

Histone deposition and chromatin assembly by RSF

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Abstract

It is becoming clear that the structure of cellular chromatin is dynamic and capable of undergoing rapid changes to respond to the metabolic requirements of the cell. These changes have a direct impact on gene expression and, therefore, the chromatin context must be considered when biochemical reactions that involve DNA are studied. Over the past several decades, a number of methods for assembling chromatin in vitro have been described. Some of them use chemical compounds to deposit histone octamers onto the DNA. Others take advantage of cellular protein complexes that have the ability to assemble chromatin. Some of these complexes have been identified and purified. This article focuses on one of these factors, RSF (remodeling and spacing factor), which was identified in our laboratory. We describe how the chromatin assembly reaction is performed and how it can be monitored to evaluate its efficiency.

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1. Introduction

In eukaryotic cells, DNA is packaged into chromatin. The chromatin unit, the nucleosome, is composed of 146 bp of DNA wrapped around the core histone octamer, which is formed by two copies of each of histones H2A, H2B, H3, and H4 [1,2]. An array of nucleosomes, known as “beads on a string” or the 11-nm fiber, is compacted into a 30-nm fiber, which undergoes further levels of compaction [3].

The packaging of DNA into chromatin has a significant influence on DNA metabolism. Efforts to understand how cellular proteins are able to efficiently bypass this barrier and carry out their functions came largely from research in the transcription field, although it is now clear that chromatin affects other processes that require access to DNA, such as DNA replication and repair [4].

Several decades of studies have demonstrated that chromatin is highly dynamic. Changes in chromatin structure occur as a consequence of the metabolic re-

quirements of the cell and directly affect and modulate gene expression. Therefore, to characterize DNA-related cellular processes in a test tube, it is necessary to have methods that allow one to imitate the state of chromatin in vivo. Such methods can be used to efficiently package DNA into chromatin prior to carrying out the biochemical process of interest.

Several different methods to assemble nucleosomes have been described and are briefly outlined below:

1. Salt and polyamides: When DNA is mixed with histones, the two entities quickly interact to form aggregates. This is a result of the electrostatic interactions between the highly positively charged histone tails and the negative charges present in the phosphates of the DNA backbone. However, if DNA and histones are mixed in high salt (2 M NaCl) and the salt concentration is slowly decreased, the correct association between DNA and histones will be favored and nucleosomes will be formed [1]. Polyamides have the same effect, neutralizing the charges of histones and, therefore, favoring the formation of nucleosomes [5]. As a result, the DNA is fully compacted into chromatin. The major disadvantage of these methods is that they produce chromatin that lacks regular spacing between nucleosomes; this is a

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serious disadvantage for many types of experiments, because consistent spacing between nucleosomes is a characteristic of chromatin within the cell. Therefore, nucleosome-positioning sequences are often inserted into the target DNA to circumvent this problem. The best-characterized nucleosome-positioning sequence is that of 5S rDNA [6].

2. Histone chaperones: Several histone chaperones have been identified and their function is to guide histone proteins to DNA. It is likely that the chaperones facilitate the association between histones and DNA by reducing nonspecific interactions between positively charged histones and negatively charged DNA, in a manner similar to salt and polyamides. Chaperones interact with the entire core histone, yet they show preferential affinity for H3/H4-containing tetramers or the H2A/H2B-containing dimers. For example, NAP-1 [7] and nucleoplasmin [8] preferentially interact with H2A/H2B dimers. On the other hand, CAF-1 [9], RCAF [10], HIRA [11], and RSF [12] prefer H3/H4 tetramers [12]. The major disadvantage of these methods is, again, that they yield chromatin with irregularly spaced nucleosomes.

3. Chromatin assembly factors: Several chromatin assembly factors have been identified and, in general, consist of multiple proteins. Some of the chromatin assembly factor complexes, such as CAF-1 and RCAF [13], assemble chromatin in a reaction that depends on DNA replication. In contrast, the reaction carried out by other chromatin assembly factors does not depend on DNA replication (e.g., ACF, CHRAC, and RSF). ACF and CHRAC are very similar in composition; in fact, the two largest subunits are shared: Acf-1 and hSNF2H (or ISWI in *Drosophila*). CHRAC is postulated to have two additional small subunits named CHRAC-16 and CHRAC-14, yet it seems that they do not affect nucleosome deposition. To carry out chromatin assembly, ACF and CHRAC require ATP and the presence of the histone chaperone NAP-1 [14].

The RSF chromatin assembly factor was initially identified in human cells as a “remodeling” factor that allows the formation of competent transcription initiation complexes on chromatin templates [15]. It is composed of two subunits, the ATPase hSNF2H and Rsf-1 (p325), a protein encoded by a novel human gene. Later, RSF was shown to have chromatin assembly activity *in vitro*, but, in contrast to ACF and CHRAC, it does not require an additional histone chaperone [12].

The mechanism of chromatin assembly is still under investigation. The current model proposes that the first, and probably rate-limiting, step is the deposition of H3/H4 tetramers onto the DNA, followed by the rapid association of two H2A/H2B dimers. Both reactions are assisted by histone chaperones. The last step is the wrapping of the DNA around the histone octamers [16,17]. Once the nucleosomes have been assembled, spacing activities such as ACF, CHRAC, and RSF

move the nucleosomes along the DNA to keep a regular spacing between nucleosomes. However, this is a simplified view of chromatin assembly. In fact, histone chaperones have been shown to work in conjunction with chromatin spacing activities [18]. Additional mechanistic analysis of chromatin assembly revealed that ACF is a processive enzyme, so that once ACF is bound to the DNA template, it becomes committed to it [19]. The analysis of the RSF-mediated chromatin assembly proposed that RSF binds first to core histones through its interaction with H3/H4 tetramers; the RSF–histone complex then binds to the DNA template. We have suggested that DNA binding is the rate-limiting step in the RSF reaction [12].

This article focuses on the formation of chromatin by RSF. Several different methods used to characterize the reaction are described, as is the purification scheme used to obtain RSF.

2. Purification of factors

2.1. RSF

2.1.1. Native RSF

The purification of RSF from the nuclear pellet fraction of HeLa cells [20] was described previously [15]. Briefly, HeLa cells are harvested by centrifugation at 3000 rpm (rotor: H6000A, Sorvall) for 10 min at 4°C, and washed with phosphate-buffered saline (PBS). The cells are osmotically swollen by incubation in 5 vol (with respect to the cell pellet) of buffer A (10 mM Tris–HCl, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF) for 10 min on ice. The cells are then centrifuged at 10,000 rpm (rotor: SLA-3000, Sorvall) for 10 min at 4°C, resuspended in 2 vol (with respect to the cell pellet) of buffer A, and homogenized with 10 strokes using a glass pestle B homogenizer. The homogenized suspension is centrifuged at 10,000 rpm (SLA-3000 rotor) for 10 min at 4°C. The supernatant is the cytosolic fraction and the pellet contains the nuclei. Nuclei are resuspended in 3 mL of buffer C per 10⁹ cells (20 mM Tris–HCl, pH 7.9, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT, 25% glycerol) and homogenized with 10 strokes using a glass pestle B homogenizer. The suspension is stirred for 30 min at 4°C and centrifuged at 15,000 rpm (SLA-3000 rotor) for 30 min at 4°C. The supernatant is the nuclear extract fraction and the pellet is the nuclear pellet fraction.

Approximately 5 g of the nuclear pellets is resuspended in 36 mL of buffer B (50 mM Tris–HCl, pH 7.9, 5 mM MgCl₂, 0.5 mM EDTA, 5 mM DTT, 0.2 mM PMSF, 25% glycerol). A 3 M stock solution of ammonium sulfate is added to a final concentration of 0.3 M, and the solution is mixed quickly by inverting the tubes several times. The solution becomes dense; after this

occurs, the solution is solubilized by sonication on ice (Sonic Dismembrator 550, Fisher Scientific), with 8–12 stroke cycles of 33 s each. The suspension is cleared of debris by centrifugation at 40,000 rpm (rotor: T-647.5, Sorvall) for 1 h at 4 °C. A saturated ammonium sulfate solution, pH 7.9, is then added drop by drop, with continuous stirring to a final concentration of 68% saturation. The solution is then stirred for 30 min and centrifuged at 35,000 rpm (T-647.5 rotor) for 1 h at 4 °C. The supernatant is discarded, and the pellet is resuspended in buffer D (50 mM Tris–HCl, pH 7.9, 0.1 mM EDTA, 2 mM DTT, 0.2 mM PMSF, 25% glycerol) to adjust the ammonium sulfate concentration to 0.05 M. The solubilized nuclear pellets are then loaded onto a 600-mL DEAE-52 column (Whatman, Catalog No. 4057200, capacity 10 mg/mL) equilibrated with buffer D containing 0.05 M ammonium sulfate. The column is washed with buffer D/0.05 M ammonium sulfate, and the proteins are eluted with a 4-column-volume, linear gradient of ammonium sulfate (from 0.1 to 0.6 M, in buffer D). About 50–70% of the proteins flow through the column, while RSF elutes between 0.1 and 0.25 M ammonium sulfate. The RSF fractions, assayed as described below, are pooled and dialyzed against 0.2 M ammonium sulfate in buffer D (buffer D now contains 10% glycerol from this point on). Proteins are then loaded onto a

140-mL heparin–agarose type I column (Sigma, Catalog No. H6508, capacity 5 mg/mL) equilibrated with buffer D/0.2 M ammonium sulfate. The column is washed with buffer D/0.2 M ammonium sulfate and proteins are eluted with a 5-column-volume, linear ammonium sulfate gradient (from 0.2 to 0.7 M, in buffer D). At this point in the purification, about 50% of the proteins flow through the heparin–agarose column, and RSF is eluted between 0.23 and 0.3 M ammonium sulfate.

The fractions containing RSF activity are then pooled and dialyzed against buffer D/0.05 M ammonium sulfate and loaded onto a Tosoh Biosep FPLC TSK-DEAE-5PW (21.5 mm × 15 cm, capacity 10 mg/mL) column. The column is washed with buffer D/0.1 M ammonium sulfate and eluted with a 7-column-volume, linear ammonium sulfate gradient (from 0.1 to 0.7 M, in buffer D). RSF elutes in a sharp peak at a conductivity equivalent to that of 0.18 M ammonium sulfate. The RSF fractions are pooled and concentrated to 250 µL with a Millipore microconcentrator (10-kDa cutoff, Catalog No. UFV4BGC00) and then loaded onto a gel filtration column (Amersham Pharmacia Biotech FPLC Superose 6 HR 10/30). The column is equilibrated with buffer BC500 (20 mM Tris–HCl, pH 7.9, 0.2 mM EDTA, 10 mM β-mercaptoethanol, 0.2 mM PMSF, 10% glycerol, 0.5 M KCl). RSF elutes with a relative molec-

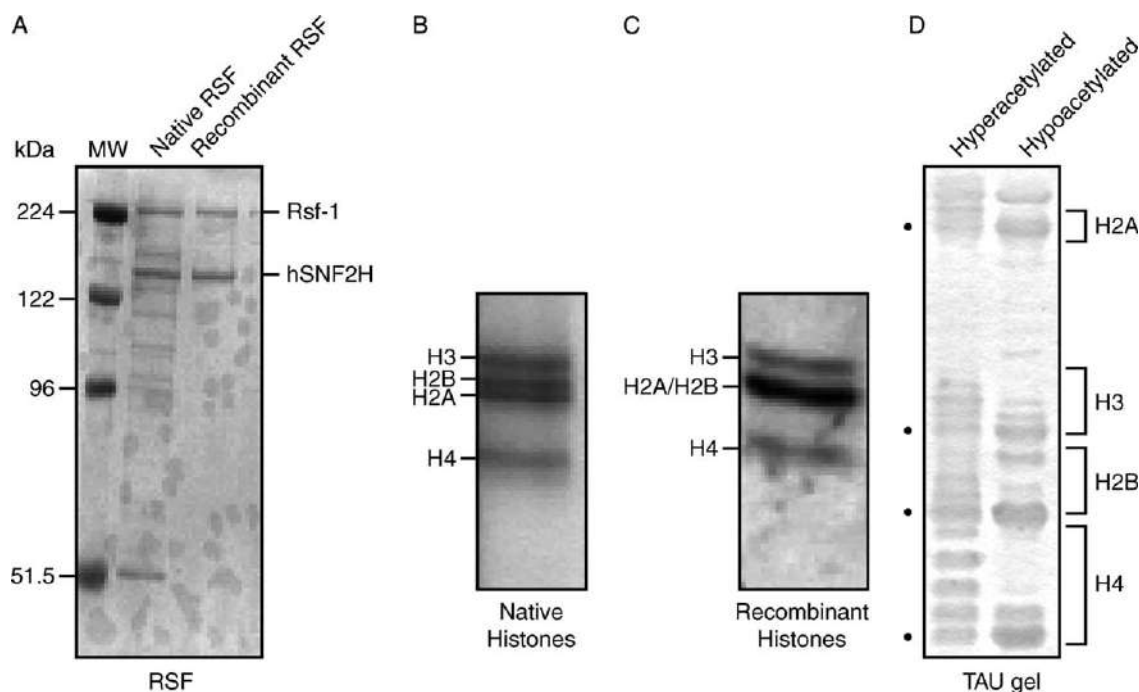


Fig. 1. Coomassie blue staining of purified RSF and core histones. (A) RSF purified from a HeLa cell nuclear pellet fraction and from SF9-baculovirus-infected cells is shown. The purification procedure was carried out as explained in the text. The migration of the RSF subunits Rsf-1 and hSNF2H is indicated on the right. (B) Native core histones purified from HeLa cells or purified recombinant *Xenopus* core histones expressed in bacteria (C) are shown. The purification procedure was carried out as explained in the text. The migration of each of the core histones is indicated on the left. Note that recombinant H2A and H2B migrate together due to high similarity in molecular weight and the lack of posttranslational modifications. (D) TAU gel of native unacetylated and acetylated core histones purified from HeLa cells. The migration of each of the histones is indicated in the right. The dots represent the migration of unacetylated histones.

ular mass of 500 kDa. The final yield is approximately 0.5 mg from 2×10^{11} cells, and the preparation is 90% homogeneous (Fig. 1A).

Each purification step is assayed by Western blot with antibodies to the RSF subunits hSNF2H and Rsf-1. The proteins fractionated during the final column are visualized by silver staining and also assayed for chromatin assembly. The final pool is made according to the results of these three assays.

2.1.2. Recombinant RSF

Recombinant RSF is purified from SF9 cells co-infected with Flag-tagged Rsf-1 and hSNF2H baculoviruses. Log-phase SF9 cells are grown in a 500-mL flask to a confluence of 10^6 cells/mL. These cells are infected with the Rsf-1 and hSNF2H baculoviruses at a multiplicity of infection (m.o.i.) of 10. Forty-eight hours after infection, the cells are harvested, pelleted, and washed twice with cold PBS. The cells are resuspended in 10 mL lysis buffer (20 mM Tris-HCl, pH 7.9, 0.6 M NaCl, 4 mM $MgCl_2$, 0.4 mM EDTA, 2 mM DTT, 20 mM β -glycerophosphate, 20% glycerol, 0.4 mM PMSF, 1 mM benzamidinium-HCl, 4 μ g/mL leupeptin, 2 μ g/mL aprotinin) and disrupted with three quick freeze/thaw cycles. The insoluble material is removed by centrifugation at 11,000 rpm (rotor: SLA-600TC, Sorvall) for 10 min at 4°C. Anti-FLAG M2-agarose beads (1 mL, Sigma, Catalog No. A-1205), equilibrated with lysis buffer, are added to the supernatant. After incubation for 4 h at 4°C, the beads are washed four times with 15 mL of the lysis buffer, and the bound proteins are eluted by incubation for 30 min with 1 mL of 0.2 mg/mL Flag peptide (Sigma Catalog No. F3290) dissolved in lysis buffer. The elution process is repeated three times. The eluate is analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie blue staining as well as by Western blot using antibodies against the RSF subunits. The RSF-containing fractions are pooled, concentrated to 250 μ L with a Millipore microconcentrator (10-kDa cutoff, Catalog No. UFV4BGC00), and then loaded onto a gel filtration column (Amersham Pharmacia Biotech FPLC Superose 6 HR 10/30). The column is equilibrated with buffer BC500 (20 mM Tris-HCl, pH 7.9, 0.2 mM EDTA, 10 mM β -mercaptoethanol, 0.2 mM PMSF, 10% glycerol, 0.5 M KCl). Recombinant RSF elutes with a relative molecular mass of 500 kDa. The final yield is approximately 0.3 mg of RSF per 0.5×10^8 SF9-infected cells (Fig. 1A).

2.2. Core histones

2.2.1. Native histones

Native histone octamers are purified from HeLa cells by a method adapted from Bulger and Kadonaga [21]. Briefly, HeLa nuclei are isolated by incubation with 5×

pellet volume of hypotonic buffer as described [21] and are digested with 200 U/mL micrococcal nuclease to obtain oligonucleosomes ranging between 1 and 2 kb. The oligonucleosomes are purified away from the nuclear proteins through a 5 to 39% sucrose gradient of 36 mL centrifuged at 20,000 rpm (rotor: SW28, Beckman) for 16 h at 4°C. Fractions of 2 mL are collected and an aliquot (20 μ L) is analyzed by SDS-PAGE followed by coomassie blue staining. Fractions containing histone polypeptides are pooled. Finally, histones are removed from the DNA by chromatography on a hydroxyapatite column (Bio-Gel HT Gel, Bio-Rad, Catalog No. 130-0150, capacity 5 mg/mL). This purification scheme yields highly purified, hypoacetylated core histones (Fig. 1B). To obtain hyperacetylated histones, the cells are treated with 10 mM butyric acid and/or 0.1 μ M trichostatin A (TSA) for 24 h prior to harvesting. In addition, buffers must contain histone deacetylase inhibitors (10 mM butyric acid and/or 0.1 μ M TSA) during all of the purification steps described above. Analysis of the extent of acetylation of the different histone polypeptides is performed by electrophoresis on a Triton-acetic acid-urea (TAU) gel, which separates molecules on basis of charge and mass (Fig. 1D), following the protocol described by Lennox and Cohen [22] [for review on histone acetylation, see [23]]. Core histone octamers can be separated into H2A/H2B dimers and H3/H4 tetramers by MonoS column chromatography [24].

2.2.2. Recombinant histones

2.2.2.1. Histone polypeptides. Recombinant *Xenopus laevis* histones were expressed and purified by a method adapted from Luger et al. [25]. Although the original purification scheme has three chromatographic steps, we found that the isolation of inclusion bodies gives rise to a highly enriched histone fraction that can then be used to reconstitute core histone octamers in vitro. After the final sizing column, the core histones are highly purified.

BL21 (DE) pLysS bacteria are transformed with the pET-histone expression plasmids and plated on Luria broth (LB) with 100 μ g/mL ampicillin and 25 μ g/mL chloramphenicol. The plates are incubated at 37°C overnight. The next day, 5 mL of LB medium containing ampicillin and chloramphenicol is inoculated with a single bacterial colony, and the culture is grown overnight at 37°C. It is recommended that induction be tested on five different cultures. The next morning, a 5-mL aliquot of LB medium containing ampicillin and chloramphenicol is inoculated with 50 μ L of the overnight cultures, and the suspension is then grown until the OD_{600} is 0.5–0.7. At this point, histone expression is induced with 0.2 mM IPTG, and the cultures are incubated for an additional 2 h at 37°C. The level of induction is checked on an 18% SDS-polyacrylamide gel, where the expression levels of histones in the IPTG-induced cultures are compared with uninduced levels.

Once a culture with high levels of induction is selected, 1 L of LB medium containing ampicillin and chloramphenicol is inoculated with 1 mL of the overnight suspension of that culture. The 1 L culture is then induced for 2 h with 0.2 mM IPTG when it reaches an OD₆₀₀ of 0.5–0.7. The bacteria are then harvested by centrifugation at 5000 rpm (rotor: SLA-3000, Sorvall) for 10 min, and the pellet is resuspended in 100 mL wash buffer (50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 1 mM Na–EDTA, 1 mM benzamidine) and frozen in liquid nitrogen. The solution is then thawed quickly in a warm water bath. The viscosity resulting from the bacterial lysate is reduced by 3 s of sonication (Sonic Dismembrator 550, Fisher Scientific), and the resulting solution is centrifuged at 12,000 rpm (rotor: SLA-600TC, Sorvall) for 20 min at 4°C. The pellet, which contains the inclusion bodies of the corresponding histone proteins, is washed twice with 100 mL wash buffer plus 1% Triton X-100, washed twice with wash buffer without Triton, and centrifuged at 12,000 rpm (rotor: SLA-600TC, Sorvall) for 10 min at 4°C. The pellet is resuspended in 30 mL of unfolding buffer (7 M guanidinium–HCl; 20 mM Tris–HCl, pH 7.5, 10 mM DTT) and mixed gently for 1 h at room temperature. The unsolubilized material is removed by centrifugation at 12,000 rpm (rotor: SLA-600TC, Sorvall) for 15 min at 20°C. The final yield is approximately 100 mg.

2.2.2.2. Histone octamers. Histone octamers are obtained by mixing the four unfolded *X. laevis* recombinant histones isolated as described above in equimolar amounts with approximately 4 mg of total protein (in about 1 mL volume). The mixture is dialyzed at 4°C against 2 L of refolding buffer (2 M NaCl, 10 mM Tris–HCl, pH 7.5, 1 mM Na–EDTA, 5 mM β -mercaptoethanol) with at least three buffer changes. Either the second or third dialysis step should be performed overnight. The sample is centrifuged to remove any precipitate material and proteins are concentrated to 250 μ L with a Millipore microconcentrator (10-kDa cutoff, Catalog No. UFV4BGC00). Then the sample is loaded onto a HiLoad Superdex 200 HR 10/30 column (Amersham Pharmacia Biotech) equilibrated with refolding buffer. Recombinant core histone octamer elutes with a relative molecular mass of 100 kDa. An aliquot of the column fractions is analyzed by SDS–polyacrylamide (18%) gel electrophoresis followed by Coomassie blue staining. After the histone octamer-containing fractions are pooled, the concentration is determined spectrophotometrically ($A_{276} = 0.45$ for a solution of 1 mg/mL core octamer). The purified octamers are stored at 4°C, at a concentration of 1 mg/mL. They are stable for at least 6 months (Fig. 1C).

The H2A/H2B dimers and the H3/H4 tetramers can be obtained using the same procedure, by mixing equimolar amounts of H2A and H2B or equimolar amounts

of H3 and H4 followed by dialysis in the refolding buffer and purification using Superdex 200. Recombinant H2A/H2B dimers elute with a relative molecular mass of 25 kDa, while H3/H4 tetramers elute with a relative molecular mass of 50 kDa.

3. Chromatin assembly and analysis

3.1. Chromatin assembly

Chromatin assembly reactions are performed as described previously [12]. In a 0.6-mL siliconized Eppendorf tube, a 3-kb supercoiled DNA template (2 μ g, pG5MLP) is combined with 1.8 μ g of native or recombinant core histones (or equimolar amounts of H2A/H2B plus H3/H4), 0.3 μ g of RSF, 150 μ g of bovine serum albumin, 3 mM ATP, 30 mM phosphocreatine (Sigma, Catalog No. P-6502), 0.2 μ g of phosphocreatine kinase (Sigma, Catalog No. C-3755), 5 mM MgCl₂, 50 mM KCl, 10 mM Hepes, pH 7.6, 0.2 mM EDTA, and 5% glycerol in a final reaction volume of 150 μ L. The reaction typically is incubated at 30°C for 5 h, but it can also be incubated overnight without affecting the efficiency of chromatin assembly. Chromatin assembled by this method can be stored at 4°C for at least 2 weeks without loss of nucleosomes.

The ratio between DNA and core histones in the chromatin assembly reaction is critical and needs to be titrated for every new DNA and histone preparation. If the ratio is not correct, chromatin assembly will not be successful.

When recombinant histones are used to assemble chromatin, the RSF-dependent reaction requires the acetylation of histones H2A and H2B [12]. Therefore, the RSF-dependent chromatin assembly reaction is not efficient when recombinant histones are used. A way around this problem is to acetylate H2A/H2B dimers using histone acetyl transferase (HAT) p300 and purify the acetylated dimers on a size exclusion column (Superdex 200, Amersham Pharmacia Biotech). Chromatin assembly can then be performed as described above, except that, rather than using core histone octamers, acetylated recombinant H2A/H2B dimers are combined with equimolar amounts of recombinant H3/H4. Another strategy is to mix in equimolar amounts native MonoS-purified H2A/H2B dimers with recombinant H3/H4. Both methods result in efficient chromatin assembly.

3.2. Analysis of the chromatin assembly reactions

The efficiency of the chromatin assembly reaction can be evaluated by partial micrococcal nuclease digestion, electron microscopy, and supercoiling assays. The results from each of these assays gives specific information

about the quality of the assembly reaction. Micrococcal nuclease digestion evaluates the spacing between nucleosomes in such a way that if nucleosomes are regularly spaced, a DNA ladder is observed. Electron microscopy evaluates the efficiency of chromatin assembly by offering the investigator a visual image of the complete population of DNA molecules. Moreover, the amount of assembly on an individual DNA molecule can also be analyzed. Due to the fact that not all the laboratories have an electron microscopy facility for day-by-day experiments, this assay can be replaced by supercoiling experiments. Supercoiling assays examines the efficiency of histone deposition in the entire population of DNA molecules, but do not provide information on nucleosome spacing. The information garnered from these assays is necessary to evaluate the efficiency and quality of the chromatin assembly reaction.

3.2.1. Partial micrococcal nuclease digestion

Micrococcal nuclease digestion is performed with 1 μ g of DNA in the following buffer: 10 mM Hepes, pH 7.6, 50 mM KCl, 5 mM MgCl₂, 0.2 mM EDTA, 10% glycerol supplemented with 3 mM CaCl₂ (final reaction volume: 80 μ L). To obtain the most information out of this type of experiment, typically, two different empirically determined concentrations of micrococcal nuclease are used to characterize a single chromatin preparation. For example, one can use 10 μ L of a 20 unit/mL prep-

aration of micrococcal nuclease (Sigma, Catalog No. N-5386) in one reaction and an order of magnitude less enzyme in a second reaction.

The micrococcal nuclease digestion reaction is carried out for 10 min at 30 °C and is stopped by the addition of 80 μ L of stop buffer (20 mM EDTA, 0.2 M NaCl, 1% SDS, 0.25 mg/mL glycogen), containing 0.07 mg/mL proteinase K. The reaction is then incubated for an additional 15 min at 37 °C. The DNA is then extracted with 160 μ L phenol/chloroform, and 120 μ L of the aqueous phase is collected. Ammonium acetate (5 μ L of a 2.5 M stock) is added to the solution and the DNA is precipitated with 600 μ L of ethanol (ethanol is stored at -80 °C). The pellet is then washed with 70% ethanol, air-dried, and resuspended in 10 μ L of sample buffer (10 mM Tris, pH 7.9, 1 mM EDTA, 0.1% bromophenol blue, 10% glycerol). The DNA products are resolved on a 1.3% agarose gel (Bio-Rad, Sub-Cell Model 192, 25 \times 15-cm trays) in 50 mM Tris/0.38 M glycine buffer. The gel is subjected to electrophoresis at 170 V for 4 h or at 50 V overnight. After electrophoresis, the gel is rapidly stained for 10 min with ethidium bromide and then destained with several washes of running buffer.

When chromatin is regularly spaced, the DNA fragments appear as a DNA ladder. If chromatin is not formed or it is not periodically spaced, DNA fragments appear as a smear, with strong bands at the bottom of the gel, representing mononucleosomes (Fig. 2).

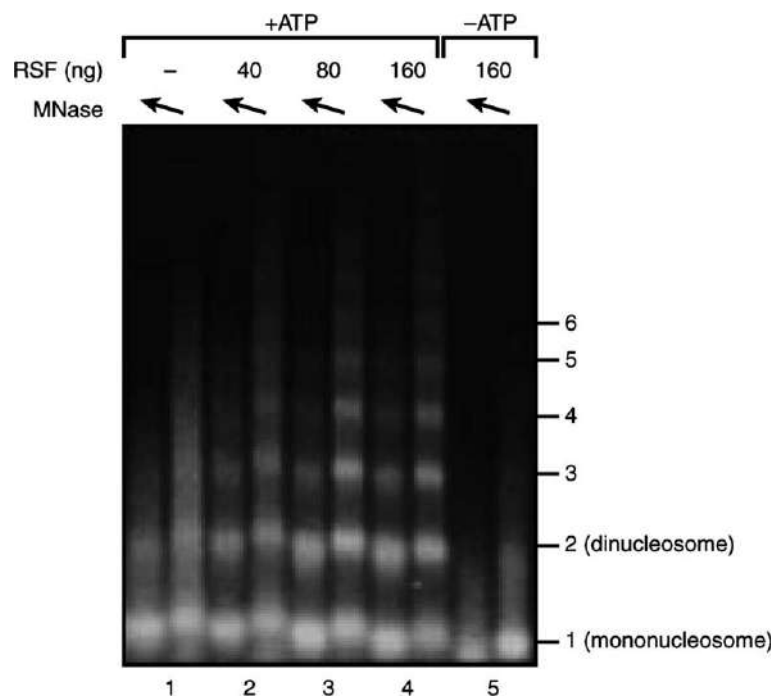


Fig. 2. Micrococcal nuclease digestion of RSF-assembled chromatin. Ethidium bromide staining of micrococcal nuclease digestions of chromatin assembled by RSF. Chromatin assembly was performed with increasing amounts of RSF. The largest amount of RSF was also assayed in the absence of ATP (lane 5), as indicated on the figure. Products of the assembly reaction were partially digested with two different concentrations of micrococcal nuclease. The migration of nucleosomes is indicated on the right.

3.2.2. Electron microscopy

A nucleosomal array quickly folds in the presence of salt, forming a higher-order chromatin structure [3]. To avoid this phenomenon, chromatin is desalted before mounting for electron microscopic analysis.

Chromatin is desalted by using a gel filtration column (1 mL of Bio-Gel A5M gel, Bio-Rad) that has been equilibrated with HE buffer (20 mM Hepes, pH 7.9, 0.5 mM EDTA). The desalted chromatin is fixed with 0.6% glutaraldehyde for 10 min at room temperature, and the DNA–protein complexes are purified using gel filtration on A5M column (1 ml resin, Bio-Rad) that has been equilibrated with buffer TE (10 mM Tris, pH 7.9, 0.1 mM EDTA). Fractions of 200 μ L are collected, and chromatin is eluted in fraction 5. The protein–DNA complexes are mixed in a buffer containing 2 mM spermidine, adsorbed to glow-charged carbon-coated grids, washed with a water/graded ethanol series, and rotary shadowed cast with tungsten. Samples are examined using a Philips 420 electron microscope. Micrographs are shown in reverse contrast. A Cohu CCD camera attached to a Macintosh computer programmed with National Institutes of Health (NIH) IMAGE software was used to form the images (Fig. 3).

3.2.3. Supercoiling assay

To investigate the efficiency of the chromatin deposition, the products of the chromatin assembly reaction

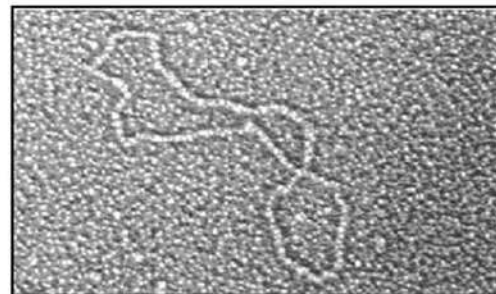
can also be analyzed in a supercoiling assay. The reaction is performed with relaxed DNA, and the deposition of one nucleosome introduces one negative superhelical change in the DNA. Efficient deposition results in the accumulation of supercoiled DNA, which is visualized after deproteinization by electrophoresis on an agarose gel. The gel is able to resolve the various topoisomers present in the preparation.

Supercoiling assays are performed in a final volume of 40 μ L by incubating 0.5 μ g of relaxed DNA (pG5MLP) with 0.45 μ g of a native or recombinant core histone preparation, 75 ng of RSF, 40 μ g of bovine serum albumin, topoisomerase I (from Promega, the amount to be used in the reaction must be determined by titration), 3 mM ATP, 30 mM phosphocreatine (Sigma, Catalog No. P-6502), 0.2 μ g of phosphocreatine kinase (Sigma, Catalog No. C-3755), 5 mM $MgCl_2$, 50 mM KCl, 10 mM Hepes, pH 7.6, 0.2 mM EDTA, 5% glycerol. The reaction is incubated at 30 °C for 2 h. The DNA topoisomers are purified as described in the section for micrococcal nuclease digestion and resolved by electrophoresis in a 1% agarose gel. The gel is quickly stained with ethidium bromide for 10 min.

3.3. Chromatin purification

Some experiments require clean chromatin. While RSF-mediated chromatin assembly yields highly puri-

A Without RSF



B With RSF

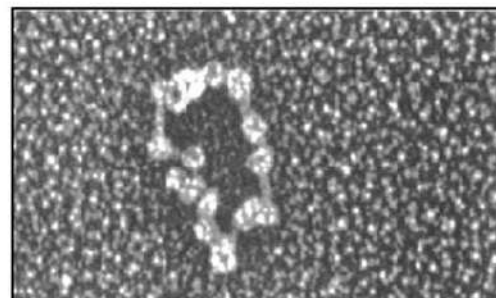
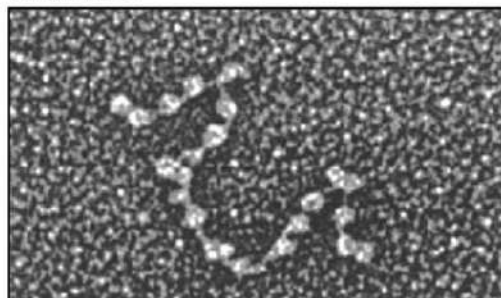


Fig. 3. Chromatin assembled by RSF as visualized by electron microscopy. (A) Electron microscopic visualization of the chromatin assembly reaction in the absence of RSF. DNA size is 3 kb. (B) The chromatin assembly reaction was carried out in the presence of RSF as described in the text using hyperacetylated (left) and hypoacetylated (right) histone polypeptides. The pictures are representatives of the majority of the molecules visualized on the grid.

fied chromatin, RSF is still present in the reaction along with ATP and, in some cases, free histones. By using size exclusion columns, chromatin can be purified from these unwanted components. Of the many options available, we prefer to use small gel filtration columns.

Chromatin is purified over a Sepharose CL-4B column (Amersham Pharmacia Biotech). The resin (4 mL) is packed in a Bio-Rad 0.5 × 20-cm glass Econo-column and equilibrated with 10 mM Hepes, pH 7.5, 10 mM KCl, and 0.5 mM EGTA. The column can be run at room temperature. Chromatin (4 µg in a volume of 300 µL) is loaded onto the column bed and allowed to sink into the resin completely. The buffer is then collected, and 200 µL of buffer is loaded onto the column and collected as one fraction. Buffer aliquots of 250 µL are then added sequentially and collected in different test tubes. This process is repeated six times. The chromatin usually elutes after 1.4 mL of buffer has been added to the column. To analyze the profile of chromatin elution, 5 µL aliquots from each column fraction are mixed with 5 µL H₂O, 0.7 µL 10% SDS, and 2 µL 5× sample buffer (50 mM Tris, pH 7.9, 5 mM EDTA, 0.5% bromophenol blue, 50% glycerol) and incubated for 10 min at 65 °C. The samples are analyzed by electrophoresis in 1% agarose gel.

4. Concluding remarks and future directions

The advantage of the RSF-mediated chromatin assembly reaction over previously described techniques (e.g., salt dialysis, polyglutamic acid treatment, and use of *Drosophila* and *Xenopus* extracts) is the formation of regularly spaced chromatin in a highly defined system. Given that the components of the reaction are well characterized and that the recombinant histones lack posttranslational modifications, this system can be used to decipher subtle chromatin alterations that play a regulatory role in DNA metabolism. For example, as mentioned above, we found that RSF-mediated chromatin assembly requires the acetylation of H2A and H2B for the reaction to be efficient [12]. One can also use this system to study the role of various histone tail modifications in chromatin-mediated transcription as well as other biological processes that use DNA as a substrate.

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