histone variants, or specifically modified histones (for recent review, see David and Muir 2017). Such customized chromatin would allow the investigation of specific biological phenomena.

• Core histone octamer to DNA mass ratio. This is the single most critical component of the assembly reaction. It is essential to determine the optimal core histone octamer to DNA mass ratio for chromatin assembly because there may be some error in the measurements of the concentrations of DNA and histones. A 10% error in either of these measurements could lead to a significant reduction in the quality of the chromatin. Therefore, for each new preparation of histones and DNA, it is necessary to perform a series of reactions with a range of core histone octamer to DNA mass ratios that each vary by only about 10%. In this manner, the optimal ratio would be identified. As a reference, in the absence of histone H1, the optimal core histone octamer to DNA mass ratio is about 0.9:1 (which corresponds to approximately 1 core histone octamer per 180 bp DNA), whereas in the presence of histone H1, the longer repeat length results in a lower optimal core histone octamer to DNA mass ratio of about 0.8:1.

• Purification of the DNA template. We normally purify the DNA by two successive CsCl density gradient centrifugation steps. This strategy yields highly purified DNA with minimal contamination by other species that might inhibit the assembly reaction or affect the accuracy of the determination of the DNA concentration. Chromatin assembly can

work with DNA purified by other methods, but we recommend the use of CsCl density gradient centrifugation when possible.

• DNA supercoiling assay. This assay provides an indication of the efficiency of chromatin assembly. Moreover, if the different topoisomers are analyzed by two dimensional agarose gel electrophoresis (Peck and Wang 1983), it is possible to obtain quantitative data on the efficiency of chromatin assembly (see, for example: Fyodorov and Kadonaga 2002; Lusser et al. 2005). In general, we recommend analyzing the chromatin by one dimensional agarose gel electrophoresis, as in this work, to estimate the efficiency of nucleosome assembly.

• Partial MNase digestion assay. This assay reveals the periodicity of the nucleosomes. In addition, if a distinct MNase ladder is achieved, it is likely that the chromatin is efficiently assembled because inefficiently assembled nucleosomes would yield a diffuse and smeary digestion pattern. However, the DNA supercoiling assay gives more reliable information on the efficiency of nucleosome assembly.

• Topoisomerase I. Because topoisomerase I is needed only for the DNA supercoiling assay, one might be tempted to bypass the purification of the topoisomerase I and the use of the DNA supercoiling assay. We recommend, however, that initial work on the use of the dNLP-ISWI system include the DNA supercoiling analysis, as it provides an indication of the overall success of the assembly reactions.

• Linear versus circular DNA. The dNLP-ISWI system works with linear as well as circular DNA. In initial experiments, we recommend using circular DNA so that the success of the assembly reaction can be monitored with the DNA supercoiling assay. Then, once the

assembly system is established, linear templates could be assembled and analyzed with the partial MNase digestion assay. Also, as noted above, topoisomerase I is not needed for the assembly of linear DNA templates.

• ATP regeneration system and BSA. We recommend that initial work with the dNLP-ISWI system include these components, which might increase the efficiency of chromatin assembly under some conditions. After the dNLP-ISWI system is successfully established, then the omission of these components could be tested.

• The minimal assembly system lacking the ATP regeneration system, topoisomerase I, and BSA. If the standard assembly system with all of the components is established, then the omission of the ATP regeneration system, topoisomerase I, and BSA could be tested. It is important to remember that this minimal system would work best with either linear DNA or negatively supercoiled plasmid DNA, as positive supercoils are generated during the assembly process. One potential advantage of the minimal system is that the resulting chromatin is of higher purity, as there are fewer components in the reaction medium. This could be useful for some applications.

In conclusion, we are entering an exciting new era of chromatin research with the advent of new technologies such as techniques for the synthesis of customized histones (see, for example, David and Muir 2017). The dNLP-ISWI assembly system could be used in conjunction with conventional or customized histones for the assembly of many different types of chromatin that could be used for a diverse range of potential applications.

Experimental procedures

Purification of recombinant His_6-tagged dNLP

The full-length dNLP coding sequence (Ito et al. 1996 - JBC) was subcloned into a pET21-based vector to give pET21-His6dNLP, which encodes N-terminally His_6-tagged dNLP (termed dNLP). The coding sequence, which was reconfirmed by DNA sequence analysis, is provided in Supplemental Table S1. For expression, freshly transformed *Escherichia coli* strain BL21(DE3) was grown in LB medium (0.5 L volume; 40 µg/mL ampicillin) at 37°C to $A_{600\,nm}$ of approximately 0.6, and the synthesis of dNLP was induced by the addition of isopropyl-β-thiogalactopyranoside (IPTG)[1] to 1 mM final concentration. The culture was incubated for an additional 3 h at 37°C, and the cells were pelleted by centrifugation (Fiberlite F14S-6x250y rotor; 6,000 rpm; 5 min; 4°C). Unless stated otherwise, all subsequent operations were performed at 4°C. The cells were resuspended in 20 mL of Buffer N [50 mM phosphate (Na^+), pH 7.0, containing 0.5 M NaCl, 1.5 mM $MgCl_2$, 0.1% (v/v) Nonidet P-40, 15% (v/v) glycerol, 0.2 mM phenylmethylsulfonyl fluoride, 0.5 mM benzamidine-HCl, and 10 mM sodium metabisulfite] containing 15 mM imidazole, and the bacteria were lysed by sonication (Branson Sonifier 450 with a 0.25 inch microtip; 20% output; 4 to 8 cycles of sonication of 45 s on and 60 s off). The mixture was immersed in water at 80°C for 15 min, and the insoluble material was removed by centrifugation (Fiberlite F21S-8x50y rotor; 10,000 rpm; 10 min). The lysate was combined with 2 mL (50% slurry in Buffer N containing 15 mM imidazole) of Ni-NTA agarose (Qiagen) and incubated for 3 h on a nutator. The dNLP-bound Ni-NTA beads were transferred into a Poly-Prep chromatography column (Bio-Rad, cat. no. 731-1550) and washed with 30 mL of Buffer N containing 15 mM imidazole. Then, protein was eluted with a step gradient of 2 mL each of Buffer N containing 50 mM, 100 mM, 200 mM, and

400 mM imidazole. The protein fractions were analyzed by 10% polyacrylamide-SDS gel electrophoresis and staining with Coomassie Brilliant Blue R-250. Nuclease activity in the fractions was detected by incubation of plasmid DNA with the samples at 37°C for 1 h in the presence of 10 mM $MgCl_2$ followed by 1% agarose gel electrophoresis and staining with ethidium bromide. Peak dNLP-containing fractions lacking nucleases were dialyzed (molecular weight cut off: 10 kDa) against 2 L of Buffer R [10 mM Hepes (K^+), pH 7.6, containing 10 mM KCl, 1.5 mM $MgCl_2$, 10% glycerol, 10 mM β-glycerophosphate, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine-HCl, and 1 mM sodium metabisulfite] for 2 h, frozen in liquid nitrogen, and stored at -80°C. A typical yield of dNLP from 0.5 L of bacterial culture is approximately 21 mg of purified protein. In the course of the purification and use of dNLP, the frozen protein should not be subjected to slow thawing, such as on wet ice. Instead, the frozen dNLP should be thawed quickly in room temperature water.

Purification of recombinant Drosophila ISWI

The coding sequence of full-length *Drosophila* ISWI fused to an intein and chitin-binding domain (Corona et al. 1999) was subcloned into a pET24-based vector to give the plasmid pET24-ISWI-iCBD (the coding sequence, which was reconfirmed by DNA sequence analysis, is provided in Supplementary Table S1). For expression, freshly transformed *E. coli* strain Rosetta(DE3) was grown in LB medium (1 L volume; 40 µg/mL kanamycin; 34 µg/mL chloramphenicol) at 37°C to $A_{600\ nm}$ of approximately 0.6, and the synthesis of the ISWI fusion protein was induced by the addition of IPTG to 1 mM final

concentration. The culture was incubated for an additional 16 to 18 h at 16°C, and the bacteria were pelleted by centrifugation (Fiberlite F14S-6x250y rotor; 6,000 rpm; 5 min; 4°C). The cells were resuspended in cold (4˚C) PBS (250 mL) and repelleted by centrifugation (Fiberlite F14S-6x250y rotor; 6,000 rpm; 5 min; 4°C). Unless stated otherwise, all subsequent operations were performed at 4°C. The cells were resuspended in 50 mL of Buffer M [20 mM Hepes (K^+), pH 7.6, containing 1 M NaCl, 1 mM EDTA, 0.1% (v/v) Nonidet P-40, 15% (v/v) glycerol, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5 mM benzamidine-HCl], subjected to two freeze-thaw cycles (frozen in liquid nitrogen and quickly thawed with room temperature water), and then lysed by sonication on ice (Branson Sonifier 450 with a 0.25 inch microtip; 20% output; 8 cycles, 15 s each; it is important to keep the cells cold during sonication). The insoluble material was removed by centrifugation (Fiberlite F21S-8x50y rotor; 16,000 rpm; 20 min). The lysate was then combined with 2 mL (50% slurry in Buffer M) chitin resin (NEB) and incubated for 1 h on a nutator. The chitin beads were washed in batch twice with 35 mL each of Buffer M (for each wash, the resin should be allowed to settle by gravity, followed by removal of the supernatant by decanting), resuspended in 2 mL of Buffer M, and transferred into a Poly-Prep chromatography column (Bio-Rad, cat. no. 731-1550). The column was washed four times each with 8 mL of Buffer M. This was followed by a quick wash (~1 min total time) with 1.5 mL Buffer E (Buffer M containing 50 mM dithiothreitol). The bottom of the column was sealed, 0.5 mL of Buffer E was added, and the beads were incubated for 16 to 20 h. [In this step, the dithiothreitol mediates the cleavage and release of ISWI from the chitin resin-bound ISWI-iCBD fusion protein.] The bottom of the column was opened,

and the ISWI-containing eluate was collected. Three additional fractions of 0.25 mL (in Buffer E) were collected. The ISWI-containing fractions were dialyzed (molecular weight cutoff: 10 kDa) against 2 L of Buffer D [20 mM Hepes (K^+), pH 7.6, containing 200 mM NaCl, 2 mM $MgCl_2$, 1 mM EDTA, 15% (v/v) glycerol, 0.02% (v/v) Nonidet P-40, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5 mM benzamidine-HCl] for 4 h, frozen in liquid nitrogen, and stored at -80°C. A typical yield of ISWI is 0.1 to 0.2 mg of purified protein from 1 L bacterial culture.

Purification of recombinant Drosophila core histones

The coding sequences of *Drosophila melanogaster* S phase-regulated histones H2A and H2B were each individually subcloned into pET11a. In the H2B expression plasmid, an additional Ile codon (ATA) was inserted after the initiating Met codon. This modification was found to increase the level of expression of H2B and to have no adverse effects on chromatin assembly (Hamiche et al. 2001). The S phase-regulated histones H3 and H4 were cosynthesized from a single pET11a-based expression plasmid. Whereas histone H3 alone as well as histone H4 alone are each individually insoluble in aqueous solution, the cosynthesized histones H3 and H4 are soluble in standard aqueous buffers and can be purified as H3-H4 tetramers (Levenstein and Kadonaga 2002). For expression, freshly transformed *E. coli* strain BL21(DE3) was grown in LB medium (40 µg/mL ampicillin) at 37°C to $A_{600\ nm}$ of approximately 0.6, and protein synthesis was induced by the addition of IPTG to 0.4 mM final concentration. The cultures were incubated for an

additional 3 h at 37°C, and the cells were pelleted by centrifugation (Fiberlite F14S-6x250y rotor; 6,000 rpm; 5 min; 4°C).

Histones H3 and H4. Histones H3 and H4 were purified by modification of the method of Levenstein and Kadonaga (2002). Unless stated otherwise, all operations were performed at 4°C. The pelleted cells (from 4 x 0.5 L of culture) were resuspended in 40 mL of Buffer D [10 mM Hepes (K^+), pH 7.6, 1 mM EDTA, 10% (v/v) glycerol, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM sodium metabisulfite, and 2 mM benzamidine-HCl] containing 0.1 M NaCl, and then lysed by sonication on ice (Branson Sonifier 450 with a 0.25 inch microtip; 20% output; 3 cycles, 30 s each). The insoluble material (including the histones) was pelleted by centrifugation (Fiberlite F21S-8x50y rotor; 13,200 rpm; 20 min, and the supernatant was discarded. The histone-containing pellet was suspended in 20 mL of 0.25 N HCl, and the dissolution of the material was facilitated by dispersion of the sample with a Wheaton Dounce homogenizer (A pestle). The mixture was then incubated at -20°C for 30 min, and then subjected to centrifugation (Fiberlite F21S-8x50y rotor; 13,200 rpm; 20 min). The supernatant was neutralized with 0.125 volumes of 2 M Tris base and dialyzed (molecular weight cutoff: 3500 Da) for 2 to 3 h against Buffer D containing 0.1 M NaCl. The insoluble material was removed by centrifugation (Fiberlite F21S-8x50y rotor; 13,200 rpm; 20 min), and the supernatant was applied to a Source 15S column [GE Healthcare; column dimensions (length x diameter) = 5.0 cm x 0.5 cm; column volume = 1 mL; flow rate = 1 mL/min] that was equilibrated with Buffer D containing 0.1 M NaCl. The column was washed with 15 mL of Buffer D containing 0.1 M NaCl, and the H3-H4 was eluted with a linear gradient

of 0.1 M to 2 M NaCl in Buffer D over 10 column volumes. The peak fractions containing H3-H4 tetramers, which typically elute at about 1 M NaCl, were identified by 15% polyacrylamide-SDS gel electrophoresis and staining with Coomassie Brilliant Blue R-250. The H3-H4 peak fractions were pooled, and 2 M Tris-HCl, pH 7.6, was added to a final concentration of 10 mM. Then, three volumes of 8 M urea (deionized immediately before use by gentle mixing for 1 h at room temperature with Bio-Rad AG 501-X8 mixed bed resin) were added to the H3-H4 peak fractions to a final urea concentration of 6 M. The sample was incubated with nutation for 1 to 2 h at 22°C before being dialyzed (molecular weight cutoff: 3500 Da) extensively against three changes of 2 L water containing 5 mM 2-mercaptoethanol. The first and third dialysis steps were performed for 2 h, and the second dialysis step was overnight. The insoluble material was removed by centrifugation (Fiberlite F21S-8x50y rotor; 13,000 rpm; 20 min). The absorbance at 276 nm was measured, and the concentration of H3-H4 was determined by using the molar extinction coefficient of 10,430 cm/M at 276 nm and a molecular mass of 26,719 g/mol. The H3-H4 was lyophilized to a dry pellet with medium heat, and then stored at -80C. A typical yield of H3-H4 from 2 L of bacterial culture is about 8 mg of purified protein.

Histones H2A and H2B. Histones H2A and H2B were each synthesized and purified separately by using the same method, which is a modified version of the protocol described by Luger at al. (1999). [It is also useful to note that histones H3, H4, H2A.V, and H3.3 can be purified individually by this same method.] Unless stated otherwise, all operations were performed at 4°C. The pelleted cells (from 4 x 0.5 L of culture) were resuspended in 20 mL of Buffer W [50 mM Tris-HCl, pH 7.5, 0.1 M NaCl, 1 mM EDTA,

5 mM 2-mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride, and 1 mM benzamidine-HCl] and then frozen in liquid nitrogen. [If desired, the cells could be stored at -80˚C at this point.] The sample was thawed in warm (42˚C) water, and lysed by sonication on ice (Branson Sonifier 450 with a 0.25 inch microtip; 20% output; 6 to 10 cycles, 15 s each). The insoluble material was pelleted by centrifugation (Fiberlite F21S-8x50y rotor; 13,000 rpm; 20 min). The resulting pellet was resuspended in 50 mL of Buffer W containing 1% (v/v) Triton X-100, dispersed and washed by using a Wheaton Dounce homogenizer (A pestle), and repelleted by centrifugation (Fiberlite F21S-8x50y rotor; 13,000 rpm; 20 min). This process of dispersion, washing, and recentrifugation of the histone-containing pellet was repeated two to four times until the appearance of the pellet lightened to an off-white color. At this stage, the pellet can be frozen in liquid nitrogen and stored at -80C. The further purification of H2A or H2B was carried out as follows. The pellet was sliced into smaller pieces with a spatula, and immersed in 0.7 mL of DMSO for 30 min at 22˚C. To extract the histones from the pellet, 15 mL of Buffer UM [10 mM Tris-HCl, pH 8.0, 7 M urea (before use, freshly deionized as a 8 M urea solution in water with Bio-Rad AG 501-X8), 0.1 M NaCl, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, and 5 mM 2-mercaptoethanol] was added, and the mixture was dispersed with a Wheaton Dounce homogenizer (A pestle) and then incubated with nutation at room temperature for 1 hour. The insoluble material was pelleted by centrifugation (Fiberlite F21S-8x50y rotor; 13,000 rpm; 22°C; 20 min). The histone-containing supernatant was saved, and the pellet was subjected to an additional round of extraction with Buffer UM (15 mL), incubation, and centrifugation. The two histone-containing supernatants were combined, and small

particulate matter was removed by ultracentrifugation (Beckman SW41 rotor; 35,000 rpm; 22˚C; 35 min). The resulting sample was filtered through a Millipore Millex-HN nylon syringe filter (0.45 µm) and then applied to tandem Source 15Q [GE Healthcare; column dimensions (length x diameter) = 5.0 cm x 0.5 cm; volume = 1 mL; flow rate = 1 mL/min] followed by Source 15S [GE Healthcare; column dimensions (length x diameter) = 10 cm x 1 cm; volume = 4 mL; flow rate = 1 mL/min] chromatography columns that were equilibrated with Buffer UD [10 mM Tris-HCl, pH 8.0, 7 M urea (before use, freshly deionized as a 8 M urea solution in water with Bio-Rad AG 501-X8), 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol] containing 0.1 M NaCl. After the tandem columns were washed with one volume (5 mL) of Buffer UD containing 0.1 M NaCl, the Source 15Q column was removed. The remaining Source 15S column was washed with 5 column volumes of Buffer UD containing 0.1 M NaCl, and protein was eluted with a 0.1 M to 0.35 M NaCl gradient in Buffer UD over 7.5 column volumes followed by a 0.35 M to 0.4 M NaCl gradient in Buffer UD over 2.5 column volumes and a step to 1.0 M NaCl in Buffer UD for one column volume. H2A and H2B typically elute from the column at approximately 0.3 M NaCl. Peak fractions were identified by 15% polyacrylamide-SDS gel electrophoresis and staining with Coomassie Brilliant Blue R-250, and then combined and dialyzed (molecular weight cutoff: 3500 Da) extensively against three changes of 2 L water containing 5 mM 2-mercaptoethanol. The first and third dialysis steps were performed for 2 h, and the second dialysis step was overnight. The insoluble material was removed by centrifugation (Fiberlite F21S-8x50y rotor; 13,000 rpm; 20 min). The absorbance at 276 nm was measured, and the histone concentration was

determined by using the molar extinction coefficients at 276 nm and molecular masses as follows: H2A, 4470 cm/M, 12,231 g/mol; H2B with additional Ile, 7450 cm/M, 13,678 g/mol; H3, 4470, 15,356 g/mol; H4, 5960 cm/M, 11,363 g/mol. The histone was lyophilized to a dry pellet, and then stored at -80C. A typical yield of H2A or H2B from 2 L of bacterial culture is about 20 to 40 mg of purified protein.

Reconstitution of the core histone octamer

The core histone octamer was reconstituted by a modified version of the protocol described by Luger et al. (1999). Each of the lyophilized histones (~2 mg of each individual histone) was dissolved in 0.1 mL of Buffer G [10 mM Hepes (K^+), pH 7.6, 6 M guanidine-HCl, 1 mM EDTA, 10% (v/v) glycerol, and 1 mM dithiothreitol] at 22˚C for 30 min. The concentrations of the dissolved histones were determined by the absorbance at 276 nm, as described above. Then, equimolar amounts of histones H3 and H4 were combined with a 25% molar excess of each of H2A and H2B to give a 1:1:1.25:1.25 molar ratio of H3:H4:H2A:H2B. If necessary, add Buffer G to give a final volume of 0.4 mL. The mixture was dialyzed (molecular weight cutoff: 3500 Da) against 1 L of Buffer RF [10 mM Tris-HCl, pH 7.5, 2 M NaCl, 1 mM EDTA, 5 mM 2-mercaptoethanol] overnight at 4˚C. The insoluble material was removed by centrifugation (Eppendorf 5415R; 13,200 rpm; 10 min; 4°C), and the supernatant was loaded onto a Superose 12 column [GE Healthcare; column dimensions (length x diameter) = 30 cm x 1 cm; volume = 24 mL; flow rate = 0.2 mL/min] that was equilibrated in Buffer RF. The core histone octamer elutes with a K_{av} of ~0.25, and the excess histones H2A and H2B elute with a K_{av} of ~0.4.

Fractions containing equimolar ratios of core histones were identified by 15% polyacrylamide-SDS gel electrophoresis and staining with Coomassie Brilliant Blue R-250. The peak fractions were combined and dialyzed (molecular weight cutoff: 3500 Da) against 1 L of Buffer CH [10 mM Hepes (K^+), pH 7.6, containing 10 mM KCl, 1 mM EDTA, 10% (v/v) glycerol, and 1 mM dithiothreitol] overnight at 4°C. The concentration of the histones was determined by the BCA assay (Pierce) with bovine serum albumin as the reference. The histones were frozen in liquid nitrogen and stored at -80°C.

Purification of native core histones, recombinant human HMGN2, and recombinant Drosophila topoisomerase ND423 catalytic domain

Native *Drosophila* core histones were purified from *Drosophila* embryos as described (Bulger and Kadonaga 1994; Fyodorov and Levenstein 2002) and dialyzed into Buffer CH, frozen in liquid nitrogen, and stored at -80C. Native human histones were purified by the method of Bulger and Kadonaga (1994) and Fyodorov and Levenstein (2002), except that HeLa cell nuclei were used instead of *Drosophila* embryo nuclei, and then dialyzed into Buffer CH, frozen in liquid nitrogen, and stored at -80C. The human HMGN2 bacterial expression construct from Rattner et al. (2007) (phHMGN2) was transformed into *E. coli* strain Rosetta(DE3), and HMGN2 protein was purified as described in Paranjape et al. (1995). The purified protein was dialyzed into Buffer CH, frozen in liquid nitrogen, and stored at -80C. The ND423 catalytic domain of *Drosophila* topoisomerase I was synthesized and purified as described by Fyodorov and Kadonaga (2003).

Purification of recombinant human histone H1.0

The full-length sequence of human histone H1.0 was the kind gift of Dr. Woojin An (USC School of Medicine). It was slightly modified and subcloned into a pET24 vector to give pET24-H1.0. The coding sequence, which was confirmed by DNA sequence analysis, is provided in Supplemental Table S1. Recombinant human H1.0 was purified by using a modified version of a method for the purification of native histone H1 (Croston et al. 1991). For expression, freshly transformed *E. coli* strain Rosetta(DE3) was grown in LB medium (40 µg/mL ampicillin) at 37°C to $A_{600\ nm}$ of approximately 0.6, and protein synthesis was induced by the addition of IPTG to 1 mM final concentration. The culture was incubated for an additional 2 h at 30°C, and the cells were pelleted by centrifugation (Fiberlite F14S-6x250y rotor; 6,000 rpm; 5 min; 4°C). Unless stated otherwise, all subsequent operations were performed at 4°C. The bacterial pellet was resuspended in 20 mL of Buffer L [20 mM Tris-HCl, pH 7.9, containing 0.5 M NaCl, 0.2 mM EDTA, 10%(v/v) glycerol, 0.2 mM phenylmethylsulfonyl fluoride, 10mM 2-mercaptoethanol, 1 µg/mL pepstatin, 1 µg/mL leupeptin, and 1 µg/mL aprotinin] and lysed by sonication on ice (Branson Sonifier 450 with a 0.25 inch microtip; 20% output; 6 to 8 cycles, 45 s each). The insoluble material was pelleted by centrifugation (Fiberlite F21S-8x50y rotor; 11,000 rpm; 20 min). Pulverized ammonium sulfate was slowly added to the lysate to 2.1 M final concentration, and the mixture was incubated with nutation for 15 min. The insoluble material was pelleted by centrifugation (Fiberlite F21S-8x50y rotor; 15,000 rpm; 20 min), and the supernatant was applied to a Phenyl Sepharose CL-4B chromatography column

[GE Healthcare; column dimensions (length x diameter) = 10 cm x 1.6 cm; column volume = 20 mL; flow rate = 0.5 mL/min]. H1.0 was eluted with a linear gradient from 2.1 to 0.1 M ammonium sulfate in HEMG buffer [25 mM Hepes (K^+), pH 7.6, 0.1 mM EDTA, 12.5 mM $MgCl_2$, 10% (v/v) glycerol, and 1 mM dithiothreitol] containing 0.5 mM phenylmethylsulfonyl fluoride over 7.5 column volumes. Fractions containing H1.0 were identified by 15% polyacrylamide-SDS gel electrophoresis and staining with Coomassie Brilliant Blue R-250. The peak fractions were combined and dialyzed against HEG buffer [25 mM Hepes (K^+), pH 7.6, 0.1 mM EDTA, 10% (v/v) glycerol, and 1 mM dithiothreitol] containing 0.1 M KCl until the conductivity of the sample was identical to that of HEG buffer containing 0.15 M KCl. Nonidet P-40 was added to a final concentration of 0.01% (v/v), and HEG buffer (lacking KCl) was added until the conductivity of the sample was identical to that of HEG buffer containing 0.1 M KCl. The sample was subjected to centrifugation (Fiberlite F21S-8x50y rotor; 10,000 rpm; 10 min), and then applied to a Source 15S column [GE Healthcare; column dimensions (length x diameter) = 5 cm x 0.5 cm; volume = 1 mL; flow rate = 1 mL/min). H1.0 was eluted with a linear gradient of 0.1 M to 1 M KCl in HEG buffer containing 0.01% (v/v) Nonidet P-40 and 0.5 mM phenylmethylsulfonyl fluoride over 15 column volumes. H1.0 containing fractions were identified by 15% polyacrylamide-SDS gel electrophoresis and staining with Coomassie Brilliant Blue R-250. The peak fractions were combined and dialyzed (molecular weight cutoff: 3500 Da) for 2 h against 2 L of HEMG buffer containing 0.1 M KCl, 0.01% (v/v) Nonidet P-40, and 0.5 mM phenylmethylsulfonyl fluoride, frozen in liquid nitrogen, and stored at -80°C. A typical yield from 1 L bacterial culture was about 0.1 mg of purified

protein. Histone H1 proteins adhere to glass and plastic; hence, it is generally useful to include 0.01% (v/v) Nonidet P-40 in buffers containing histone H1.

Chromatin assembly reactions

Chromatin assembly reactions and DNA supercoiling and partial MNase digestion analyses were generally performed as described by Fyodorov and Levenstein (2002) and Fyodorov and Kadonaga (2003) with ACF and dNAP1. Here, with the dNLP-ISWI system, we have included different variations of the chromatin assembly method that could be used to assemble chromatin for different specific applications. The different steps should be performed in the order listed below. This protocol is sufficient for 6 chromatin assembly reactions in which 0.54 µg of DNA is assembled into chromatin per reaction.

Determination of the correct amount of topoisomerase I to use for the relaxation of supercoiled plasmid DNA (optional: for analysis of the chromatin by the DNA supercoiling assay). If the chromatin is to be analyzed by the DNA supercoiling assay, then the amount of topoisomerase I to use for the relaxation of supercoiled plasmid DNA should be determined. Otherwise, the topoisomerase I step can be omitted. [Note also that topoisomerase I should not be included in chromatin assembly reactions with linear DNA.] The purified ND423 topoisomerase I (Fyodorov and Kadonaga 2003) should have a concentration of about 1 to 2 mg/mL. Before use, the enzyme should be diluted about 100-fold into Buffer S [10 mM Hepes (K^+), pH 7.6, 0.1 mM EDTA, 50% (v/v) glycerol, 50 mM NaCl, 0.2 mg/mL recombinant insulin, 0.01% (v/v) Nonidet P-40, 10 mM 2-mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride, 0.5 mM benzamidine-HCl, and

1 µg/mL leupeptin]. In the context of this work, we define 1 unit to be the amount of topoisomerase I that is sufficient to relax 5 µg of template DNA completely in 10 min at 30˚C in 1x Topo I buffer [50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 0.1 mM EDTA, 50 µg/mL BSA, and 0.5 mM dithiothreitol]. By analyzing the activity of different dilutions of the topoisomerase I stock, it should be possible to determine the appropriate dilution that would give a concentration of 0.5 units/µL. It is also useful to note that excess topoisomerase I can inhibit the assembly reaction. We therefore recommend the addition of only 1 unit of topoisomerase I to the DNA relaxation reactions described below.

Dilution of dNLP, ISWI, and core histones. Before use, the proteins are diluted to the following working concentrations: dNLP to 2.0 mg/mL (22 µM of dNLP pentamers) in HEG buffer (12 µL of 2.0 mg dNLP/mL is sufficient for 6 reactions); ISWI to 0.12 mg/mL (1.0 µM) in HEG buffer (18 µL of 0.12 mg ISWI/mL is sufficient for 6 reactions); core histones to 0.35 mg/mL (3.3 µM of histone octamers) in Buffer CH (12 µL of 0.35 mg core histones/mL is sufficient for 6 reactions). Note that frozen stocks of dNLP, ISWI, and core histones should be thawed quickly in room temperature (22˚C) water immediately before use, kept on ice during use, and then frozen in liquid nitrogen before storage at -80˚C.

Preparation of ATP mix (two versions). An ATP regeneration system (phosphoenolpyruvate and pyruvate kinase) is usually not necessary for chromatin assembly, but it is typically included to ensure that sufficient ATP is available for the reaction. Two versions of the ATP mix are as follows. Version 1 (7x scale ATP mix with ATP regeneration system): 63 µL of water; 37.5 µL of 100 mM $MgCl_2$; 4.5 µL of 0.5 M

ATP, pH 7.0; 4.5 µL of 0.5 M phosphoenolpyruvate, pH 7.0 (Millipore Sigma; cat. no. 860077); and 3 µL of 5000 U/mL pyruvate kinase [Millipore Sigma; cat. no. P9136; in 10 mM Hepes (K^+), pH 7.6, containing 10 mM KCl and 50% (v/v) glycerol] to a final volume of 112.5 µL (use 15.8 µL per reaction, as indicated below). Version 2 (7x ATP mix without ATP regeneration system): 70.5 µL of water; 37.5 µL of 100 mM $MgCl_2$; and 4.5 µL of 0.5 M ATP, pH 7.0, to a final volume of 112.5 µL (use 15.8 µL per reaction, as indicated below). The ATP mixes are prepared on ice and stored on ice before use.

Preparation of the dNLP mix. A 7x scale dNLP mix is prepared with the following components: 290 µL of HEG buffer; 123 µL of 600 mM KCl; 147 µL of a solution of 5% (w/v) polyethylene glycol (Millipore Sigma; cat. no. P2139) and 5% (w/v) polyvinyl alcohol (Millipore Sigma; cat. no. P8136); 8 µL of 2 mg/mL bovine serum albumin (BSA; Pierce cat. no. 23209); and 9.1 µL of 2 mg/mL dNLP to a final volume of 577 µL (use 82.5 µL per reaction). Mix the tube by gentle flicking, and consolidate the liquid by low speed centrifugation (2000 rpm or less). It should be noted that the BSA is not required for the chromatin assembly reaction, but is included to maximize the consistency and reproducibility of the reactions. If desired, the 8 µL of BSA could be replaced with 8 µL of water. The dNLP mix is prepared on ice and store on ice before use.

Formation of dNLP-core histone complexes. For each reaction (1x scale), 1.5 µL of the core histones (0.35 mg/mL) is added to 82.5 µL of the dNLP mix on ice. The tube was mixed by gentle flicking or vortexing and kept on ice for 20 min.

Preparation of the DNA mix (two versions). Version 1 (relaxation of supercoiled DNA with topoisomerase I). If DNA supercoiling analysis is to be performed, it would be

necessary to relax supercoiled plasmid DNA with topoisomerase I. While the dNLP-core histone mixture is incubating on ice, relax the plasmid DNA by combining the following components (9x scale): 18 µL water; 3 µL of 10x Topo I buffer; 6 µL DNA (0.90 µg DNA/µL in water or TE); and 3 µL of topoisomerase I (0.5 units/µL, as defined above) to 30 µL final volume. Mix and incubate at 30˚C for 10 min. Store at room temperature (22˚C) before use. Version 2 (use of DNA without treatment with topoisomerase I). Combine the following: 21 µL water; 3 µL 10x Topo I buffer; 6 µL DNA (0.90 µg DNA/µL in water or TE) to 30 µL final volume. Mix and incubate at 30˚C for 10 min. Store at room temperature (22˚C) before use.

Chromatin assembly reactions. For each reaction (1x scale), place the tube containing the dNLP-core histone complexes (84 µL) in a 22˚C water bath. (Note that the samples will remain at 22˚C until the 27˚C incubation below.) To the tube, add 15.8 µL of the ATP mix and 3.3 µL of the DNA mix. Blend the components by gentle vortexing for about 3 seconds. Then, initiate the chromatin assembly reaction by the addition of 2.1 µL of the 0.12 mg/mL ISWI. Mix the sample by gentle flicking, and incubate the tube at 27˚C in a water bath for 1.5 h. Terminate the reactions by the addition of 11.4 µL of 0.5 M EDTA (Na^+), pH 8.0, to 50 mM final concentration of EDTA. Further analyses, such as DNA supercoiling or MNase digestion assays, could be performed as described by Fyodorov and Levenstein (2002) and Fyodorov and Kadonaga (2003). In the case of MNase digestion assays, the chromatin assembly reactions should not be terminated with 50 mM EDTA, which would inhibit MNase activity.

Additional notes on chromatin assembly conditions. Some additional notes regarding the chromatin assembly reactions are as follows. First, the core histone to DNA mass ratio is a critical factor in the successful assembly of chromatin. For each new preparation of histones and DNA, it is necessary to determine the optimal histone:DNA ratio empirically by careful titration, such as by variation of the ratio from 0.7 to 1.2 in increments of 0.1. Second, in the assembly of chromatin with histone H1, we reduce the histone:DNA mass ratio by about 10-20% to adjust for the longer repeat length of the nucleosomes. Third, in reactions containing histone H1 or HMGN2, we add 0.01% (v/v) Nonidet P-40 and 1 mM dithiothreitol to the reaction medium.

Native gel electrophoresis of mononucleosomes

In this assay, the newly-assembled chromatin is digested to mononucleosomes by MNase, and the resulting particles are analyzed by native gel electrophoresis. For each sample, an aliquot from the assembly reaction (32 µL; from the step prior to the addition of EDTA to 50 mM final concentration) was combined with 100 mM $CaCl_2$ (1 µL). MNase (0.1 units/µL) was added to a final concentration of about 0.003 units MNase/mL. The reaction was carried out for 1 min at 22˚C, and then terminated by the addition of 0.5 M EDTA (Na^+), pH 8.0, to a final concentration of 60 mM. The sample was applied to a 5% nondenaturing polyacrylamide gel (height x width x thickness: 21 cm x 19 cm x 1 mm) that was made from freshly prepared 0.8:30 bisacrylamide:acrylamide and 0.5x TBE. The gel (which was pre-run for 1 h prior to the application of the samples) was run at 60 V at 4˚C for about 5.5 h.

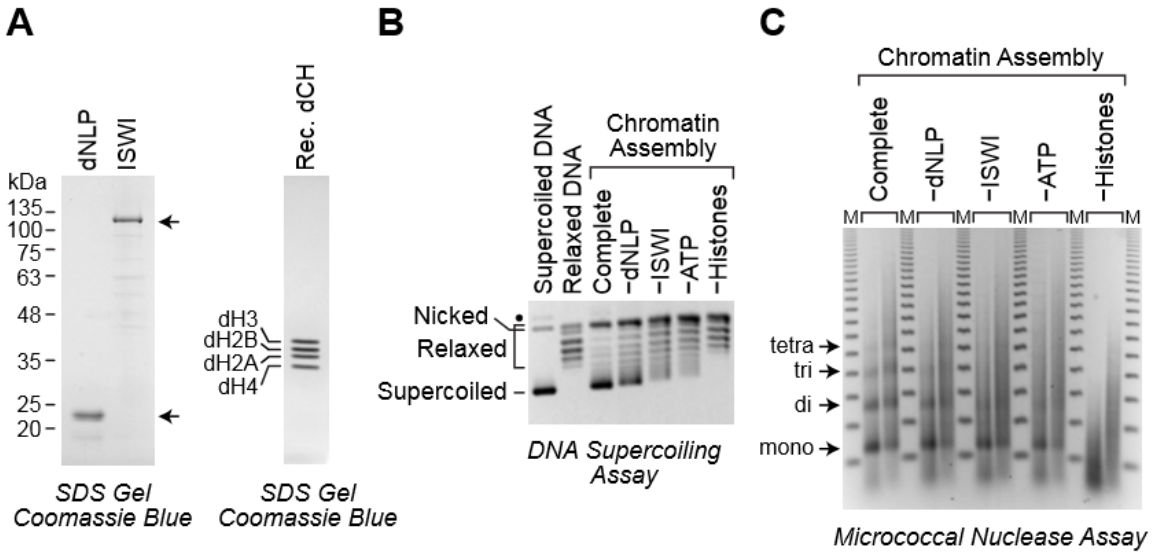


Figure 4.1. ATP-dependent assembly of periodic nucleosome arrays with the dNLP-ISWI system. *A*, Purification of recombinant *Drosophila* dNLP, ISWI, and S phase-regulated core histones. The proteins were synthesized in *E. coli*, purified, and analyzed by 10% (left) or 15% (right) polyacrylamide-SDS gel electrophoresis and staining with Coomassie Brilliant Blue R-250. The dNLP is the N-terminally His_6-tagged version of the protein. The sizes of molecular mass markers (in kDa) and the positions of the individual histones are indicated. *B*, DNA supercoiling analysis reveals efficient nucleosome assembly by dNLP and ISWI. Complete chromatin assembly reactions were carried out with dNLP, ISWI, core histones, ATP, and relaxed plasmid DNA in the presence of purified topoisomerase I, an ATP regeneration system, and bovine serum albumin (BSA) as a stabilizing agent. Reactions lacking individual components are indicated. The reaction products were deproteinized, subjected to 0.8% agarose gel electrophoresis, and stained with ethidium bromide. Samples of supercoiled DNA and relaxed DNA were included as references. The positions of nicked DNA, relaxed DNA, and supercoiled DNA are shown. The black dot corresponds to a minor unknown contaminant, which may be supercoiled dimeric plasmid DNA. *C*, Partial MNase digestion analysis indicates the assembly of periodic nucleosome arrays by dNLP and ISWI. Chromatin assembly reactions were performed as in *B*. The reaction products were partially digested with two different concentrations of MNase, deproteinized, and subjected to 1.3% agarose gel electrophoresis. The resulting DNA fragments were detected by staining with ethidium bromide. The DNA bands that correspond to mono-, di-, tri-, and tetra-nucleosomes are shown. The DNA size markers (M) are the 123 bp ladder (Invitrogen).

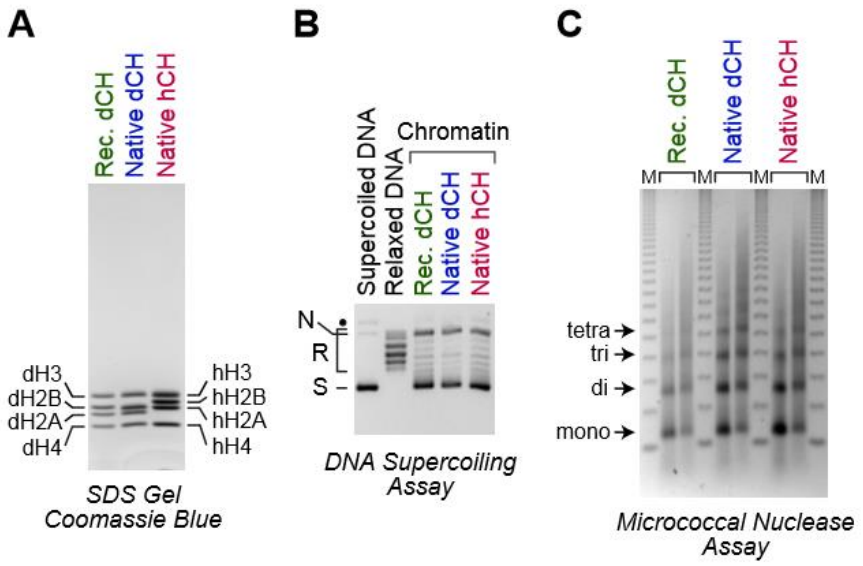


Figure 4.2. Assembly of native *Drosophila* and human histones with dNLP and ISWI. *A*, Purification of core histones from *Drosophila* embryos and human HeLa cells. The proteins were analyzed by 15% polyacrylamide-SDS gel electrophoresis and staining with Coomassie Brilliant Blue R-250. The positions of the individual *Drosophila* (d) and human (h) core histone (CH) are indicated. *B*, DNA supercoiling analysis. Chromatin assembly reactions were carried out and analyzed, as in Fig. 1*B*, with the indicated core histones. The positions of nicked DNA (N), relaxed DNA (R), and supercoiled DNA (S) are shown. *C*, Partial MNase digestion analysis. Chromatin was assembled and analyzed, as in Fig. 1*C*, with the indicated core histones.

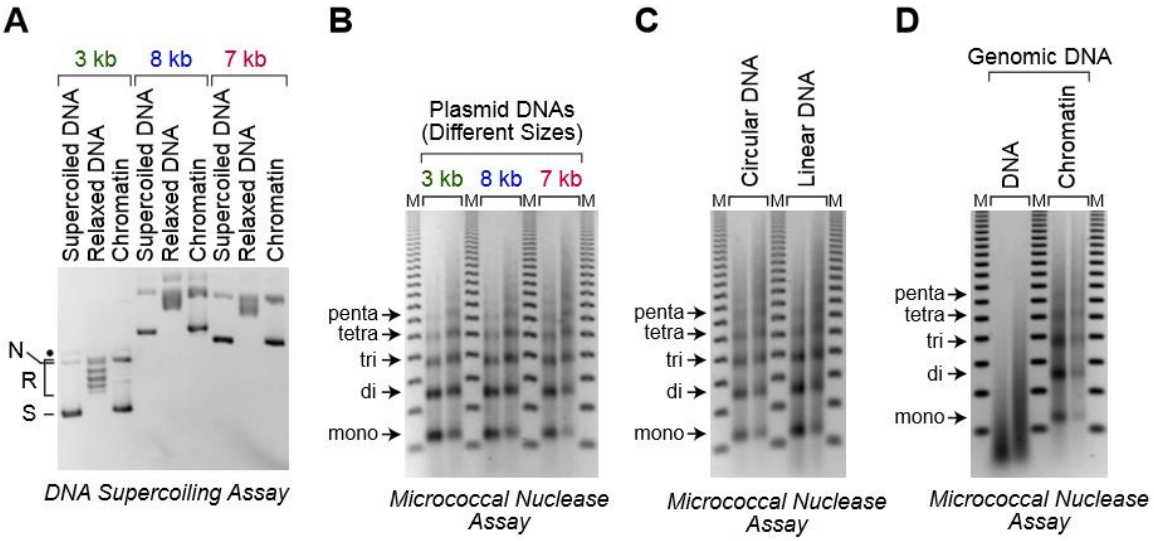
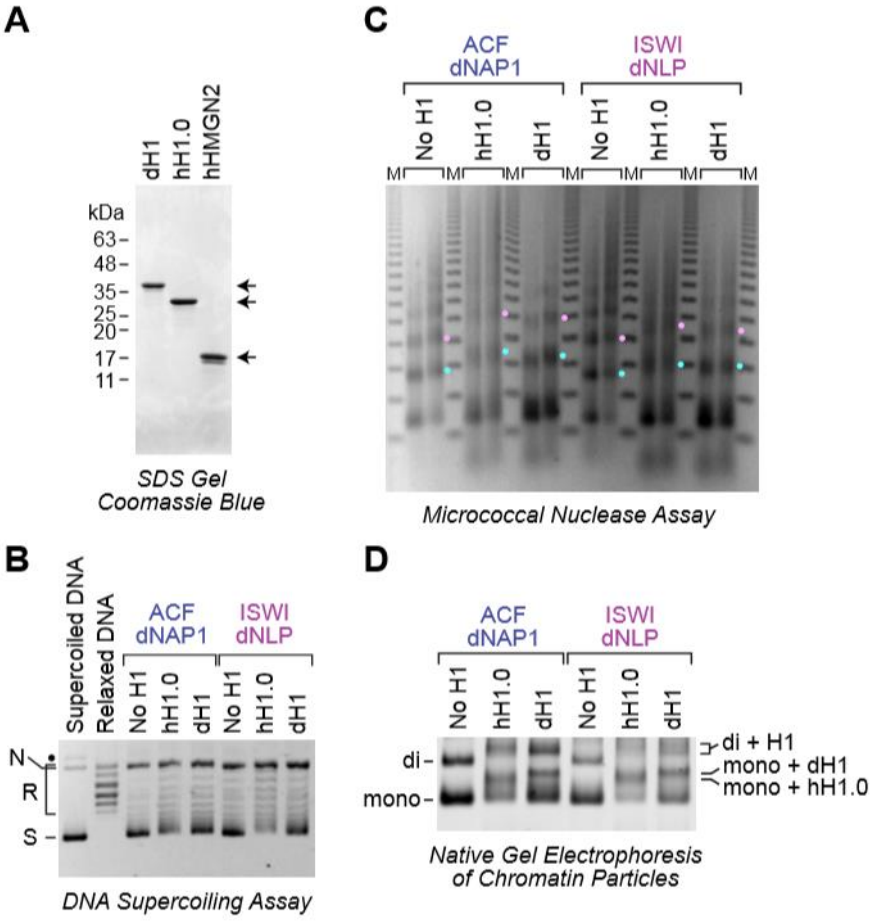


Figure 4.3. Assembly of various DNA templates with the dNLP-ISWI system. *A*, DNA supercoiling analysis shows the efficient assembly of three different plasmid DNAs. Chromatin assembly reactions were carried out with three plasmid DNAs: pGIE-0 (3 kb) (Pazin et al., 1994), which contains sequences from the adenovirus E4 promoter; pJH187 (8 kb), which contains sequences from the *Drosophila Antennapedia* gene; and pLA4 (7 kb) (Albright et al., 1988), which contains sequences from bacteriophage T4. Chromatin assembly and analysis were performed as in Fig. 1*B*, except that native *Drosophila* core histones were used instead of recombinant *Drosophila* core histones. *B*, Partial MNase digestion analysis reveals the assembly of periodic nucleosome arrays on three different plasmid DNAs. Reactions were performed as in *A*, and the samples were analyzed as in Fig. 1*C*. *C*, Chromatin can be assembled with either circular or linear DNA. Reactions were performed as in *A* with relaxed circular or linearized pLA4 (7 kb), and the resulting samples were subjected to partial MNase digestion analysis. *D*, The dNLP-ISWI system can be used for the assembly of genomic DNA into periodic nucleosome arrays. Chromatin assembly reactions were carried out as in *A* with native calf thymus genomic DNA that is mostly greater than 10 kb in length (Fig. S4). The chromatin (right) and genomic DNA (left) were subjected to partial MNase digestion analysis in parallel

Figure 4.4. The dNLP-ISWI system can be used to assemble histone H1-containing chromatin. *A*, Purification of native *Drosophila* histone H1, recombinant human H1.0, and recombinant human HMGN2. The proteins were subjected to 15% polyacrylamide-SDS gel electrophoresis and staining with Coomassie Brilliant Blue R-250. *B*, DNA supercoiling analysis of chromatin assembled with histone H1. Reactions were performed either with the dNAP1-ACF system (Fyodorov and Kadonaga 2003) or the dNLP-ISWI system, as in Fig. 3. Where indicated, *Drosophila* histone H1 and human H1.0 were included at a ratio of 0.75 molecules of H1 per nucleosome. The positions of nicked DNA (N), relaxed DNA (R), and supercoiled DNA (S) are shown. The black dot corresponds to a minor unknown contaminant. *C*, Partial MNase analysis shows an increase in the nucleosome repeat length upon incorporation of histone H1 into chromatin. Reactions were performed as in *B*, and the resulting samples were subjected to partial MNase digestion analysis. The blue dots correspond to the DNA bands derived from dinucleosomes, and the pink dots designate DNA fragments from trinucleosomes. *D*, Native gel electrophoresis of mono- and di-nucleosomes obtained by extensive MNase digestion of chromatin either lacking or containing histone H1. The chromatin particles were detected by staining of the DNA with SYBR Gold. The positions of mononucleosomes (mono), dinucleosomes (di), H1-containing mononucleosomes (chromatosomes; mono + H1), and H1-containing dinucleosomes (di + H1) are indicated.


A
dH1
hH1.0
hHMGN2
kDa
63
48
35
25
20
17
11
SDS Gel
Coomassie Blue
C
ACF
dNAP1
ISWI
dNLP
No H1
hH1.0
dH1
No H1
hH1.0
dH1
M
Micrococcal Nuclease Assay
B
Supercoiled DNA
Relaxed DNA
ACF
dNAP1
ISWI
dNLP
No H1
hH1.0
dH1
No H1
hH1.0
dH1
N
R
S
DNA Supercoiling Assay
D
ACF
dNAP1
ISWI
dNLP
No H1
hH1.0
dH1
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mono + hH1.0
Native Gel Electrophoresis
of Chromatin Particles

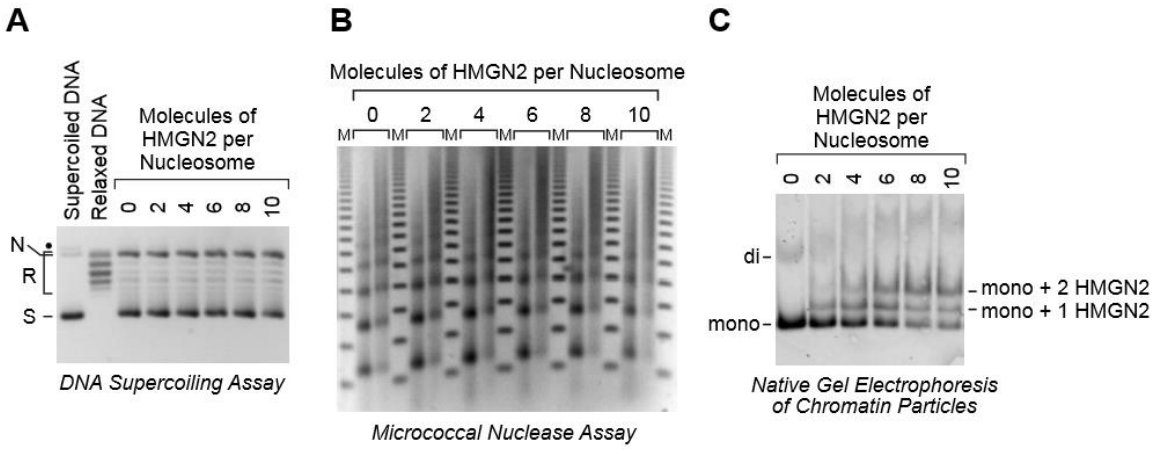

Figure 4.5. The dNLP-ISWI system can be used to assemble HMGN2-containing chromatin. *A*, DNA supercoiling analysis of chromatin assembled with HMGN2. Chromatin assembly reactions were performed as in Fig. 3 in the absence or presence of purified human HMNG2 (shown in Fig. 4*A*) at the indicated molar ratios of HMGN2 to nucleosomes. *B*, Partial MNase analysis of HMGN2-containing chromatin assembled as in *A*. *C*, Native gel electrophoresis of mono- and di-nucleosomes obtained by extensive MNase digestion of chromatin either lacking or containing HMGN2. The chromatin particles were detected by staining of the DNA with SYBR Gold. The positions of mononucleosomes (mono), dinucleosomes (di), mononucleosomes containing one molecule of HMGN2 (mono + 1 HMGN2), and mononucleosomes containing two molecules of HMGN2 (mono + 2 HMGN2) are indicated.

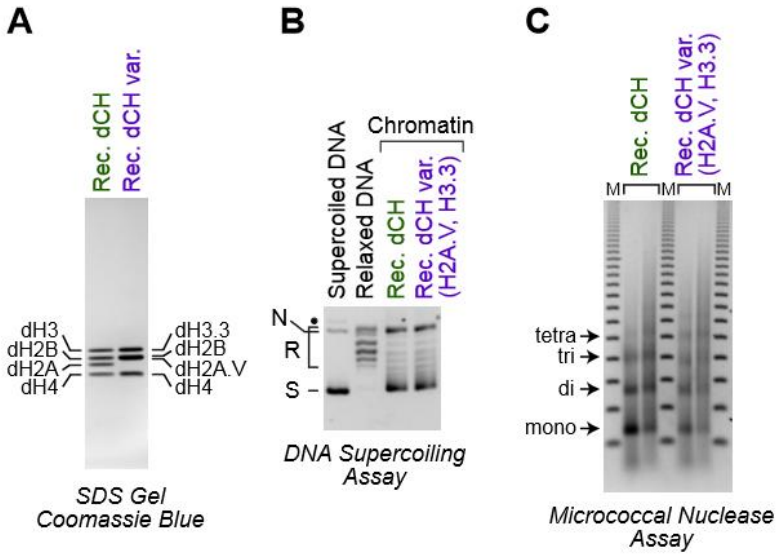

Figure 4.6. The dNLP-ISWI system can assemble containing histone variants H3.3 and H2A.V. *A*, Purification of *Drosophila* core histones with the H3.3 and H2A.V variants. The core histone proteins were synthesized in *E. coli*, purified, and analyzed by 15% polyacrylamide-SDS gel electrophoresis and staining with Coomassie Brilliant Blue R-250. The positions of the individual histones are shown. *B*, DNA supercoiling analysis of chromatin assembled with histones H3.3 and H2A.V. Chromatin assembly reactions were performed as in Fig. 1*B* except that the H3.3 and H2A.V variants were used instead of the S phase-regulated H3 and H2A histones. *C*, Partial MNase digestion analysis of chromatin assembled with histones H3.3 and H2A.V.

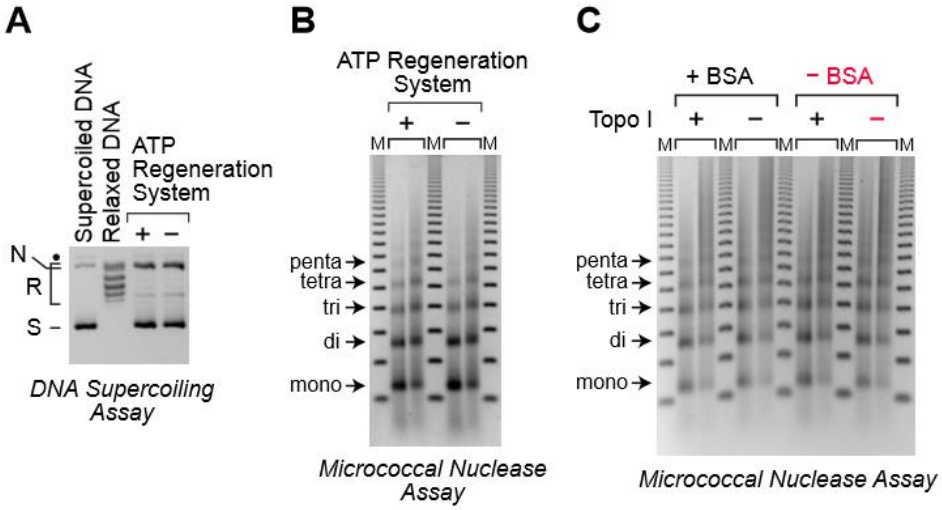

Figure 4.7. A simplified minimal chromatin assembly system. *A*, An ATP-regeneration system is not essential for chromatin assembly by dNLP and ISWI. Chromatin assembly reactions were performed in the presence or absence of the ATP regeneration system, which consists of pyruvate kinase and phosphoenolpyruvate. The reactions were carried out as in Fig. 3. DNA supercoiling analysis reveals that the efficiency of nucleosome assembly is not affected by the inclusion of the ATP-regeneration system. *B*, Partial MNase digestion analysis of the chromatin assembled in *A* shows that the ATP regeneration system is not essential for the assembly of periodic nucleosome arrays. *C*, Topoisomerase I and bovine serum albumin (BSA) are not essential for the assembly of periodic nucleosome arrays with supercoiled plasmid DNA. Reactions were carried out in the absence of the ATP regenerating system, as in *A*, with supercoiled plasmid DNA in the presence or absence of topoisomerase I and BSA, as indicated. The samples were analyzed by the partial MNase digestion assay. The minimal system that consists only of dNLP, ISWI, core histones, DNA, and ATP is highlighted in red type.

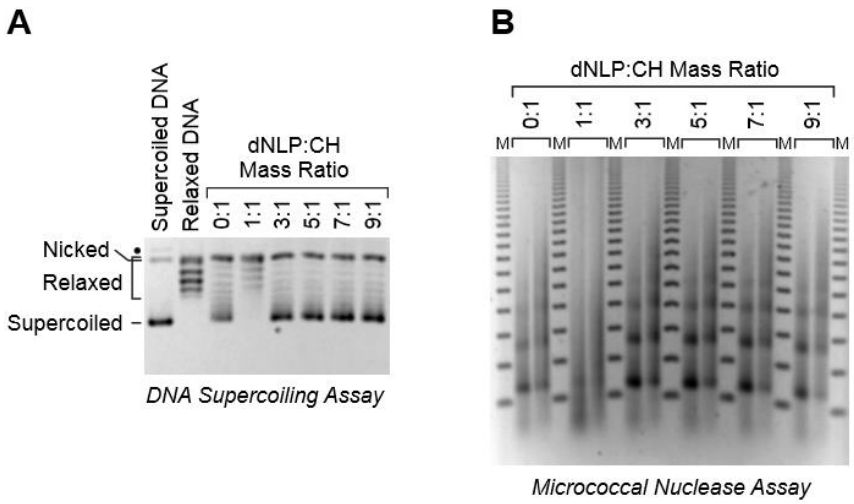

Figure 4.S1. Determination of the optimal dNLP to core histone mass ratio for chromatin assembly. Reactions were performed as in Fig. 1 of the main text with the indicated dNLP to core histone mass ratios. A, DNA supercoiling analysis. B, Partial MNase digestion analysis. Based on these results, a 5:1 mass ratio of dNLP to core histones was used throughout this work unless stated otherwise. The low efficiency of chromatin assembly at a 1:1 mass ratio of dNLP to core histones was consistently and reproducibly observed.

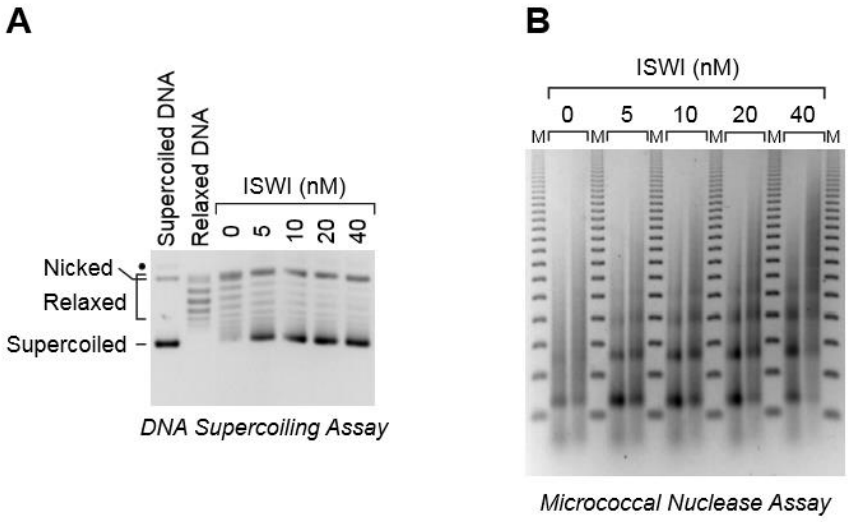

Figure 4.S2. Determination of the optimal ISWI concentration for chromatin assembly. Reactions were performed as in Fig. 1 of the main text with the indicated concentrations of ISWI. A, DNA supercoiling analysis. B, Partial MNase digestion analysis. Based on these results, a concentration of 20 nM ISWI was used throughout this work unless stated otherwise.

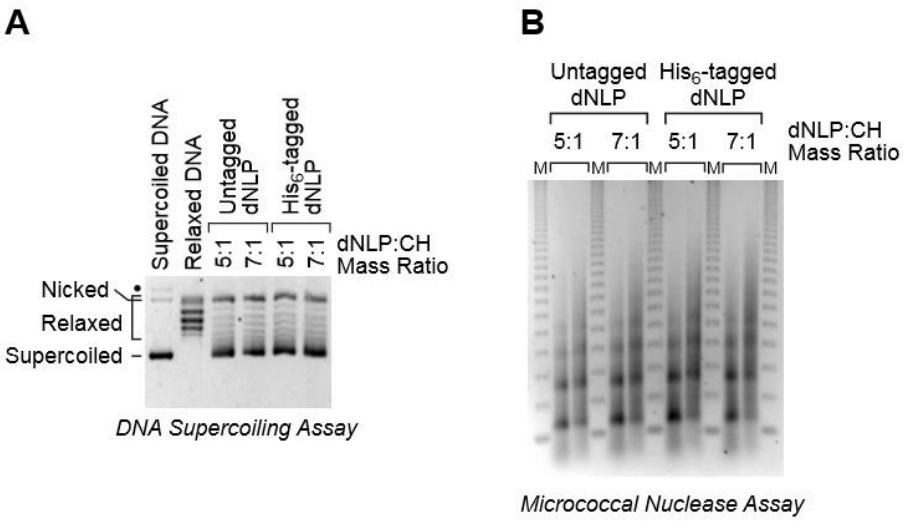

Figure 4.S3. The N-terminally His6-tagged version of dNLP has chromatin assembly activity that is comparable to that of untagged dNLP protein. Chromatin assembly reactions were carried out as in Fig. 1 of the main text with the indicated amounts of either N-terminally His6-tagged dNLP or untagged dNLP. A, DNA supercoiling analysis. B, Partial MNase digestion analysis.

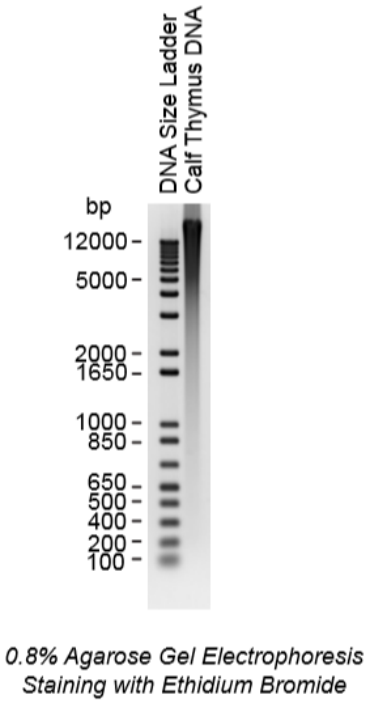

Figure 4.S4. Agarose gel electrophoresis of calf thymus genomic DNA. Purified, unsheared calf thymus DNA (Millipore-Sigma; D4764-1UN) was analyzed by 0.8% agarose gel electrophoresis and staining with ethidium bromide.

FOOTNOTES

[1]The abbreviations used are: ACF, ATP-utilizing chromatin assembly and remodeling factor; BSA, bovine serum albumin; Chd1, chromodomain helicase DNA-binding protein 1; dNAP1, *Drosophila* nucleosome assembly protein 1; dNLP, *Drosophila* nucleoplasmin-like protein; HMG, high mobility group; HMGN, high mobility group, nucleosome-binding; IPTG, isopropyl-β-thiogalactopyranoside; ISWI, imitation switch; MNase, micrococcal nuclease; RSF, remodeling and spacing factor

[2]The chromatin assembly system described in this article could potentially be sold commercially as a research reagent.

Chapter 4, in full, is being prepared for submission for publication of the material. Khuong, Mai T.; Fei, Jia; Kadonaga, James T. The dissertation author was the primary investigator and author of this paper.

CHAPTER 5:

Summary and Perspectives

Chromatin is a nucleoprotein complex that is extremely dynamic. It must disassemble and reassemble during DNA-utilizing processes such as replication, transcription, and repair. It is suggested that chromatin's dynamic property is derived from the presence of non-nucleosomal chromatin particles. These particles are often found at regions of active chromatin, and it is believed that they poise the regions for activation of DNA-utilizing processes. For part of my dissertation, I sought to investigate two of these non-nucleosomal chromatin particles, the H3C110 particle and the prenucleosome. The latter half of my dissertation focuses on a simple and versatile method to assemble chromatin *in vitro*.

Chapter 2 of this dissertation discusses the methods and the challenges faced in isolating the non-nucleosomal chromatin particles *in vivo*. The main challenge was not knowing whether these particles were salt-labile and/or fragile. H3C110 particles were also difficult to isolate due to the unavailability of the original resin used. Alternative Hg(II) resin were used, but the analysis of the particles isolated were inconclusive. The prenucleosome or prenucleosome-like particles (H3K56ac particles) were difficult to isolate in cells due to its low abundance (0.04%) in mammalian cell lines and the lack of antibody that is sufficiently specific for H3K56ac relative to other histone modifications such as H3K9ac.

The work presented in chapter 3 reveals that prenucleosomes, which were originally formed by the deposition of histone chaperone NAP-1, could also be formed by histone chaperone NLP. The prenucleosomes formed by NLP exhibited the same properties as those formed by NAP-1. Prenucleosomes exhibited template commitment, were formed

within seconds, and were stable for at least two hours before being converted into mature canonical nucleosomes. This chapter also reveals that presence of prenucleosomes or prenucleosome-like particles at promoters and enhancers of active genes.

From my work with NLP, I developed a simple and versatile method to assemble chromatin *in vitro*, which is discussed in chapter 4. The substitution of NLP for NAP-1 and ISWI for ACF removes the need for a baculoviral expression system. This lowers costs and time investment. NLP is also thermostable, which is useful when removing nuclease activity by heat treatment. The new system is as versatile as the older system, but is much easier to set-up.

Future Directions

Non-nucleosomal chromatin particles are likely to be biologically important and efforts should be continued to study them in cells. However, replicating the original experiments with Hg(II) nucleosomes would not be possible because Affi-Gel 501 has been discontinued. Two separate attempts with alternative Hg(II) affinity resins were also unable to enrich for active chromatin. This might suggest that with current experimental conditions, the Hg(II) affinity resin is simply unable to access the H3C110 particle. Efforts should be put into testing other thiol-specific reagents. Maleimides were primarily used to try and isolate H3C110 particles, but it could be possible that maleimides also were unable to access the exposed thiol group. Very brief work was done with iodoacetamides and it is possible that they may be more effective in accessing the thiol.

It is inadvisable to pursue isolating the H3K56ac particles by using antibodies. Due to their low abundance in mammalian cells and the high likelihood that antibodies against

H3K56ac cross react with H3K9ac due to H3K9ac, H3K56ac particles would be difficult to isolate. An alternative to studying active chromatin and possibly non-nucleosomal chromatin particles could be to enrich for active chromatin using methylation sensitive enzymes. These enzymes would cleave unmethylated CpG sequences leaving the methylated sequences intact. This would result in an enrichment of active chromatin and mass spectrometry could be used to characterize the particles isolated. It could be that by searching more broadly, H3C110, prenucleosome-like particles, and other novel non-nucleosomal chromatin particles could be found.

Regarding the new simplified *in vitro* chromatin assembly system, many more avenues of research can now be explored. The low cost, time investment, and simplicity of the system will allow a broad range of scientists to assemble customized chromatin and analyze its effects on DNA-utilizing processes. Companies like Active Motif are now creating custom histones and with this new system, designer chromatin templates can be assembled and structural and functional analysis can be performed.

Concluding Remarks

Chromatin is a dynamic nucleoprotein complex that is responsible for vital genomic processes. Over the past 50 years, we have made significant progress in understanding how these processes have been regulated by chromatin and its associated factors. Further investigation into the non-nucleosomal chromatin particles and how processes are affected by customized chromatin will likely add to that understanding.

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