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BIOCHEMICAL ANALYSES OF TRANSCRIPTIONAL REGULATORY MECHANISMS IN A CHROMATIN CONTEXT

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Abstract

We have optimized a recombinant chromatin assembly system that properly incorporates core histones and histone H1 into a chromatin template containing a natural promoter sequence. This article provides a step-by-step procedure for expression and purification of the proteins required for assembling well-defined chromatin templates. We describe how the degree of chromatin assembly in the absence and presence of histone H1 is measured using topological analysis and the use of micrococcal nuclease digestion performed to confirm H1 incorporation and determine the quality of *in vitro* chromatin templates. Further we describe the use sucrose gradient ultracentrifugation to verify that no unincorporated H1 remains as a second means for deciding on the proper H1 to core histone ratio during assembly. Additionally, we discuss the use of both yeast and *Drosophila* NAP-1 (yNAP-1 and dNAP-1, respectively) in the assembly of H1-containing chromatin. Finally, we provide detailed description of functional assays for investigating the mechanism of transcriptional regulation in a chromatin context (transcription, histone acetyltransferase activity, and protein association with promoter-bound complexes using immobilized chromatin templates).

INTRODUCTION

Eukaryotes package their DNA into chromatin to accommodate the tight quarters within a cell's nucleus and to regulate DNA-dependent cellular processes. Structurally, the core histones combine with DNA to form the basic repeating unit of chromatin, the nucleosome (1). Histone H1 interacts with the nucleosome and functions to stabilize higher order structures within the chromosome (2). How cells perform biological functions in the complex, but organized, context of chromatin remains a central question in biology.

Development of recombinant systems for assembling chromatin has contributed greatly to our understanding of the role of the core histones in transcriptional regulation by providing a well defined template and as a means to compare the numerous possibilities for combinations of histone variants, amino acid substitutions and modifications (3,4). Recently, a recombinant system has been used to incorporate histone H1 into a chromatin template (5). Prior to this development, H1 was typically incorporated into chromatin using either salt dialysis or crude extracts that contain chromatin assembly factors from *Drosophila* (e.g. S-190 extracts). The use of a recombinant system to assemble chromatin that contains histone H1 greatly facilitates studies on the role of H1 in chromatin structure and transcriptional regulation and generally

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provides a more physiological platform from which to study the mechanism of transcriptional regulation.

To examine the functional role of histone H1 at the HTLV-1 promoter, we optimized H1 incorporation into chromatin using the recombinant assembly factors dNAP-1 and ACF. We found H1 addition to chromatin templates increased the nucleosome repeat length about 20 base pairs, a characteristic associated with chromatosome formation. Further optimization revealed a slight molar excess of histone H1 relative to core histone octamer was necessary to achieve incorporation at roughly one molecule of H1 per nucleosome. Additionally, we compared the ability of yNAP-1 to dNAP-1 in assembling H1-containing chromatin. While dNAP-1 readily promoted incorporation of linker histone, only a C-terminally truncated form of yNAP-1 produced H1-chromatin. Further, chromatin assembled with even the truncated yNAP-1 failed to repress transcriptional activation, a functional characteristic of chromatin assembled using dNAP-1. Therefore, functional experiments must be performed on H1-chromatin assembled using dNAP-1 as the histone chaperone.

Here we describe the methods for generating the reagents required for assembly of chromatin templates and their use in chromatin assembly. We also describe procedures for analyzing the quality of the chromatin templates. Finally, we provide the methods for conducting a range of functional experiments to investigate the mechanism of transcriptional repression by chromatin components and the mechanism of transcriptional activation in the context of a chromatin template.

PROTEIN EXPRESSION AND PURIFICATION

Native *Drosophila* Histones

Drosophila core histones (Figure 1) can be purified from *Drosophila* embryos as described previously (6). Essentially the same procedure can be used to purify core histones for culture mammalian cells if so desired. Recombinant core histones from various species can be expressed in *E. coli* and purified as described by Dyer et al. (7) Histone H1 (Figure 1) can be isolated and purified from *Drosophila* embryos as described by Croston et al. (8). The advantage of using *Drosophila* H1 is the presence of only a single histone H1 isoform. In comparison, mammalian cells contain as many as seven different isoforms (9).

Baculovirus Clone Care and Feeding

When expressing proteins from baculovirus, special care must be taken to ensure minimal passaging of each viral stock. If this guideline is not followed, mutations can occur in the baculovirus that ultimately cause a significant loss of protein expression. The first step in maintaining a low passage viral stock is to isolate new baculovirus clones through plaque assays. A detailed protocol in *Baculovirus Expression Vectors: A Laboratory Manual* (10) can be followed for the plaque assays and picking of individual, isolated clones (pages 125–126). Clones should be generated for all of the baculoviruses to be used, which included dNAP-1_{His6}, Acf1, ISWI_{FLAG} and p300_{His6}. If the baculoviruses have been obtained elsewhere, as is the case here, you need only perform one round of plaque assays for acquiring each clone.

Amplification of Viral Clones: Passage One Viral Stock—Passage one viral stocks are generated as described (10) (pages 128–129) with the following modification. Typically, viral supernatant is collected 7–9 days post infection as the cells begin to lyse, instead of collecting at 4 days post infection. After harvesting the viral supernatant, there should be 1.5 to 2 ml of passage one virus. At this time, small-scale infections (described later) can be

performed to select for one or two clones that produce high levels of the target protein. Ideally, the passage one virus is further amplified to a passage two stock before expression testing.

Amplification of Viral Clones: Passage Two Virus and Working Stocks—

Generation of a second passage viral stock is necessary to further amplify the virus, both in titer and volume. The amplification is performed as described (pages 129–130) (10), but the infection is allowed to proceed until the cells began to lyse. After collecting the viral supernatant, plaque assays are performed to determine the titer of each virus. To better visualize and count plaques the vital stain neutral red can be overlaid as described (10). However, neutral red should not be used when initially picking clones from which to generate new baculovirus stocks, as neutral red can mutate the virus. Results from a typical plaque assay are shown in Figure 2.

The best time to perform small-scale infections is after calculating the titer of each passage two clone. Because the titer is known, cells can be infected with equivalent plaque forming units (pfu) of virus. For the small-scale infections, seed cells in log phase growth at a density of 1.0×10^6 cells/ml in 6-well plates in a final volume of 2 ml of serum-free TNM-FH medium. Let the cells attach for 30 minutes to 1 hour in the serum-free medium and then aspirate the medium containing any cells that did not attach. Infect the cells with virus to a multiplicity of infection (MOI) of 5–10 and rock the plates at room temperature for 1 hour to ensure even infection. The virus may need to be diluted, as the cells should be covered with 0.5 – 1 ml of virus. After 1 hour, add complete TNM-FH media to 5 ml and place the cells at 27°C until they start to show signs of infection (typically 2–5 days, depending on the virus). The cells should appear visibly swollen and larger than control (uninfected) cells. Harvest the cells by scraping them off of each plate and collecting them into 15 ml conical tubes. Centrifuge at $1000 \times g$ for 5 minutes to pellet the cells, aspirate the supernatant, and resuspend the cells in 50 μ l SDS-PAGE loading dye and analyze protein expression by Western blot.

Importantly, if small-scale infections are to be performed with passage one virus, use 0.2 ml of virus to infect the cells. Since there is not enough passage one virus for both titer determination and amplification, the infections may not be of equal pfu. In addition, because passage one virus is very low in titer, visualizing cell growth is more difficult. Therefore, it is best to use passage two viruses for these initial infections.

After expression testing, the best clone from each virus is further amplified by infecting the cells at a low MOI of 0.1 to 1 to produce the working baculovirus stock as described (page 130) (10). It is important for production of baculovirus working stocks to infect at very low MOIs of 0.1 to 1 and for protein expression to infect at high MOIs of 5 to 10. With our viruses, we found it necessary to carry this infection until the cells began to lyse (often days 7 to 9) before harvesting the viral supernatant for the working stock. Special care must be taken in storing working stocks, as they must be protected from light to avoid compromising the viral titer (11). After working stocks are generated, perform another round of plaque assays to determine the virus titer. Working stocks are stable for six months, after which, a plaque assay should again be performed to determine any loss in viral titer. After a year, a new working stock can easily be made from an aliquot of passage two virus that is stored in liquid nitrogen. It is critical at this stage to store aliquots of the passage two clones that yielded the most protein at both -80°C and in liquid nitrogen. This step is vital in maintaining low passage working stocks over several years and bypasses the need to isolate clones every year.

To further characterize each virus for optimal protein expression, cells should be infected at various MOIs between 5 and 10. This is readily accomplished using 100 ml spinners containing 50 ml of log phase cells at 1×10^6 cells/ml for each MOI. Allow the infections to proceed until the cells show signs of lysis. In the meantime, remove 1 ml of cells every day until the cells

begin to lyse. These cells will be used to determine the hours of infection required for optimal protein production and the best MOI. After removing the cells, pellet them, aspirate the media, lyse the cells in 50 μ l SDS-PAGE dye, and store at -20°C . When the last sample has been collected, analyze for maximum protein expression via western blot. Congratulations! You have finally determined the best MOI and infection time for optimal protein expression of one virus. Continue this process for every baculovirus clone in the laboratory.

***Drosophila* NAP-1 Expression and Purification**

For dNAP-1 expression, one liter of Sf9 insect cells in log phase growth at 1×10^6 cells/ml is infected with dNAP-1_{His6} baculovirus to a MOI of five (the MOI will vary between 5–10, depending on the virus). The infection is allowed to proceed in spinner culture for 60–72 hours. The cells are then harvested by centrifugation at 1000 x g. Each cell pellet is washed with 30 ml 1X PBS, re-centrifuged, and either used immediately or frozen in liquid nitrogen and stored at -80°C for later use. To purify dNAP-1, follow the protocol described by Fyodorov and Kadonaga exactly (5). Note: be certain to pH the buffers containing imidazole to pH 7.6. After isolation of a new dNAP-1 baculovirus clone, typical yields were 5–6 mg of purified dNAP-1 per liter of Sf9 cells. The expected Source 15Q elution profile and purified dNAP-1 protein are shown in Figure 3A and 3B, respectively.

ACF Expression and Purification

For ACF expression, log phase Sf9 cells are seeded at a density of 2×10^7 cells/plate and four plates are seeded per infection. Alternatively, log phase cells from spinner culture can be diluted to 1×10^6 cells/ml and a total of 8×10^7 cells infected. Cells are co-infected with Acf1 (untagged) and ISWI_{FLAG} baculoviruses. Typically cells are infected with Acf1 baculovirus at a MOI of 5 and ISWI_{FLAG} at an MOI of 10. To ensure proper one-to-one stoichiometry of the purified ACF complex, the MOIs of each virus are adjusted accordingly depending on the clones. Cells are infected for 44–46 hours and harvested by centrifugation at 1000 x g, washed with 30 ml 1X PBS and can be frozen for later purification. ACF purification is performed as described (5) and typical yields are 2–5 μ g ACF per 2×10^7 cells (Figure 3C).

p300 Expression and Purification

p300 was expressed and purified using a protocol defined by Kraus and Kadonaga (12). For p300 expression, four 15 cm plates are seeded with cells at a density of 2×10^7 cells/plate. The cells are then infected with p300_{His6} baculovirus to a MOI of 5–10 and incubated at 27°C for about 72 hours. The cells are harvested by removing them from the plates into a 50-ml conical and pelleted by centrifugation at 1000 x g for 5 minutes. The cells are then washed with 30 ml 1X PBS and pelleted to remove the PBS. Cells pellets can either be used immediately or flash frozen and stored at -80°C for later use.

The cell pellet is resuspended in 1 ml of homogenization buffer per 15 cm plate (10 mM Tris-HCl, pH 7.5, 500 mM NaCl, 10 % glycerol, 0.1 % NP-40, 15 mM imidazole, 2 mM β -mercaptoethanol, 2 mM PMSF, 20 μ g/ml leupeptin, and 20 μ g/ml aprotinin). The cells are transferred to a “Dounce” glass homogenizer and lysed with 10 strokes using a tight pestle on ice. The lysate is incubated 15 minutes on ice followed by clarification by centrifugation for 10 minutes at 4°C . The supernatant is transferred to a 15-ml conical containing 30 μ l of a 50% Ni-NTA resin slurry per 15 cm plate that has been washed previously with H_2O and equilibrated in homogenization buffer. The lysate is incubated with the equilibrated Ni-NTA resin for 2 hours on a rotator at 4°C . The resin is then pelleted by centrifugation at 1000 x g and the unbound proteins in the supernatant removed. Four successive washes are performed, each with 1 ml wash buffer per 15 cm plate (10 mM Tris-HCl, pH 7.5, 200 mM NaCl, 10 % glycerol, 0.2 % NP-40, 15 mM imidazole, 2 mM β -mercaptoethanol, 2 mM PMSF). As much as possible of the last wash buffer is removed before eluting the purified p300. The p300 is eluted with

100 μ l elution buffer per 15 cm plate (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 % glycerol, 0.1 % NP-40, 250 mM imidazole, 2 mM β -mercaptoethanol, 2 mM PMSF), incubated on ice for 10 minutes and the elution is repeated. The purified p300 is divided into 5–10 μ l aliquots, frozen in liquid N₂ and stored at –80°C. A 4-plate infection typically produces 10–50 μ g of purified p300 (Figure 3D).

Topoisomerase I Expression and Purification

The topoisomerase I catalytic domain (ND423) that contains a C-terminal His6 tag and N-terminal truncation (13) is expressed and purified as described (5). The amount of topoisomerase I required to relax a supercoiled plasmid DNA template was empirically determined for each preparation.

CHROMATIN ASSEMBLY AND QUALITY CONTROL

H1-Chromatin Template Assembly

Core histones are deposited onto plasmid DNA using the dNAP-1/ACF recombinant chromatin assembly system (5).

We use a 6:1 dNAP-1:core histone (wt/wt) ratio and 25 ng (79 fmoles) of ACF (Acf1/ISWI) per 150 ng (68 fmoles) plasmid DNA. To begin the assembly, core histones are pre-incubated with dNAP-1 on ice for 30 minutes in 25 mM HEPES (K⁺, pH 7.6), 0.05 mM EDTA and 5% glycerol. ACF is added to the core histone/dNAP-1 mix and mixed immediately, followed by the addition of an ATP-regenerating system (3 mM ATP, 30 mM phosphocreatine, 1 μ g/ml creatine phosphokinase). Reactions are adjusted for assembling 150 ng (67 fmol) of DNA in a 7 μ l volume (amount added to a single *in vitro* transcription reaction). However, the actual assembly volumes can range from 50 to 600 μ l. Either supercoiled or relaxed plasmid DNA can be added to the assembly reactions and incubated for 4–18 hours at 27°C under final conditions of 10 mM HEPES pH 7.6, 50 mM KCl, 5 mM MgCl₂, 5% (v/v) glycerol, 1 % (wt/v) polyethylene glycol and 0.01% (v/v) NP-40. Histone H1 is added 1.5 hours post initiation of assembly to the chromatin template at a 1.25 H1:octamer molar ratio. The order of protein addition is crucial for proper assembly, as is thorough mixing after each addition. When necessary, ACF and creatine phosphokinase are freshly diluted in assembly buffer (10 mM HEPES pH 7.6, 50 mM KCl, 5 mM MgCl₂, 5% v/v glycerol, 1 % wt/v polyethylene glycol) and histones and dNAP-1 are diluted in buffer R (10 mM HEPES K⁺ pH 7.6, 10 mM KCl, 1.5 mM MgCl₂, 10% glycerol, 10 mM β -glycerophosphate, 1 mM DTT, 0.2 mM PMSF).

Histone H1 can be added to the assembly reaction either at the beginning of assembly or two hours post-assembly and not interfere with chromatin assembly, as measured by topological analysis. However, the same degree of supercoiling occurs at a lower core histone to DNA ratio in the presence of H1 as compared to that from templates without H1 (Figure 4A, compare top & bottom panel). In the presence of H1, fewer nucleosomes occupy the plasmid DNA template due to the increased linker DNA length, resulting in more DNA associated with each nucleosome. Therefore, the core histone to DNA ratio at which templates are best assembled in the presence of linker histone should be less than the ratio from chromatin assembled in the absence of H1. Topological analysis can be used as indirect assay for incorporation of H1, as binding of the linker histone increases the DNA associated with each nucleosome (14, 15).

Topological (1-D) Supercoiling Assays

Chromatin is assembled as described above with the following modifications. Supercoiled plasmid DNA (700 ng) is relaxed first using recombinant *Drosophila* topoisomerase I (expressed from a plasmid containing clone ND423 producing *Drosophila* topoisomerase I with the N-terminal 423 amino acids deleted and purified as previously described in ref. (13))

in a 70 μ l reaction volume with topoisomerase buffer (50 mM Tris-Cl pH 7.5, 50 mM KCl, 10 mM MgCl₂, 1 mM DTT, 0.5 mM EDTA, 30 μ g/ml BSA). Typically, each reaction contains 3.5 μ l of diluted topoisomerase I. The dilution of topoisomerase I stock in topoisomerase buffer varies between 1:10 to 1:100 depending on each enzyme preparation. The 70 μ l relaxation reactions are incubated for 1 hour at 37°C. Immediately before adding relaxed DNA to the assembly, the DNA is “spiked” with an additional 20 μ l of the appropriate topoisomerase I dilution. After chromatin assembly, the degree of supercoiling is measured by phenol/chloroform extraction of the DNA and electrophoresis on a 1% TBE agarose gel followed by SYBR gold (Invitrogen) staining and STORM (Molecular Dynamics) imaging. Relaxed DNA was only used in supercoiling assays and all other assembly reactions are performed with supercoiled plasmid DNA.

Micrococcal Nuclease Digestion

Micrococcal nuclease (MNase) digestion is performed on 2.1 μ g of assembled DNA (98 μ l assembled chromatin). First, 135 μ l of MNase buffer (1.74 mM HEPES pH 7.6, 121 mM KCl, 12 % v/v glycerol, 2.4 % wt/v PEG, 11.2 mM CaCl₂) are added to chromatin samples and incubated for 5 minutes at 37°C. Digestion is initiated by addition of 0.12 U MNase (Worthington) in a 7 μ l volume to templates lacking histone H1 and 0.48 U MNase to H1-containing templates at 37°C. After 1, 2, 4, & 8 minutes, 60 μ l aliquots are removed and digestion stopped with 12 μ l TE/EDTA (6 μ l 10 mM Tris-HCl pH 7.9 1 mM EDTA/6 μ l 0.5 M EDTA). Samples are deproteinized with 9.6 μ l 2.5 mg/ml proteinase K and 96 μ l chromatin stop (20 mM EDTA, 200 mM NaCl, 1 % wt/v SDS, 0.25 mg/ml glycogen) for 30 minutes at 37°C. The DNA is extracted with phenol/chloroform followed by ethanol precipitation. Nucleic acids are resolved on 1.2 % TBE agarose gels and visualized with SYBR gold (Figure 4B). Gene ruler 100 bp plus markers (Fermentas) can be used as DNA size standards. The nucleosome repeat length can be calculated using Image Quant 5.1 software. Briefly, mobilities are measured for each standard and plotted against their corresponding size in base pairs. The data are then fit to the semi-logarithmic curve and nucleosome repeat lengths extrapolated from the curve. The position of marker and nucleosomal DNA is determined as the pixel peak for each band. In the example, nucleosome repeat lengths are calculated using DNA digested for 4 min and data corresponding to mononucleosomes was omitted in the calculations. Three independent experiments should be performed to determine the repeat length for chromatin assembled in the absence and presence of H1.

Since a nucleosome containing H1 protects 10–20 additional base pairs from micrococcal nuclease digestion (14), increased nucleosome repeat length is used to verify proper addition of linker histone. The repeat length increased from 169 (+/- 6) base pairs in the absence of H1 to 190 (+/- 4) base pairs upon H1 incorporation (see Fig. 1A, Konesky et al. *J. Virology in press*). In this assay, the increased nucleosome repeat length only occurred if H1 is added 1.5–2 hours post-assembly to the reactions, establishing that the time H1 was added to the assembly is crucial for proper incorporation. Furthermore, the presence of several nucleosomes (greater than six) on each chromatin template confirmed the quality of chromatin formed using this system (see Fig. 1A, Konesky et al. *J. Virology in press*).

We have tested whether mammalian H1 isolated from an uninfected T-cell line (CEM) can be incorporated into chromatin using this protocol. The results shown in Figure 4B demonstrate CEM H1 is incorporated into the chromatin template, producing a nucleosome repeat length similar to that observed with incorporation of *Drosophila* H1.

Sucrose Gradient Analysis

For sucrose gradient analysis, 4.2 μ g of DNA is assembled into chromatin with varying H1:octamer molar ratios. Chromatin samples are resolved using 13 ml 15–40 % sucrose

gradients (10 mM Tris-Cl pH 7.8, 0.1 mM EDTA, 1mM DTT, 0.1 mM PMSF, 0.1 mM benzamidine). Gradients are centrifuged at 4°C in an SW41Ti rotor (Beckman) at 40,000 rpm for 4 hours. Gradient fractions (11 tubes, each containing 1.2 ml) are collected from top to bottom. Proteins from 800 µl of each fraction are TCA/DOC (add 200 µl 100% w/v trichloroacetic acid, 4 mg/ml deoxycholate) precipitated by incubating on ice 20 min, centrifuging at 4 °C 15 min and washing with 1.5 ml acetone. Proteins are then resolved on 15% SDS-PAGE and visualized with SYPRO ruby and STORM imaging or silver staining. DNA is deproteinized and precipitated as described for MNase digestion analysis from 200 µl of each gradient fraction, resolved on a 1% TBE agarose gel and visualized with SYBR gold. Results from such an analysis are shown in Figure 5.

One molecule of H1 typically binds per core histone octamer *in vivo* (16). Therefore, it is critical to assemble chromatin at histone H1 to core histone ratios to allow for all nucleosomes to contain H1 while avoiding the presence of excess, unincorporated H1. H1 incorporation is optimized by assembling chromatin samples at different H1 to core histone octamer ratios. The resulting samples are analyzed using 15–40% sucrose gradients and ultracentrifugation. We find that at histone H1 to core histone octamer ratios up to 1.25 (H1:CH) the repeat length is fully shifted to approximately 199 bp, but no free H1 is observed (see Fig. 1B Konesky et al., *J. Virology in press*). At ratios greater 1.25, significant levels of incorporated H1 is seen (not shown). The addition of H1 at ratios of 1.75 or greater resulted in an aggregate of chromatin that sediment to the bottom of the gradient. As a result, we assemble chromatin templates at a 1.25 H1:CH ratio.

Comparison of the Histone Chaperones yNAP-1 and dNAP-1 in Chromatin Assembly

Because yNAP-1 is much easier and cheaper to purify in large quantities than dNAP-1, the ability of yNAP-1 to assemble chromatin that contains linker histone was compared to dNAP-1. Yeast NAP-1 was expressed and purified as described previously (17). Interestingly, full-length yNAP-1 failed to form H1-chromatin with an increased nucleosome repeat length (not shown). Non-specific interactions between full-length yNAP-1 and the core histones are minimized upon truncation of yNAP-1 by deletion of amino acids within the N- and C-terminal regions (truncated yNAP-1 containing amino acids 74–365 expressed and purified as described previously) (18). Therefore, truncated yNAP-1 was tested for its ability to assemble chromatin containing linker histone. There appeared to be no difference between dNAP-1 and truncated yNAP-1 in their ability to assemble chromatin containing H1 as measured by MNase (not shown) and sucrose gradient analysis (Figure 5). However, there were functional differences between templates assembled with these histone chaperones. Specifically, H1-chromatin assembled with truncated yNAP-1 failed to further repress Tax-mediated transcriptional activation, a characteristic of H1-chromatin assembled with dNAP-1 (not shown). Thus dNAP-1 must be used as the histone chaperone protein to assemble H1-chromatin for functional assays.

BIOCHEMICAL FUNCTIONAL ASSAYS ON CHROMATIN TEMPLATES

Transcription

Following chromatin assembly, 150 ng (67 fmol) of DNA in a 7 µl volume is added to transcription reactions containing TM buffer (25 mM Tris-Cl pH7.9, 50 mM KCl, 6.25 mM MgCl₂, 0.5 mM EDTA, 10% glycerol, 1 mM DTT) and 50 µM acetyl-coenzyme A (AcCoA) in a 30–40 µl final volume. Exogenous transcriptional activator proteins are added after or during the chromatin assembly where appropriate. p300 (0.3 pmol) or other transcriptional coactivators can also be added to transcription reactions. Preinitiation complexes are formed with the addition of 40 µg CEM (HTLV-1 negative T-cell line) nuclear extract (19) and samples are incubated for 60 minutes at 30°C. RNA synthesis is initiated with addition of 2.4 µl NTP

mix (250 μ M ATP, 250 μ M CTP, 12 μ M UTP & 0.8 μ M [α - 32 P] UTP 3000Ci/mmol) and incubated for 30 minutes at 30°C. Following RNA synthesis, 4 μ l of RNase T1 (25U/ μ l) are added and samples are incubated for 30 minutes at 37°C. Proteins are digested for 30 minutes at 50°C with 8.5 μ l proteinase K (10 mg/ml) and 10 μ l transcription stop (250 mM NaCl, 1 % SDS, 20 mM Tris-Cl pH 7.5, 5 mM EDTA). An end-labeled 622-bp DNA fragment, isolated from an *Hpa*II digest of pBR322 should be added for a recovery standard. The amount of recovery standard added will vary depending upon isotope decay (number of days since end-labeling). RNA products and labeled DNA recovery standards are precipitated with 165 μ l of a salt, ethanol and tRNA mixture (140 μ l 100% EtOH, 20 μ l 4M NH_4OAc , 5 μ l 10 mg/ml tRNA). The addition of tRNA helps prevent the loss of pellets. After precipitation by centrifugation and a 70% EtOH wash, the pellets are briefly air dried on the bench top, resuspended in 7 μ l formamide loading dye, and resolved on 6.5% sequencing urea-PAGE. Radiolabeled *Hpa*II-digested pBR322 can serve as molecular weight size markers. The gel is imaged using STORM phosphorimaging and transcript levels quantified with Image Quant 5.1 software. Experiments should be performed a minimum of three times. For examples of several transcriptional experiments see Figure 6 from Konesky et al. (20).

Histone Acetyltransferase (HAT) Assays

For *in vitro* acetylation assays, 2 μ g of core histones are assembled into chromatin as described previously. Typically, reactions consist of 2.8 μ g of assembled DNA in a 40–60 μ l volume. Transcriptional activator proteins are included in the chromatin assembly reaction at the same levels used in transcription experiments, relative to the total DNA binding sites. A slight excess of p300 is used (8.4 pmol), compared to that used in transcription studies, relative to the DNA binding sites. Final reaction volumes can vary from 100–150 μ l and contain 75 μ M ^{14}C -labeled AcCoA (56 mCi/mmol specific activity) and TM buffer (25 mM Tris-Cl pH7.9, 50 mM KCl, 6.25 mM MgCl_2 , 0.5 mM EDTA, 10% glycerol, 1 mM DTT) with 10 mM sodium butyrate and 1 mM PMSF. Reactions are incubated for 60 minutes at 30°C. Proteins are then methanol-chloroform precipitated and resolved on 18% SDS-PAGE. Free (unassembled) core histones are acetylated to serve as a marker in a final volume of 20 μ l. For examples of HAT assays used to complement transcriptional analyses see Konesky et al. (20).

Deoxyribonuclease I (DNase I) Primer Extension Footprinting

Experiments are performed as described (21) with the following modifications. Supercoiled plasmid DNA should be separated from nicked DNA using agarose gel preparative-cell electrophoresis (Bio-Rad). The resulting fully supercoiled (un-nicked) DNA (56 fmol) is assembled into chromatin in a 25 μ l volume. CREB and Tax or your favorite sequence-specific DNA-binding proteins are added as appropriate, either during or after chromatin assembly in a final volume of 50 μ l in TM buffer. Binding is generally allowed to occur through incubation for 1 hour at 30°C. DNA digestion is performed as described (21), except digestion is stopped by placing samples in a dry ice/EtOH bath for 10 min. Digested DNA (10 fmol) is incubated with 50 fmol of [γ - ^{32}P] ATP-labeled primer and primer extension is performed as described (21). As an example, see the footprint of the distal and middle vCREs see Fig. 7 (reproduced from (20)). Following primer extension, the DNA is precipitated and resolved on an 8% sequencing gel. The percent accessibility to cleavage is quantified using the primary band that disappears within each core vCRE region. Experiments should be performed a minimum of three times.

Protein Association Using Immobilized Chromatin Templates

Biotinylated DNA fragments containing the full promoter (P) including the regulatory elements and the core promoter alone (C) are prepared by standard PCR using biotinylated forward primers. PCR products are purified using preparative-cell electrophoresis (Bio-Rad) according

to the manufacturers recommendations with a 0.25% agarose stacking gel, a 1% agarose running gel, and running at 50 volts (constant current) in 0.5% TBE at 4°C and collecting fractions at 50 µl per min. Purified biotinylated DNA are bound to M 280 streptavidin Dynabeads (Dyna Biotech ASA, 10 µl beads/pmol DNA) as described by the manufacturer. Chromatin templates are assembled using 2 pmol biotinylated DNA. Transcription regulatory proteins and H1 (1.25 molar excess per octamer) are added to the assembly reactions in a final volume of 50 µl in TM buffer. Chromatin is assembled for 4 hours at 27°C with constant rotation. After assembly, additional factors can be added (for example 0.75 pmol p300) and the volume adjusted to 100 µl in TM buffer. Binding reactions are performed for 45 minutes at 27°C with constant rotation. Immobilized templates are washed twice with 100 µl TM buffer containing 100 mM KCl, resuspended in SDS- PAGE loading dye and the proteins associated with the DNA assessed by western blotting. Experiments should be performed at least three times. H3 (Ab 1791) and H1 (V7013) antibodies are available from Abcam and Biomed, respectively. An example an experiment using a chromatin-assembled bead-bound template and be seen in Fig. 8 (reproduced with permission from (20)).

DISCUSSION

Prior to 2001, histone H1 had only been incorporated into chromatin using salt dialysis (14) or S-190 cell extracts from *Drosophila* (6). While these methods are still widely used today, we feel it is best to take advantage of a recombinant system for assembling chromatin. Therefore, we tested whether the recombinant assembly factors, dNAP-1 and ACF developed in the Kadonaga laboratory (5), could properly assemble chromatin that contained linker histone.

We have found that histone H1 addition to the assembly reaction an hour or two after initiation of chromatin assembly to be a crucial for proper H1 incorporation. This requirement is not apparent through topological analysis, only through micrococcal nuclease digestion analysis (see above). During our optimization of H1 incorporation using dNAP-1 and ACF, the Kadonaga laboratory published a method for assembling chromatin containing H1 using the recombinant assembly system (5). They obtained proper incorporation with addition of histone H1 at the same time as the core histones to the assembly reaction. One possible explanation may be that the ACF purified in our laboratory is not quite as active as that in the Kadonaga laboratory. As a result, we only observed evenly spaced nucleosomes and increased repeat length if the assembly was allowed to proceed initially in the absence of histone H1. We find that we are able to use this system to incorporate H1 isolated from mammalian T-cells. Interestingly, NAP-1 was recently identified as a linker histone chaperone in *Xenopus*, as well, depositing H1 onto the linker DNA at physiological pH and ionic strengths (22), which supports a role for dNAP-1 as an H1 chaperone in our system.

Since dNAP-1 is expressed and purified from Sf9 cells, we tested the ability of yNAP-1, which can be expressed in bacteria, to assemble chromatin with histone H1. We found truncated yNAP-1 (74–365) as effective as dNAP-1 for assembling histone H1 into chromatin, even though yeast only contain a putative H1 (23). However, H1-chromatin assembled with truncated yNAP-1 does not further repress transcriptional activation past that level obtained with core histones alone, whereas H1-chromatin assembled with dNAP-1 does. We attribute the difference seen with yNAP-1 to be related to the ability of yNAP-1 to exchange histone H2A/H2B dimers (24). We propose that yNAP-1 increases the off-rate of histone H1, thereby alleviating the additional transcriptional repression by H1 incorporation observed with dNAP-1. As a result, extensive optimization of H1 incorporation using yNAP-1 was not performed, and we recommend the use of dNAP-1 for chromatin template assembly.

A concern, when using a recombinant assembly system to form chromatin containing H1 is whether H1 is properly incorporated. MNase digestion confirms an increase in the nucleosome repeat length and that H1 is incorporated every nucleosome when added in slight molar excess relative to histone octamer. At this ratio, the final concentration of H1 in our assembly reactions was approximately 170 nM, which is higher than the optimal concentration of 50–100 nM found by other researchers (25). However, in their study chromatin containing linker histone was formed using *Drosophila* S-190 extracts containing endogenous H1, which could skew the amount of exogenously added H1 required for apparent saturation of the chromatin template. Additionally, the dynamic behavior of H1 may play a role, and addition of slight excess of H1 could help ensure that most of the nucleosomes contain a molecule of linker histone at any given time. Thus, we are confident that the chromatin formed by our protocol contains the *in vivo* H1 stoichiometry of approximately one histone H1 per nucleosome, and produces chromatin templates that better resemble those found *in vivo* as compared to chromatin assembled in the absence of linker histone.

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Figure 1. Purified native core histones and histone H1 from *Drosophila* embryos
Histones were resolved using 15% SDS-PAGE and visualized with coomassie brilliant blue. Positions of each histone are indicated and the sizes of molecular weight markers (MW) in kilodaltons are labeled on the left. The actual molecular weight of *Drosophila* H1 is 26343 daltons. This histone runs higher than expected due to its high lysine content.

Figure 2. Plaque visualization is enhanced by neutral red staining

Serial dilutions of recombinant hSNF (RSF subunit) baculovirus were used in this plaque assay to calculate virus titer. Virus dilutions are indicated to the left of each plate. The titer was calculated from plaque counts at virus dilutions of 1×10^{-7} and 1×10^{-6} .

Figure 3. Recombinant dNAP-1_{His6}, ACF, and p300 purification

(A) A typical elution profile from Source 15Q column purification (top). Fractions in peak two of the elution step were pooled and concentrated. While peak one contains dNAP-1, some of the fractions also contain contaminating bands and were not pooled with the other fractions. (B) The resulting dNAP-1 protein was resolved using 10% SDS-PAGE and visualized with coomassie brilliant blue. Molecular weight markers (MW) in kilodaltons are indicated to the left. (C) Acf1 and ISWI_{FLAG} were co-expressed in Sf9 cells and the resulting ACF complex was affinity purified. ACF was resolved using 6.5% SDS-PAGE and visualized with coomassie brilliant blue. Molecular weight markers (MW) in kilodaltons are indicated to the left. (D) Sf9 cells were infected with p300_{His6} baculovirus and purified using Ni-NTA resin. p300 was

resolved using 6.5% SDS-PAGE and visualized with coomassie brilliant blue. Molecular weight markers (MW) in kilodaltons are indicated to the left.

Figure 4. Chromatin assembles at a lower core histone to DNA ratio upon addition of histone H1 and mammalian H1 can be incorporated into chromatin using a recombinant assembly system (A) Relaxed plasmid DNA was assembled into chromatin without (–) or with (+) histone H1. Core histones were titrated onto the DNA template to form nucleosomes. The core histone to DNA (CH/DNA) mass ratios are indicated. The resulting change in DNA topology was resolved using 1%TBE agarose gel electrophoresis. Nucleic acids were visualized with SYBR gold staining and STORM imaging. The positions of nicked, relaxed and supercoiled (SC) DNA are illustrated. (B) Chromatin was assembled in the absence (–) or presence (+) of histone H1 and digested with micrococcal nuclease as described previously. The nucleosome repeat lengths have been calculated for each assembly and are as follows: no H1 - 170 (+/- 9 bp),

dH1 - 186 (+/- 8 bp), and CEM H1 - 189 (+/- 10 bp). Base pair markers from Fermentas (100 bp DNA ladder plus) are indicated to the right.

Figure 5. Truncated yNAP-1 (74–365 aa) assembles chromatin containing H1

Chromatin was assembled with histone H1 using the truncated histone chaperone yNAP-1 and purified using 15–40% sucrose gradients as previously described. Proteins were TCA/DOC precipitated and visualized by silver staining. The DNA was extracted, precipitated and resolved using agarose gel electrophoresis. Molecular weight markers (M) in kilodaltons are indicated, as are the positions of H1, core histones and supercoiled DNA. The fractions corresponding to the chromatin peak are illustrated.

A. **B.**

Figure 6. Tax counteracts the H1-chromatin repression of transcriptional activation

(A) Timeline of chromatin assembly and factor addition for *in vitro* transcription reactions. (B) Tax and CREB, but not CREB alone, overcome H1 repression of transcriptional activation. Chromatin (67 fmols p-306/G-less DNA) was assembled without or with histone H1. Equivalent amounts of Tax and CREB (1.6 pmols each) were added either after (AA) or during chromatin assembly (DA) as indicated for each chromatin template. Transcript levels were averaged between duplicate lanes and calculated relative to lane 3, which was set to 100. (C) Addition of p300 does not overcome H1 repression of CREB-mediated transcriptional activation. Chromatin was assembled as described above. Tax and CREB (1.6 pmols each) were added during chromatin assembly (DA), while p300 (0.3 pmols) was added after

chromatin assembly (AA) to the transcription reactions as indicated. Transcript levels were averaged between duplicate lanes and calculated relative to lane 4, which was set to 100. (D) Graphical representation of three independent transcription experiments from chromatin lacking (open bars) or containing (gray bars) histone H1 assembled with factors added during chromatin assembly (DA). The fold activation was calculated relative to CREB-alone transcription from chromatin assembled without H1, which is set to 1. This figure has been reproduce with permission from (20).

Figure 7. H1-chromatin repression is not through inhibition of CREB binding

DNase I primer extension footprinting analysis of CREB binding on chromatin templates (56 fmols p-306/G-less DNA) assembled in the absence and presence of histone H1. CREB was added after chromatin assembly to each template and titrated from a 4 to 40-fold molar excess as indicated by triangles (1.3, 2.7, 6.7 & 13.4 pmols of CREB, respectively). Undigested DNA (UN) and no protein (-) lanes are labeled for both chromatin templates. ^{32}P -labeled DNA products from the primer extension reaction were resolved on an 8% sequencing gel. Protection was observed at both the middle and distal vCRE core regions as CREB was titrated onto the DNA. Protein binding was measured as a percent of DNA accessibility with the digested, no protein (-) lanes set as 100 percent accessible. The percent accessibility was calculated for the

distal vCRE, although similar levels of protection were calculated for the middle and proximal vCREs. This figure has been reproduce with permission from (20).

Figure 8. Recruitment of p300 is not affected by chromatin containing H1

(A) Factor binding is vCRE-dependent. Chromatin was assembled in the absence of linker histone on promoter (P, three vCREs) or control (C, no vCREs) DNA (2 pmols biotinylated fragment DNA). Recombinant Tax (18 pmols), P-CREB (12 pmols) and p300 (0.75 pmols) were added to the assembled chromatin templates. Upon isolation and washing of each template, the bound proteins were analyzed by western blot. Antibodies are indicated to the left of each blot. (B) Factor binding to the promoter fragment assembled into chromatin either in the absence (–) or presence (+) of histone H1. Tax and P-CREB were added during (DA) or after (AA) chromatin assembly, as indicated. All reactions contained p300, which was added after chromatin assembly. Bound proteins were detected using western blot analysis with the

indicated antibodies. (C) Graphical representation of p300 binding from either Tax/P-CREB or P-CREB-mediated recruitment in the context of chromatin lacking (open bars) or containing linker histone (gray bars). Data was averaged between three independent experiments and p300 binding is relative to chromatin assembled without H1 for each condition, which are set to 1. This figure has been reproduce with permission from (20).