13 Studying Chromatin Dynamics In Vitro: Chromatin Assembly, Remodeling, and Transcription

IMPORTANT ISSUES

- *Can an endogenous chromatin structure be recapitulated in vitro?*
- *The template, source of histones, and nucleosome assembly method are critical choices when attempting to reproduce specific chromatin structures in vitro.*
- *Several approaches can be taken to evaluate the integrity and quality of chromatin reconstitutions.*
- *Immobilized template assays allow dissection of the roles of multiple chromatin remodeling/modification enzymes in transcriptional regulation in vitro.*
- *ATP-dependent chromatin remodeling enzymes have distinct biochemical activities that can influence transcriptional activity.*
- *Recombinant histones can be altered by site-specific chemistry to generate novel reagents for analysis of chromatin structure and function.*

INTRODUCTION AND OVERVIEW

As outlined in the preceding chapters, an outstanding problem in the field of eukaryotic gene regulation is understanding the biochemical mechanism by which gene-specific activators regulate transcription. In Chapters 11 and 12, we discussed biochemical strategies to dissect how these transcriptional regulators interact with their cognate DNA sequences and enhance transcription from DNA templates. But given that eukaryotic genomes within cells are assembled into nucleosomal arrays, complete recapitulation of the pathways governing complex gene regulatory events also requires mechanistic studies to be performed on DNA templates that have been reconstituted into chromatin.

As outlined in Chapter 1, nucleosome assembly and chromatin fiber formation create potent barriers for nearly every step in the pathway leading to transcription initiation and elongation in vivo and in vitro. Consequently, the regulation of

chromatin dynamics plays an integral role in controlling gene expression. In the majority of cases, gene-specific activators or repressors play a central role in this process by coordinating the recruitment of a multitude of chromatin remodeling and modification enzymes that regulate transcription in the context of chromatin. Adenosine triphosphate (ATP)–dependent remodeling enzymes can alter nucleosome positions, evict histone octamers, displace H2A/H2B dimers, or change the composition of chromatin by introducing or eliminating histone variants. A specific subset or the full constellation of these activities may be used at a particular target gene. Likewise, there are a plethora of site-specific histone modifications that are directed by gene-specific regulators, but how these histone modifications contribute to different steps in a gene regulatory pathway is largely unclear. Thus, development of an in vitro system that recapitulates both an endogenous chromatin structure and the full spectrum of transcription-associated nucleosome rearrangements and modifications is crucial for a detailed understanding of biochemical mechanism.

Protocol 13.3 Reconstitution of nucleosomal arrays using recombinant *Drosophila* ACF and NAP1, 589

The goal of this chapter is to describe strategies for assembling and analyzing chromatin reconstitutions for subsequent analysis in transcription assays, factor binding, and/or analysis of chromatin remodeling/modification enzymes. Assays for chromatin remodeling and histone modification enzymes are also described, as are strategies for in vitro transcription analysis with chromatin templates. The advantages and disadvantages of each assay are discussed, and the investigator is alerted to technical problems that may arise during a study. Each strategy also differs in commitment of time and resources. Our overall goal is to provide investigators with all of the experimental tools and strategies to dissect each step of a complex transcriptional pathway in vitro on a physiological template, chromatin. Several general considerations when planning a study requiring chromatin reconstitutions are as follows.

- *What will be used for the source of core histones?* Histones are abundant proteins, and thus they can be purified from a variety of cell sources. However, recombinant histones have the advantage that they lack all posttranslational modifications and their sequence can be manipulated by altering the cloned genes. However, purification of recombinant histones requires significant effort and resources. Although histones are highly conserved among eukaryotes, sequence variability does exist (especially with the H2A and H2B histones), and thus the cell source (e.g., yeast or human) of histones should be considered before starting a study.
- *Does the study require assembly of mononucleosomes or nucleosomal arrays?* A mononucleosome length of DNA (150–220 bp) may be sufficient to harbor a core promoter as well as promoter proximal regulatory elements. These simple substrates may be suitable for studies that analyze binding of factors to nucleosomal sites or enzymatic activities of histone-modifying or -remodeling enzymes, or for studying initial steps in transcription initiation or elongation. Clearly, mononucleosomes are important tools in the chromatin arsenal, but one must always bear in mind that mononucleosomes do not exist in cells. Indeed, in the absence of adjacent nucleosomes, the histone amino-terminal domains rearrange significantly (Usachenko et al. 1994), and the H4 amino-terminal domain blocks the accessibility of DNA at the nucleosomal dyad axis (Vettese-Dadey et al. 1994, 1996). Reconstituted nucleosomal arrays more closely mimic physiological chromatin, and thus these substrates are most useful if the long-range goal of a project is to reconstruct specific regulatory events in vitro.
- *Will chromatin assembly use purified components or will a crude assembly system be sufficient?* Crude cytoplasmic extracts can efficiently assemble large DNA molecules into regularly spaced nucleosomal arrays; however, these extracts contain abundant chromatin-remodeling enzymes that can complicate further analyses. In this case, extensive purification of chromatin templates may be required. In contrast, the salt dialysis method is quite simple and

requires only histones and DNA. Although this method works well for mononucleosome assemblies, salt dialysis reconstitutions generate nucleosomal arrays with very closely packed, irregularly spaced nucleosomes. Thus, with this method, physiological positioning of nucleosomes on longer DNA templates requires the inclusion of nucleosome positioning sequences.

EXPERIMENTAL STRATEGIES

Strategies for Assembling Chromatin

Methods for the assembly of purified histones and DNA into chromatin-like particles were first developed in the early 1970s (Oudet et al. 1975), close to the time of the official discovery of the nucleosome by Thomas and Kornberg (Kornberg 1974; Kornberg and Thomas 1974). Those early methods used dialysis of a histone–DNA mixture from high-salt or high-urea into low-salt buffers. Such salt dialysis reconstitution methods remain one of the simplest and most widely used protocols for reconstitution of chromatin. Subsequently, Laskey et al. (1977) developed an ATP-dependent, cell-free system from unfertilized eggs of *Xenopus laevis* that could assemble nucleosomal arrays with periodic, physiological spacing of nucleosomes (~200 bp). Similar systems were subsequently described using cytoplasmic extracts from *Xenopus* oocytes (Glikin et al. 1984) and *Drosophila* embryos (Becker et al. 1994; Kamakaka and Kadonaga 1994). More recently, the Kadonaga group purified the active components of the *Drosophila* embryo extract system, and they developed a fully recombinant assembly system that generates long, periodic nucleosomal arrays in an ATP-dependent reaction (Ito et al. 1999; Fyodorov and Kadonaga 2003). In the following section, we introduce these different methods and discuss their advantages and disadvantages.

Chromatin Assembly by Salt Dialysis

The histone octamer is a stable oligomeric complex in buffers containing high salt (i.e., 2 M NaCl), reflecting the fact that the molecular interactions that drive assembly of the octamer are dominated by hydrophobic interactions (Arents et al. 1991; Luger et al. 1997). The high salt stability of the histone octamer facilitated the determination of its X-ray structure at 3.1 Å resolution by the Moudrianakis group (Arents et al. 1991). Transfer of the octamer into buffers containing low salt (0–200 mM NaCl) leads to dissociation of the octamer into two H2A/H2B dimers and an H3/H4 tetramer. Further dissociation of these oligomers into individual histones requires treatment with extremes of pH or high concentrations of urea (Eickbush and Moudrianakis 1978). Histones are highly charged proteins, and thus they tend to adhere to glass and plastic tubes in low-salt buffers; therefore, siliconized tubes are recommended when working with histones.

If a solution of histone octamer is rapidly diluted into low salt in the presence of DNA, nonspecific aggregates are formed. However, bona fide nucleosomes can be assembled if a solution of DNA and histone octamer is slowly diluted to low-salt conditions by gradient dialysis, step dialysis, or step dilution (also called salt jumping). Chromatin assembly by salt dialysis exploits the disassembly of the histone octamer at salt concentrations below 2 M NaCl, as well as the distinct DNA-binding properties of the H3/H4 tetramer and H2A/H2B dimers. For instance, as the salt is lowered from 2 M to 1 M NaCl, the octamer disassembles and the H3/H4 tetramer binds to DNA, organizing about 90 bp. At this salt concentration, the H2A/H2B dimers do not bind stably to DNA. As the salt concentration is further lowered, the H2A/H2B dimers bind to the tetrasome particle, with complete assembly occurring by dialysis to 0.6 M NaCl (Hansen et al. 1991). The assemblies are then dialyzed or diluted into a low-salt buffer (0.05–0.2 M NaCl), where the chromatin is stable on ice for several weeks (do not freeze).

Mononucleosome assembles often contain some free DNA or aggregated histone–DNA complexes, and in this case mononucleosomes can be easily purified by sedimentation in a linear sucrose

gradient. Nucleosomal array reconstitutions do not usually require purification. Proponents of the salt dialysis reconstitution method have suggested that the stepwise assembly of nucleosomes by this method may closely mimic the in vivo assembly of nucleosomes behind replication forks, where it is known that H3/H4 tetramers are assembled onto daughter strands prior to addition of the H2A/H2B dimers.

In addition to using salt dialysis to deposit purified histones onto DNA, one can also use high salt concentrations to "donate" nucleosomes from cellular chromatin fragments onto ^{32}P-labeled DNA fragments. The reagents for this method are very easy to prepare; purified histones are not required, and, in general, we have never had to purify mononucleosomes assembled by this method. The Workman group has also used this donor transfer method to assemble small quantities of radiolabeled nucleosomal arrays for use in transcription assays and analyses of histone acetyltransferase complexes (Steger et al. 1998). This method requires preparation of short oligonucleosomes (4–10-mers) from isolated nuclei by limited micrococcal nuclease (MNase) digestion and gel filtration fractionation (Owen-Hughes et al. 1999). Nanogram amounts of a ^{32}P-labeled DNA fragment (usually mononucleosome length) are mixed with a large molar excess of the oligonucleosomes, and the salt concentration is raised to 2 M NaCl to dissociate the histone octamers. The reaction is then successively diluted with buffer containing lower salt concentrations, and the octamers redistribute between the labeled probe and cellular DNA. By varying the ratio of probe DNA to oligonucleosomes, the investigator can assemble 100% of the probe into mononucleosomes.

One disadvantage of the octamer transfer technique is that subsequent assays will contain cellular oligonucleosomes. Note that purification of the labeled mononucleosomes by sucrose gradient centrifugation will generate very dilute solutions of chromatin that are not very stable. An alternative strategy that has been used successfully by the Workman group is to assemble mononucleosomes (or nucleosomal arrays) on a biotinylated DNA fragment. The resulting biotinylated mononucleosomes can then be purified from the cellular oligonucleosomes by capture on streptavidin agarose beads. Mononucleosomes can be released in a small volume from the beads by restriction enzyme digestion.

Advantages and disadvantages. The salt dialysis method for nucleosome reconstitution has several advantages over other reconstitution methods. First and foremost, only a few reagents are required for this method—namely, purified histones and DNA. Furthermore, the density of nucleosomes on a long DNA fragment can be easily controlled by changing the ratio of histone octamers to DNA. For instance, a ratio of approximately 1 octamer per 160 bp of DNA will yield assemblies where the DNA is fully loaded (saturated) with nucleosomes, whereas the use of much less histone octamer yields subsaturated chromatin fragments. However, it is important to realize that whereas a DNA fragment can become saturated with nucleosomes (i.e., 1 nucleosome per 160 bp), deposition of histone octamers does not appear to be a reaction that saturates (for discussion, see Carruthers et al. 1999). Thus, if an investigator uses a high ratio of histone octamers to DNA, a large number of octamers will be deposited onto DNA that do not represent bona fide nucleosomes. These nonspecifically bound octamers can interfere with subsequent reactions, and they can also lead to aggregation of nucleosomal arrays in the presence of low concentrations of divalent cations (e.g., Mg^{++}). For this reason, concentrations of histone octamer and DNA should be determined just prior to assembly of each reconstitution reaction so that accurate ratios can be calculated. Note that an assembly that contains even 10% excess histone octamer can sometimes lead to a disproportionately high level of aggregation.

There are several applications where salt dialysis reconstitution may not be the best method for chromatin assembly. When octamers are deposited by salt dialysis onto long DNA fragments, the resulting nucleosomes are often closely packed with nonphysiological spacing (see Box 13.1). Variable lengths of nucleosome-free DNA may also exist between patches of close-packed nucleosomes. Thus, if the goal is to reconstruct physiologically relevant nucleosome positions on a long DNA fragment, salt dialysis reconstitution may not be appropriate. One strategy to avoid this issue is to use model nucleosomal array templates where a target DNA fragment (200–400 bp) is inserted between tandem,

head-to-tail repeats of a nucleosome positioning element (NPE), such as a 5S rRNA gene (Simpson et al. 1985) (Box 13.1). During nucleosome assembly, the positioning of nucleosomes on the 5S gene forces the assembly of positioned nucleosomes on the inserted regulatory region. Workman and colleagues have used this method to assemble nucleosomal arrays where nucleosomes are positioned over a human immunodeficiency virus type-1 (HIV-1) promoter (Steger and Workman 1997) or an adenoviral E4 promoter that contains five binding sites for the Gal4p–VP16 activator (Utley et al. 1998).

Box 13.1. Effects of DNA Template and Reconstitution Method on Positioning of Nucleosomes within Arrays

Bulk chromatin within cells is typically composed of long arrays of nucleosomes that are spaced at regular intervals (Figure 13.1). When histone octamers are deposited onto a DNA template by the salt dialysis method, a nonphysiological, closely packed arrangement of nucleosomes is generated that can leave large regions of free DNA within the array (Fig. 13.1, bottom left panel). An alternative strategy uses a DNA template that contains head-to-tail repeats of a nucleosome positioning element (NPE), such as the sea urchin 5S rDNA gene. If a set of NPEs flank a target sequence (shown is a dinucleosome-length fragment harboring the adenovirus E4 promoter), nucleosomes that are positioned by the NPE elements will create boundaries that direct positioning of nucleosomes over the central promoter fragment (Fig 13.1, top panel). By varying the position of the promoter within the central fragment, the investigator can control the nucleosomal positioning of the promoter elements. In a third strategy, the ATP-dependent, ACF/NAP1 assembly method is used to reconstitute a regularly spaced nucleosomal array onto the heterologous DNA fragment (Fig. 13.1, bottom right panel). Note that in this case, the nucleosomes are regularly spaced, but each array in the population has a different set of translational positions. The random positioning of nucleosomes leads to an accessible promoter region in a subset of arrays (note position of arrowheads).

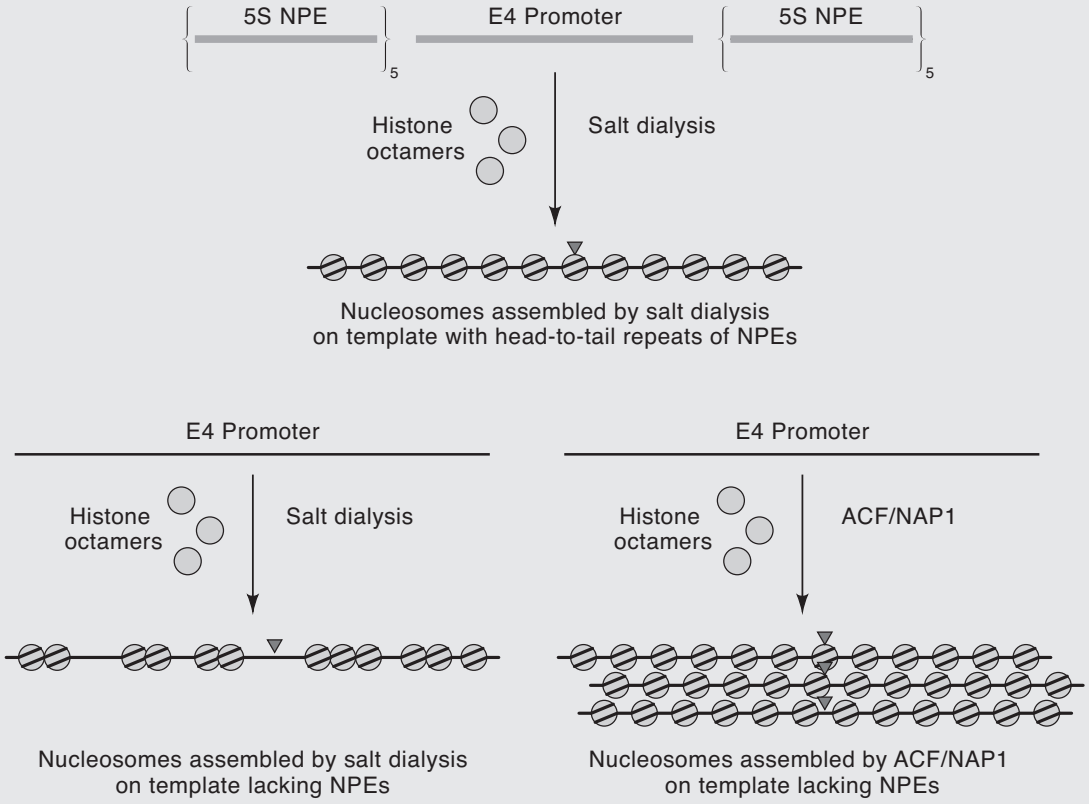

FIGURE 13.1. Reconstitution of nucleosomal arrays.

These templates were used successfully for monitoring activator-dependent recruitment of histone acetyltransferase complexes (Utley et al. 1998; see Figure 13.9, below), targeting of the SWI/SNF chromatin remodeling complex (Yudkovsky et al. 1999), and analyzing in vitro transcription (Steger et al. 1998; Utley et al. 1998; Ikeda et al. 1999; Neely et al. 1999).

In vivo studies may indicate that one or more sequence-specific gene regulatory proteins are bound to nucleosome-free regions prior to transcriptional activation. To assemble this type of structure in vitro, one would need to prebind these factors prior to nucleosome assembly. However, exposure to salt concentrations above 0.35 M will often strip such factors off their sites. For these types of assays, alternative nucleosome assembly methods must be used in which nucleosomes can be deposited in low-salt buffers. Some DNA–protein complexes are remarkably salt-resistant and are thus compatible with salt dialysis reconstitution. Most notably, Luse and colleagues (Chang and Luse 1997) preformed an RNA polymerase II (RNAPII, or Pol II) elongation complex on plasmid DNA prior to assembly of plasmid chromatin by rapid salt dialysis. These studies were the first to fully demonstrate the inhibitory effects of nucleosomes on transcriptional elongation.

Finally, it is our experience that salt dialysis reconstitution is not the method of choice when using yeast histone octamers to assemble nucleosomal arrays. With both recombinant yeast histones or histone octamers purified from yeast nuclei, the efficiency and reproducibility of nucleosomal array reconstitution are very low. Similar observations have been made by the Laybourn group (Wongwisansri and Laybourn 2004). The molecular basis for these effects are not known, but it may be that the yeast H2A/H2B dimers bind better than vertebrate histones to DNA at higher salt concentrations and thereby interfere with proper stepwise assembly. In contrast, the Wu lab has published success with the rapid salt-dilution method for assembly of yeast mononucleosomes (Shen et al. 2003).

Chromatin Assembly with Histone Chaperones

The direct mixing of histones and DNA at physiological salt concentrations leads to the formation of DNA–histone aggregates. This reaction can be avoided by including a negatively charged "assembly factor" in the reaction. These factors have been termed histone chaperones, although in essence they are simply histone-binding factors. Historically, polyglutamate was one of the first negatively charged polymers to be used for nucleosome assembly (Stein and Mitchell 1988). Subsequently, acidic histone binding proteins, such as NAP1 (Ishimi and Kikuchi 1991; Ito et al. 1996), were identified that were also able to assembly nucleosomes in physiological salt buffers.

Typically, purified histone octamers (in high salt) are mixed with an excess of chaperone (4:1 ratio for yNAP1) and then dialyzed into a low-salt buffer. This histone–chaperone complex is then incubated with the target DNA for several hours to complete nucleosome assembly. When a histone–chaperone complex is added to DNA, the octamer is transferred from the chaperone to the DNA in what appears to be a single-step reaction, yielding an assembled nucleosome and free chaperone. For yNAP1, assembly is efficient with linear or circular DNA templates, whereas *Drosophila* and mouse NAP1 function best with supercoiled plasmid DNA. Because yNAP1 is a homodimer with a native molecular mass of approximately 90 kD, it can usually be purified from nucleosome assemblies by sucrose gradient sedimentation.

Advantages and disadvantages. The primary utility of chaperone-mediated nucleosome assembly is the ability to assemble nucleosomes under conditions of physiological salt. Laybourn and colleagues have used yNAP1 to assemble yeast octamers onto plasmid DNA that contains the PHO5 promoter, and in this case they were able to reconstitute physiological nucleosome positions over the promoter elements (Terrell et al. 2002). Indeed, chaperone-dependent assembly appears to be the method of choice for reconstitutions using yeast histones. However, similar to the case of salt dialysis reconstitution, chaperone-mediated assembly usually generates closely packed, irregularly spaced nucleosomes on long DNA fragments that lack nucleosome positioning sequences. In addition, it may be difficult to remove the histone chaperone entirely from the assembled nucle-

osomes. Contaminating chaperone may interfere with subsequent assays. This may be especially problematic for yNap1, as it is sufficient to catalyze the loss of H2A/H2B dimers from nucleosomes and enhance nucleosome mobility (Park et al. 2005).

ATP-Dependent Chromatin Assembly

As outlined above, the largest disadvantage of salt dialysis and chaperone-mediated nucleosome assembly methods is that the deposition of nucleosomes on long DNA fragments or plasmids does not lead to periodic arrays but instead results in closely packed, irregularly spaced nucleosomes. However, there are several cell-free systems that will assemble plasmids into chromatin templates where nucleosomes are regularly spaced at physiological intervals of about 200 bp. These assembly systems are composed of histone chaperones and ATP-dependent chromatin remodeling activities that combine to deposit and space nucleosomes, even on very large plasmids. Early studies used crude cytoplasmic extracts (S150 or S190) from *Xenopus* oocytes (Glikin et al. 1984) or *Drosophila* embryos (Becker et al. 1994; Kamakaka and Kadonaga 1994). Plasmid chromatin assembled in these extracts has been used extensively for biochemical studies of transcriptional regulation. However, most researchers do not have access to the necessary quantities of frog oocytes or fly embryos, and purification of plasmid chromatin from the crude extracts can be problematic. Consequently, these types of assembly extracts are not currently recommended.

In 1997, Kadonaga and colleagues reported the purification of ACF, the ATP-utilizing component of the *Drosophila* embryo extract nucleosome assembly system (Ito et al. 1999; Fyodorov and Kadonaga 2003). ACF is a two-subunit enzyme composed of Acf1 and the ISWI ATPase. Both subunit genes have been cloned into baculovirus vectors where they can be expressed individually or in combination as FLAG-tagged polypeptides (see Protocol 13.3). These constructs are made generally available by the Kadonaga lab. Reaction mixtures containing plasmid DNA, ACF, a histone chaperone (NAP1 or Caf1), core histones, and ATP are sufficient to program the assembly of long, periodic nucleosomal arrays under physiological salt conditions (see Protocol 13.3). To limit the ATP-independent deposition of nucleosomes by the NAP1 chaperone, plasmid DNA is first relaxed by treatment with a eukaryotic topoisomerase I (Topo I). Topo I may also be included in the assembly reaction to remove positive supercoils that accumulate during nucleosome assembly. These assembly reactions are quite robust; ACF can program nucleosome assembly at an optimal ratio of 1 ACF per 50 histone octamers.

Advantages and disadvantages. ATP-dependent chromatin assembly generates periodic nucleosomal arrays that closely resemble bulk cellular chromatin. It is the method of choice when large DNA fragments or plasmid DNAs are to be reconstituted into chromatin. Because assembly is carried out in low-salt buffers, sequence-specific DNA-binding proteins can be included during the assembly reaction. These proteins can cause localized nucleosome positioning and formation of chromatin structures that mimic endogenous loci (Barton et al. 1993; Pazin et al. 1997). Numerous investigators have used the ACF-based assembly system to assemble plasmid chromatin for studies of transcriptional regulation (Jiang et al. 2000; An et al. 2004; An and Roeder 2004; Angelov et al. 2004; Guermah et al. 2006).

One of the disadvantages of the ACF-dependent assembly method is that it requires purification and functional analysis of multiple components. In particular, the activity of ACF must be carefully titrated, as high concentrations actually disrupt the spacing of nucleosomal arrays. Once assembly is achieved, the investigator must also decide whether to purify the chromatin from the assembly reaction. Contaminating assembly components may be problematic for interpretation of subsequent assays, as NAP1 can catalyze histone H2A/H2B dimer displacement and ACF can function as an ATP-dependent chromatin remodeling enzyme. Most studies, however, have not purified the chromatin, and investigators simply acknowledge the possibility that ACF and NAP1 activities may contribute to the observed results. In general, this assembly method requires a great deal more commitment of time and resources than does assembly by salt dialysis.

Although the ATP-dependent assembly method generates periodic nucleosomal arrays, it does not assemble positioned nucleosomes (see Box 13.1). Thus, within the population of chromatin molecules, the nucleosomes will be randomly positioned with respect to a particular DNA sequence. This contrasts to the positioned arrays reconstituted on DNA templates that harbor head-to-tail repeats of nucleosome positioning sequences (Box 13.1). One consequence of random positioning is that transcription factor–binding sites, restriction enzyme recognition sites, and promoter elements will often be located in the linker region between nucleosomes. Consequently, nucleosome assembly may not be inhibitory to several steps in the transcription process that might be blocked by positioned nucleosomes in vivo. Furthermore, as template utilization in many in vitro transcription assays can be less than 1%, one needs to consider these caveats when interpreting results.

Incorporation of Linker Histones

In addition to regularly spaced nucleosomes, physiological chromatin from metazoan species contains approximately 1 molecule of a linker histone per nucleosome core. Recent studies indicate that the ratio of linker histone to nucleosome core particle actually varies among different tissues, with values as low as 0.5 linker histone per core particle in embryonic stem cells to 0.83 per core particle in thymocytes (for review, see Fan et al. 2003, 2005; Woodcock et al. 2006). Less complex eukaryotes, such as the budding yeast *Saccharomyces cerevisiae*, have extremely low levels of linker histone (estimated at 0.03–0.25 per core particle). In general, the level of linker histone incorporation may need to be varied to achieve the desired physiological chromatin structure.

Linker histones can be incorporated during either a salt dialysis or octamer transfer reconstitution (Juan et al. 1994) or during ATP-dependent assembly by the ACF/NAP1 system (Fyodorov and Kadonaga 2003). If salt dialysis reconstitution is used, linker histone is added to the reconstitution reaction after the salt concentration is lowered to 0.6 M NaCl. After addition of linker histone, dialysis is continued to a low salt concenration. In the case of ATP-dependent assembly by ACF/NAP1, linker histone is simply added with the core histones. Importantly, nucleosomal arrays that contain linker histone are quite prone to self-association or "aggregation" reactions in buffers containing divalent cations, and thus the solubility of arrays should be carefully monitored in different buffer solutions by a quick spin in a microcentrifuge.

Source of Histones

Core Histones

With the exception of crude extract systems, all of the recommended chromatin assembly methods require exogenous, purified histones. The histones are very well-conserved among eukaryotes, with the sequences of rodent, chicken, human, fly (*Drosophila melanogaster*), and frog (*X. laevis*) histones being nearly identical (Marino-Ramirez et al. 2006). Thus, each of these recombinantly expressed histones or histones purified from these cell sources can be considered interchangeable. In contrast, histones from budding yeast (*S. cerevisiae*), fission yeast (*Schizosaccharomyces pombe*), or plants (*Arabidopsis thaliana*) show significant differences, especially for histones H2A and H2B. Biochemically, histones from all sources assemble into histone octamers that organize approximately 147 bp of DNA, but the stability of the histone octamer does vary. Histone octamers and mononucleosomes assembled with recombinant yeast histones are notoriously less stable than recombinant vertebrate histones, and stocks should be used within 1 week of assembly. In contrast, plant octamers appear to be even more stable than vertebrate histones such that dissociation of the octamer into dimers and tetramers requires much lower salt concentrations (≤0.6 M NaCl) than typically used for other octamer sources (Moehs et al. 1992). This may make reconstitution of plant chromatin by salt dialysis problematic.

Historically, histones were first isolated by acid extraction of nuclei (Phillips and Johns 1965). This method is still in use and can yield isolated H2A/H2B dimers and H3/H4 tetramers (Chang

and Luse 1997). We have commonly purified histones from solubilized chromatin released from nuclei prepared from chicken erythrocytes or HeLa tissue culture cells (see Protocol 13.1). Briefly, nuclei are treated with micrococcal nuclease to release long oligonucleosomes. These chromatin fragments are mixed with CM-Sephadex resin in 0.35 M NaCl to strip oligonucleosomes of linker histones. Stripped oligonucleosomes are trimmed to smaller pieces by further MNase digestion, and histone octamers are purified from cellular DNA by chromatography on hydroxyapatite (HTP) resin. Gradient elution from the HTP column can also be used to separate H2A/H2B dimers from H3/H4 tetramers (Simon and Felsenfeld 1979). Note that the short oligonucleosomes that are generated by the second MNase digestion can also be used as donors for octamer transfer reconstitutions. Typically, we obtain about 50 mg of purified histone octamer from 200 ml of chicken blood. Our solutions of chicken histone octamer are stable at 4°C for more than 1 year.

One caveat of using histones isolated from bulk chromatin is that they harbor a spectrum of posttranslational modifications that may complicate subsequent assays (Garcia et al. 2007). Furthermore, there are no means to eliminate modifications from purified histones. The abundance of these modifications can sometimes be modulated by treating cells with enzymatic inhibitors; for instance, sodium butyrate is a potent histone deacetylase inhibitor, and it has routinely been used to increase the levels of lysine acetylation in histones isolated from HeLa cells (Tse et al. 1998).

Alternatively, individual histones can be expressed in bacteria, and the recombinant versions lack posttranslational modifications. Typically, each histone is purified as a denatured polypeptide from bacterial inclusion bodies by gel-filtration and ion-exchange chromatography (Luger et al. 1999). Tsukiyama and colleagues have published a modified version of this protocol in which sequential MonoQ and MonoS chromatography is used to purify the individual histones in urea-containing buffers (Vary et al. 2004). All four denatured core histones are then mixed together in an equimolar ratio, and histones are refolded and octamers reconstituted by dialysis into buffer containing 2 M NaCl. Histone octamers are then purified by gel-filtration chromatography. A detailed protocol for purification and analysis of recombinant histones has been published (Luger et al. 1999). In our hands, recombinant octamers assembled by the Luger method are not as stable as native histone octamers, and we generally store them on ice for only about 1–2 months. Octamers can also be dialyzed into buffer containing 50% glycerol and stored at –80°C.

Gloss and colleagues have reported that histone polypeptides tend to form helical aggregates during the refolding reaction (Gloss and Placek 2002). To diminish this reaction and to enhance the overall efficiency of octamer reconstitution, these authors have developed a modified version of the octamer assembly method in which H3/H4 tetramers and H2A/H2B dimers are refolded and assembled as independent units, prior to octamer assembly. The key to this method is the rapid dilution of denatured histones into a buffer that lacks denaturant. In some cases, this method is so efficient that purification of the octamer by gel-filtration chromatography is not necessary (L. Gloss, pers. comm.). Although not yet tested, this octamer reconstitution method may yield recombinant yeast octamers with enhanced stability.

The use of recombinant histones has numerous advantages over histones obtained from cellular chromatin. Of course, the lack of posttranslational modifications on recombinant histones makes them the reagent of choice for in vitro transcription assays. But first and foremost, the recombinant clones can be manipulated at will by mutagenesis strategies (see Chapter 10). Histones can be expressed that harbor amino acid substitutions that might have been identified through genetic studies, and these can then be analyzed in vitro. For instance, yeast genetics had identified single-amino-acid substitution alleles of histones H3 and H4 (Sin alleles) that alleviated the transcriptional requirement for the SWI/SNF remodeling complex (Kruger et al. 1995). It had been hypothesized that these histone alterations might create a chromatin structure that mimicked the remodeled state. We (Horn et al. 2002b) and others (Flaus et al. 2004) tested this idea by creating Sin^- recombinant histones and assembling mononucleosomes and nucleosomal arrays. Remarkably, the Sin^- mononucleosomes were found to slide along DNA at much lower tempera-

tures than wild-type nucleosomes, and Sin⁻ nucleosomal arrays were unable to fold into 30-nm fibers. These data led to the proposal that SWI/SNF may function in vivo to control nucleosome mobility and chromatin folding.

In addition to testing the functional importance of histone residues, novel cysteine residues can be added to histones to facilitate postsynthetic addition of crosslinking agents (Lee et al. 1999; Kan and Hayes 2007), fluorescent compounds (Bruno et al. 2003b), or affinity purification agents, such as biotin (Sinha and Peterson 2008). As discussed in more detail below, engineered cysteine residues also allow investigators to use chemical methods to introduce site-specific histone posttranslational modifications. Finally, we have had excellent success using the denatured, recombinant histones as antigens to produce polyclonal antibodies to unmodified histones. The only disadvantage of recombinant histones is that their large-scale preparation requires a significant expenditure of time and resources.

Linker Histones and Histone Variants

Linker histones have been purified from a wide variety of sources, including chicken erythrocytes, cultured mammalian cells (HeLa), and *Drosophila* embryos. In most cases, linker histones can be isolated in a single step during the purification of core histones (see Protocol 13.1). For instance, linker histone H1 or H5 (a erythrocyte-specific variant of H1) can be purified by gradient salt elution from the CM-Sephadex resin used during purification of chicken octamers, or partially purified linker histones can be isolated from the flowthrough fraction of the HTP column during purification of HeLa core histones (Workman et al. 1991). Kadonaga and colleagues have also published a general method for purification of linker histone H1 using hydrophobic interaction chromatography (Croston et al. 1991).

In most cases, native linker histone preparations are suitable for chromatin reconstitution and in vitro analyses. However, such preparations are most always a mix of several different linker histone variants or subtypes. Mice contain six somatic linker histone H1 subtypes, H1a–e and H1(0) (Woodcock et al. 2006). Each H1 subtype has a similar overall structure and each binds to nucleosomes and stabilizes higher-order chromatin structures; however, each variant appears to have a distinct spectrum of posttranslational modifications and tissue distribution (Woodcock et al. 2006; Wisniewski et al. 2007). It seems likely that linker histone subtypes will have distinct protein-binding partners. For example, Reinberg and colleagues have reported that methylation of the human H1b subtype at lysine 26 mediates higher-order folding of nucleosomal arrays via an interaction with the malignant brain tumor (MBT) protein L3MBTL1 (Trojer et al. 2007). Other linker histone subtypes, such as H1(0), lack the lysine at position 26 and thus they do not interact with L3MBTL1 (Trojer et al. 2007). In general, linker histones are not well conserved among species in comparison to the core histones, so investigators should pay close attention to the species source. Thus, as in the case with the core histones, the use of recombinant histone H1 subtypes is becoming more prevalent.

Reconstitution of an endogenous chromatin structure may also require assembly of nucleosomes that harbor a core histone variant. For instance, nearly all yeast RNAPII promoters are flanked by positioned nucleosomes that harbor the histone H2A variant Htz1 (called H2A.Z or H2AZ in other species) (Raisner et al. 2005; Zhang et al. 2005). Many *Drosophila* and mammalian genes appear to have a similar (although not identical) structure (Mavrich et al. 2008; Schones et al. 2008). Similarly, the macro-H2A variant is enriched on the inactive X chromosome of female mammals (Chadwick and Willard 2001b), whereas the H2A-Bbd variant is depleted from the inactive X chromosome and may associate with transcriptionally active chromatin (Chadwick and Willard 2001a). In general, histone variants are purified as recombinant proteins, and octamers are assembled from the unfolded proteins just like core histones. The H2A-Bbd variant, however, does not assemble into a stable histone octamer in vitro (Bao et al. 2004). Thus, in this case, H2A/H2B dimers and H3/H4 tetramers must be assembled individually, and the octamer assembly method outlined above by Gloss and colleagues may be most appropriate.

Biochemical Characterization of Chromatin Reconstitutions

Perhaps the most important decision that a researcher must make is whether a chromatin analysis can be performed adequately with a mononucleosome substrate or whether nucleosomal arrays are required. If an in vivo study indicates that a promoter region or a binding site for a gene regulatory factor is encompassed by a single positioned nucleosome, then a mononucleosome (150–250 bp) may suffice. Alternatively, the regulatory region may be located between positioned nucleosomes or be too large (>250 bp) for mononucleosome assembly. Furthermore, gene regulatory regions are generally organized as long nucleosomal arrays in vivo, and thus this is the physiological context that the investigator may want to recapitulate in vitro. Nucleosomal arrays assembled in vitro undergo complex chromatin folding dynamics that mimic cellular chromatin condensation, and thus, studies with nucleosomal arrays allow the researcher to investigate how chromatin higher-order structures impact transcription factor binding and transcriptional activity.

If an investigator decides to reconstitute a nucleosomal array, then two general types of arrays can be assembled. First, one can use the ACF/NAP1 assembly system to reconstitute nucleosomal arrays in which the nucleosomes are regularly spaced, but randomly positioned with respect to particular DNA sequences. Thus, in this case, nucleosomes are not positioned specifically over gene regulatory sequences. Consequently, the binding of sequence-specific DNA-binding proteins to regulatory sites is generally not inhibited by nucleosome assembly because these sites will be contained in the linker DNA between nucleosomes in much of the population (Pazin et al. 1998). However, these randomly positioned nucleosomes are quite effective at inhibiting transcription initiation and elongation, and, consequently, these types of nucleosomal arrays have been used extensively for in vitro transcription assays (Jiang et al. 2000; An et al. 2004; An and Roeder 2004; Angelov et al. 2004; Guermah et al. 2006). Kadonaga and colleagues have shown that the ACF/NAP1 assembly system can still be used to generate localized regions of positioned nucleosomes if a site-specific DNA-binding protein is bound to the DNA template prior to nucleosome assembly. For instance, they found that the binding of the bacterial LacI protein forced the positioning of a few adjacent nucleosomes by acting as an assembly boundary (Pazin et al. 1997).

The second strategy is to use salt dialysis reconstitution or chaperone-dependent assembly (e.g., NAP1) to assemble nucleosomal arrays on DNA templates that harbor tandem, head-to-tail repeats of a NPE. Simpson et al. (1985) were the first to develop such model nucleosomal array templates that contained ten or more repeats of a 5S rDNA sequence from sea urchin. Each 5S rDNA repeat can position a nucleosome after in vitro salt dialysis reconstitution, yielding a positioned array of nucleosomes. These model nucleosomal arrays undergo complex hierarchical structural changes in vitro in the presence of divalent cations (Fletcher and Hansen 1996). Low concentrations of Mg^{++} ions (<2 mM) induce intramolecular compaction of individual nucleosomal arrays through association of neighboring nucleosomes ("folding"), whereas progressively higher concentrations of Mg^{++} (>2 mM Mg^{++}) or low concentrations of polyamines (~200 μM) (Pollard et al. 1999) induce nucleosomal arrays to oligomerize reversibly. The intramolecular folding of model arrays at low Mg^{++} concentrations is believed to mimic the formation of 30-nm chromatin fibers, whereas intermolecular oligomerization generates relatively defined, soluble structures that sediment in the thousands of Svedberg units and are believed to mimic the fiber–fiber interactions that stabilize higher-order chromosomal domains, such as chromonema fibers (Belmont and Bruce 1994).

During the past 10 years, several groups have created modified 5S array templates that facilitate analyses of transcription factor binding and transcriptional control mechanisms. Workman and colleagues created a modified 5S array where they inserted a mononucleosome length of DNA that harbored five Gal4-binding sites into the middle of the 5S array. They were then able to investigate binding of the Gal4 transcriptional activator to nucleosomal sites (Owen-Hughes and Workman 1996). Roeder and colleagues also created similar 5S templates for monitoring nucleosomal binding of other site-specific transcription factors as well as transcription driven by these factors from chromatin templates (Malik et al. 2002; Wallberg et al. 2002). Subsequently, a dinu-

cleosome-length fragment encompassing the adenoviral E4 promoter (Utley et al. 1998) or the HIV promoter (Steger and Workman 1997) was inserted into the 5S array, and Zaret and colleagues successfully inserted a trinucleosome length of DNA that included the albumin promoter (Cirillo et al. 2002). In all cases, the positioning of nucleosomes on the surrounding 5S NPEs led to the positioning of nucleosomes over the inserted DNA fragments (see Box 13.1). Because these nucleosomal arrays are assembled from only core histones and DNA, they are quite amenable for extensive biophysical analyses, as well as in vitro transcription assays.

Once the decision has been made regarding whether to reconstitute mononucleosomes or nucleosomal arrays, and the method of nucleosome assembly has been chosen, the investigator must devote considerable time and energy to analyze the quality of the reconstituted chromatin prior to performing the desired experiment (e.g., in vitro transcription and chromatin remodeling). In particular, the investigator must ensure that (1) bona fide nucleosomes were assembled, (2) nucleosomes are positioned as expected or desired, and (3) the target number of assembled nucleosomes has been achieved.

Analysis of Mononucleosomes and Chromatosomes

Has a bona fide nucleosome or chromatosome been assembled? Reconstitution of mononucleosomes is typically performed by salt dialysis, salt jump, or octamer transfer (see above) and involves a DNA fragment of 150–250 bp. In all cases, the first step in the analysis is to electrophorese the assembly reaction on a native TBE polyacrylamide gel (6% acrylamide; 30:0.8 acrylamide to *bis*-acrylamide ratio). A mononucleosome migrates much slower than the free DNA, and it generates a well-defined protein–DNA complex that migrates near a 300–400-bp DNA marker. At increasing ratios of histone octamer (or nucleosomal donor) to DNA, the amount of residual free DNA can be minimized. Note that high levels of histone octamer lead to extraneous deposition onto DNA, and these products are detected as slowly migrating smears on the native gel. If a linker histone is included in the reconstitution reaction, an assembled chromatosome (nucleosome core plus one molecule of linker histone) will migrate slower than the mononucleosome particle. As in the case for core histones, multiple assembly reactions should always be performed at different ratios of linker histone to nucleosome core, and reconstitution efficiency should be monitored by native gels. Even after a chromatosome is formed, linker histones will continue to bind, generating aberrant complexes that migrate slower on the native gel.

If the native gel analysis indicates that a mononucleosome or chromatosome has been assembled, then two simple methods are used to ensure that the nucleosomes have a normal stoichiometry of histones and correct DNA content. To assess histone stoichiometry, mononucleosomes or chromatosomes must first be purified by 5–30% glycerol gradient sedimentation to remove unassembled histone octamers or nucleosomal donors. Peak fractions are then analyzed by 18% SDS–polyacrylamide gel electrophoresis (SDS-PAGE), followed by Coomassie staining to show the histones. In general, histones stain poorly and heterogeneously by most silver-staining methods, which is why these methods are generally avoided. Most core histones migrate as a set of four closely spaced polypeptides, migrating in the order of H3, H2B, H2A, and H4 (slowest to fastest). In the case of yeast histones, H2B and H2A migrate as a single species. A properly assembled nucleosome contains equal levels of all four histones.

To assess DNA content, samples are digested extensively with micrococcal nuclease, and the products are resolved on high-percentage agarose gels or native PAGE. A canonical mononucleosome protects about 147 bp of DNA from extensive MNase digestion, but eventually even nucleosomal DNA will be digested. Likewise, in the case of a chromatosome, MNase digestion yields a transient, approximately 166-bp "chromatosome stop" during the digestion time course prior to accumulation of the more resistant 147-bp product.

Analysis of nucleosome positioning: Mononucleosomes. When attempting to reconstruct an endogenous chromatin structure or to perform mechanistic studies with chromatin, it is often

essential to assemble mononucleosomes that are precisely positioned with respect to the underlying DNA sequence. Such "translational" positioning can ensure whether or not the recognition sequence for a DNA-binding protein of interest is wrapped onto the histone octamer. In addition to translational positioning, DNA can also assume a specific rotational position on the surface of the histone octamer. A rotationally positioned nucleosome can have one or several different translational positions, but all molecules in the population have a fixed orientation of the DNA helix on the octamer; that is, a particular segment of DNA always faces away from the octamer surface, whereas another stretch of DNA may always be facing inward toward the octamer.

Nucleosome positions that are physiologically relevant can sometimes be reconstituted using the native gene sequence (Roberts et al. 1995; Terrell et al. 2002), but in many cases, well-defined, heterologous NPEs are used to position nucleosomes with respect to other target sequences. NPEs are high-affinity histone octamer-binding sites whose base composition favors the bending of DNA around the octamer (Lowary and Widom 1998). One of the first NPEs to be identified was the Shrader–Crothers sequence element, which is composed of 4–10 tandem copies of a 10-bp A/T-rich DNA sequence with the consensus, $(A/T)_3NN(G/C)_3NN$ (Shrader and Crothers 1989). When a Shrader–Crothers element is included within a DNA fragment of interest, this sequence will favor formation of a nucleosome that is translationally and rotationally positioned. Changing the position of this NPE with respect to a target DNA sequence can allow an investigator to orient the target either "in" or "out" from the octamer surface. For example, Imbalzano et al. (1994) used multiple copies of the Shrader–Crothers sequence to orient the TATA box of a RNAPII promoter on the nucleosome surface. Furthermore, by changing the spacing between the positioning sequences and the TATA box, these authors were able to position this promoter element either pointing away or toward the octamer surface.

Two of the more widely used NPEs are the 5S rRNA gene from either sea urchin (Simpson et al. 1985; Hansen et al. 1989; Dong et al. 1990) or *Xenopus borealis* (Hayes et al. 1990) and a synthetic DNA sequence called 601 (Lowary and Widom 1998). The 601 NPE is unique in that it can precisely position a nucleosome even if the 601 sequence is embedded within a large DNA fragment (Shundrovsky et al. 2006). In contrast, the 5S rDNA NPE positions about 50% of the octamers at one primary position, with the remaining 50% of the molecules positioned at minor positions that differ in 10-bp integrals from the major position (Dong et al. 1990). Such heterogeneity of nucleosome positioning is often apparent during native PAGE analysis of the reconstituted mononucleosomes (for an example, see Fig. 13.2). For instance, when a 5S mononucleosome assembly is analyzed on a 5% native PAGE gel, the different translational positions are clearly shown as a series of bands of differing mobilities—nucleosomes that are positioned in the center of the fragment migrate the slowest, whereas nucleosomes closer to the DNA ends migrate faster. In contrast, a 601 mononucleosome generates a well-defined, single species in native PAGE analysis. In general, the 601 and 5S NPEs show the greatest utility when investigators wish to place a promoter or transcription factor–binding site adjacent to a positioned nucleosome. To date, there have been few attempts to insert binding sites or promoters within a 601 or 5S, although in one case, Pederson and colleagues were able to successfully embed a binding site for yeast heat shock factor (HSF) within a 5S NPE without disrupting nucleosome positioning (Pederson and Fidrych 1994).

Nucleosome positions can be mapped by digestion with a variety of nucleases (see also Chapter 11). First, investigators can use a battery of restriction enzymes to provide a qualitative assessment of nucleosome positioning. Nucleosome assembly inhibits the rate of restriction enzyme cleavage by 10^3–10^5-fold, with stronger inhibitory effects at the nucleosomal dyad compared to more peripheral locations (Polach and Widom 1995). Quantification of the amount of substrate cleaved can provide simple estimates for both nucleosome density and positioning. Restriction enzyme digestion can provide even more positioning information if mononucleosomes are first trimmed to 147-bp core particles by MNase, and then the restriction enzyme digestion is performed on the purified DNA. Electrophoresis of the small digestion products on a native polyacrylamide gel maps the nucleosome edge with respect to the restriction enzyme site. Note that this method

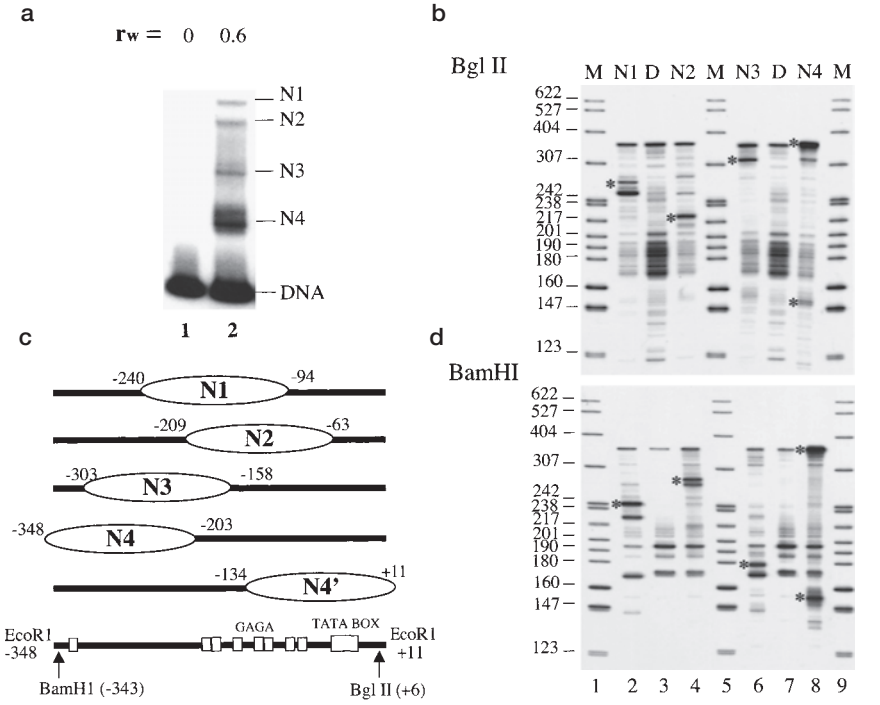

FIGURE 13.2. Probing translational positioning of mononucleosomes by native gel and ExoIII analyses. (*a*) Native polyacrylamide gel electrophoresis of mononucleosomes reconstituted with a histone:DNA weight ratio (rw) of 0.6 on a radiolabeled 359-bp EcoRI fragment carrying the *hsp70* promoter. The four major nucleosome species are indicated as N1, N2, N3, and N4. (*b*) The bands corresponding to each nucleosome species (DNA radiolabeled either at BglII or BamHI positions) were excised from the gel, and nucleosomes were eluted and digested with 400 units/ml of ExoIII for 2 min at 37°C. DNAs were analyzed by electrophoresis on a sequencing gel. Lanes represent N1–N4 nucleosomes: (*d*) Free DNA; (M) pBR322 HpaII digestion markers; (*asterisks*) nucleosome boundaries. (*c*) Nucleosome positions on the 359-bp EcoRI fragment (*bars*) are represented as (*ovals*); positions of nucleosome boundaries are given ±2 bp. The GAGA and TATA promoter elements are indicated. (Reprinted, with permission of Elsevier, from Hamiche et al. 1999, ©1999.)

requires either a uniformly labeled DNA fragment, Southern blot analysis, or reconstitution in sufficient quantities for ethidium bromide detection.

A common means to map nucleosome positions at single-base-pair resolution is by digestion with exonuclease III (see Fig. 13.2). As described in detail in Chapter 11, ExoIII is a processive 3′ to 5′ exonuclease whose activity is blocked when it encounters a DNA-bound protein, such as a nucleosome. After electrophoresis of the ExoIII reaction products on a high-resolution denaturing polyacrylamide gel, the location for one edge of the nucleosome can be determined with respect to a 5′ ^{32}P label. To determine a nucleosome's translational position accurately, ExoIII analysis must be performed on two different nucleosome preparations where the ^{32}P label is on different ends.

As DNA wraps around the histone octamer, the minor groove is exposed on the nucleosome surface with a periodicity of 10 bp. If DNA is positioned rotationally on the octamer surface, agents that cleave DNA in the minor groove will generate a 10-bp ladder of hypersensitive sites. Typically, DNase I or hydroxy radicals (Chapter 11) are used to map the rotational setting of nucleosomal DNA, using a typical footprinting reaction that uses limited digestion of an end-labeled nucleosome (Fig. 13.3). In this way, one can determine whether a factor-binding site is exposed on the nucleosome or whether the site is juxtaposed to the octamer surface. This type of analysis has also been instrumental for investigating how the SWI/SNF family of ATP-dependent chromatin remodeling enzymes disrupts nucleosome structure (Cote et al. 1994; Imbalzano et al. 1996; see below).

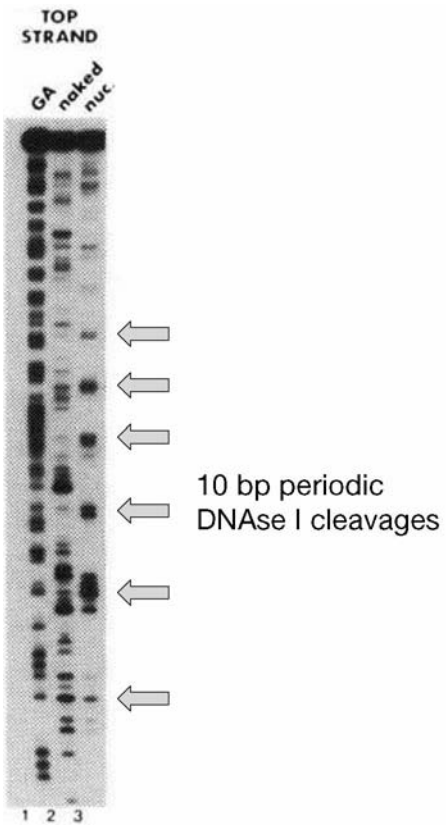

FIGURE 13.3. Probing rotational positioning of nucleosomal DNA by DNase I digestion. Digestion with DNase I is used to evaluate the rotational positioning of DNA on a reconstituted mononucleosome. In this experiment, a mononucleosome is assembled by either the salt dialysis or octamer transfer method onto a ^{32}P-end-labeled DNA fragment. The reconstituted mononucleosome is subjected to a limited digestion with DNase I, and the DNA products are purified and then separated on a denaturing polyacrylamide gel. If DNA is rotationally positioned on the surface of the histone octamer, this DNase I digestion will yield a diagnostic pattern of 10-bp cleavages and protections, indicative of the periodic exposure of the DNA minor groove on the nucleosomal surface. Note that DNase I exhibits considerable sequence preference, and a DNase I digestion of free DNA should always be analyzed in parallel. (Modified, with permission of MacMillan Publishing Ltd., from Imbalzano et al. 1994, ©1994.)

Analysis of Nucleosomal Arrays

Nucleosome positioning. Reconstitution of linear or circular nucleosomal arrays is typically accomplished by one of two methods: salt dialysis or the ATP-dependent ACF/NAP1 system. Salt dialysis reconstitution is most suited to DNA templates that harbor head-to-tail repeats of nucleosome positioning sequences, because this assembly method yields closely packed, nonphysiologically spaced nucleosomes on heterologous DNA fragments (see Box 13.1). ACF/NAP1 reconstitution can generate uniformly spaced arrays on both linear or circular DNAs, with the caveat that reconstitutions may need to undergo further purification to remove the assembly factors. In both cases, the monitoring of nucleosome positioning is normally assessed by either restriction enzyme or MNase digestions. The accessibility of array DNA to restriction enzyme digestion can probe one or more nucleosomes within the array, whereas limited MNase digestion can provide information on the spacing of nucleosomes. If a reconstitution yields an array of positioned nucleosomes, limited MNase digestion will yield a repeating "ladder" of MNase digestion products that reflect cleavages within each of the exposed linker DNAs (Box 13.2; see also Box 9.2). The distance between "rungs" indicates the spacing between positioned nucleosomes, and the number of "rungs" on the ladder provides an indication of the quality of the positioned array (Box 13.2). For instance, salt dialysis reconstitution on a heterologous DNA fragment may only yield one to three bands, because such reconstitutions contain patches of closely packed nucleosomes as well as nucleosome-free gaps. On the other hand, ACF/NAP1 assembly on this same fragment may yield more than ten bands. By varying the salt concentration in an ACF/NAP1 assembly reaction, the spacing between nucleosomes can also be modulated (Blank and Becker 1995). These same MNase digests can also be used to determine the positioning of nucleosomes within the array (Box 13.2). If an array is reconstituted on an end-labeled DNA fragment, then the rungs of the MNase ladder will provide a low-resolution view of nucleosome positions. Alternatively, Southern blot

Box 13.2. MNase Analysis of Nucleosomal Arrays

Micrococcal nuclease preferentially cleaves nucleosomal DNA within the linker DNA between nucleosomes. Three different strategies are shown for using MNase to probe nucleosome positioning within reconstituted arrays. In Figure 13.4A, arrays are subjected to increasing concentrations (or time of digestion) of MNase, and the deproteinized samples are electrophoresed on an agarose gel. When gels are stained with ethidium bromide to show DNA, a regular series of bands at intervals of about 150 bp indicate that the nucleosomes are uniformly spaced within the array. In contrast to the example shown, a reconstitution that did not show uniform spacing would be characterized by the appearance of a mononucleosome band (fastest migrating species of ~150 bp) but little to no larger N-mer products. Figure 13.4B illustrates an indirect end-labeling experiment that tests whether nucleosomes within the array are positioned translationally. In this case, the array is reconstituted on end-labeled DNA, or Southern analysis with an end-positioned probe (bar shown over array) is used to map nucleosome positions. The array is subjected to a partial MNase digest, and the positions of MNase cleavages are mapped relative to the directly or indirectly labeled end. A series of protected regions are indicative of positioned nucleosomes, whereas a uniformly spaced, but randomly positioned array will yield a free DNA pattern of MNase cleavage. Note that if an end-labeled array is used, higher concentrations of MNase will remove the labeled end, leading to loss of signal. The strategy in Figure 13.4C is used to determine if a small target sequence (<100 bp) is located within a nucleosome in the array population. In this case, an MNase digest is performed as in Figure 13.4A, and Southern blot analysis is performed with an oligonucleotide that probes the target DNA of interest. If the target DNA is encompassed by nucleosomes within most arrays in the population (Probe 1), then the oligonucleotide will hybridize to mononucleosomal DNA and a ladder of MNase products (left). However, if the target DNA is primarily located with the linker DNA between nucleosomes (Probe 2), then the oligonucleotide will detect DNA fragments smaller than 150 bp and the nucleosomal ladder will be less distinct. Note that if nucleosomes are randomly positioned within the population, the result would be a combination of the two extremes shown.

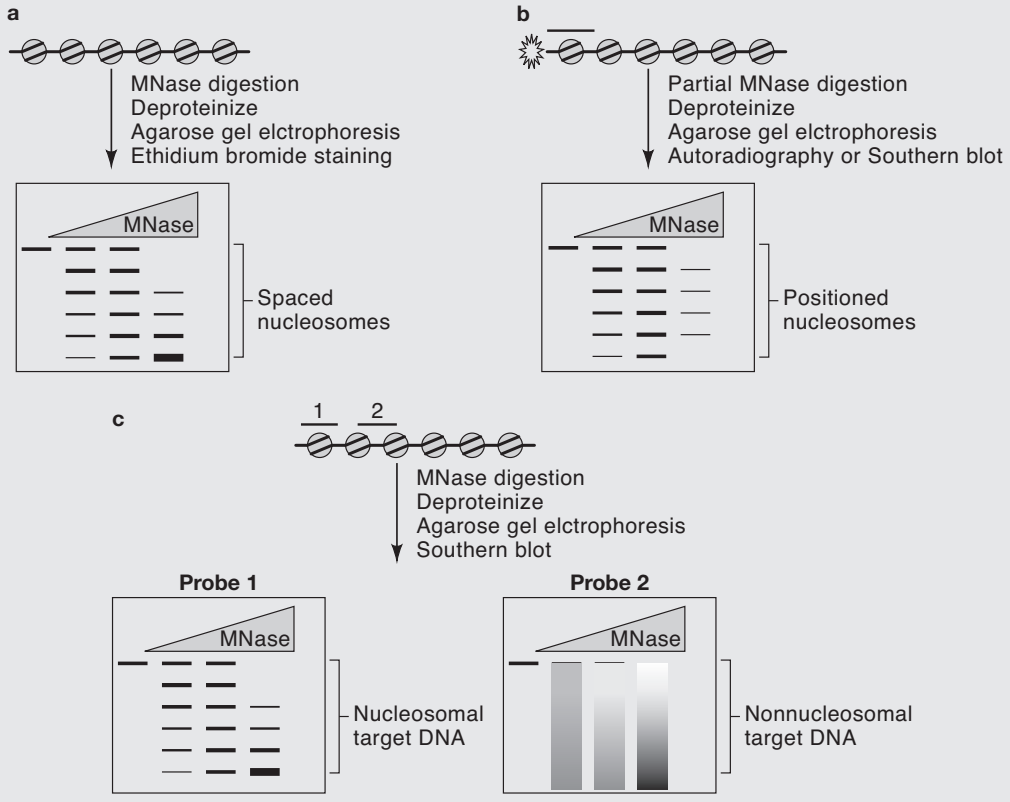

FIGURE 13.4. MNase analysis of nucleosomal arrays.

analysis of the MNase digest can be used, utilizing oligonucleotides as hybridization probes to determine if a particular sequence is encompassed within a nucleosome (Box 13.2; see also Sinha and Peterson 2008). One can also map MNase cut sites at a higher resolution using either a ligation-mediated polymerase chain reaction (LM-PCR) approach or cyclic primer extension. For instance, Laybourn and colleagues used a primer extension assay to map MNase cut sites at the single-nucleotide level to follow positioning of nucleosomes on the yeast PHO5 promoter following NAP1-dependent nucleosome assembly on a long DNA fragment (Terrell et al. 2002).

Analysis of nucleosome density. When assembling nucleosomal arrays, it is most critical to determine the extent to which a DNA template is saturated with nucleosomes. On linear arrays of 12 nucleosomes, even a single nucleosome-free gap can block the folding of these arrays into 30-nm-like fibers (Hansen and Lohr 1993). Undersaturation can also yield nucleosomal templates in which promoter elements are nucleosome-free in many molecules in the population. On the other hand, if too many octamers are added to an assembly reaction, or the amount of DNA is too low, arrays can become oversaturated so that excess octamers bind nonspecifically to nucleosomal DNA and interfere with chromatin folding or subsequent functional assays.

The evaluation of nucleosome saturation usually involves several techniques, but the overall strategy is dependent on the reconstitution method. When arrays are assembled on DNA templates that lack repeats of nucleosome-positioning sequences, nucleosome saturation is determined by a combination of MNase digestion and topological analysis. When such arrays are reconstituted by an ATP-dependent assembly reaction, limited MNase digestion is useful for both linear and circular DNA templates, and the number of "rungs" on the digestion ladder provides an indication of the average number of reconstituted nucleosomes. Detection of MNase products of less than 147 bp indicates a significant amount of nucleosome-free DNA. This assay is less informative for salt dialysis or chaperone-mediated assemblies, because the heterogeneous spacing of reconstituted nucleosomes will disrupt the MNase ladder. Importantly, MNase activity does not appear to be inhibited by nonspecifically bound histone octamers, and thus MNase digestion can provide a misleading view of the quality of an assembly reaction; i.e., even though one obtains a regular ladder of MNase products, the sample may have a very high level of nonspecifically bound histone octamers that may interfere with subsequent analyses.

The best means to assess the degree of nucleosome saturation on circular DNA templates is by topological analysis. The wrapping of DNA around the histone octamer constrains one negative supercoil and generates one positive supercoil in the adjacent linker DNA. If a eukaryotic Topo I is present during the assembly reaction, then the degree of nucleosome saturation can be assessed directly by monitoring the number of negative supercoils that are introduced into the closed, circular plasmid. Typically, an investigator would begin a nucleosome reconstitution with plasmid DNA that had been previously relaxed by treatment with Topo I, ensuring that any supercoils that are subsequently detected are due to nucleosome assembly. One-dimensional gel analysis can provide an approximate measurement of the extent of nucleosome assembly, but a two-dimensional analysis, in which samples are run in the absence or presence of the DNA-intercalating drug chloroquine, can provide a more accurate assessment of nucleosome density (for a detailed protocol, see Fyodorov and Kadonaga 2003). Such topological analyses are best done in concert with an MNase analysis, as the wrapping of DNA around many types of DNA-binding proteins can alter plasmid topology in this type of assay.

DNA templates that contain head-to-tail repeats of nucleosome positioning elements have been engineered so that a restriction enzyme cleavage site is located in the linker DNA between each positioned nucleosome. For instance, 5S rDNA arrays contain EcoRI sites between each 5S repeat, whereas arrays of 601 sequences contain ScaI sites between repeats. When these array templates are reconstituted into a nucleosomal array, the extent of nucleosome saturation can be easily determined by a simple restriction enzyme digest followed by native gel electrophoresis (Box 13.3). In essence, this type of analysis is very similar to how one monitors assembly of mononucleosomes. For instance, when a reconstituted array contains only a few nucleosomes (undersatu-

rated), the restriction digest/gel analysis will show a large amount of released free DNA repeats relative to released mononucleosomes. In contrast, a reconstituted array that is fully saturated with nucleosomes will yield mostly mononucleosomes by this assay. In general, we find that a reconstituted array that contains about 4% free DNA repeats represents a "perfect" saturated nucleosomal array (note that the wrapping of DNA around the histone octamer quenches ethidium bromide fluorescence by ~2.5-fold). Attempts to eliminate free DNA completely usually lead to arrays that harbor an excess of nonspecifically bound octamers, which are shown as a general smearing of the digestion products in the lane.

The most rigorous method for analyzing the quality of reconstituted nucleosomal arrays is a velocity sedimentation experiment in an analytical ultracentrifuge. This is an old-fashioned technique that is making a comeback with the advent of state-of-the-art analytical ultracentrifuges, such as the Beckman XL-A and XL-I (for reviews, see Hansen et al. 1994, 1997; Ausio 2000). Analytical ultracentrifuge protocols allow investigators to determine sample purity, molecular weight, sedimentation coefficient, diffusion coefficient, frictional coefficient (shape), and association/dissociation reactions. Indeed, this type of analysis is essential for any in-depth analysis of the solution state behavior of a macromolecule. Typically, we use velocity sedimentation to determine

Box 13.3. EcoRI Analysis of Nucleosomal Arrays

Model nucleosomal array templates composed of head-to-tail repeats of nucleosome positioning elements (NPEs) contain restriction enzyme recognition sites between each NPE (Fig. 13.5, left panel). Templates that contain 5S rDNA genes harbor EcoRI sites between each repeat, whereas templates that harbor 601 NPEs contain ScaI sites. Following salt dialysis reconstitution, nucleosomal arrays are digested with EcoRI (or ScaI) to determine the percentage of NPEs that are occupied by nucleosomes. Digestions are electrophoresed on a native 5% polyacrylamide gel and stained with ethidium bromide (Fig. 13.5, right panel). At lower ratios (*r*) of histone octamer to NPE ($r = 0.6$), many of the NPEs are nucleosome-free (Fig. 13.5, right panel). As the *r* value is increased, the majority of NPEs are assembled into mononucleosomes. Note that EcoRI digestion does generate some dimer and trimer products because of alternative nucleosome positions that block access to EcoRI at a subset of NPEs. A reconstitution that is fully saturated with nucleosomes will contain about 4% free DNA by the EcoRI analysis (note that DNA in mononucleosomes bind ~2.5 times less ethidium bromide). In this set of reconstitutions, the $r = 1.0$ sample is very close to a saturated array. At higher *r* values, additional octamers bind to the nucleosomal array nonspecifically, generating more slower-migrating smears in this analysis (e.g., at $r = 1.2$ for these reconstitutions).

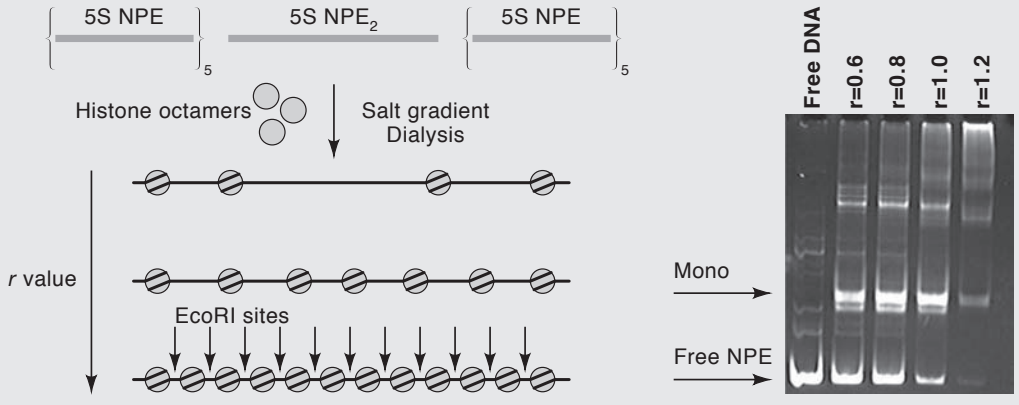

FIGURE 13.5. EcoRI analysis of nucleosomal arrays.

the degree of nucleosome saturation accurately, identify arrays of poor quality and/or homogeneity, and characterize the higher-order folding of arrays induced by buffer conditions or nonhistone proteins. For instance, a 208-12 5S array (12 repeats) in low-salt TE buffer will sediment in the XL-I ultracentrifuge as a uniform population of 29S macromolecules when it is saturated with nucleosomes, but an array with only 11 nucleosomes will sediment at approximately 27S. No other method provides the same degree of sensitivity. Furthermore, when nucleosomal arrays fold into higher-order structures, this leads to a change in shape that alters the frictional coefficient and therefore alters the sedimentation rate. Indeed, sedimentation velocity analysis is one of the few available methods for analysis of chromatin-folding dynamics in solution (Hansen et al. 1989; Hansen and Lohr 1993; Hansen and Wolffe 1994; Carruthers and Hansen 2000; Horn et al. 2002a; Dorigo et al. 2003; Shogren-Knaak et al. 2006). Although we are able to analyze arrays routinely in the analytical ultracentrifuge, most researchers do not have access to such an instrument. Consequently, simple EcoR1 or ScaI assays remain the method of choice for analysis of linear nucleosomal arrays assembled on 5S or 601 repeats.

Strategies to Test the Role of Histone Modifications In Vitro

As early as 1964, Allfrey et al. (1964) proposed a link between the reversible acetylation of histone amino-terminal domains and transcription. Numerous studies supporting a correlation between histone hyperacetylation (especially of H3 and H4) and transcriptionally active chromatin followed this proposal. These early studies identified several other posttranslational modifications on the core histones, but work during the past 10 years has elucidated an enormous number of core histone modifications, including serine or threonine phosphorylation, arginine methylation, proline isomerization, and lysine methylation, ubiquitylation, acetylation, or sumoylation (for a recent review, see Kouzarides 2007). Indeed, the regulation of site-specific histone modifications is believed to be one of the primary means by which chromatin regulates transcription.

As outlined in detail in Chapter 1, various patterns of histone modifications are associated with different transcriptional states. General features of genes that are transcribed by Pol II include acetylation of several lysines within H3 (mainly K9, 14); acetylation of H4K4, 8, 12, and 16; dimethylation of H3K79; and di- or trimethylation of H3K4. Some transcribed genes may also be distinguished by dual H3K14 acetylation and H3S10 phosphorylation. In contrast, repressed loci tend to lack these marks and instead be enriched for histones that harbor several methylated lysines, such as H3K9me2, H3K27me2, or H4K20me3. However, there does not appear to be a "code" per se for active or repressed transcriptional states, and the investigator's favorite regulated gene may have its own pattern of modifications. How that pattern of modifications is "read" into a transcriptional decision is the type of question that can be addressed by a biochemical approach. In this regard, a major challenge for the chromatin community has centered on the preparation of purified histones that harbor homogeneous, defined patterns of posttranslational modifications for investigations into biochemical mechanisms of transcriptional activation or repression. The following sections consider three strategies: (1) enzymatic addition of histone modifications, (2) native chemical ligation, and (3) introduction of methyl lysine analogs. Applications of modified histones are discussed in a later section.

Enzymatic Addition of Histone Modifications

Since the discovery of the first nuclear histone acetyltransferase Gcn5 in 1996 by Allis and colleagues (Brownell et al. 1996), a large number of histone-modifying enzymes have been identified and characterized biochemically. In many cases, recombinant enzymes function well on histone substrates (e.g., Gcn5, P/CAF, CBP, hMOF, PR-SET7, G9A), and several studies have used such enzymes to create nucleosomes or nucleosomal arrays that harbor site-specific acetylated or methylated histones for use in functional assays (Hassan et al. 2001; An et al. 2004; Chandy et al. 2006; Trojer et al. 2007) (see also Fig. 13.7, below). For instance, Workman and colleagues have

acetylated nucleosomal arrays with several yeast histone acetyltransferase (HAT) complexes and monitored the effect on HIV-1 transcription (Steger et al. 1998). Likewise, Roeder and colleagues have used the p300 HAT in immobilized template assays to determine which steps during transcriptional activation are stimulated by histone acetylation (An et al. 2004). In principle, enzymatic modification of histones can use either free histones or nucleosomal substrates, but in either case the amount of enzyme and the time of the reaction must be titrated to limit formation of minor modification products. Indeed, one of the major limitations of this approach is that enzymatic modification can produce heterogeneity of site specificity. An additional disadvantage of the enzymatic addition strategy is that these types of reactions are very difficult to drive to completion. Furthermore, the desired histone-modifying enzyme may simply not be available or be difficult to purify in sufficient quantities. Furthermore, this strategy is unlikely to be feasible if an investigator wishes to create a complex pattern of histone modifications because this would require multiple enzymatic additions. Thus, although this strategy can be useful in some applications, the limitations are numerous.

Native Chemical Ligation

In the past couple of years, native chemical ligation has emerged as a powerful tool for the creation of recombinant histones that have amino-terminal modifications (He et al. 2003; Shogren-Knaak et al. 2003, 2006; Shogren-Knaak and Peterson 2004; Ferreira et al. 2007). In principle, this method is well-suited for creating recombinant histones that have multiple types and combinations of modified residues. Furthermore, unlike enzymatically modified histones or histones purified from cellular lysates, ligation provides homogeneously modified histones. This native chemical ligation strategy generates full-length proteins from unprotected, synthetic peptides and expressed protein fragments, and it is well-suited for including amino acids that are not directly incorporated into proteins via the genetic code. Specifically, this technique requires an amino-terminal polypeptide fragment ending in a carboxy-terminal thioester and a carboxy-terminal polypeptide fragment beginning with an amino-terminal cysteine (Fig. 13.6). When mixed together, these fragments can form a reversible covalent association via *trans*-thioesterification of the carboxy-terminal thioester by the amino-terminal cysteine thiol. This reaction intermediate can then rearrange irreversibly via an *S*- to *N*-acyl shift, resulting in a canonical amide peptide bond at the ligation site. This method is compatible with proteins containing other cysteines, because thioester products with these cysteines cannot rearrange to form an amide bond and thioesterification is reversible.

To incorporate modified residues of interest into the amino-terminal domain of a ligated histone, the amino-terminal thioester-containing fragment is generated by solid-phase peptide synthesis and the carboxy-terminal cysteine-containing protein fragment is expressed and purified from bacteria. Importantly, we have found that the initiator methionine is removed efficiently in bacteria from histones that contain an adjacent cysteine residue. This eliminates the need to express histones harboring site-specific protease cleavage sites. In theory, it should also be possible to study carboxy-terminal histone modifications by expressing recombinant amino-terminal protein fragments that contain a carboxy-terminal thioester (obtained by intein cleavage; David et al. 2004) and by synthesizing carboxy-terminal tail peptides containing an amino-terminal cysteine.

Generation of peptides with a reactive carboxy-terminal thioester group requires the selective thioesterification of the terminal peptide α-carboxylic acid group in the presence of other potentially reactive functional groups. To achieve this selectivity, peptides are synthesized by standard Fmoc-based solid-phase peptide synthesis on an acid-hypersensitive resin. We have found that double coupling of all residues improves the yield and purity of the histone peptide. Synthesized peptides are then cleaved from the synthetic support with a weak acid cleavage cocktail to expose the free terminal α-carboxylic acid while maintaining the side-chain protecting groups. The terminal α-5 carboxylic acid of the peptide is then thioesterified using standard peptide-coupling

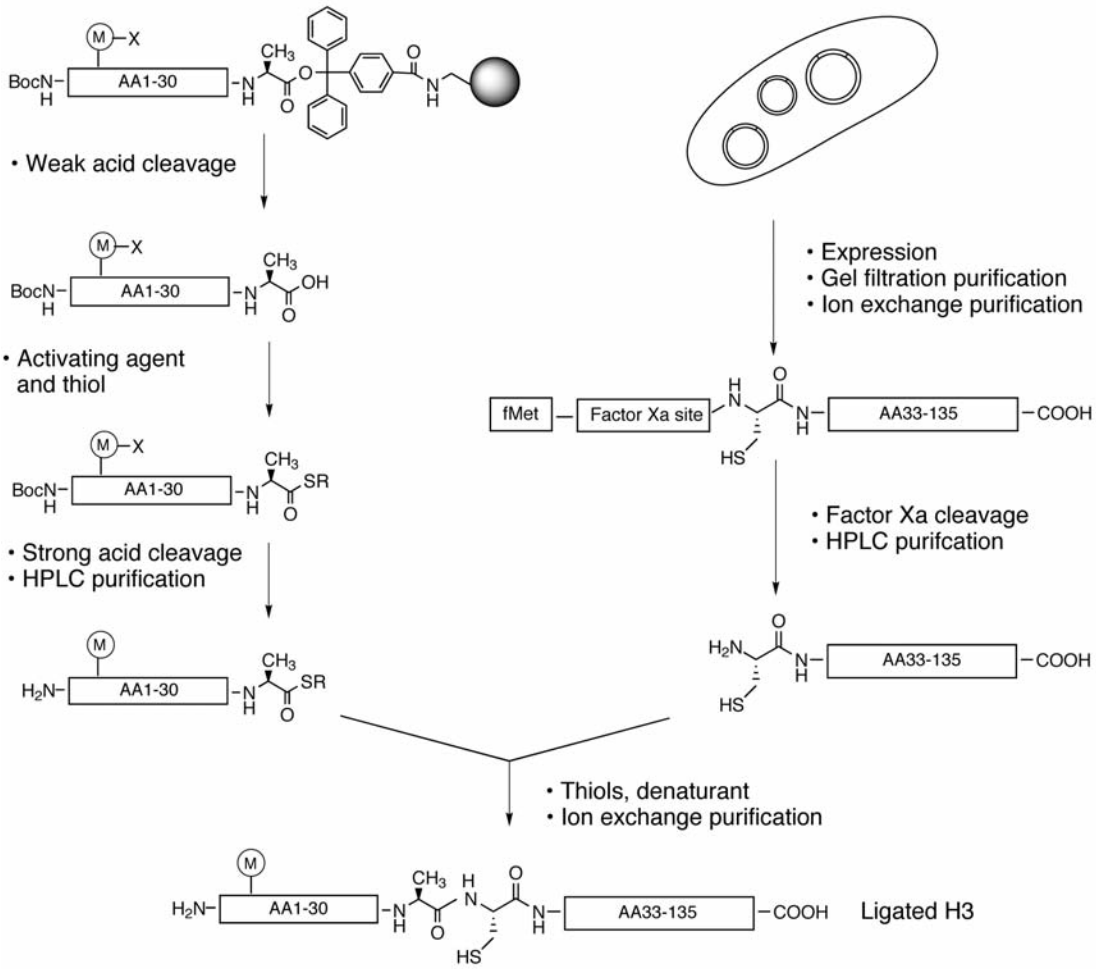

FIGURE 13.6. Native chemical ligation strategy for generating "designer" histones. In the example shown, an amino-terminal peptide fragment of histone H3 that contains a specifically modified amino acid residue (residue denoted by an encircled M), and a carboxy-terminal thioester moiety (COSR), is produced by standard solid-phase peptide synthesis on an acid-hypersensitive support (*left*). A carboxy-terminal protein fragment of histone H3 containing an amino-terminal cysteine residue is generated by expression and purification from bacteria (*right*). The amino-terminal cysteine can be exposed by proteolytic cleavage as shown or the cysteine can be positioned adjacent to the initiating methionine residue, which is cleaved efficiently inside bacterial cells. Reaction of these two fragments in the presence of thiol reagents produces native full-length histone H3 containing the modification(s) of interest. (Reprinted, with permission of Elsevier, from Shogren-Knaak and Peterson 2004, ©2004.)

reagents. Subsequent treatment of the peptide with a strong acid cleavage cocktail removes the side-chain protecting groups while maintaining the carboxy-terminal thioester. Finally, high-pressure liquid chromatography (HPLC) is used to obtain the thioester ligation substrate in pure form. For a detailed description of this method, see Shogren-Knaak and Peterson (2004).

The product of the ligation reaction contains a cysteine residue that is not present in the native histone. Several criteria must be considered when engineering the position in which this cysteine will be introduced. First, the ligation must occur within the first 30–35 residues of the amino terminus, because of the inability to synthesize larger peptides efficiently. Second, one should try to identify an amino-terminal residue that is not well-conserved among eukaryotes. An alternative strategy would be to insert a new cysteine residue within a histone amino-terminal domain at the desired location, with the caveat that this may change the spacing of potential functional elements. Finally, proline, valine, or isoleucine are known to inhibit ligation and should not be located adjacent to the cysteine residue (carboxy-terminal) in the recombinant protein.

Advantages and disadvantages. The native chemical ligation method has several clear advantages over other strategies. First, the investigator has complete control over which modified residues are incorporated, as well as their position within the amino-terminal domain. Homogeneity of product is guaranteed, and the number of modifications that can be incorporated within a single peptide is not limited. However, this method does have several disadvantages. First, the investment of time and resources can be huge. Unless an investigator has an in-house peptide synthesis facility, obtaining high-quality peptides that are synthesized on the nonconventional, acid-labile resin can be a difficulty. In particular, we have found it difficult to obtain peptides longer than 30 residues, because they are often truncated or of low purity. An additional problem is that the charged nature of histone amino-terminal peptides can create solubility problems once they are cleaved from the resin in preparation for the thioesterification reaction. This is presumably due to the continued presence of the hydrophobic blocking groups that remain on the amino terminus and on amino acid functional groups. Consequently, most reactions must be performed in the presence of high concentrations of denaturants (e.g., 6 M guanidine-HCl). In the case of some methylated H3 peptides, we have been unable to perform the ligation reaction because of severe solubility issues. An additional problem is that this method is not applicable for modifications that occur carboxy terminal to approximately residue 30, because of the limitations of peptide synthesis. However, given the powerful possibilities of the ligation strategy, it is hoped that at least some of these problems will be addressed and minimized by commercial peptide synthesis facilities.

Introduction of Methyl Lysine Analogs

Within the past couple of years, it has become clear that histone modifications are not restricted to the amino-terminal domains of the core histones, but rather there appear to be a host of modifications located within the carboxy-terminal globular domains. One of the more notable of these markers is H3K79me3, which appears to be an abundant mark in bulk cellular chromatin and may play a key role in recruitment of DNA-repair proteins during repair of DNA double-strand breaks (Huyen et al. 2004). Many other carboxy-terminal modifications have been identified by mass spectrometric analysis of cellular histones (Zhang et al. 2003; Garcia et al. 2007), and we await their functional analyses.

As stated above, the native chemical ligation strategy is not applicable for modifications that occur carboxy terminal to approximately residue 30, and for novel marks the modifying enzyme may not be available. How can investigators study these new marks in vitro? Recently, Shokat and colleagues have solved this problem, at least for methylated lysine residues, by using an old bit of chemistry and applying it to recombinant histone biochemistry. In their methyl analog approach, an aminoethylation reaction is used to convert a cysteine residue into an *N*-methylated aminoethylcysteine residue, which is a methyl lysine analog (MLA) (Simon et al. 2007). Notably, the only difference between a methylated lysine and an MLA is the replacement of the lysine γ-methylene with a sulfide, which yields neglible structural changes. This strategy takes advantage of the fact that histones contain only one cysteine residue, H3C110, which can be replaced by alanine without impacting nucleosome structure. A lysine of interest can then be replaced with a unique cysteine residue that can then be targeted for aminoethylation. Importantly, the MLA technique allows specific synthesis of all three methylation states as a homogeneous population within the modified histone molecules. Thus, using specific reaction conditions and commercially available (2-haloethyl) amines, milligram amounts of histones can be converted to a 1-me, 2-me, or 3-me MLA with yields of more than 90% and at low cost. In general, the number of reagents and expertise that are required seem reasonable for most molecular biology or biochemistry groups. For a detailed protocol, see Simon et al. (2007).

There have been a couple of pilot studies reported in which MLAs have been used to analyze the role of methylated histone residues in chromatin structure or function. Recently, Luger and colleagues have used this strategy to investigate the structural properties of nucleosomes and

nucleosomal arrays that harbor H4K20me3 or H3K79me2 (J. Hansen, pers. comm.). They find that H4K20me3 enhances the salt-dependent condensation of nucleosomal arrays, whereas H3K79me2 alters the surface charge features of individual nucleosomes. This latter effect may influence the binding of nonhistone proteins. In the original report, Shokat and colleagues created recombinant histones that harbor H3K9me2, H4K20me2, and H3K79me2 (Simon et al. 2007). Surprisingly, each of these MLAs is reactive against the corresponding methyl lysine–specific antibody; H3Kc9-2me is able to interact specifically with the histone binding protein HP1. The MLAs do not appear to disrupt nucleosome structure grossly, as mononucleosomes bearing aminoethylcysteine at H3K9 can still be methylated by SUV39H1 and such mononucleosomes bearing H3K9, H3K20, or H3K79 MLAs can be mobilized by the ACF remodeling complex.

Advantages and disadvantages. The MLA strategy is currently the method of choice for studies focused on site-specific histone methylation. The chemistry appears to be robust, the reagents are inexpensive, and there are no limitations on where the MLA can be introduced into a recombinant histone. One of the few limitations of this method is that investigators can only introduce one type of methylation state per histone (i.e., introduction of one or more Kme3, but not a Kme2 and Kme3 on the same histone), although multiple methylation states can be introduced on different histones (i.e., H3Kme2 and H4Kme3). Thus, investigators can generate many patterns of methylated lysines, but with some limitations. Furthermore, by combining the MLA approach with native chemical ligation, investigators can combine site-specific lysine methylation with other histone marks. We note, however, that there is still concern among investigators that an MLA may not faithfully mimic a methylated lysine and that the extra sulfide group may interfere or decrease protein–histone interactions. As more investigators use this technique, additional pros and cons of MLAs are likely to be elucidated.

Analysis of Chromatin Remodeling/Modification Enzymes

When early studies revealed that DNA in the eukaryotic nucleus was compacted via its association with histones, researchers immediately recognized that this condensed structure would have a major impact on the regulation of gene expression. The discovery that active loci exhibited a general increase in sensitivity to nucleases quickly led to the view that chromatin structure, and therefore transcription, might be regulated by enzymatic activities (Weintraub and Groudine 1976). Although the search for such enzymes began in the early 1980s, it was not until 1994 that the first of the "chromatin-remodeling" enzymes was identified from yeast and human cells. This was the ATP-dependent remodeling enzyme SWI/SNF (Cote et al. 1994; Kwon et al. 1994), a discovery that was followed by the identification of many related families of ATP-dependent remodeling enzymes. Likewise, despite intense interest in histone acetylation, the purification of a nuclear histone acetyltransferase had long eluded researchers. This breakthrough was finally achieved through the development of an in-gel assay for HAT activity that allowed identification of the first nuclear HAT from *Tetrahymena* macronuclei (Brownell and Allis 1995; Brownell et al. 1996). When the gene that encoded this HAT was cloned and sequenced, it was found to be homologous to the yeast gene *GCN5*, which had previously been identified as a transcriptional coactivator (Georgakopoulos and Thireos 1992; Marcus et al. 1994). As in the case for SWI/SNF, the identification of *GCN5* opened the floodgates for the cloning and characterization of human homologs of *GCN5*, as well as the identification and characterization of many other HATs that had been previously linked to transcriptional activation. Since those early studies, researchers have identified histone deacetylases, histone kinases, histone methylases, and histone demethylases that all contribute to complex dynamics of chromatin structure and transcriptional regulation. Many of these enzymes are available as recombinant enzymes or protocols have been published for their purification from various cell sources. The following section focuses on biochemical assays for monitoring and quantifying enzymatic activities, with the goals of either preparing for transcriptional assays or for characterizing a new member of this large family of enzymes.

Biochemical Analysis of Chromatin-Modifying Enzymes

Histone acetyltransferases. The detection and characterization of HAT activity is relatively straightforward and requires few reagents. All previously identified HATs can use free histones as substrates, and a subset of recombinant HATs (p300/CBP, SRC1, PCAF) are active on nucleosomes. In general, however, optimal nucleosomal HAT activity requires other proteins within multisubunit HAT complexes, such as the Ada2 and Ada3 subunits of the endogenous yeast *GCN5* HAT complex SAGA (Spt-Ada-Gcn5-acetyltransferase) (Balasubramanian et al. 2002). Histones purified from chick erythrocytes are an excellent endogenous histone source because they contain very few aceylated lysines. If histones are purified from other sources, it is recommended that deacetylase inhibitors, such as sodium butyrate or trichostatin, be omitted from all buffers so that endogenous deacetylases will remove most of the aceylated lysines during the purification. However, recombinant histones are preferred as they lack endogenous lysine acetylation that might mask enzymatic activity. For nucleosomal substrates, the short oligonucleosome arrays that are prepared for octamer transfer reconstitutions are excellent (see above). Alternatively, histone amino-terminal peptides can also be synthesized and used as substrates for HAT assays. Peptides can prove to be useful for quickly identifying lysine residues that are targeted for modification by synthesizing derivatives harboring various substitution alleles.

Assays for the detection and quantification of HAT activity rely on the transfer of a radioactive acetyl group from acetyl-coenzyme A (CoA) to a histone substrate. We typically use [^{3}H]acetyl-CoA (~5 Ci/mmole), but [^{14}C]acetyl-CoA can also be used. [^{3}H]acetyl-CoA is stable for at least 1 year stored at –20°C, and it is less expensive than [^{14}C]acetyl-CoA. In our assays, we use 3.3 μM [^{3}H]acetyl-CoA and 4.7 μM unlabeled acetyl-CoA to yield a final concentration of 8 μM acetyl-CoA, a value that is near or above the K_m for characterized acetyltransferases (Langer et al. 2001; Berndsen et al. 2007). This provides a good starting point for analysis of an unknown enzyme, but concentrations of acetyl-CoA should be titrated to determine concentrations where enzymatic activity is in the linear range.

Liquid HAT assays provide the most sensitive means for detecting HAT activity. Reactions contain histone substrate (100–1000 nM), acetyl-CoA (8 μM), variable amounts of enzyme, 10 mM sodium butyrate to inhibit contaminating histone deacetylases, and a low-salt (<100 mM NaCl) buffer (pH 7.0–8.0). Reaction time points are stopped by spotting aliquots onto phosphocellulose (P-81, Whatman) filter disks (15 mm x 15 mm). Disks are rapidly washed in 50 mM sodium carbonate (pH 9.0) to remove unreacted acetyl-CoA and air-dried; [^{3}H]acetyl-CoA incorporation is then quantified by scintillation counting. A no-enzyme control should always be included so that the background level of [^{3}H]acetyl-CoA binding can be determined. As with all enzymatic assays, care must be taken to ensure that timepoints are within the linear range of the reaction. We prefer less than 10-minute time points to calculate initial velocities accurately at different histone and enzyme concentrations (Fry et al. 2004).

The liquid HAT assay is excellent for detecting total acetyltransferase activity, but it does not yield information on which histone polypeptide has been modified. This information is obtained by electrophoresis of a portion of a liquid HAT assay on a 15% SDS-PAGE gel and detecting ^{3}H-labeled histones by fluorography. This method is not very sensitive, and thus we typically use higher concentrations of histones (>1000 nM) in such reactions so that suitable gel exposures can be obtained within a few days.

The liquid- and gel-based assays provide information on the specific activity and substrate specificity of a HAT, but how does an investigator determine which residues are modified? One strategy involves liquid HAT assays using peptide substrates in which specific lysine residues have been substituted with alanine. This type of assay determines which lysines are key for HAT activity, which may or may not be equivalent to the site of modification. Alternatively, the site of ^{3}H-labeled acetyl incorporation can be determined by amino-terminal microsequencing of the labeled protein (for details, see Mizzen et al. 1999). However, this method relies on the availability of a peptide sequencing facility that is willing to analyze radioactive protein samples. A more com-

mon strategy is to carry out HAT reactions with unlabeled acetyl-CoA and then analyze the products by mass spectrometry to identify labeled peptides and sites of modification.

Histone deacetylases. Histone lysine acetylation is a dynamic marker that has long been known to show rapid reversibility in vivo. In 1996, Schreiber and colleagues purified and identified the first histone deacetylase (HDAC), a homolog of the yeast transcriptional regulator, Rpd3 (Taunton et al. 1996). Since that time, large families of HDACs have been identified in yeast as well as in mammals. These enzymes often show both histone and lysine site specificity, and in many cases their activity is linked to transcriptional repression pathways. Several strategies have been developed for analysis of HDAC activity in vitro, with each having its advantages and disadvantages. Several methods monitor HDAC activity by the release of [^{3}H]acetate from a labeled histone peptide or polypeptide substrate. In all of these assays, reactions are stopped by addition of acid (0.1 M HCl, 0.16 M acetic acid), the [^{3}H]acetic acid is extracted with ethyl acetate, and the organic phase is quantified by scintillation counting. Several companies (e.g., Sigma-Aldrich and BIOMOL International) also market a deacetylase assay kit that uses fluorogenic or chromogenic substrates. Note that yeast *SIR2* and the sirtuin family of HDACs are unique from other HDACs in that they require nicotinamide adenine dinucleotide (NAD) as a cofactor for HDAC activity (Imai et al. 2000; Vaziri et al. 2001). In other regards, though, assays for *SIR2* and sirtuins involve the same strategy as other HDAC enzymes.

The radioactive HDAC assays differ in how the substrate is prepared. An early method involved the metabolic labeling of cultured HeLa or chick erythrocytes with [^{3}H]acetate, followed by histone purification by acid extraction. Of course, to maintain the acetyl groups, histone deacetylase inhibitors, such as sodium butyrate or trichostatin, must be added to all buffers. The advantage of this method is that histones are acetylated on physiologically relevant sites, but the disadvantage is that the labeled histones have low specific activities. This can limit detection of HDAC activity. This method has been used successfully by many groups, including Grunstein and colleagues (Carmen et al. 1996), to purify several HDAC enzymes from crude yeast lysates.

An alternative to in vivo labeling is to acetylate individual histone polypeptides or synthetic histone peptides chemically (Inoue and Fujimoto 1969). This method generates labeled substrates with high specific activity, but labeling of intact polypeptides can generate acetyl lysines at positions that are not physiological. Chemical acetylation of amino-terminal histone peptides are preferred, and, as in this case, acetylation events will be representative of physiological modifications. The only disadvantage with the peptide substrates (which is true for all studies with histone modification enzymes) is that the acetylated lysines are not presented to the enzyme in a physiological context—that is, a folded histone or histone–histone pair.

A third [^{3}H]acetate-labeling strategy involves the acetylation of individual histones or histone complexes with recombinant HATs in vitro. For instance, recombinant HAT1 will acetylate all four lysines within the amino-terminal domain of histone H4 with high specific activity, whereas recombinant *GCN5* will acetylate the majority of lysines within the amino-terminal domain of H3. Labeled histones are then purified from the reaction components by column chromatography or HPLC (for a detailed method, see Wade et al. 1999). This technique has no large disadvantage.

HDAC assays with [^{3}H]acetyl-lysine substrates require a great deal of commitment of time and resources. HATs may need to be purified and radioactive histones prepared and purified; the HDAC assay requires organic extraction of ^{3}H-labeled proteins. Furthermore, some HDACs show exquisite site specificity, and thus if one wishes to assess the deacetylation of one particular residue, this activity may not be easily assayed with substrates labeled at multiple positions. One very simple strategy for monitoring HDAC activity relies on the plentiful supply of commercial antibodies that are specific for particular acetylated histone lysine residues. In this case, nonradioactive, acetylated histones are either purified from a cell source or generated by an in vitro HAT reaction. When histones are purified from cells, sodium butyrate or trichostatin are added to cells prior to cell lysis to promote histone hyperacetylation in vivo. These HDAC inhibitors are then included in all subsequent buffers. Hyperacetylated histones are then incubated with a purified or crude HDAC

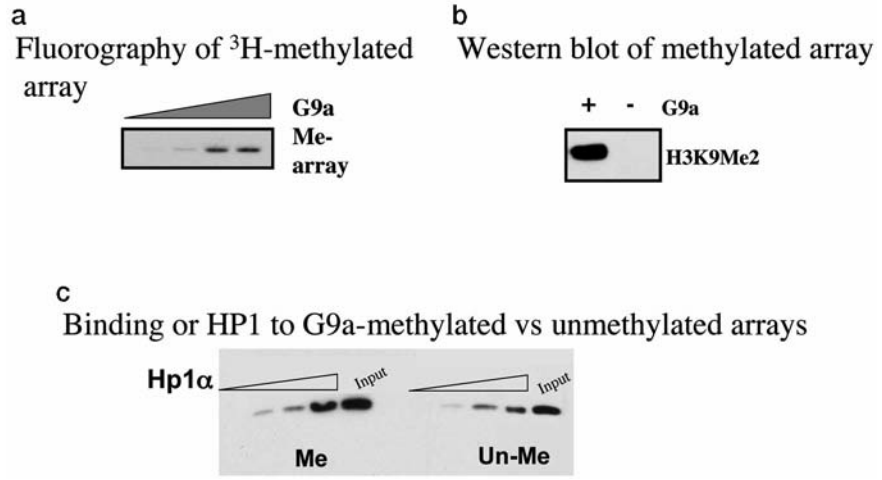

FIGURE 13.7. G9a methylation of nucleosomal arrays enhances the binding of HP1. Biotinylated G5E4T-5S arrays (see legend to Fig. 13.9) were methylated with increasing amounts of recombinant G9a histone methyltransferase in a reaction containing ^{3}H-labeled SAM as the methyl donor. (*a*) Methylation of histone H3 detected by fluorography. (*b*) Methylation of histone H3 detected by western blot with an antibody specific for H3K9me2. (*c*) Methylated or unmethylated arrays were incubated with recombinant HP1α, arrays were captured on steptavidin magnetic beads, and the amount of bound HP1α bound to the arrays was detected by western blot.

preparation, and the reaction products are analyzed by SDS-PAGE and western analysis to monitor the time-dependent and HDAC-dependent loss of a specific acetylated lysine epitope.

Histone methylases and demethylases. The core histones can be mono-, di-, or trimethylated on lysine residues (mainly H3K4, K9, K36, K79, and H4K20) or mono- or dimethylated on arginine residues (mainly H3R2, R17, R26, and H4R3). Prior to 2004, it was widely believed that methylation of histone lysine and arginine residues was an irreversible marker that was removed only by histone proteolysis or diluted from cellular chromatin by cell division. During the past few years, however, two broad families of histone lysine demethylases have been identified—the LSD1/BHC110 and JmjC histone demethylases (Klose and Zhang 2007). Interestingly, one member of the JmjC family, JMJD6, has also been shown to catalyze demethylation of both H3R2 and H4R3 (Chang et al. 2007). Furthermore, Kouzarides and colleagues discovered that some methylated arginine residues could also be converted to citrulline by peptidylarginine deiminase 4 (PADI4) (Cuthbert et al. 2004). Thus, like histone acetylation, histone methylation is also subject to dynamic regulation.

The strategies that are commonly used for analysis of histone methylases and demethylases are quite similar to those used for HATs and HDACs. For histone methyltransferase (HMT) assays, one simply substitutes *S*-adenosyl-L-[methyl-^{3}H]methionine ([^{3}H]SAM, ~80 Ci/mmole) for the [^{3}H]acetyl-CoA in the enzymatic reactions. Incorporation of [^{3}H]methyl groups can be monitored either by binding to P81 filter paper (liquid HMT assay) or by electrophoresis of products on 15% SDS-PAGE and fluorography (see Fig. 13.7). As is the case for HAT assays, the choice of histone substrate is also key for HMT assays. For each enzyme, investigators should test both core histones and nucleosomal arrays. For instance, identification of the Set8 HMT that methylates H4K20 was delayed for several years until it was discovered that it can only use nucleosomal substrates (Fang et al. 2002). Furthermore, some HMTs, like the PRC2 complex, prefer to methylate nucleosomal arrays isolated from endogenous sources (e.g., calf thymus or HeLa nuclei), rather than arrays assembled with recombinant histones (Kuzmichev et al. 2002). This enhanced activity is presumably due to the presence of other histone marks that stimulate HMT activity.

Monitoring histone demethylase activity is also similar to strategies used for HDACs. Commonly, a demethylase assay uses core histones isolated from a cell source (i.e., HeLa, calf thy-

mus, or chick erythrocytes) and activity is monitored by western analysis with methyl lysine–specific antibodies. Peptide substrates can also be used, and products of the demethylation reaction assayed by matrix-assisted laser desorption/ionization-time of flight/time of flight (MALDI-TOF/TOF) to determine which methyl groups have been removed. Unlike HDACs, members of the JmjC family of histone demethylases require novel cofactors. These enzymes use a hydroxylation-based reaction mechanism to remove the methyl group from a lysine, and this reaction requires Fe(II) (50 μM) and α-ketoglutarate (1 mM) (Tsukada et al. 2006; Whetstine et al. 2006). The Fe(II) is typically added to reactions as $(NH_4)_2\ Fe(SO_4)_2 + 6(H_2O)$. Consequently, iron chelators such as EDTA and deferoxamine (DFO) are often used as enzyme inhibitors. Obviously, buffers used in purification and analysis of these enzymes should lack EDTA. In contrast, members of the LSD1/BHC110 family use a flavin-dependent oxidation reaction to demethylase histone lysines, a reaction that generates formaldehyde (Shi et al. 2004). Note that the flavin cofactor, flavin adenine dinucleotide (FAD), copurifies with the enzyme even when prepared from bacteria. The formation of formaldehyde can also be measured in a coupled spectrometric assay that is amenable to kinetic analyses (for details, see Shi et al. 2004).

DNA methyltransferases. Whereas histone marks are dynamically regulated, methylation of cytosines within DNA is a stable mark that can be transmitted to progeny, and it is associated with long-term transcriptional silencing. DNA methylation is carried out by DNA methyltransferases (DNMTs), which transfer a methyl group from SAM to the 5′ position of cytosine residues, often within CpG dinucleotides (Klose and Bird 2006). There are two classes of DNMTs: (1) the de novo DNMTs DNMT3a and DNMT3b are able to modify unmethylated DNA and (2) the maintenance DNMT DNMT1, which prefers to methylate hemimethylated DNA substrates, although it can also modify unmethylated substrates (Jair et al. 2006). DNMT1 is believed to be a component of the replication fork, where it may play a key role in propagating patterns of DNA methylation through multiple cell divisions (Rountree et al. 2000; Esteve et al. 2006). The de novo enzymes function early in embryonic development to establish domains of methylation. Because these enzyme both establish and propogate methylation states that correlate with transcriptional activity, they represent factors that control stable epigenetic transcriptional memory during development.

Analysis of DNMT activity in vitro is relatively straightforward. All three enzymes can be expressed and purified from bacteria, and simple assays exist for monitoring methyltransferase activity. As in the case for HMT assays, the DNMTs can use [^{3}H]SAM as a cofactor to transfer [^{3}H]methyl groups to a DNA substrate such as poly(dI-dC). Incorporation is followed by binding the reaction components to DE81 paper (Whatman), washing with ammonium bicarbonate, and scintillation counting (Pradhan et al. 1999). This assay is very similar to liquid HAT and HMT assays.

Bacterial DNA methyltransferases are also becoming more and more useful as site-specific probes of nucleosome positioning and chromatin structure. In particular, Kladde and colleagues have described methyltransferase methods for mapping in vitro and in vivo nucleosome positions in populations of nucleosomes, as well as in single molecules (Kilgore et al. 2007; see also Chapter 9). Like restriction enzymes and other DNA-binding proteins, nucleosome assembly inhibits the methyltransferase activity of MTases. However, unlike restriction enzymes or DNA-binding proteins, the frequency of MTase target sites (usually CpG or GpC dinucleotides) is very high in most sequences, allowing excellent resolution of nucleosome positioning. Most useful are bacterial enzymes such as M.SssI and M.CviPI, which modify the 5′ position of cytosines within the dinucleotides CpG and GpC, respectively, because 5-methyl cytosine can be easily detected in DNA through PCR-based bisulfite sequencing.

ATP-Dependent Chromatin-Remodeling Enzymes

There are four different classes of ATP-dependent chromatin-remodeling enzymes, SWI/SNF, ISWI, INO80, and Mi-2/CHD, and each group is composed of several multisubunit complexes that all contain a catalytic ATPase subunit that belongs to the SWI2/SNF2 subfamily of DNA-dependent

ATPases (for recent reviews, see Cairns 2005; Durr et al. 2006). Consequently, each of these enzymes has comparable turnover numbers for ATP hydrolysis (~200–800 ATPs hydrolyzed per minute per complex), and significant ATP hydrolysis requires the presence of a nucleic acid cofactor. Whereas members of the SWI/SNF family can use double-stranded DNA, single-stranded DNA, or nucleosomes as cofactors in ATPase assays with nearly equal efficiencies, members of the ISWI group require a nucleosome cofactor to activate significant ATP hydrolysis; members of the INO80 and Mi2/CHD group can use either type of cofactor, but they show a preference for nucleosomes (Boyer et al. 2000). Several studies indicate that ATP hydrolysis is coupled to DNA translocation, which can generate superhelical torsion and cause repositioning of nucleosomes in *cis* (Cairns 2007; see below). Because all nucleosome remodeling activities require ATP hydrolysis, we have routinely used cofactor-stimulated ATPase activity to determine the specific activities of different remodeling enzymes or to calculate the concentration of active enzyme in different preparations.

ATPase assays. One simple method to monitor ATP hydrolysis is by measuring the amount of [^{32}P]pyrophosphate that is released from [γ-^{32}P]ATP by binding the remaining ATP to activated charcoal in 20 mM phosphoric acid and quantifying radioactivity in the supernatant by scintillation counting (Cote et al. 1994). Unfortunately, this method generally has a high background in the absence of enzyme, and the method is hindered by the fact that one can only measure one product of the reaction. Consequently, we typically use thin-layer chromatography (TLC) on polyethyleneimine (PEI)–celluose plates to quantify both [γ-^{32}P]ATP and the amount of released ^{32}P (Logie and Peterson 1999). This method yields very reproducible results and it is amenable to detailed kinetic analyses. Cairns et al. (1994) have also presented a simple, colorimetric ATPase assay in which formation of inorganic phosphate is quantified by the addition of an acidic malachite green–sodium molybdate solution to the reaction.

For members of the SWI/SNF family of enzymes (e.g., yeast SWI/SNF and RSC, mammalian BAF and PBAF), "chromatin remodeling" refers to numerous in vitro ATP-dependent changes in a chromatin substrate, including disruption of histone–DNA contacts within mononucleosomes, movement of histone octamers in *cis* and in *trans*, loss of negative supercoils from circular minichromosomes, eviction of H2A/H2B dimers, and increased accessibility of nucleosomal DNA to transcription factors and restriction endonucleases (for review, see Smith and Peterson 2005). In contrast, enzymes of the ISWI, Mi-2/Chd, and INO80 families have generally been characterized by their ability to mobilize nucleosomes in *cis* or to enhance restriction enzyme accessibility of nucleosomal arrays. Interestingly, the SWR1 enzyme, a member of the INO80 family, does not appear to mobilize nucleosomes in *cis*, but its activity appears to be dedicated to the ATP-dependent exchange of an H2A/H2B dimer for an Htz1/H2B dimer (Mizuguchi et al. 2003).

Restriction enzyme accessibility assays. As an initial measure of ATP-dependent nucleosome remodeling activity, we typically use a restriction enzyme accessibility assay (Logie and Peterson 1997, 1999). This assay is based on the fact that nucleosome assembly inhibits restriction enzyme cleavage by several orders of magnitude, and ATP-dependent remodeling can enhance the rate of cleavage 10–30-fold. Thus, this is often a very sensitive measurement of remodeling activity. In principle, one can use mononucleosomes, linear nucleosomal arrays, or circular nucleosomal arrays in this type of assay. However, as the positioning of a mononucleosome can impact the activity of some enzymes (see below), nucleosomal array substrates are preferred.

Although nearly any DNA fragment can be reconstituted into a nucleosomal array and the efficiency of restriction enzyme cleavage monitored, we developed a quantitative remodeling assay that uses a DNA template composed of a tandem array of 11 5S rDNA nucleosome positioning sequences (Logie and Peterson 1997, 1999). The key feature of this template is that the central repeat has been modified to include a unique Sal1/HincII restriction enzyme recognition site near the center of the positioned nucleosome (the 208-11-Sal template). This DNA fragment is typically reconstituted into a positioned array of nucleosomes either by salt dialysis, octamer transfer, or rapid dilution methods. When these reconstituted nucleosomal arrays are exposed to SalI (or HincII), the

cleavage kinetics are biphasic. The first phase is very rapid, where 25–50% of the arrays are cleaved at a rate very close to that of naked DNA. This fraction of array cleaved in the first phase represents the percentage of arrays in the population that do not harbor a nucleosome at the central repeat, because of either alternative nucleosome positions or subsaturation of the template. The second phase is much slower and represents the occlusion of the SalI/HincII site by the positioned nucleosome. Typically, arrays are precleaved for 20 minutes in the absence of remodeling enzyme to eliminate the first phase of cleavage. When a remodeling enzyme and ATP are then added to the reaction, the second phase of restriction enzyme cleavage is greatly enhanced. To begin an experiment, an equimolar ratio of remodeling enzyme to nucleosomes (11 molecules of enzyme per array) is recommended to detect weaker activities. Reactions with the yeast SWI/SNF enzyme usually use ratios of one SWI/SNF per 11-mer array, and much lower concentrations of SWI/SNF have demonstrated catalytic remodeling events (Logie and Peterson 1997). This two-enzyme coupled reaction has been used to measure several kinetic parameters of an SWI/SNF remodeling reaction (Logie and Peterson 1997) and recruitment of SWI/SNF activity by transcriptional activators (Yudkovsky et al. 1999); it has been used to directly compare the remodeling activities of five different enzymes, yeast SWI/SNF, human SWI/SNF, CHRAC, Mi-2, and ACF (Boyer et al. 2000).

Nucleosome mobilization assays. All ATP-dependent chromatin remodeling enzymes tested to date (with the possible exception of Swr1) use the energy derived from ATP hydrolysis to mobilize nucleosomes along DNA in *cis*. A direct comparison of seven different remodeling complexes on a mononucleosome substrate has shown that each complex has distinct nucleosome positioning characteristics (Rippe et al. 2007). For instance, the ACF and Mi-2 enzymes prefer to move nucleosomes from the ends toward the center of the DNA fragment, whereas ISWI tends to move nucleosomes from the middle to the DNA ends. Several other studies have shown that the SWI/SNF and RSC remodeling enzymes can mobilize nucleosomes off the ends of DNA fragments such that less than 147 bp of DNA remains associated with the histone octamer (Kassabov et al. 2003). These differences are attributed to a combination of different ATPase subunits as well as enzyme-specific noncatalytic subunits. The mononucleosome mobilization assay is based on the fact that a nucleosome that is positioned at the center of a DNA fragment will migrate slower in a polyacrylamide gel compared to a nucleosome positioned toward the DNA ends. For instance, a mononucleosome that is reconstituted onto a 350-bp Hsp70 promoter fragment generates as many as five nucleosome complexes with distinct mobilities that represent alternative translational positions (see Fig. 13.2). If this mononucleosome preparation is incubated with a remodeling enzyme (usually equimolar ratios of enzyme to nucleosome), and the products are analyzed by PAGE, one finds that the distribution of species has been changed or that complexes with new mobilities are generated. Note that for some remodeling enzymes (e.g., SWI/SNF), we find that addition of excess DNA just prior to loading the samples is essential for dissociating enzyme–nucleosome complexes and yielding a discrete nucleosome banding pattern. These experiments are quite simple to perform, and they are amenable to rough kinetic analyses. Furthermore, one can isolate the different mononucleosome species from the gel and map nucleosome positions with ExoIII as described earlier (Fig. 13.2).

In addition to mobilizing nucleosomes in *cis*, members of the SWI/SNF family of remodeling enzymes (e.g., yeast SWI/SNF and RSC) can also displace histone octamers and transfer them to DNA fragments in *trans* (Lorch et al. 1999, 2006). This activity is very inefficient in most cases, although the yeast RSC complex appears to be the most proficient of enzymes tested (Fig. 13.8). Furthermore, binding of multiple activator proteins to the underlying nucleosome or recruitment of SWI/SNF by an activator can enhance this reaction (Owen-Hughes et al. 1996; Gutierrez et al. 2007). Octamer displacement assays differ in a few respects from nucleosome mobilization assays. Typically, mononucleosomes are assembled on shorter, radiolabeled DNA fragments that are close to core particle length (147–160 bp), and reconstitutions should be assembled so that a small amount of naked DNA remains. A key addition to the remodeling reaction is an excess of unlabeled acceptor DNA that will capture the histone octamer as it is displaced by the remodeling enzyme (Fig. 13.8). The most effective acceptor DNA contains one or more copies of a high-affinity nucle-

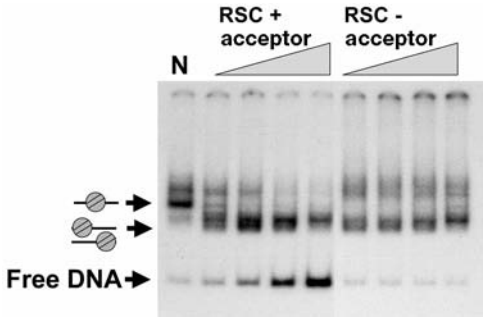

FIGURE 13.8. RSC-mediated histone octamer eviction requires acceptor DNA. Native gel analysis of a centrally positioned mononucleosome prior to (lane N) and after remodeling by the RSC complex. In the presence of ATP, the RSC complex mobilizes the mononucleosome and generates products in which the nucleosome is positioned at either of the two ends of the DNA fragment. When acceptor DNA is added to the reaction, RSC also mediates eviction of the histone octamer, leading to the appearance of free DNA.

osome-binding site, such as the 601 nucleosome positioning sequence. In other respects, the assay is similar to nucleosome mobilization reactions. Following incubation with the mononucleosome substrate and the acceptor DNA, reactions are analyzed by PAGE. Successful octamer displacement is monitored by the ATP-dependent increase in radiolabeled free DNA. Ensuring that this reaction is ATP-dependent is key, because several factors can destabilize dilute solutions of mononucleosome in vitro. For instance, we find that addition of bovine serum albumin (BSA; 100 μg/ml) is an essential component of remodeling reactions that use nanomolar concentrations of nucleosomes, as nucleosomes can spontaneously dissociate at very low protein concentrations (Godde and Wolffe 1995).

Dimer eviction and replacement assays. In addition to mobilizing nucleosomes in *cis* and in *trans*, some ATP-dependent remodeling enzymes (e.g., SWI/SNF and SWR1) can displace one or both H2A/H2B dimers or catalyze the exchange of a canonical H2A/H2B dimer with a dimer that carries an H2A variant. For instance, yeast SWI/SNF can displace H2A/H2B from a mononucleosome assembled on an MMTV promoter fragment in a reaction that requires a histone-binding domain of the Swi3p subunit (Yang et al. 2007). On the other hand, the SWR1 complex can replace a canonical H2A/H2B dimer with an Htz1/H2B dimer in a reaction that requires either the Nap1 or Chz1 histone chaperone (Luk et al. 2007). In the case of human SWI/SNF, this dimer displacement reaction appears to play a key role in transcriptional activation from the MMTV LTR in vivo (Yang et al. 2007), and SWR1 is essential for deposition of Htz1 in nucleosomes that surround nearly every RNAPII promoter in yeast (Raisner et al. 2005; Zhang et al. 2005).

Several assays have been developed to monitor the ATP-dependent displacement of H2A/H2B dimers by the SWI/SNF complex. Bruno and Owen-Hughes described a simple assay using recombinant histone H2A that is covalently labeled with a fluorescent group, Oregon Green, at an engineered cysteine residue within its carboxy-terminal domain (Bruno et al. 2003b). In this assay, the Oregon Green labeling is performed with recombinant octamers, which are then assembled by salt dialysis onto an unlabeled DNA fragment to yield fluorescent mononucleosomes. This labeling reaction is quite simple and appears to be efficient. The mononucleosomes are then incubated in a remodeling reaction, and the products are analyzed by PAGE analysis. For detection of the fluorescence mononucleosomes, we have used a Kodak Imaging scanner, with an excitation filter of 465 nm and an emission filter of 535 nm (Yang et al. 2007). Typically, one measures the ATP-dependent loss of fluorescence from the mononucleosome complex. In some cases, addition of an array of H3/H4 tetrasomes may facilitate this reaction by functioning as an acceptor for the displaced dimers (Bruno et al. 2003a), although we have found this to be dispensable when using intact yeast SWI/SNF. One drawback of the Oregon Green assay is that detection of the fluorescent group requires a high concentration of mononucleosomes in the remodeling reaction (~30–100 nM nucleosomes), which can quickly deplete stocks of remodeling enzymes. A potentially easy alternative to the Oregon Green assay is to use an in vitro chromatin immunoprecipitation (ChIP) assay to monitor the ATP-dependent loss of H2A or H2B (Yang et al. 2007). This assay is severely limited, however, by the lack of commercial H2A or H2B antibodies.

The establishment of a dimer exchange assay requires several additional reagents. First, a DNA fragment is biotinylated on one or both ends by the Klenow reaction using biotinylated nucleotides. This labeled DNA fragment is then reconstituted into a nucleosomal array by salt dialysis using either recombinant histones or histone octamers purified from a cell source. The second reagent is purified H2A/H2B or H2A-variant/H2B dimers that will be analyzed for exchange activity. For instance, in SWR1 assays, Htz1/H2B dimers are purified from yeast cells (using a strain harboring a Htz1-FLAG fusion protein) or prepared as recombinant proteins. When purified from yeast, the Htz1/H2B dimers are associated with either the Nap1 or Chz1 histone chaperone whose activity is greatly stimulatory for the exchange reaction (Luk et al. 2007). Chz1 can also be prepared as a recombinant protein and added to the reaction. Thus, a SWR1-dependent exchange reaction contains biotinylated nucleosomal arrays, Htz1/H2B dimers, Chz1 or Nap1 chaperone (usually as a complex with the dimers), ATP, and SWR1 remodeling enzyme. At different time points, the nucleosomal array is captured on streptavidin magnetic beads, the beads are washed with 0.4 M KCl buffer to remove nonspecifically bound histones, and the amount of Htz1 that has been exchanged onto the array is quantified by western analysis with commercial Htz1 antibodies. As one might imagine, this assay requires a greater commitment of effort than other remodeling assays. The amount of H2A/H2B dimers, histone chaperone, and remodeling enzyme must be titrated for each set of assays. In addition, to follow exchange of canonical H2A/H2B dimers, one must purify an epitope-tagged form of H2A (vectors for recombinant H2A-FLAG are available), given the lack of commercial H2A antibodies.

In Vitro Transcription with Chromatin Templates

As discussed in detail in the previous sections of this chapter, two general types of DNA templates are typically used for in vitro transcription studies. Model nucleosomal array templates contain a dinucleosome- or trinucleosome-length promoter region embedded within an array of nucleosome positioning sequences (usually tandem copies of a 5S rDNA gene). In this type of template, nucleosome positions can be controlled so that binding sites for key transcriptional regulators or the core promoter are encompassed by nucleosomes. Alternatively, large DNA fragments or circular plasmid DNAs can be assembled into regularly spaced nucleosomal arrays using extract-based assembly systems or the recombinant ACF/NAP1 system. These chromatin templates do not contain positioned nucleosomes, and, consequently, many factor-binding sites will be accessible and chromatin will not impede these initial steps.

Overall, the design of an in vitro transcription study with a chromatin template involves many of the same considerations as assays that use naked DNA. A source of Pol II and general transcription factors must be identified and their activities quantified and buffer conditions optimized. Relevant sequence-specific activators or repressors must be purified and binding sites incorporated into DNA templates. However, there are several different approaches to using chromatin templates in transcription assays. One approach is to determine whether the activator facilitates transcription during the process of chromatin assembly. For example, the activator alone or in the presence of the general machinery would be incubated with a DNA template in an embryo extract capable of assembling the template into chromatin (or a purified ACF/NAP1 recombinant system). If activation is observed, one might imagine two mechanisms. In one, the activator could be blocking access of the promoter to chromatin, thereby passively facilitating assembly of the basal transcription complex. Alternatively, the activator could be directly stimulating complex assembly by interaction with the transcriptional machinery.

An alternative approach is to preassemble the chromatin onto the template and the activator and general machinery are added later. If the activator stimulates transcription, one might imagine two possible mechanisms. The activator might gain access to promoters preassembled into chromatin and actively remove the chromatin by recruiting chromatin-remodeling enzymes. This remodeling would allow the general machinery to bind passively. Alternatively, the activator might facilitate chromatin remodeling and also actively promote transcription complex assembly. Some

activators are known to recruit components of the chromatin remodeling and general transcription machinery actively and independently. For example, GAL4–VP16 directly recruits both the SWI/SNF complex and histone acetylases to DNA, while also interacting directly with transcription activation factors, other coactivators, and general transcription factors to stimulate transcription.

Immobilized Template Assays/Order of Addition Assays

Numerous examples have been described in which the transcriptional activation of a gene involves a linear sequence of regulatory events. For instance, a gene-specific activator may first bind to one or more sites upstream of an RNAPII-dependent promoter region. The activator may then recruit a histone acetyltransferase complex that acetylates lysine residues within promoter proximal nucleosomes. The activator might next recruit an ATP-dependent remodeling enzyme such as SWI/SNF whose binding to promoter nucleosomes is stabilized by the acetylated lysine residues. SWI/SNF remodeling could mobilize or evict a nucleosome that occludes the transcription start site, and this event would subsequently lead to preinitiation complex (PIC) assembly. Of course, this is only one particular example and there are likely to be a multitude of possible events that sequence together to control transcriptional activation (or repression).

One powerful strategy that allows investigators to dissect a regulatory pathway and to determine the interdependencies among different steps is the use of immobilized nucleosomal templates coupled with order of addition assays. The key to this strategy is to reconstitute nucleosomes on a DNA fragment that has been labeled with one or more biotinylated nucleotides at one terminus, generally by the Klenow reaction. The biotinylated nucleosomal array can then be immobilized on a steptavidin-coupled resin. In this regard, magnetic beads are preferred because of their lower nonspecific protein-binding properties compared to various types of agarose beads.

Once the nucleosomal template is immobilized, individual regulatory components can be reacted with the template, and washes between each factor addition can be used to remove proteins, cofactors, or exchange buffers. For instance, if an activator is added, excess factor can be removed by capturing the template on the magnetic bead, followed by a mild wash with buffer. The template can also be incubated in a chromatin remodeling or histone-modification reaction. Once that reaction is completed, the template is captured and remodeling/modification enzymes are removed by a wash with buffers that contain approximately 400 mM NaCl. Alternatively, recombinant histones that harbor site-specific histone modifications can be used to bypass the need for various modification enzymes. A source of RNAPII and general transcription factors can then be added after a selected addition to monitor transcriptional capacity.

Establishing Recruitment of Remodeling Enzymes

All chromatin-remodeling and -modification enzymes have a general, nonspecific affinity for DNA and chromatin. Consequently, incubation of such enzymes with a nucleosomal template will lead to random remodeling or modification of nucleosomes. In vivo, however, these events are generally targeted to a selected few nucleosomes or to a nucleosomal domain, and this "targeting" of activity is generally directed by sequence-specific regulatory proteins. Although remodeling enzymes such as SWI/SNF or histone acetyltransferases such as yeast SAGA can interact directly with acidic transcription activation domains, these interactions are of relatively low affinity. Thus, one typically cannot target remodeling/modification activity simply by lowering the concentration of enzyme. One exception is the p300 histone acetyltransferase, which shows quite robust activator targeting of HAT activity (Fig. 13.9). One approach that has been successful for many enzymes is to supplement remodeling/modification reactions with competitor nucleosomes that do not contain binding sites for a relevant activator (Neely et al. 1999; Yudkovsky et al. 1999). Thus, when an activator is prebound to a test nucleosomal array, the remodeling/modification enzyme is targeted to this array, rather than to the competitor. If the activator is eliminated from the reaction,

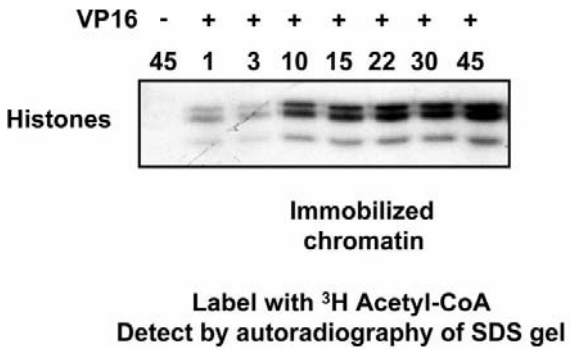

FIGURE 13.9. Recruitment of p300-mediated histone acetylation by the Gal4–VP16 activator on G5E4T-5S arrays. Nucleosomes were reconstituted by salt dialysis on the DNA template G5E4T-5S, which contains two sets of five tandem copies of a 208-bp 5S rDNA nucleosome positioning sequence flanking a dinucleosome-length DNA fragment harboring five Gal4-binding sites upstream of an E4 promoter. The DNA fragment was biotinylated on each end by Klenow reaction, using biotin-dUTP, and the biotinylated nucleosomal array was immobilized on streptavidin magnetic beads. The p300 HAT was added to [^{3}H]acetyl-CoA histone acetylation reactions that contained or lacked the Gal4–VP16 activator. Reactions were stopped at the indicated times (minutes), immobilized templates were washed and eluted with sample buffer, and histones were separated by SDS-PAGE. Acetylation was detected by fluorography. Note that in the case of p300, robust acetylation is activator-dependent.

the remodeling/modification enzyme will interact with all nucleosomes equally. Typically, the concentration of competitor is titrated so that no remodeling or modification of the immobilized template occurs in the absence of prebound activator.

In vitro ChIP assays have been quite useful for monitoring the targeting of remodeling/modification enzymes by activators (Vignali et al. 2000). In the case of histone acetyltransferases, in vitro ChIP has also been used to delineate how far nucleosome modifications spread upstream or downstream from the bound activator. As in the case for a typical ChIP assay, protein–protein and protein–DNA crosslinking is induced with formaldehyde. Instead of shearing DNA by sonication, nucleosomal arrays are fragmented by restriction enzyme cleavage of MNase digestion. Individual nucleosomes can then be recovered and quantified by PCR after immunoprecipitation with a relevant antibody.

Monitoring the Role of Chromatin Condensation in Transcription Assays

As discussed above, nucleosomal arrays can undergo complex folding hierarchies as a function of monovalent and divalent cations (Fletcher and Hansen 1996). These folding transitions have been well-characterized for nucleosomal arrays assembled on DNA templates that contain head-to-tail repeats of nucleosome positioning sequences, such as 5S NPEs or the synthetic 601 sequence. In low-salt buffers (e.g., TE), these arrays exist as extended fibers that resemble the "beads-on-a-string" structures observed by electron microscopy, whereas addition of 1–2 mM $MgCl_2$ induces formation of condensed fibers that are believed to mimic physiologically relevant 30-nm fibers. At higher concentrations of divalent cations (>3 mM), arrays interact with each other to form very large oligomers that may mimic large interphase chromatin fibers greater than 30 nm in diameter. A typical transcription reaction contains 50–75 mM NaCl and 3–6 mM $MgCl_2$, conditions that are likely to induce formation of 30-nm fibers or larger oligomeric states. Recently, Tremethick and colleagues monitored in vitro transcription alongside of condensation of nucleosomal arrays by sedimentation velocity analysis in the analytical ultracentrifuge (Zhou et al. 2007). These authors found that nucleosomal arrays in transcription buffer formed large oligomers and, furthermore, that histone alterations that influenced the extent of chromatin condensation had dramatic consequences for transcription in vitro.

It seems clear that one must think about strategies to evaluate the role of chromatin condensation in the regulatory properties of a gene of interest. Ideally, one strategy would reconstitute

nucleosomal arrays with altered histones that are defective in formation of higher-order structures. For instance, histones that lack the H4 amino-terminal tail domain or contain an acetyl group at H4K16 are unable to form 30-nm-like fibers in vitro and are less apt to form oligomers as well (Shogren-Knaak et al. 2006). However, such alterations of the H4 amino terminus may also disrupt other regulatory events. Alternatively, mutations of specific acidic residues in histone H2A will also disrupt folding of arrays in vitro (Zhou et al. 2007). Finally, it is known that formation of 30-nm-like fibers requires a continuous array of nucleosomes. Thus, assembling slightly subsaturated arrays (i.e., arrays with one or more gaps) will also block formation of higher-order structures (Hansen and Lohr 1993). With such tools in hand, investigators can evaluate whether a regulatory factor plays a role in disrupting chromatin higher-order folding or whether it functions at other steps.

TECHNIQUES

Protocol 13.1

Chicken Erythrocyte Histone Octamer Preparation

Core histones can be purified from a variety of cell sources, including *Drosophila* embryos, HeLa tissue culture cells, calf thymus, or chicken erythrocytes. We have routinely used chick erythrocytes as a source of cellular histones, because it is simple to obtain large quantities of chicken blood and the purified histones have low levels of posttranslational modifications. Linker histones H1 and H5 can also be purified from the same cell sample. A protocol for H1 and H5 purification is included here as an optional step. Note that avian histones have an amino acid sequence identical to that of human histones. Typical yields of core histones from 200 ml of blood are usually in excess of 50 mg. A protocol for the preparation of H3/H4 tetramers and H2A/H2B dimers is also presented as an optional step at the end of this procedure. Histone stocks have been successfully stored in TE/2 M NaCl buffer for more than a year at 4°C and for several years at –20°C. (Protocol courtesy of Jeff Hansen, Colorado State University.)

TIME LINE AND ORGANIZATION

The purification of core histones from chick erythrocytes takes a total of 3 days. Blood must be processed immediately. We have found that addition of heparin to the blood enhances its stability for a short period. Samples can be stored on ice for several days after the second MNase digest (Step 6) and prior to the hydroxyapatite chromatography step (Step 7). All buffers should be prepared and chilled prior to receipt of the blood.

OUTLINE

Step 1: Prepare buffers and chill (2 hr).
Step 2: Preparation of nuclei (1 hr).
Step 3: Micrococcal nuclease I digestion (2 hr).
Step 4: CM Sephadex C-25 fractionation (3.5 hr).
Step 5: Overnight dialysis.
Step 6: Linker histone H1/H5 preparation (optional).
Step 7: Micrococcal nuclease II digestion (2–5 hr).
Step 8: Hydroxyapatite chromatography (6 hr).
Step 9: H21/H2B dimer and H3/H4 tetramer preparation (optional).

MATERIALS

CAUTION: See Appendix for appropriate handling of materials marked with <!>.

Reagents

Agarose gel (1%)
Buffer 1
  1X Stock buffer
  2.5 mM EDTA
  0.5 mM EGTA

Buffer 2
- 1x Stock buffer
- 2.5 mM EDTA
- 0.5 mM EGTA
- 0.5% Nonidet P-40 (NP-40)

Buffer 3
- 1x Stock buffer

Buffer 4
- 15 mM sodium citrate (citric acid) <!>
- 150 mM NaCl

Buffer 5
- 0.1 M K^+ phosphate (pH 6.7)
- 0.1 M NaCl

Buffer 6
- 0.1 M K^+ phosphate (pH 6.7)
- 1.1 M NaCl

Buffer 7
- 0.1 M K^+ phosphate (pH 6.7)
- 2.2 M NaCl

$CaCl_2$ (0.1 M) <!>
Chicken blood (fresh 250 ml; Pel-Freeze, 33132-1)
Coomassie Blue dye <!>
ddH_2O
EDTA (0.25 mM and 25 mM)
Elution buffer
- 0.1 M potassium phosphate (pH 6.7)
- 2.2 M NaCl

Ethanol <!>
Ethidium bromide <!>
5x HTP loading buffer
- 0.5 M K^+ phosphate buffer
- 0.5 M NaCl

Micrococcal nuclease (MNase; Worthington)
Milli-Q H_2O
NaCl (0.35: 1.6 M in 10 mM Tris <!>, pH 8.8)
NaCl (0.6: 1.2 M in 10 mM Tris <!>, pH 8.8)
NaCl (5 M)
NaOH (0.1 M) <!>
pBR322 MspI standard
Phenylmethylsulfonyl fluoride (PMSF) <!> stock (0.1 M)
Protease inhibitors (aprotinin <!>, leupeptin <!>, PMSF <!>)
SDS gel (18% separating gel, 6% stacking gel) <!>
5x SDS loading dye <!>
- 250 mM TrisHCl (pH 6.8)
- 10% SDS <!>
- 5% β-mercaptoethanol <!>
- 0.02% Bromophenol Blue <!>

Starting column buffer (either 10 mM Tris <!>, pH 8.8, 0.35 M NaCl, or 10 mM Tris <!>, pH 8.8, 0.6 M NaCl)
2x Stock buffer
- 30 mM Tris base <!>

30 mM NaCl
120 mM KCl <!>
1 mM spermidine <!>
0.3 mM spermine <!>
680 mM sucrose

TE buffer
10 mM Tris (pH 7.8) <!>
0.25 mM EDTA
0.1 mM PMSF <!>

Tris (1 M , pH 7.8) <!>

Wash buffer
0.1 M K^+ phosphate (pH 6.7)
0.1 M NaCl

Equipment

Amicon concentrator with XM50 membrane
Bottles (250 ml)
Centrifuge (GSA and HB6 rotors)
CM-Sephadex C-25 column chromatography apparatus
Dialysis apparatus
Electrophoresis apparatus
Eppendorf tubes (1 ml)
Pipette (25 ml)
Spectrophotometer
Water bath set at 37°C
Water purification system (Millipore)

PROCEDURE

Step 1: Prepare buffers and chill

Store all buffers at 4°C. **IMPORTANT:** Add PMSF (stock 0.1 M) to all buffers *immediately prior to use* to a final concentration of 0.1 mM. (Buffers for hydroxyapatite chromatography are described in Step 7.)

2x Stock buffer (4 liters needed for 250 ml of chicken erythrocyte chromatin)

	m.w. (g/mole)	**Grams needed**	
30 mM Tris base	121.14	14.54	
30 mM NaCl	58.44	7.01	
120 mM KCl <!>	74.56	35.79	
1 mM spermidine <!>	145.25	0.628 ml	0.925 g/ml
0.3 mM spermine <!>	348.20	0.418	
680 mM sucrose	342.30	931.06	

Adjust pH to 7.5 with HCl; bring to final volume of 4 liters with Milli-Q H_2O.

Buffer 1 (2 liters needed for 250 ml of chicken erythrocyte chromatin)

1x Stock buffer	1.0 liter of 2x stock
2.5 mM EDTA	10 ml of 0.5 M EDTA
0.5m M EGTA	5.0 ml of 0.2 M EGTA

Adjust to 2 liters with Milli-Q H_2O.

Buffer 2 (4 liters needed for 250 ml of chicken erythrocyte chromatin)

1X Stock buffer	2.0 liters of 2X stock
2.5 mM EDTA	20 ml of 0.5 M EDTA
0.5 mM EGTA	10 ml of 0.2 M EGTA
0.5% NP-40	20 ml of 100% NP-40

Adjust to 4 liters with Milli-Q H_2O.

Buffer 3 (2 liters needed for 250 ml of chicken erythrocyte chromatin)

X Stock buffer	1.0 liter of 2X stock

Adjust to 2 liters with Milli-Q H_2O.

Buffer 4 (2 liters needed for 250 ml of chicken erythrocyte chromatin)

	m.w. (g/mole)	**Grams needed**
15 mM sodium citrate (citric acid) <!>	294.1	8.82
150 mM NaCl	58.44	17.53

Adjust to pH 7.2 with HCl; bring to final volume of 2 liters with Milli-Q H_2O.

Step 2: Preparation of nuclei

This step involves washing and lysing of chicken erythrocytes to obtain a clean pellet of nuclei. PMSF is added to each buffer immediately prior to use. Keep the solutions on ice during all steps.

1. Prepare a 0.1 M stock of PMSF in advance by dissolving 0.436 g in 25 ml of ethanol, wrapping the bottle in foil, and storing at –20°C.
2. Divide 250 ml of fresh chicken blood into four 250-ml bottles at 62.5 ml per bottle.
3. Add 0.1 M PMSF to buffer 4 (add 1 ml/liter; final concentration is 0.1 mM). Fill the 250-ml bottles of chicken blood with this buffer, and centrifuge in a GSA rotor at 3000 rpm (1464*g*) for 5 minutes at 4°C.

   CAUTION: The pellets are soft so decant carefully.
4. Resuspend the pellet in buffer 4 and centrifuge in a GSA rotor at 3000 rpm (1464*g*) for 5 minutes at 4°C for a total of three times.

   At this stage, try to decant only yellowish liquid from the bottles.
5. Add 0.1 M PMSF to buffer 1 (add 1 ml/liter; final concentration is 0.1 mM). Resuspend the pellet in buffer 1 and centrifuge in a GSA rotor at 3000 rpm (1464*g*) for 5 minutes at 4°C. Repeat this step twice.
6. Add 0.1 M PMSF to buffer 2 (add 1 ml/liter; final concentration is 0.1 mM). Resuspend the pellet in buffer 2 and centrifuge in a GSA rotor at 3000 rpm (1464*g*) for 5 minutes at 4°C. Repeat this step three to four times.

   At the end of this step, a very white pellet of nuclei should appear. All red/pink color from the heme should be removed from the repeated resuspensions. Drawing the nuclei in and out of a 25-ml pipette when resuspending the pellet helps break up the clumps of nuclei and remove the red/pink color.
7. Add. 0.1 M PMSF to buffer 3 (add 1 ml/liter; final concentration is 0.1 mM). Resuspend the pellet in buffer 3 and centrifuge in a GSA rotor at 3000 rpm (1464*g*) for 5 minutes at 4°C. After the first spin, inspect the pellet for dark-colored specks and resuspend the chunks thoroughly to remove. Repeat resuspension and spin twice.

   This final wash is critical because it removes EDTA from the mixture. If some EDTA remains, the MNase digest will not work.
8. Resuspend the nuclei in 60 ml of buffer 3 (combining all into one bottle). Avoid a greater volume because of concentration needed for next step.

9. Quantify DNA by diluting nuclei into 0.1 M NaOH and measuring the A_{260} (5 µl nuclei + 995 µl 0.1 M NaOH). (A_{260} x 200) divided by 20 = mg/ml; mg/ml x 60 ml = total mg.

Step 3: Micrococcal nuclease digestion I

This part of the preparation involves a limited MNase digestion, lysis of the nuclei, and removal of the nuclear membranes.

1. Rehydrate MNase in ddH_2O so that the stock is 45,000 units/ml. (A 500-fold dilution of this stock should give the appropriate concentration for the digestion, 90 units/ml.)
2. Dilute the suspension of nuclei with buffer 3 and 0.1 M $CaCl_2$ stock solution so that the final concentration of DNA is 3 mg/ml, and the final concentration of $CaCl_2$ is 1 mM (100-fold dilution of the 0.1 M stock).
3. Place the nuclei in a 37°C water bath and swirl for 5 minutes to warm.

   A flask works well for swirling.
4. Add MNase stock so that the MNase concentration is 90 units/ml (30 units/mg DNA). Swirl for an additional 5 minutes in 37°C water bath.
5. Place on ice and add 0.25 M EDTA to a final concentration of 2.5 mM (1 ml/100 ml).
6. Centrifuge the suspension in a GSA rotor at 6500 rpm (~6800*g*) for 5 minutes at 4°C.
7. Resuspend the pellet in a volume of 0.25 mM EDTA, 0.1 mM PMSF equal to one-fourth of the volume of the digest (1000-fold dilution of 0.25 M EDTA and 1000-fold dilution of 0.1 M PMSF in H_2O).

   Avoid greater volumes because of the concentration needed in the step in which CM-Sephadex is added.
8. Break up the pellet and stir for 1 hour in the cold room.

   This step lyses the nuclei.
9. Centrifuge the pellet mixture in a GSA rotor at 6500 (~6800*g*) for 20 minutes at 4°C. Collect the supernatant.
10. Quantify the DNA by diluting the supernatant in H_2O and reading the A_{260} (5 µl supernatant + 995 µl ddH_2O). (A_{260} x 200) divided by 20 = mg/ml; mg/ml x ml of supernatant = total mg DNA.

Step 4: CM-Sephadex C-25

This part of the preparation removes the H1 and H5 histones from the oligonucleosomes.

1. Dilute the supernatant from Step 3:9 to 5 mg/ml with H_2O, but include 1 M Tris (pH 7.8), 25 mM EDTA, and 5 M NaCl so that the final concentrations are 10 mM Tris, 0.25 mM EDTA, and 0.35 M NaCl.

   This step is performed to get the chromatin back into buffer. The MNase digestion in Step 3, to lyse the nuclei, was in H_2O and EDTA, no buffer. Using TE instead of 1 M Tris causes the final EDTA concentration to be 0.5 mM instead of 0.25 mM, but this does not matter and helps chelate all of the Ca^{++}. This is important because some MNase is still present in the preparation.
2. Add 0.1 M PMSF to maintain the 0.1 mM PMSF concentration in the preparation.
3. Add CM-Sephadex, using 60 mg of dry resin/ml of chromatin (which is 12 mg resin/mg chromatin).
4. Stir the CM-Sephadex mixture for 3 hours in the cold room. Soak the dialysis tubing for the next step during this time.

   If the CM-Sephadex will be saved for the recovery of bound H1/H5 (see optional Step 7 (below), it should *not* be stirred. Instead, *rock* the CM-Sephadex slurry to mix it during the 3-hour incubation.

5. Centrifuge the mixture in an HB6 rotor at 6500 rpm for 30 minutes at 4°C. Carefully remove the supernatant with a pipette.

   If the CM-Sephadex will be used to recover the H1 and H5, it must be continued at this time during dialysis of the supernatant. Refer to Step 5 for the H1/H5 preparation.

Step 5: Overnight dialysis

1. Dialyze the supernatant from Step 4:5 against 2 liters of TE buffer overnight at 4°C.
2. In the morning, dialyze the supernatant against fresh TE (~2 hr).

Step 6: Linker histone H1/H5 preparation (optional)

The first section of this step is the same as the histone octamer preparation through the CM-Sephadex steps. If H1 and H5 will be prepared at the same time as the octamers, the H1 and H5 should be eluted from the CM-Sephadex while the oligonucleosomes supernatant is being dialyzed overnight in TE. Refer to the original paper by Garcia-Ramirez et al. (1990).

This step elutes the histone H1/H5 from the CM-Sephadex as two separate peaks. The following issues are important:

- pH is critical.
- Initial washes are critical (to remove dimers and oligonulceotides that are interspersed in the Sephadex).
- The slope of gradient is important. Decreasing the slope of the salt gradient (0.6–1.2 M NaCl) can also assist in separating contaminating dimers with the front of the H1 peak. If the 0.6–1.2 M NaCl gradient is used, consider washing the column with 1.5 volumes of the 1.6 M NaCl after the gradient is finished to elute anything that may still be bound to the column.
- Add PMSF to a final concentration of 0.1 M to all buffers, and work as quickly as possible to avoid protein degradation.

1. Wash the CM-Sephadex pellet from Step 4:5 well two or three times in Starting column buffer (either 10 mM Tris at pH 8.8, 0.35 M NaCl or 10 mM Tris at pH 8.8, 0.6 M NaCl), centrifuging after each wash in an HB6 rotor at 6500 for 10 minutes at 4°C (also continue to be gentle with the CM-Sephadex).
2. Layer the washed CM-Sephadex on top of a CM-Sephadex column (2.5 cm x ~26 cm) equilibrated with the chosen Starting column buffer.
3. Elute with a 1-liter gradient of either 0.35–1.6 M NaCl in 10 mM Tris (pH 8.8) or 0.6–1.2 M NaCl in 10 mM Tris (pH 8.8). Collect 120-drop (4-ml) fractions. Collect fractions for the whole gradient. Locate the peaks by reading the A_{230} of the fractions. Expect H1 to elute around fraction 90 and H5 to elute around fraction 140 for the gradient starting with 0.35 M NaCl.
4. Add protease inhibitors to the fractions.

aprotinin	10 µg/ml final concentration
leupeptin	10 µg/ml final concentration
PMSF	0.1 mM final concentration

   Add from 1000x stock (1 µl/ml) so as not to dilute the fractions.
5. Run SDS-PAGE with an 18% separating gel and a 6% stacking gel to determine the quality of the histones. Load approximately 1 µg of histone from each fraction collected in Step 7:3 and treated in Step 7:4. Stain with Coomassie dye.
6. Pool fractions from Step 7:3 that contain linker histone, aliquot, and store at 4°C.

Step 7: Micrococcal nuclease digestion II

This step digests the oligonucleosomes into small pieces: trimers, dimers, and monomers. If large oligonucleosomes are loaded onto the hydroxyapatite (HTP) column, the eluted peak of octamers will be too spread out.

1. The concentration of the chromatin preparation should still be approximately 5 mg/ml. Double check by reading the A_{260}.
2. Add 0.1 M $CaCl_2$ to a final concentration of 1 mM $CaCl_2$.
3. Prepare the test digest as follows:

Chromatin (5 mg/ml)	30 µl (150 µg)
MNase (4500 units/ml; tenfold dilution of stock)	1 µl (4.5 units or 0.03 units/mg chromatin)

4. Incubate the mixture at 37°C, and take a sample at time points of 5, 10, 20, and 30 minutes. At each time point, remove 1 µl (~5 µg) and add to an Eppendorf tube containing 99 µl of 2.5 mM EDTA.
5. After all the time points are collected, prepare the samples for a 1% agarose gel as follows:
   a. Remove 10 µl (~0.5 µg) from each time point.
   b. Add 2 µl of 5x loading dye/5% SDS.
   c. Heat for 30 minutes at 37°C and run a 1% agarose gel at 80 V for about 1 hour; use pBR322 MspI as a standard.
   d. Stain with ethidium bromide and visualize.
6. Set up a large-scale digest at the test digest time point that gave the best results. Stop the reaction by adding 0.25 M EDTA to a final concentration of 2.5 mM.
7. Concentrate the digest in an Amicon Concentrator (using an XM 50 membrane) to approximately one-tenth of its volume (i.e., to ~50 mg/ml).

   Even after the MNase digest, the concentration should be approximately 5 mg/ml. The desired concentration is about 50 mg/ml, because an aliquot is mixed with 5x HTP column buffer before it is loaded on the HTP column.

Step 8: Hydroxyapatite chromatography

This step removes DNA to yield free octamers.

1. The HTP column is poured according to the Bio-Rad instruction pamphlet.
2. Make 5x HTP loading buffer (0.5 M K^+ phosphate buffer, 0.5 M NaCl, with the desired working concentration of 0.1 M K^+ phosphate at pH 6.7, 0.1 M NaCl). Check the potassium phosphate bottle for molecule weight. This will vary depending on hydration state.

Solute	Grams (anhydrous)	Grams (trihydrate)
K_2HPO_4 (dibasic)	17.42	22.82
KH_2PO_4 (monobasic)	13.62	
NaCl	5.84	

   Dissolve in Milli-Q H_2O to make 0.2 liter (200 ml).
3. Make 500 ml of 1x wash buffer (0.1 M K^+ phosphate at pH 6.7, 0.1 M NaCl). Take 100 ml of loading buffer and add Milli-Q H_2O to make 0.5 liter (500 ml).

4. Make the Eluting column buffer (0.1 M potassium phosphate at pH 6.7, 2.2 M NaCl).

K_2HPO_4 (dibasic)	8.70 g (11.42 g for K_2HPO_4 trihydrate)
KH_2PO_4 (monobasic)	6.80 g
NaCl	64.28 g

Dissolve in Milli-Q H_2O to make 0.5 liter (500 ml).

5. Mix 800 µl to 4 ml of concentrated nucleosomes with 200 µl to 1 ml of 5x HTP loading buffer. Remove the wash buffer from the top of the HTP column and layer the sample carefully on top. After the sample has run into the column, gently layer 1x wash buffer on top and attach the reservoir. Wash with 0.5–1 column volume of 0.1 M K^+ phosphate (pH 6.7) and 0.1 M NaCl buffer (wash buffer). Then attach the reservoir containing 1x Eluting column buffer (0.1 M K^+ phosphate at pH 6.7, 2.2 M NaCl) and collect 180-drop fractions (~6 ml per fraction; ~30 drops/ml). The column should flow at approximately 1 ml/min. Collect 80 fractions, although the peak should elute somewhere between fractions 35 and 45.
6. Locate the peak by reading the A_{230} of each fraction. Use a 50-µl aliquot for the absorbance reading and then discard it. One mg/ml of octamer has an $A_{230} = 4.3$. Therefore, dividing the A_{230} of each fraction by 4.3 will give the concentration of the fraction in mg/ml.
7. Add protease inhibitors to the fractions.

aprotinin	10 µg/ml final concentration
leupeptin	10 µg/ml final concentration
PMSF	0.1 mM final concentration (added from 1000x stock so as not to dilute the fractions)

8. Run SDS-PAGE with an 18% separating gel and a 6% stacking gel to determine the quality of the histones. Load about 4 µg of histone from each fraction and run the gel for 1–1.5 hours at 100 V. Stain with Coomassie dye.
9. Store the histones at 4°C. Aliquot each fraction into six Eppendorf tubes (1 ml each).
10. To reuse the HTP column, remove several centimeters of HTP resin from the top and replace it with fresh HTP. Wash the column with several column volumes of 0.5 M K^+ phosphate buffer (pH 6.7) to elute the DNA. If the column will not be used for long periods of time, store it in 1x wash buffer containing 0.02% NaN_3; 0.77 g/liter. Just before use, equilibrate with 1x buffer without NaN_3.

Step 9: H2A-H2B dimer and H3/H4 tetramer preparation (optional)

The first section of this preparation is the same as the histone octamer preparation through Step 7. However, the HTP chromatography is done differently. In the octamer preparation, the column buffer has a 2.2 M NaCl concentration, which disrupts the ionic interaction between all of the positively charged histones and the negatively charged DNA. All of the histones comprising the octamer elute while the DNA remains bound. In this procedure, a step elution is done with buffers containing increasing NaCl concentrations. The H2A/H2B dimers elute at a lower NaCl concentration, and the H3/H4 tetramers elute at a higher concentration.

1. Prepare buffers as follows:

Buffer 5 (0.1 M K^+ phosphate, pH 6.7, 0.1 M NaCl)

K_2HPO_4 (dibasic)	17.42 g (22.82 g for K_2HPO_4 trihydrate)
KH_2PO_4 (monobasic)	13.61 g
NaCl	5.85 g

Add H_2O to make 1 liter.

Buffer 6 (0.1 M K^+ phosphate at pH 6.7, 1.1 M NaCl)

K_2HPO_4 (dibasic)	17.42 g (22.82 g for K_2HPO_4 trihydrate)
KH_2PO_4 (monobasic)	13.61 g
NaCl	64.3 g

Add H_2O to make 1 liter.

Buffer 7 (0.1 M K^+ phosphate at pH 6.7, 2.2 M NaCl)

K_2HPO_4 (dibasic)	17.42 g (22.82 g for K_2HPO_4 trihydrate)
KH_2PO_4 (monobasic)	13.61 g
NaCl	128.6 g

Add H_2O to make 1 liter.

2. Pour the HTP column according to the Bio-Rad instruction pamphlet. Use a glass column 1.5 cm x 30 cm and pour the column approximately 25 cm tall. Equilibrate the column in buffer 5.
3. Mix 800 µl of concentrated nucleosomes (~40 mg) with 200 µl of 5x Column loading buffer (0.5 M K^+ phosphate at pH 6.7, 0.5 M NaCl).
4. Allow the sample to flow into the column and then attach the buffer 5 reservoir. Collect a total of 15 fractions of 90 drops each (~3 ml).
5. Change to the buffer 6 reservoir and collect at least 30 fractions of 90 drops each (~3 ml). Change to the buffer 7 reservoir and collect at least 30 fractions of 90 drops each (~3 ml). The dimer should elute approximately 10–12 fractions after buffer 6 is started, and the tetramer should elute 10–12 fractions after buffer 7 is started. Locate the peaks by reading the A_{260}.
6. Add protease inhibitors to the fractions.

aprotinin	10 µg/ml final concentration
leupeptin	10 µg/ml final concentration
PMSF	0.1 mM final concentration (added from 1000x PMSF stock so as not to dilute the fractions)

7. Run SDS-PAGE with an 18% separating gel with a 6% stacking gel to determine the quality of the histones. Load approximately 2 µg of histone from each fraction.
8. Aliquot the fractions and store at 4°C.
9. To reuse the HTP column, remove several centimeters of HTP from the top and replace with fresh HTP. Wash the column with several column volumes of 0.5 M K^+ phosphate (pH 6.7). If the column is not used for long periods of time, store it in 1x wash buffer (add 0.02% NaN_3 if storing for long periods).

ADDITIONAL CONSIDERATIONS

1. Other cell sources. The nuclear pellet from a Dignam and Roeder nuclear extract preparation (see Chapter 12) can be used as a substitute for the chicken erythrocyte nuclear pellet in Step 2:7.
2. Octamer concentration. Typically, the histone octamer will elute from the HTP column at a concentration of about 2 mg/ml. If the final octamer pool has a concentration much less than 1 mg/ml, then the histones should be concentrated. Dilute solutions of octamers appear to be less stable and may reconstitute chromatin less efficiently. We typically use Centricon-type microconcentrators with a 1ow-molecular-weight cutoff (4500).

3. We have routinely stored histone octamers at 4°C for greater than 1 year. Octamers can also be dialyzed into HTP elution buffer that contains 50% glycerol and stored at –80°C.

TROUBLESHOOTING

Nuclei are hard to resuspend prior to the first MNase digest

Possible cause: The nuclei may have lysed during the wash steps.

Solutions: Be sure to be very gentle during each resuspension step. Do not vortex, but gently pipette with a large-bore glass pipette. Check to ensure that buffers were made correctly and that glassware does not contain residual detergent.

Histones elute from the HTP column as a broad peak

Possible cause: Incomplete MNase digestion leads to loading of very long oligonucleosomes onto the HTP column.

Solution: Histones can be pooled and concentrated. Check an aliquot of the HTP load on an agarose gel to confirm size of oligonucleosomes.

Protocol 13.2

Salt Gradient Dialysis Reconstitution of Nucleosomes

In the salt gradient dialysis protocol, purified core histones are incubated with a DNA template in a buffer containing high concentrations (2 M) of NaCl. As the salt is slowly dialyzed away, nucleosomes spontaneously assemble on the DNA, and their translational positioning along the DNA is directed by the DNA sequence. In the absence of nucleosome-positioning elements (e.g., 5S rDNA genes), the nucleosomes can adopt a nonphysiological, closely packed structure with little space between the nucleosomes. Removal of remaining free histones, as well as templates with closely packed nucleosomes, can be achieved by fractionation over sucrose gradients. The chromatin assembled in these reactions is often subjected to micrococcal nuclease (MNase) digestion analysis. (Protocol courtesy of Lee Kraus, Cornell University.)

TIME LINE AND ORGANIZATION

Salt dialysis reconstitutions are quite easy to set up, but they are time-consuming because of multiple changes of the dialysis buffer. This reconstitution method takes a total of 1.5–2 days for completion. All dialysis buffers should be prepared and chilled prior to the start of reconstitution.

OUTLINE

Step 1: Prepare buffers and chill (1 hr).
Step 2: Set up the chromatin assembly reactions (30 min).
Step 3: Salt gradient dialysis and sample collection (30–36 hr).
Step 4: MNase digestion analysis (4 hr).

- MNase digestion.
- Preparation of the DNA samples.
- Agarose gel electrophoresis.

MATERIALS

CAUTION: See Appendix for appropriate handling of materials marked with <!>.

Reagents

Agarose gel
Ammonium acetate (2.5 M)
Bromophenol Blue dye <!>
Buffer R
10 mM HEPES-potassium hydroxide (KOH <!>, pH 7.5) containing:
10 mM KCl <!>
1.5 mM $MgCl_2$ <!>
0.5 mM EGTA
10% (vol/vol) glycerol
Store in aliquots at –20°C. Can freeze-thaw multiple times.

Dialysis buffer stocks
  NaCl (5 M)
  Tris-Cl (1 M, pH 8.0)
  EDTA (0.5 M)
Dithiothreitol (DTT; 0.25 M) <!>
DNA template
Ethanol <!>
Ethidium bromide <!>
Histone octamer stock
Micrococcal nuclease stock solution (200 units/ml)
  200 units of micrococcal nuclease (Sigma-Aldrich N5386) in 1 ml of 5 mM sodium phosphate buffer (pH 7.0) containing 2.5 μM $CaCl_2$.
    Store in aliquots at –20°C. Can freeze-thaw multiple times.
MNase stop solution
  27.5 μl of TE <!>
  17.5 μl of 0.5 M EDTA
  5 μl of RNase
NaCl stock (5 M)
Phenol:chloroform:isoamyl alcohol (25:24:1 v/v/v) <!>
Phenylmethylsulfonylfluoride (PMSF; 0.1 M) <!>
PK digestion solution
  20 mM EDTA
  Na^+, pH 8, containing:
    0.2 M NaCl
    1% (w/v) SDS <!>
    0.25 mg/ml glycogen (Sigma-Aldrich G0885).
    Store at room temperature.
Proteinase K stock
  2.5 mg/ml proteinase K (USB 20818) in TE buffer <!>
    Store at –20°C. Can freeze-thaw multiple times.
RNase, DNase-free, available as a solution containing 0.5 mg/ml protein (Boehringer Mannheim)
1× Tris/borate/EDTA (TBE) <!>
TE buffer (1.0 M, 0.8 M, 0.6 M, 0.0025 M)
5× TG loading buffer
  50% (v/v) glycerol
  5 mM EDTA (pH 8.0)
  0.1% (w/v) Bromophenol Blue <!>

Equipment

Beckman SW41 rotor
Centrifuge
Dialysis tubing (3000 m.w. cutoff) or MWCO minidialysis cups (Pierce Biotechnology) or Slidelyzer cassettes (Pierce Biotechnology)
Eppendorf tubes (1.5 ml)
Microcentrifuge
Microfuge tubes (siliconized, 1.5 ml)
SDS-PAGE apparatus
Spectrophotometer
SpeedVac or other rotary concentrator
UV transilluminator

PROCEDURE

Step 1: Prepare buffers and chill

Store all buffers at 4°C.

Dialysis buffers (final volume for each is 1 liter)

Reagent stocks	TE 1.0 M	TE 0.8 M	TE 0.6 M	TE 0.0025 M
5 M NaCl	200 ml	160 ml	120 ml	0.5 ml
1 M Tris-Cl, pH 8.0	10 ml	10 ml	10 ml	10 ml
0.5 M EDTA	0.5 ml	0.5 ml	0.5 ml	0.5 ml

Add 2 ml each of 0.1 M PMSF and 0.25 M DTT just prior to using.

Step 2: Set up the chromatin assembly reactions

Salt dialysis reconstitutions are generally assembled at a DNA concentration of 100 µg/ml, although we have successfully assembled chromatin using concentrations of 50 µg/ml and 200 µg/ml. The amount of core histone octamers used in a reconstitution depends on the desired final level of nucleosomal occupancy. For instance, to assemble a chromatin substrate that is fully occupied with nucleosomes, use a ratio of one histone octamer per 200 bp of DNA template. On a mass basis, this is equivalent to 0.793 g of histone octamer per gram of DNA. In cases where the DNA template is composed exclusively of nucleosome positioning sequences (e.g., tandem repeats of the 208-bp 5S rDNA gene), use a ratio of one histone octamer per nucleosome positioning element. For instance, for 208-bp 5S rDNA arrays, use a mass ratio of 0.77 g of octamer per gram of array template.

Note that in this example, the reaction will have a final volume of 500 µl and contain 50 µg of a 2.4-kb plasmid DNA (1,584,000 daltons). To saturate this DNA with nucleosomes, add 12 histone octamers per molecule of plasmid DNA (1 octamer per 200 bp; $r = 1$), which will require 39.65 µg of octamer. However, we recommend always preparing three different assembly reactions that contain molar ratios (r value) of 0.9, 1.0, and 1.1 of histone octamer to 200 bp of DNA. Although these reactions vary by only 10%, these variations are often required to obtain a nucleosomal array that has the proper level of assembly.

Step 3: Salt gradient dialysis and sample collection

1. Thaw DNA template and recheck concentration.
2. Centrifuge aliquot of histone octamer stock for 10 minutes in microcentrifuge to remove aggregates. Recheck the concentration by reading the absorbance at 230 nm.
3. Mix required amounts of histones and DNA in a 1.5-ml Eppendorf tube and adjust to 500 µl. Mix gently by tapping the bottom of the tube, but do not vortex. Adjust NaCl to 2 M with 5 M NaCl stock.
4. Cut a small piece of 3000 MWCO dialysis tubing and rinse it in H_2O. Set up the chromatin assembly reaction in the dialysis tubing.

   Alternatively, we have used 10 MWCO minidialysis cups (Pierce Biotechnology) for small (<1 ml) reconstitutions or Slidelyzer cassettes (Pierce Biotechnology) for larger reconstitutions.
5. Dialyze in a stepwise fashion as described below.

Step	Dialysis buffer	Time
1	1 liter of TE + 1 M NaCl	4 hours to overnight
2	1 liter of TE + 0.8 M NaCl	4 hours to overnight
3	1 liter of TE + 0.6 M NaCl	4 hours to overnight
4	1 liter of TE + 0.0025 M NaCl	4 hours to overnight
5	1 liter of TE + 0.0025 M NaCl	4 hours

6. Carefully collect the sample into a 1.5-ml siliconized microfuge tube (histones are very sticky!!!!) and store at 4°C.

   Salt-dialyzed chromatin can be stored at 4°C for at least 1 month.

   To remove free histone, the chromatin samples can be fractionated on approximately 12-ml 15–40% sucrose gradients (~200 µg of DNA assembled into chromatin per gradient; 30,000 rpm in a Beckman SW41 rotor for 16 hours at 4°C). Take 300-µl fractions and assay for DNA (by agarose gels with ethidium bromide staining) and core histones (by SDS-PAGE). The peak DNA- and histone (i.e., chromatin)-containing fractions should be relatively free of unincorporated histones.

Step 4: Micrococcal nuclease analysis of chromatin assembled in vitro

The MNase digestion assay is based on the fact that MNase preferentially cleaves the linker DNA between nucleosomes, and, thus, it can generate DNA fragments containing different numbers of nucleosomes when used under limiting digestion conditions. The deproteinized DNA fragments can be resolved by agarose gel electrophoresis, and, if chromatin assembly has occurred, a repeating pattern of increasing DNA fragment sizes will be visible. This assay can be used to determine changes in nucleosome spacing, as seen upon the incorporation of linker histones, for example.

MNase digestion

1. Set up the chromatin samples as follows:
   a. Dilute 2 µl of salt-dialyzed chromatin sample in 150 µl of Buffer R.
   b. Add 4.5 µl of 0.1 M $CaCl_2$ (which is required for the activity of MNase) to the 150-µl dilution. Mix immediately by flicking the tube gently.
   c. Make two 50-µl aliquots (containing ~300 ng of DNA each).

Sample no.	Sample	MNase dilution
1	Salt dialyzed chromatin	1:20,000
2	Salt dialyzed chromatin	1:10,000

   IMPORTANT: Make up the MNase Stop solution before beginning the digests!!!! (See part B.)

2. Add 5 µl of diluted micrococcal nuclease (see table above and table below). For each sample of chromatin, prepare the following dilutions of the micrococcal nuclease stock in Buffer R. Make immediately before use.

Salt dialyzed chromatin		
MNase dilution	MNase volume	Buffer R
1:400	2 µl of stock	798 µl
1:10,000	2 µl of 1:400	48 µl
1:20,000	2 µl of 1:400	98 µl

3. Mix immediately by flicking the tube gently. Start multiple samples consecutively, 15 seconds apart.

   In this protocol, the use of several concentrations of MNase to achieve differing degrees of digestion is described. However, a constant concentration of MNase could be used, and the time of digestion could be varied.

4. Digest for 10 minutes at room temperature.

5. Add 5 µl of MNase stop solution. Mix immediately by flicking the tube gently. Stop multiple samples consecutively, 15 seconds apart.

   The EDTA in the MNase stop solution chelates the calcium and inhibits the reaction.

Preparation of DNA samples

6. Add 100 µl of PK digestion solution. Vortex thoroughly. Incubate for 30 minutes at 37°C.

   Add the proteinase K to the PK digestion solution before use as follows: Combine proteinase K stock with the solution listed above in a 1:10 (v/v) ratio before adding to the samples (i.e., dilute the proteinase K stock 1:10 in the buffer listed). Make a fresh dilution for each use.

7. Extract with 150 µl of phenol:chloroform:isoamyl alcohol. Centrifuge in a microcentrifuge for 5 minutes at room temperature.

8. Transfer the aqueous phase to a new tube (avoid the interface!), and add 15 µl of 2.5 M ammonium acetate and 340 µl of ethanol. Vortex. Centrifuge in a microcentrifuge for 15 minutes at room temperature.

   Do not use sodium acetate—it will give a large, oily pellet.

9. Remove the supernatant, and dry the pellet in rotary concentrator (e.g., Speedvac).

10. Resuspend the pellet in 10 µl of H_2O. Allow it to stand for 10 minutes at room temperature. Add 2.5 µl of 5× TG loading buffer and mix.

    The samples can be stored at –20°C after this step).

Agarose gel electrophoresis

11. Run samples on a 1% (w/v) agarose gel in 1× TBE at 150 V versus a 123-bp DNA ladder and a control MNase-digested DNA sample. Stop the gel when the Bromophenol Blue dye has migrated about two-thirds of the length of the gel.

12. Stain the gel in H_2O containing 0.75 µg/ml ethidium bromide for 30 minutes at room temperature. Visualize the DNA with a UV transilluminator.

    The gel can be destained in H_2O for up to 2 hours at room temperature to reduce the background fluorescence. If better resolution is needed, rerun the gel a bit more after staining.

TROUBLESHOOTING

No DNA detected after reconstitution

Possible cause: Nuclease contamination in DNA, histone, or buffer stocks.
Solution: Test for nucleases by incubating each component with DNA for 30 minutes at 37°C and check DNA integrity on agarose gel. EDTA concentration in dialysis buffers can also be increased to 1 mM.

Possible cause: DNA aggregated during reconstitution, leading to precipitation.
Solution: Reanalyze concentration of DNA and histone stocks to confirm that the proper ratio was assembled. Because free nucleotides can influence absorbance readings of DNA, electrophorese

aliquots of DNA template on an agarose gel to confirm absorbance readings. Likewise, check histone stocks using SDS-PAGE with BSA concentration standards electrophoresed in parallel.

Possible cause: MNase stocks for the analysis are too concentrated or stop buffer not correct.
Solution: Check the integrity of recons without an MNase digest. For array templates (~2.5 kb), a reconstituted array migrates slightly faster than a free DNA fragment.

Nucleosomes reconstituted but arrays are subsaturated

Possible cause: DNA or histone octamer concentrations were not accurate.
Solution: Recheck concentrations by absorbance and by electrophoresis on either agarose gels or SDS-PAGE.

Possible cause: Octamer stock is not fully competent for assembly.
Solution: In some cases, recombinant octamer stocks contain more than 10% octamers that are incompetent for assembly, likely because of incomplete refolding. This proportion may be increased when using octamers harboring various amino acid substitutions. In this case, simply increase the *r* value (e.g., from 1.3 to 1.8 octamers per 200-bp repeat) to achieve greater reconstitution efficiency.

Protocol 13.3

Reconstitution of Nucleosomal Arrays Using Recombinant *Drosophila* ACF and NAP1

The goal of chromatin assembly procedures is to prepare extended nucleosomal arrays from cloned DNA templates and purified core and linker histones. The assembled chromatin should be highly defined in its protein content and resemble bulk chromatin isolated from living cell nuclei in terms of periodicity and nucleosome positioning. Here, we describe how to assemble minichromosome templates in an ATP-dependent fashion from circular plasmid DNA and purified core histones. This system can also be used to assemble minichromosomes from linear DNA (plasmid and λ) and can also incorporate proteins other than core histones (linker histone H1, HMG17, and DNA-binding transcription factors). The products of the chromatin assembly reaction have been used directly (or after purification) in assays to study transcription, DNA replication, recombination, and repair. The system uses purified recombinant *Drosophila* chromatin assembly factors ACF and NAP1. Protocols for their purification are also provided below. (Protocol courtesy of Jim Kadonaga and Dimitri Fyodorov, University of California, San Diego.)

TIME LINE AND ORGANIZATION

Core histones are preincubated on ice with NAP1 to form histone–chaperone complexes. The chromatin assembly reaction is then initiated at 27°C by addition of ACF, ATP, and plasmid DNA (circular supercoiled or relaxed, or linear). The extent of chromatin assembly can be monitored by analysis of circular DNA supercoiling, whereas the "quality" of chromatin is assayed by micrococcal nuclease digest assay. The assembly reactions can be completed in 1.5–2.5 hours, and analysis requires about 2 hours.

OUTLINE

Step 1: Prepare buffers (1 hr) and assemble master solutions for the chromatin assembly reactions (30 min).
Step 2: Chromatin assembly (1.5–2.5 hr) and MNase digestion analysis (2 hr).
Appendix 1: Purification of recombinant *Drosophila* ACF.
Appendix 2: Purification of recombinant *Drosophila* NAP1.

MATERIALS

CAUTION: See Appendix for appropriate handling of materials marked with <!>.

Reagents

ACF dilution buffer: Wash buffer F (see recipe below) containing 0.4 mg/ml recombinant human insulin (Roche)
Agarose gel, 1x TBE
AM master mix
- 3 µl of 0.5 M ATP
- 30 µl of 0.5 M creatine phosphate
- 16.5 µl of distilled H_2O

25 µl of 100 mM $MgCl_2$ <!>.
Add 0.5 µl of 5 mg/ml creatine kinase immediately before use.
Ammonium acetate (2.5 M)
ATP (0.5 M)
Bovine serum albumin (BSA) solution (2 mg/ml)
Buffer R
10 mM potassium HEPES, pH 7.6
10 mM KCl <!>
1.5 mM $MgCl_2$ <!>
0.5 mM EGTA
10% (v/v) glycerol
Store solution up to 24 hours at 4°C.
Add immediately prior to use:
10 mM β-glycerophosphate
1 mM DTT <!>
0.2 mM PMSF <!>
$CaCl_2$ (10 mM) <!>
Core histones (purified) dialyzed into core histone storage buffer (0.3–2.0 mg/ml)
Core histone storage buffer
10 mM potassium HEPES (pH 7.6)
1 mM EDTA
10 mM KCl <!>
10% (v/v) glycerol
Store solution up to 24 hours at 4°C.
Add 1 mM dithiothreitol (DTT) immediately before use.
Creatine kinase solution
5 mg/ml creatine kinase (Sigma-Aldrich)
10 mM potassium phosphate (pH 7.0)
50 mM NaCl
50% (v/v) glycerol
Store in 5–10-µl aliquots up to 2 years at –80°C.
Creatine phosphate (0.5 M)
106 mg/ml creatine phosphate (phosphocreatine)
20 mM potassium HEPES (pH 7.6)
Adjust pH to 7.0. Store in aliquots of 0.1–1 ml up to 2 years at –20°C.
Relaxed DNA template
7.64 µl of plasmid DNA (at 0.42 mg/ml)
2 µl of 10x topoisomerase I buffer
2.36 µl of recombinant topoisomerase I working solution
8 µl of distilled H_2O
EDTA (0.5 M)
Ethanol (100%) <!>
Ethidium bromide <!>
Glycogen stop buffer
20 mM EDTA
0.2 M NaCl
1% (w/v) SDS <!>
0.25 mg/ml glycogen
Store up to 2 years at room temperature.
HEG buffer
25 mM potassium HEPES (pH 7.6)

0.1 mM EDTA
10% (v/v) glycerol
Store in aliquots of 0.1–1 ml up to 2 years at –20°C.

KCl (300 mM) <!>
$MgCl_2$ (100 mM) <!>
Micrococcal nuclease stock solution (200 units/ml)
1.56 mg/ml (200 units/ml) micrococcal nuclease (Sigma-Aldrich)
5 mM sodium phosphate (pH 7.0)
2.5 μM $CaCl_2$ <!>
Store in aliquots of 0.1–1 ml up to 1 year at –20°C.

Phenol:chloroform:isoamyl alcohol <!>, (50:49:1 v/v/v), equilibrated with 10 mM Tris-Cl <!> (pH 8.0)
Plasmid DNA in TE buffer (0.3–2.0 mg/ml)
Proteinase solution
1.1 ml of glycogen stop buffer
55 μl of 2.5 mg/ml proteinase K
PvOH/PEG solution
HEG buffer (see recipe above) containing:
5% polyvinyl alcohol (m.w. 10,000; Sigma-Aldrich P8136)
5% polyethylene glycol (m.w. 8,000; Sigma-Aldrich P2139)
Store in aliquots of 0.1–1 ml up to 2 years at –20°C.

Recombinant ACF (0.002–0.2 mg/ml) (for preparation, see Appendix 1 below)
Recombinant NAP1 0.5–4.0 mg/ml (for preparation, see Appendix 2 below)
RNase A (10 mg/ml) <!>
Stop solution
55 μl of 0.5 M EDTA
11 μl of 10 mg/ml RNase A <!>
Topoisomerase I (*Drosophila* recommended)
10x Topoisomerase I buffer
0.5 M Tris-Cl (pH 7.5)
100 mM $MgCl_2$ <!>
1 mM EDTA
0.5 mg/ml BSA
5 mM DTT <!>
Store in aliquots of 0.1–1 ml up to 2 years at –20°C.

Wash buffer F
20 mM Tris-Cl (pH 7.9)
150 mM NaCl
15% (v/v) glycerol
2 mM $MgCl_2$ <!>
0.2 mM EDTA
0.01% (v/v) NP-40 (Nonidet P-40)
Store solution up to 24 hours at 4°C.
Add immediately prior to use:
1 mM DTT <!>
10 mM β-glycerophosphate
0.2 mM PMSF <!>
0.5 mM benzamidine-HCl <!>
2 μg/ml leupeptin <!>
1 μg/ml aprotinin <!>

Xylene cyanol dye <!>

Equipment

Electrophoresis apparatus
Tubes (siliconized polypropylene, 1.5 ml)

Step 1: Prepare master solutions for chromatin assembly

1. Thaw all buffers and proteins.

   It is recommended that all buffers be equilibrated to room temperature, and proteins must be quick-thawed (in a room-temperature water bath and transferred on ice) and quick-frozen (in liquid nitrogen) after use. ACF, NAP1, core histones, and micrococcal nuclease can withstand multiple freeze–thaw cycles.

2. Prepare the master mix of NAP1 (see Appendix 2) and core histones (NH) by combining the following in a siliconized 1.5-ml microcentrifuge tube:

   172 µl of HEG buffer

   70 µl of 300 mM KCl

   84 µl of PvOH/PEG solution

   4.2 µl of 2 mg/ml BSA solution

   6.4 µl of 2 mg/ml NAP1

   3.03 µl of 0.7 mg/ml core histones.

   Vortex gently for 2–3 seconds.

   Use siliconized 1.5-ml tubes throughout the protocol. The provided recipe is calculated for six standard reactions and should be used to perform five or fewer reactions (to allow for imprecise pipetting). The NAP1 to core histone mass ratio is 5:1 and should be sufficient to eliminate unbound histones in the reaction.

3. Pipette a 56.6-µl aliquot of the NH mix prepared in Step 1:2 (at room temperature) into each of five siliconized 1.5-ml microcentrifuge tubes. Incubate on ice for ≥20 minutes to allow histone–NAP1 binding.

4. Prepare the AM master mix of ATP and Mg^{++}.

5. Prepare the DNA template. Relax the DNA for 10 minutes at 30°C; keep at room temperature until ready to use.

   The topoisomerase I in the reaction should be in five- to tenfold excess over the amount that is necessary to completely relax the supercoiled plasmid after a 10-minutes incubation at 30°C. The purified system will also efficiently assemble chromatin on supercoiled DNA in the absence of the topoisomerase. In the latter case, the template must contain more than 95% supercoiled DNA

6. Prepare ACF dilution(s) in ACF dilution buffer (2–10 units/µl). Keep on ice (1 unit of ACF equals 22 fmoles of protein).

Step 2: Assemble chromatin and analyze by micrococcal nuclease assay

1. Start five assembly reactions as follows:

   a. To 56.6 µl of NH (Step 1:3) add 1 µl of ACF dilution buffer (2–10 units)

   b. Transfer from ice and equilibrate to room temperature.

   c. Add 10.5 µl of AM master mix (Step 1:4) and 2 µl of DNA template (relaxed with excess topoisomerase I or supercoiled; Step 1:5). Immediately vortex, gently, for 2–3 seconds.

   d. Allow the assembly to proceed at 27°C for 1.5 to 2.5 hours.

2. Immediately before use, prepare two dilutions of micrococcal nuclease in Buffer R (1:500 and 1:1500).
3. Add 17.5 µl of 10 mM $CaCl_2$ to each reaction. Divide each reaction in two equal parts ("a" and "b"). In a controlled manner (at certain time intervals, e.g., 15 sec), add 5 µl of the 1:1500 dilution prepared in Step 2:2 to each "a" tube and 5 µl of the 1:500 dilution to each "b" tube. Allow the digestion to progress for 10 minutes at room temperature for every tube.

   The assembly reaction can also be monitored by the DNA supercoiling assay. Stop one-quarter of the 70-µl assembly reaction (~0.177 µg of DNA in 17.5 µl) by addition of 3 µl 0.5 M EDTA. Deproteinate and precipitate the DNA as in Step 2:5 below. Run, along with supercoiled and relaxed DNA samples, 1-kbp DNA ladder on a 0.8% agarose, 1X TBE gel until the xylene cyanol dye front reaches the bottom third of the gel. Stain and destain with ethidium bromide.
4. Prepare Stop solution (ST). Add 6 µl of ST to each tube to stop the micrococcal digestions and then vortex. Allow samples to stand for 5 minutes at room temperature to digest the contaminating RNA.
5. Prepare proteinase solution (PR). Add 105 µl of PR to each tube and then vortex. Digest the histones and soluble proteins for ≥30 minutes at 37°C. Extract samples with 200 µl of 50:49:1 phenol:chloroform:isoamyl alcohol. Precipitate the DNA with 25 µl of 2.5 M ammonium acetate and 475 µl of 100% ethanol.
6. Perform agarose gel electrophoresis.

Appendix 1: Purification of Recombinant *Drosophila* ACF

Drosophila ACF (ATP-utilizing chromatin assembly and remodeling factor) is prepared by coexpression of the carboxylterminally FLAG-tagged Acf1 subunit with the untagged ISWI subunit in baculovirus. The complex is purified in one step by FLAG immunoaffinity chromatography. This procedure typically results in a stoichiometric complex of Acf1 and ISWI.

OUTLINE

Step 1: Infect and harvest Sf9 cells.
Step 2: Prepare and analyze ACF protein.

MATERIALS

CAUTION: See Appendix for appropriate handling of materials marked with <!>.

Reagents

Acf1-FLAG and ISWI baculovirus stocks, high titer

The sole source of these stocks at present is the J. Kadonaga laboratory at University of California, San Diego.

Bovine serum albumin (BSA) standard (2 mg/ml; Pierce, 23209)

Dilution buffer F

20 mM Tris-Cl, pH 7.9
10% (v/v) glycerol
0.02% (v/v) NP-40 (Nonidet P-40)

Store up to 24 hours at 4°C.

Elution buffer F
  Wash buffer F (see recipe below) containing:
    0.4 mg/ml FLAG peptide (Sigma-Aldrich)
    0.4 mg/ml recombinant human insulin (Roche)
  Use immediately. Add FLAG peptide from 10 mg/ml stock in STE buffer (100 mM NaCl, 10 mM Tris <!> at pH 8.0, 1 mM EDTA) and then add insulin from 50 mg/ml stock in TE buffer <!> (10 mM Tris at pH 7.4, 1 mM EDTA)
FLAG-M2 resin <!> (1:1 v/v slurry; Sigma-Aldrich), equilibrated in lysis buffer F
Liquid nitrogen <!>
Lysis buffer F
  20 mM Tris-Cl (pH 7.9)
  500 mM NaCl
  20% (v/v) glycerol
  4 mM $MgCl_2$ <!>
  0.4 mM EDTA
    Store solution up to 24 hours at 4°C.
  Add immediately prior to use:
    2 mM DTT (dithiothreitol) <!>
    20 mM β-glycerophosphate
    0.4 mM PMSF <!>
    1 mM benzamidine hydrochloride
    4 μg/ml leupeptin <!>
    2 μg/ml aprotinin <!>
Phosphate-buffered saline (PBS; ice-cold)
Sf9 cells (late-log-phase) cultured in suspension (>2 Å ~10^6 cells/ml)
Wash buffer F
  20 mM Tris-Cl (pH 7.9)
  150 mM NaCl
  15% (v/v) glycerol
  2 mM $MgCl_2$ <!>
  0.2 mM EDTA
  0.01% (v/v) NP-40
    Store solution up to 24 hours at 4°C.
  Add immediately prior to use:
    1 mM DTT <!>
    10 mM β-glycerophosphate
    0.2 mM PMSF <!>
    0.5 mM benzamidine-HCl
    2 μg/ml leupeptin <!>
    1 μg/ml aprotinin <!>

Equipment

Clinical centrifuge with swinging-bucket rotor
Conical centrifuge bottles (250 ml), appropriate for clinical centrifuge, or conical tubes (50 ml)
Conical centrifuge tubes (14 and 50 ml; disposable)
Culture plates (150 mm)
Polypropylene tubes (15-ml, capped)
Siliconized 1.5-ml polypropylene tubes
Sorvall Superspeed centrifuge with SS-34 rotor (or equivalent)
Spinner flasks (150 or 500 ml)
Wheaton dounce homogenizer ("A" pestle, 15 ml)

PROCEDURE

Step 1: Infect and harvest Sf9 cells

1. Amplify baculovirus stocks several days before the infection. Viruses are supplied as cell culture supernatants and are to be stored at 4°C. To amplify the viruses, plate the Sf9 cells on 150-mm culture plates at 2×10^7 cells/plate in a total of 25 ml of appropriate serum-containing medium, and infect at a multiplicity of infection of 0.1–0.5 (10–20 µl of viral supernatant per plate). To pass a virus stock for storage, allow the infection to proceed for 60 hours and collect the medium supernatant. For high-titer stock, allow the infection to progress until cell lysis is apparent (~72 hours for His-NAP1 virus, ~84 hours for ISWI and Acf1-FLAG). Aspirate the medium off the plates and store in sterile 50-ml tubes in the dark at 4°C (up to 12 months).
2. Grow Sf9 cells in 150- or 500-ml spinner flasks for 2–3 days after seeding at 0.5×10^6 cells/ml. Plate 5–25 plates of Sf9 cells at 2.5×10^7 to 3×10^7 cells/plate in a total of 25 ml of appropriate insect medium per plate. Allow the cells to settle for 20 minutes in the tissue culture hood and infect with recombinant Acf1-FLAG and ISWI baculoviruses at a multiplicity of infection (MOI) of 5–10 each.
3. At 44–46 hours subsequent to infection, aspirate the medium and wash the cells off the plates with 10 ml ice-cold PBS per plate. Centrifuge in a clinical centrifuge at 2000 rpm in 250-ml conical bottles or 50-ml tubes for 5 minutes at 4°C.

   Cell pellets can be frozen in liquid nitrogen and stored at –80°C for several weeks before further processing.
4. Resuspend the cell pellet in 8 ml of Lysis buffer F and disrupt with a Wheaton dounce homogenizer. Use an "A" pestle to perform three series of ten strokes over a 30-minute period, on ice.

Step 2: Prepare and analyze ACF protein

1. Pellet insoluble material by centrifuging in 14-ml conical tubes at 14,500*g* (11,000 rpm in an SS-34 rotor) for 10 minutes at 4°C. Combine the supernatant with 250 µl of FLAG-M2 resin (as a 1:1 slurry equilibrated in Lysis buffer F) and 7 ml of Dilution buffer F. Mix the slurry on a rocking platform in a 15-ml capped polypropylene tube for 3–4 hours at 4°C.
2. Wash the resin four times, each time with 12 ml of Wash buffer F by successive cycles of centrifugation at 2000 rpm in the clinical centrifuge for 3 minutes at 4°C, followed by aspiration and resuspension by inverting the tube.
3. Elute the protein as follows:
   a. Add 100 µl of Elution buffer F to resin pellet from Step 2:2 and resuspend.
   b. Transfer resin to a 1.5-ml siliconized microcentrifuge tube.
   c. Incubate on ice 10 minutes.
   d. Microcentrifuge at maximum speed for 30 seconds.
   e. Transfer the supernatant to another tube (to be pooled with subsequent elutions).
   f. Continuing in the same siliconized microcentrifuge tube, repeat Steps 2:3a–e three times, pooling all of the eluates.
4. Freeze the protein in liquid nitrogen in small aliquots (20–50 µl) and store at –80°C.

   The recombinant ACF is stable for several years and can withstand multiple (5–10) freeze-thaw cycles. Typical yields of ACF are less than 5–10 µg per 150-mm plate.

Appendix 2: Purification of Recombinant *Drosophila* NAP-1

Sf9 cells are infected with HIS6–dNA-1-expressing baculovirus. The chaperone protein is purified by Ni^{+2}-NTA affinity chromatography, followed by anion-exchange chromatography on Source 15Q resin (Pharmacia).

OUTLINE

Step 1: Infect and harvest Sf9 cells.
Step 2: Purify NAP1 by nickel affinity chromatography.
Step 3: Purify NAP1 by anion-exchange chromatography.

MATERIALS

CAUTION: See Appendix for appropriate handling of materials marked with <!>.

Reagents

Bovine serum albumin (BSA) standard (2 mg/ml; Pierce, 23209)
Buffer R
- 10 mM potassium HEPES (pH 7.6)
- 10 mM KCl <!>
- 1.5 mM $MgCl_2$ <!>
- 0.5 mM EGTA
- 10% (v/v) glycerol

  Store solution up to 24 hours at 4°C.
- Add immediately prior to use:
  - 10 mM β-glycerophosphate
  - 1 mM DTT (dithiothreitol) <!>
  - 0.2 mM PMSF <!>

Elution buffer H: Wash buffer H (see recipe below) containing 480 mM imidazole <!>
Ethanol (20% v/v) <!>
HEGD buffer with 0.1 M NaCl
- 25 mM potassium HEPES (pH 7.6)
- 1 mM EDTA
- 10% (v/v) glycerol
- 0.1 M NaCl
- 0.01% (v/v) NP-40 (Nonidet P-40)

  Store solution with above components up to 24 hours at 4°C.
- Add immediately prior to use:
  - 1 mM DTT <!>
  - 0.2 mM PMSF <!>
  - 10 mM β-glycerophosphate

His-NAP1 baculovirus stock (high-titer)

  The sole source of this stock at present is from the Kadonaga laboratory at University of California, San Diego.

Liquid nitrogen <!>
Lysis buffer H
- 50 mM sodium phosphate (pH 7.6)
- 0.5 M NaCl

15% (v/v) glycerol
20 mM imidazole <!>
0.01% (v/v) NP-40
Store solution with above components up to 24 hours at 4°C.
Add immediately prior to use:
10 mM β-glycerophosphate
0.2 mM PMSF <!>
0.5 mM benzamidine

NAP1 purification buffer
Buffer R (see recipe above) containing: 0.0, 0.1, or 1.0 M NaCl (add from 5 M NaCl stock or as solid NaCl) 0.01% NP-40 (add from 10% v/v stock). Store up to 24 hours at 4°C.

Ni-NTA agarose resin (QIAGEN) <!>

Phosphate-buffered saline (PBS; ice-cold)

SDS-PAGE gels (8% and 15%) <!>

Sf9 cells (late-log-phase), cultured in suspension (>2 x 10^6 cells/ml)

Source 15Q resin (Amersham Pharmacia Biotech) <!>

Wash buffer H
50 mM sodium phosphate (pH 7.6)
100 mM NaCl
20 mM imidazole <!>
15% glycerol (v/v)
0.01% NP-40 (v/v)
Store solution up to 24 hours at 4°C.
Add immediately prior to use:
10 mM β-glycerophosphate (add immediately prior to use)
0.2 mM PMSF (add immediately prior to use) <!>
0.5 mM benzamidine (add immediately prior to use)

Equipment

Centrifuge bottles (250-ml conical) appropriate for clinical centrifuge
Clinical centrifuge with swinging-bucket rotor
Dialysis tubing (12,000 to 15,000 MWCO)
Fast protein liquid chromatography (FPLC) apparatus
HR-5 or HR-10 FPLC column (Amersham Pharmacia Biotech)
Rotator (end-over-end)
SDS-PAGE apparatus
Sorvall Superspeed centrifuge with SS34 rotor (or equivalent)
Spinner flasks (500 or 1000 ml)
Tubes (conical, 15 and 50 ml)
Tubes (siliconized polypropylene, 1.5 ml)
Wheaton Dounce homogenizer (40-ml, with "A" pestle)

PROCEDURE

Step 1: Infect and harvest Sf9 cells

1. Grow Sf9 cells in 500- or 1000-ml spinner flasks to a density greater than 2.0 x 10^6 cells/ml in culture medium. Dilute with medium to 1.0 x 10^6 cells/ml. Infect with 25 ml of His-NAP1 virus per liter cell culture.

2. At 72 hours after infection, collect the cells by centrifuging at 2000 rpm in 250-ml conical tubes in a clinical centrifuge for 5 minutes at 4°C. Resuspend each pellet in cold PBS (one-tenth of the original culture volume). Repeat the centrifugation.

   Cell pellets can be frozen in liquid nitrogen and stored at –80°C for several weeks before further processing.

3. Resuspend the cell pellet in Lysis buffer H (1/40 original culture volume). Homogenize in a Dounce homogenizer using 40 strokes of the "A" pestle over 30 minutes. Centrifuge at 14,500*g* (11,000 rpm in an SS-34 rotor) for 10 minutes at 4°C. Pool all supernatants in a 50-ml conical tube.

Step 2: Purify NAP-1 by nickel affinity chromatography

1. Equilibrate 1 ml of Ni-NTA agarose resin in Lysis buffer H per 500 ml of original cell culture volume. Add cell extract and incubate 3–4 hours on an end-over-end rotator. Pellet the resin by centrifuging at 2000 rpm in a clinical centrifuge for 5 minutes at 4°C. Wash the resin twice with 100 ml of Lysis buffer H, and then twice with 100 ml of Wash buffer H. Resuspend the resin by inverting the tube, and pellet the resin by centrifuging at 2000 rpm in the clinical centrifuge for 3 minutes.
2. To elute the protein, resuspend the resin in 2 ml of Elution buffer H by gentle vortexing. Incubate for 5 minutes on ice. Centrifuge at 2000 rpm in a clinical centrifuge for 3 minutes, and then remove the supernatant to a fresh tube on ice. Repeat this elution cycle three more times, pooling the eluates.
3. Dialyze the eluted NAP1 twice in 12,000–15,000 MWCO tubing, each time for 2 hours against 4 liters of HEGD buffer containing 0.1 M NaCl.
4. Dialyze for an additional 2 hours against 4 liters of NAP1 purification buffer containing 0.1 M NaCl. The dialyzed protein can be frozen in liquid nitrogen and stored at –80°C before further processing.
5. Remove the precipitate by spinning in a 15-ml conical tube at 14,500*g* (11,000 rpm in an SS-34 rotor) at 4°C. Analyze the dialyzed NAP1 by SDS-PAGE with BSA standard to estimate the amount of protein.

Step 3: Purify NAP1 by anion-exchange chromatography

1. Using an FPLC, pack Source 15Q resin in an HR-5 or HR-10 column according to the manufacturer's instructions. Use 1 ml of packed resin per 5 mg of NAP1 from Step 2:5. Equilibrate the Source 15Q column in 10 column volumes of NAP1 purification buffer containing 0.1 M NaCl.
2. Load the NAP1 onto the Source 15Q column. Wash the sample with 10 column volumes of NAP1 purification buffer containing 0.2 M NaCl. Elute the protein with a 20-column-volume gradient of NAP1 purification buffer from 0.2 M to 0.5 M NaCl.

   NAP1 should elute in two distinct peaks. The early (lower-salt) peak is inhibitory toward assembly whereas the later (higher salt) peak is active. A 14-kD band elutes with the early peak. Collect fractions of 0.25 to 0.5 column volumes.

3. Run 2 µl of each fraction on a 15% SDS-PAGE gel to identify pure NAP1-containing fractions. Combine peak fractions and dialyze twice, each time for 2 hours against 2 liters of NAP1 purification buffer containing 0.1 M NaCl.
4. Analyze the dialyzed NAP1 on an 8% SDS-PAGE gel along with BSA standard to determine the concentration. Divide the material into 100–200-µl aliquots in 1.5-ml siliconized tubes

and freeze in liquid nitrogen. Store at –80°C.

The expected yield of the active NAP1 fraction is 1–3 mg/liter of cell culture.

TROUBLESHOOTING

No DNA detected after reconstitution

Possible cause: Nuclease contamination in DNA, histone, or buffer stocks.

Solution: Test for nucleases by incubating each component with DNA for 30 minutes at 37°C, and check DNA integrity on agarose gel. EDTA concentration in dialysis buffers can also be increased to 1 mM.

Possible cause: DNA aggregated during reconstitution, leading to precipitation.

Solution: Reanalyze concentration of DNA and histone stocks to confirm that proper ratio was assembled. Because free nucleotides can influence absorbance readings of DNA, electrophoreses aliquots of DNA template on agarose gel to confirm absorbance readings. Likewise, check histone stocks on SDS-PAGE with BSA concentration standards electrophoresed in parallel.

Possible cause: MNase stocks for the analysis are too concentrated or Stop buffer not correct.

Solution: Check integrity of recons without a MNase digest. For array templates (~2.5 kb), a reconstituted array migrates slightly faster than a free DNA fragment.

Nucleosomes reconstituted but arrays are subsaturated

Possible cause: DNA or histone octamer concentrations were not accurate.

Solution: Recheck concentrations by absorbance and by electrophoresis on either agarose gels or SDS-PAGE.

Possible cause: Octamer stock is not fully competent for assembly.

Solution: In some cases, recombinant octamer stocks contain >10% octamers that are incompetent for assembly, likely due to incomplete refolding. This proportion may be increased when using octamers harboring various amino acid substitutions. In this case, simply increase the *r* value (e.g., from 1.3 to 1.8 octamers per 200-bp repeat) to achieve greater reconstitution efficiency.

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