Reconstitution of Nucleosomes with Histone MacroH2A1.2†

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ABSTRACT: MacroH2A histones have an unusual hybrid structure, consisting of an N-terminal domain that is approximately 65% identical to a full-length histone H2A and a large C-terminal nonhistone domain. To develop an in vitro approach for investigating the effects of macroH2A proteins on chromatin structure and function, we reconstituted nucleosomes with recombinant macroH2A1.2, substituting for conventional H2A. Recombinant macroH2A1.2 was able to efficiently replace both of the conventional H2As in reconstituted nucleosomes. The substitution of macroH2A1.2 for H2A did not appear to grossly perturb the basic structure of the nucleosome core, as assessed by sedimentation and by digestion with micrococcal nuclease or DNase I. However, two differences were observed. First, the region around the midpoint of the nucleosomal core DNA was more resistant to digestion by DNase I in nucleosome core particles reconstituted with macroH2A1.2. Second, preparations of core particles reconstituted with macroH2A1.2 had a greater amount of material that sedimented more rapidly than mononucleosomes, suggesting that macroH2A1.2 may promote interactions between nucleosomes. Recombinant macroH2A proteins should be valuable tools for examining the effects of macroH2A on nucleosome and chromatin structure.

MacroH2A histones have an unusual hybrid structure, consisting of a large nonhistone region and a region that closely resembles a conventional histone H2A. These proteins were discovered on the basis of their copurification with rat liver mononucleosomes (*1*). The salt elution profile of macroH2As from chromatin indicated that the H2A region replaces conventional H2A in a subset of nucleosomes. We estimated that there is approximately one molecule of macroH2A for every 30 nucleosomes in rat liver (*1*).

Three macroH2A subtypes have been identified: macroH2A1.1 and macroH2A1.2, which are formed by the alternate splicing of the *macroH2A1* gene, and macroH2A2, which is encoded by the *macroH2A2* gene (*1*−*5*). All three subtypes are very similar in size and have the same general structure. The N-terminal third of these macroH2A subtypes is about 65% identical to a full-length conventional H2A histone. The majority of the nonhistone region appears to be derived from a gene of unknown function whose evolutionary history predates eukaryotes (*6*). Located between the H2A region and the nonhistone domain is a lysine-rich basic region that resembles the basic tails found in other histones (Figure 1). The pattern of macroH2A subtypes present in different tissues and cell types is different and changes during differentiation (*2*, *5*). MacroH2A proteins are highly conserved among vertebrates (*6*), but thus far, *macroH2A* genes have not been found in an invertebrate.

Recent studies showed that macroH2A1.2 (*7*) and macroH2A2 (*4*, *5*) are preferentially concentrated in the inactive X chromosome in comparison to bulk chromatin. The initiation of X inactivation requires an X-linked gene called *Xist* that is only transcribed from the inactive X (*8*−*16*). *Xist* does not appear to encode a protein but rather is transcribed to form a large nuclear RNA that coats the inactive X chromosome in cis. The localization of macroH2A1.2 to the inactive X in fibroblasts was lost when a large segment of *Xist* was deleted by Cre-mediated recombination (*17*). This suggests that *Xist* RNA participates, directly or indirectly, in the localization of macroH2A to the inactive X.

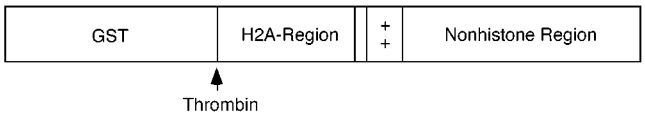

FIGURE 1: Diagram of glutathione-*S*-transferase−macroH2A1.2 fusion protein. Glutathione-*S*-transferase is on the N terminus and is marked GST. The H2A region is residues 1−122 of rat macroH2A1.2, the basic region is marked with + and is residues 133−159, and the nonhistone region is residues 160−370. The arrow marks the site of thrombin cleavage.

The structure and evolutionary conservation of macroH2As suggest that they have specialized functions that are distinct from conventional H2As. On the basis of the localization of macroH2As to the inactive X, we suggested that one function might involve gene silencing (*7*). Although macroH2A clearly appears to replace conventional H2A in the nucleosome core, there are several basic questions concerning how the unusual structural features of macroH2A might effect nucleosome core structure, including (1) is it possible to replace both H2As in a nucleosome core with macroH2As, (2) does the unusual H2A region of macroH2A alter nucleosome core structure, and (3) what effect does the large nonhistone domain have on nucleosome core structure? There are also important questions about the effects of macroH2A on higher-order chromatin structures. One approach to examining the effects of macroH2A on chromatin structure and function is to use chromatin reconstitution. This approach requires a source of highly purified macroH2A protein. Here, we report a method for expressing macroH2A1.2 in bacteria and purifying it from bacterial extracts. We also describe the use of this recombinant macroH2A1.2 protein for

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nucleosome reconstitution, and we characterize some of the features of these reconstituted nucleosomes.

EXPERIMENTAL PROCEDURES

Construction of the MacroH2A1.2 Expression Vector. The cDNA sequences encoding the non-H2A region of macroH2A1.2 were inserted into the expression vector pGEX-4T-1 (Amersham Pharmacia Biotech, Cleveland, OH) using a *Bam*H I site that is located near the end of the H2A region of rat *macroH2A1.2*. The H2A region of rat *macroH2A1.2* was amplified by polymerase chain reaction (PCR). A *Bam*H I site was added to the 5′ primer used in this reaction to facilitate insertion of this fragment into the construct. The PCR product was inserted into the *Bam*H I site of the non-H2A region pGEX-4T-1 construct. DNA sequencing was used to confirm the integrity of all of the junction sequences and to ensure that no mutations were introduced during PCR.

Expression and Purification of the MacroH2A1.2 Protein. The *macroH2A1.2* pGEX-4T-1 plasmid was used to transform *Escherichia coli* strain BL21, and the transformed bacteria were cloned. The transformed bacteria were inoculated into 300 mL of Luria−Bertani (LB) medium containing 50 μg/mL ampicillin and grown for 2−2.5 days at room temperature. The 300 mL culture was added to 1500 mL of the LB medium containing 50 μg/mL ampicillin and grown for 1 h at room temperature. Protein expression was then induced by adding isopropyl β-D-thiogalactopyranoside to a concentration of 0.1 mM, and cells were cultured for an additional 3 h at room temperature. All of the subsequent steps were performed on ice or at 4 °C. Bacteria were collected by centrifugation and resuspended in a 1/10 volume (180 mL) of 0.5% IGEPAL CA-630 (Sigma, St. Louis, MO; used as a substitute for Nonidet P-40), 20 mM Tris (pH 8.0), 100 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA),[1] 5 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1.5 μg/mL aprotinin. Samples were sonicated until there was a noticeable decrease in turbidity. Insoluble material was removed by centrifugation. The supernatant was mixed with a 1/10 volume (18 mL) of glutathione Sepharose 4B beads (Amersham Pharmacia Biotech) and rocked overnight. The beads were collected by centrifugation, washed four times with 80 mL of 0.15 M NaCl and 10 mM Tris (pH 7.5) (TBS) and once with 80 mL of 0.35 M NaCl and 10 mM Tris (pH 7.5), followed by four times with 80 mL of TBS. Beads were then resuspended in 18 mL of TBS. Forty-five NIH units of bovine thrombin (Sigma; T 7513) were added, and the beads were rocked for 4.5 h. After thrombin treatment, the volume was adjusted to 60 mL with TBS, and 12.5 mL of 5 M NaCl was added; this brought the NaCl concentration to 1 M, which improved the extraction of macroH2A1.2 from the beads. Rocking was continued for 15 min. The supernatant was collected and diluted 2-fold with 10 mM Tris (pH 7.5), and PMSF was added to a concentration of 0.2 mM and DTT to 1 mM.

The solution was then applied to a column containing 5 mL of Macro Prep High S (Bio-Rad Laboratories, Randolph, MA) that was equilibrated with 10 mM Tris (pH 7.5), 0.5 M NaCl, 1 mM DTT, and 0.2 mM PMSF. The column was washed with 300 mL of a solution containing 10 mM Tris (pH 7.5), 0.6 M NaCl, 0.2 mM PMSF, and 1 mM DTT. The column was eluted with a 300 mL gradient from 0.6 to 2.0 M NaCl, with both solutions containing 10 mM Tris (pH 7.5), 0.2 mM PMSF, and 1 mM DTT. The absorbance at 280 nm was measured, and the fractions around the major peak (peak absorbance was approximately 0.1) were analyzed by electrophoresis in a 15% acrylamide sodium dodecyl sulfate (SDS) gel (*18*). Fractions that contained appreciable amounts of macroH2A1.2 were pooled and applied to a column containing 5 mL of hydroxyapatite, which was equilibrated with 1 M NaCl, 1 mM $NaPO_4$ (pH 7.0), 0.2 mM PMSF, and 1 mM DTT. The column was washed with 300 mL of 1 M NaCl, 1 mM $NaPO_4$ (pH 7.0), 0.2 mM PMSF, and 1 mM DTT. The column was eluted with a 160 mL gradient from 1 to 100 mM $NaPO_4$; both solutions contained 1 M NaCl, 0.2 mM PMSF, and 1 mM DTT, and the phosphate was at pH 7.0. The absorbance at 280 nm was measured, and the fractions around the major peak were analyzed by electrophoresis in a 15% acrylamide SDS gel.

The purified macroH2A1.2 was relatively stable when stored for several weeks or even longer at 4 °C. Some preparations showed a slow accumulation of the degradation products. This proteolytic activity was inhibited by the addition of the protease inhibitor cocktail Complete (Roche Molecular Biochemicals, Mannheim, Germany).

Sources of Histones. The core histone pairs H2A/H2B and H3/H4 were purified from chicken red blood cells or rat liver chromatin (*19*). H2A was separated from H2B by chromatography on Bio-Gel P-100 (Bio-Rad Laboratories) in 20 mM HCl and 0.3 M NaCl (*20*). Prior to mixing with the other histones, the H2A and H2B fractions were neutralized with a solution of Tris base. The core histones were mixed in equal proportions, as estimated by Coomassie Brilliant Blue R-250 staining of bands separated by electrophoresis in 15% acrylamide SDS gels. Histone concentrations were estimated by comparison to histone standards that were stained with Coomassie Blue. In the mixtures that contained macroH2A1.2, the intensity of the macroH2A1.2 band was adjusted to be approximately 2.5 times darker than that for H2B in order to compensate for the difference in molecular weight.

Sources of DNA Used for Reconstitution. Salmon sperm DNA was from 5 Prime → 3 Prime, Inc. (Boulder, CO). Mononucleosomal DNA was isolated from rat liver chromatin. Briefly, rat liver nuclei were isolated as described previously (*5*), and an S2 chromatin fraction was prepared (*21*). H1 was extracted from the chromatin using CM-Sepharose CL-6B (*22*). The H1-stripped chromatin was digested with micrococcal nuclease for 30 min at 37 °C; the amount of nuclease added was optimized for the production of nucleosome core-particle length DNA (∼146 bp) by analyzing test digestions on 2% agarose gels. Digestions were terminated by the addition of EDTA to a concentration of 5 mM and were then centrifuged at 7000*g* for 20 min. The supernatant was collected, NaCl was added to a concentration of 1 M, SDS was added to 1%, and the mixture was incubated at 55 °C for 20 min. The solution was then extracted twice with phenol/chloroform (1:1), and the DNA was precipitated by adding two volumes of ethanol. The precipitate was dissolved in 10 mM Tris (pH 7.5) and 1 mM

[1] Abbreviations: DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; TBS, Tris buffered saline.

EDTA and then was dialyzed against the same buffer. The concentration of mononucleosomal DNA was estimated by ethidium bromide staining of bands separated by electrophoresis in a 2% agarose gel (*23*).

Nucleosome Reconstitution. DNA and histones were mixed with a final DNA concentration of approximately 150 μg/mL and a final histone concentration of approximately 80 μg/mL. The total volume of the reconstitution mixture was 5.5 mL. PMSF was added to a concentration of 0.2 mM and DTT to 1 mM. Nucleosomes were reconstituted by salt dialysis using a variation of the procedure of Tatchell and Van Holde (*24*). All of the dialysis solutions contained 10 mM MOPS (pH 7.0), 0.5 mM EDTA, and 10 mM 2-mercaptoethanol. Samples were initially dialyzed against 2 M NaCl for 6 h and then overnight against 1.5 M NaCl. This was followed by dialysis for 4 h against 1 M NaCl, 4 h against 0.75 M NaCl, 3 h against 0.5 M NaCl, and overnight against 0.1 M NaCl. After dialysis, the samples were concentrated to 1−1.5 mL by centrifugal ultrafiltration with Centriplus YM-100 filters (Millipore, Bedford, MA).

The concentrates were layered onto 5−20% sucrose gradients containing 0.1 M NaCl, 10 mM MOPS (pH 7.0), 1 mM EDTA, 1 mM DTT, and 0.2 mM PMSF. Gradients were prepared in 36 mL tubes for the AH-629 rotor (Du Pont Sorvall, Wilmington, DE) and spun for 21 h at 25 000 rpm. Fractions (1.5 mL) were collected by puncturing the bottom of the tube. The fractions were analyzed for mononucleosomal DNA by electrophoresis in 2% agarose gels stained with ethidium bromide. Prior to electrophoresis, the DNA was dissociated from the histones by adding SDS to 0.2% and incubating for 20 min at 37 °C (*25*). The protein composition of the fractions was analyzed by electrophoresis in 15% acrylamide SDS gels. Proteins were concentrated by precipitation with 20% trichloroacetic acid prior to electrophoresis (*2*). The rat liver mononucleosomes used as a standard in the sedimentation analysis were prepared by digesting rat liver nuclei with micrococcal nuclease and collecting the S1 chromatin fraction (*21*). This fraction contains predominately H1-free mononucleosomes.

In the experiments that used salmon sperm DNA, nucleosome reconstitution was carried out as described previously except that the final concentration of NaCl was 10 mM rather than 0.1 M. $CaCl_2$ was added to a concentration of 2 mM and micrococcal nuclease to 75 units/mL, and samples were digested for 20 min at 37 °C. To inhibit proteases that contaminated the commercial preparations of micrococcal nuclease that we tested, a 10× stock solution of micrococcal nuclease was preincubated with α_2-macroglobulin (Roche Molecular Biochemicals) at 1 mg/mL for 45 min at 37 °C. We then added E-64 (Roche Molecular Biochemicals) to a concentration of 10 μg/mL and Pefabloc SC (Roche Molecular Biochemicals) to a concentration of 4 mM and preincubated for an additional 15 min at 37 °C. Digestion was stopped by adding EDTA to a concentration of 5 mM. SDS was added to 0.2%, the mixture was heated to 37 °C for 20 min and run in a 2% agarose gel stained with ethidium bromide.

DNase I Digestions. Samples containing approximately 2 μg of DNA in 100 μL were prepared from selected fractions of the sucrose gradients. E-64 was added to a concentration of 2.5 μg/mL and Pefabloc SC to a concentration of 1 mM to inhibit proteases. Samples were end-labeled by adding $MgCl_2$ to a concentration of 2 mM, five units of T4 polynucleotide kinase (New England Biolabs, Beverly, MA), and 20 μCi of γ ^{32}P-ATP and by incubating at 37 °C for 30 min. Pancreatic DNase I (USB) was added to a concentration of 25 units/mL, and the digest was incubated at 37 °C. Twenty microliter samples were removed at times ranging from 15 s to 3 min of digestion and stopped by adding EDTA to a concentration of 25 mM and boiling them for 1 min. Proteinase K was added, and the samples were incubated overnight at 55 °C. Glycogen was added as a carrier, and the DNA was precipitated with ethanol. Samples were dissolved in 95% formamide containing 20 mM EDTA and were boiled and run in a 12% acrylamide gel containing 7 M urea (*23*). The gel was dried and autoradiographed. A 25 bp DNA ladder (Life Technologies, Metuchen, NJ) was used as a marker.

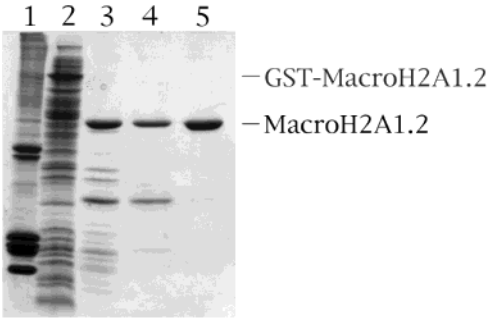

FIGURE 2: Purification of macroH2A1.2. The protein compositions of samples taken from various stages of purification were analyzed by electrophoresis in a 15% acrylamide SDS gel: (lane 1) an extract of rat liver nuclei used to mark the location of macroH2A1.2, (lane 2) an extract of *E. coli* expressing the GST−macroH2A1.2 fusion protein, (lane 3) proteins released from glutathione sepharose affinity beads by cleavage with thrombin, (lane 4) pooled fractions eluted from Macro Prep High S column, and (lane 5) peak tube from a hydroxyapatite column.

RESULTS

Expression and Purification of Histone MacroH2A1.2. MacroH2A1.2 was expressed in *E. coli* as a glutathione-*S*-transferase (GST) fusion protein (see Figure 1). The bacteria were grown at room temperature, because very little soluble fusion protein was recovered when expression was carried out at 37 °C. The fusion protein was separated from most of the other proteins in the extract by binding it to glutathione beads. MacroH2A1.2 was cleaved from GST and released from the beads by incubating the bound fusion protein with thrombin. The macroH2A1.2 released by thrombin cleavage is identical in sequence to rat macroH2A1.2 except for the addition of two amino acids (one glycine and one serine) to the N terminus of the recombinant protein. At this stage, the preparation had significant contamination from other proteins, including what appeared to be degradation products of macroH2A1.2 (Figure 2 , lane 3). There also appeared to be contamination by nucleic acids, as indicated by the ratio of absorbance at 260 nm to that at 280 nm. Additional enrichment for macroH2A1.2 was achieved by chromatography on Macro Prep High S (BioRad) and hydroxyapatite. These chromatographic steps removed most of the contaminating nucleic acid, as indicated by a reduction in the ratio of absorbance at 260 nm to that at 280 nm to about 0.6. Most of the contaminating proteins were also removed, as was indicated by electrophoresis in an acrylamide gel containing SDS (Figure 2).

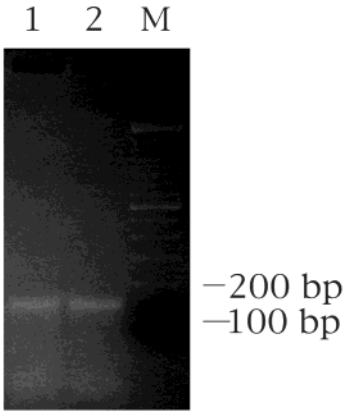

FIGURE 3: Micrococcal nuclease digestion of reconstituted nucleosomes. Nucleosomes were reconstituted with salmon sperm DNA and a mixture of H2B, H3, H4, and either macroH2A1.2 or H2A. Samples of each were digested with micrococcal nuclease, and the protected fragments were analyzed by electrophoresis in a 2% agarose gel: (lane 1) digest of macroH2A1.2-containing nucleosomes, (lane 2) digest of H2A-containing nucleosomes, and (lane M) DNA marker.

Reconstitution of Nucleosomes with MacroH2A1.2. Nucleosomes were reconstituted from mixtures of purified histones and DNA by salt dialysis (*24*). Initially the histones were reconstituted with salmon sperm DNA. After reconstitution, the mixtures were tested for the presence of nucleosomes by digestion with micrococcal nuclease. Conventional nucleosomes give rise to micrococcal nuclease-resistant fragments of approximately 146 bp. Three different histone mixtures were used: H2A, H2B, H3, and H4 (H2A mixture); macroH2A1.2, H2B, H3, and H4 (macroH2A1.2 mixture); and H2B, H3, and H4 (no-H2A mixture). The reconstitution reactions containing the H2A mixture or the macroH2A1.2 mixture both formed nucleosomes as indicated by the presence of nuclease-resistant DNA fragments of approximately 146 bp in micrococcal nuclease digests (Figure 3). As expected, no evidence of nucleosome formation was seen with the no-H2A mixture (data not shown).

Our attempts to purify nucleosome core particles produced by the micrococcal nuclease digestion of chromatin reconstituted with salmon sperm DNA produced unsatisfactory results because of proteases that contaminated the commercial preparations of micrococcal nuclease that we tested. These proteases were active on macroH2A1.2 but had no noticeable effect on the other core histones. The broad-spectrum protease inhibitor α_2-macroglobulin was effective at minimizing this activity such that little or no degradation of macroH2A1.2 was observed immediately following digestion with micrococcal nuclease (data not shown). However, we were unsuccessful at completely eliminating the proteolytic activity, and noticeable degradation of macroH2A1.2 occurred during the sedimentation of nucleosomes in sucrose gradients. We, therefore, switched to a procedure that involved the reconstitution of histones directly with DNA isolated from nucleosome core particles (*24*). This approach directly reconstitutes nucleosome core particles, thus eliminating the need for digestion with micrococcal nuclease.

Reconstitution reactions with core-particle DNA were carried out by salt dialysis using the H2A, macroH2A1.2, or no-H2A histone mixtures. The reconstitution products were concentrated by ultrafiltration and layered onto sucrose gradients for sedimentation. A fourth gradient with rat liver

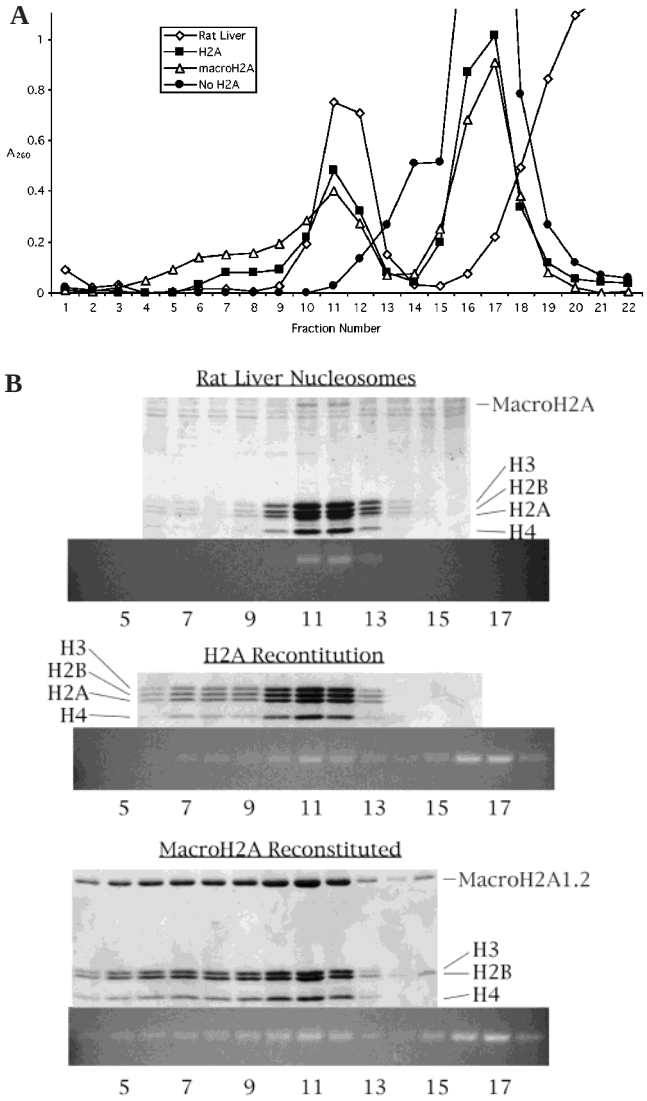

FIGURE 4: Sedimentation analysis of nucleosomes reconstituted with nucleosome core DNA. Nucleosomes were reconstituted with nucleosome core DNA and a mixture of histones H2B, H3, H4, and either conventional H2A or macroH2A1.2; a no-H2A mixture was included as a control. Following reconstitution, the mixtures were sedimented in sucrose gradients, and fractions were collected. Fraction 1 is the bottom of the gradient. A gradient with rat liver mononucleosomes was run to mark the position of mononucleosomes. (A) The absorbance at 260 nm of each fraction is plotted. (B) The analysis of proteins and DNA in selected fractions is shown. Proteins were resolved in 15% acrylamide SDS gels. Nucleosome core DNA was analyzed by electrophoresis in 2% agarose gels that were stained with ethidium bromide. The fraction numbers are indicated below the DNA gel.

mononucleosomes was included as a standard for locating the position of mononucleosomes in the gradients. After sedimentation, fractions were collected from the gradients and analyzed for DNA content by absorbance at 260 nm (Figure 4A) and by agarose gel electrophoresis (Figure 4B). The protein composition of the fractions was analyzed by SDS gel electrophoresis (Figure 4B). The DNA and protein profiles both indicated that the peak fraction for rat liver mononucleosomes was 11. The samples reconstituted with H2A and macroH2A1.2 both had a DNA and core histone peak at fraction 11. This indicates that mononucleosomes were formed from both of these mixtures. As expected, the sucrose gradient from the sample reconstituted with the no-H2A mixture did not have a peak at fraction 11. A DNA

peak at fraction 17 was present in all three sucrose gradients of the reconstituted samples. This peak appears to consist of free nucleosome core DNA, as indicated by the virtual absence of histones; a small amount of macroH2A1.2 and H2B was present in the leading edge of this peak from the macroH2A reconstitution mixture in the experiment shown in Figure 4B, while no histone was detected in this peak in an independent experiment or in gradients of reconstitutions with the H2A mixture. The no-H2A gradient had a shoulder at fraction 14 that contained mononucleosomal DNA, H3, H4, and a small amount of H2B (data not shown). This shoulder was absent from the other gradients and probably contained H3−H4 tetramers bound to nucleosome core DNA.

The samples reconstituted with the H2A mixture and the macroH2A1.2 mixture showed some material that sedimented faster than mononucleosomes. This material contained mononucleosomal DNA and all four of the core histones in ratios that were similar to what was present in the mononucleosome peak. The amount of rapidly sedimenting material was greater in reconstitutions with macroH2A1.2, and this material extended farther down the gradient than it did with the reconstitutions with conventional H2A (Figure 4).

DNase I Digestion of Reconstituted Nucleosomes. We also characterized our reconstituted nucleosomes by DNase I digestion. Samples from the mononucleosome peaks of the sucrose gradients (fraction 11) were end-labeled with ^{32}P phosphate and digested with DNase I (*26*). As a control, we also labeled and digested a sample from the free-DNA peak (fraction 17). The digestion products were analyzed on a denaturing acrylamide gel. The DNase I digest of fraction 17 showed a smear of fragments (data not shown), as expected for free DNA. In contrast, the digests of the reconstituted nucleosomes showed a highly regular pattern of fragments with peaks at approximately 10 base intervals (Figure 5), as expected for nucleosome core particles (*26*). The pattern of DNase I cutting sites in nucleosomes reconstituted with H2A was similar to that obtained from nucleosomes reconstituted with macroH2A1.2. Both patterns had prominent cutting at sites 2, 4, and 5 helical turns from the labeled 5′ end and less cutting at sites 3, 6, and 8. The relative cutting at site 7 (S7 in Figure 5) was noticeably greater for the nucleosomes reconstituted with H2A than for those reconstituted with macroH2A1.2. This difference at site 7 was observed in two independent reconstitution experiments and was largely insensitive to changes in the length of the DNase I digestion (results not shown). The DNase I digestion patterns of the reconstituted nucleosomes were similar to those obtained by the digestion of rat liver nucleosome core particles (Figure 5). The rat liver nucleosome pattern more closely resembled the pattern obtained with nucleosomes reconstituted with macroH2A1.2 in having a relatively low cutting frequency at site 7. A study by Tatchell and Van Holde also observed increased cutting at site 7 for reconstituted nucleosome core particles in comparison to nucleosome core particles prepared by the micrococcal nuclease digestion of isolated chromatin (*27*).

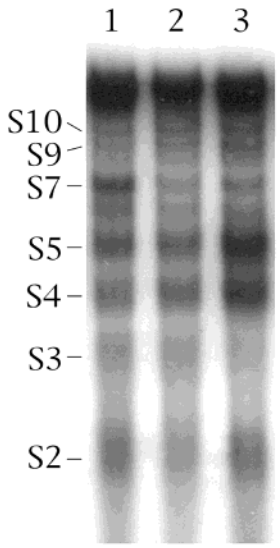

FIGURE 5: DNase I digestion patterns of reconstituted nucleosomes. Reconstituted nucleosome core particles were labeled with ^{32}P on their 5′ ends and then digested with DNase I. The DNA was denatured and run in an acrylamide gel containing 7 M urea, and the gel was dried and autoradiographed. Reconstituted nucleosome core particles were from fraction 11 of the sucrose gradients shown in Figure 4: (lane 1) nucleosomes reconstituted with H2A, (lane 2) nucleosomes reconstituted with macroH2A1.2, and (lane 3) rat liver nucleosome core particles. Sites of DNase I cutting are labeled S2, S3, S4, and so forth, with the number indicating the number of helical turns of DNA from the labeled 5′ end.

DISCUSSION

Our previous studies of histone macroH2A indicated that it replaces conventional H2A in a subset of nucleosomes (*1*). Here, we report the use of nucleosome reconstitution to directly examine the ability of macroH2A1.2 to substitute for H2A in the nucleosome core. We assessed nucleosome reconstitution by digestion with micrococcal nuclease, sedimentation in sucrose gradients, and digestion with DNase I. Nucleosomes reconstituted with macroH2A1.2 or conventional H2A both protected approximately 146 bp of DNA from micrococcal nuclease digestion. The sedimentation rate of nucleosome core particles reconstituted with macroH2A1.2 was very similar to that of core particles reconstituted with conventional H2A and to rat liver mononucleosomes. We also found that the DNase I digestion pattern of nucleosomes reconstituted with macroH2A1.2 was similar to that of rat liver nucleosomes and to nucleosomes reconstituted with conventional H2A. These results all indicate that macroH2A1.2 can occupy both of the H2A sites in the core region of the nucleosome and that the basic structure of the nucleosome core is not grossly altered by the presence of macroH2A1.2.

Our assays of the reconstituted nucleosomes detected two differences between nucleosomes that contain macroH2A1.2 and those that contain conventional H2A. One difference is in the DNase I digestion pattern. Digestion at the site that is seven helical turns of DNA from the end of nucleosome core DNA (marked S7 in Figure 5) was noticeably reduced in nucleosomes containing macroH2A1.2 as compared to those with conventional H2A. This site corresponds to the midpoint of the nucleosomal core DNA and, in the crystallographic structure of the nucleosome core particle (*28*), is referred to as superhelix location zero, or SHL0. The C terminus of conventional H2A is located close to SHL0 in the crystallographic structure of the nucleosome core particle (*28*) and can cross-link to DNA next to SHL0 in isolated core particles (*29*). This indicates that regions in or extending beyond the C terminal part of the H2A region of macroH2A1.2 could interact with DNA or with histones around SHL0. Thus, the

reduced DNase I cleavage rate at this site in nucleosomes reconstituted with macroH2A1.2 could involve a direct effect of macroH2A1.2 on the accessibility or conformation of DNA around SHL0. It is also possible that the nonhistone region shields SHL0 from DNase I without directly interacting with this region.

It is not clear whether this effect of macroH2A1.2 at SHL0 in nucleosome core particles is relevant to intact chromatin. Cross-linking studies suggest that the interaction of the C terminus of conventional H2A with SHL0 observed in core particles may be significantly reduced or absent in intact chromatin (*29*, *30*). On the other hand, the location of the C terminus of conventional H2A in core particles and chromatin may not be a reliable indicator of the location of the corresponding region of macroH2A, given the significant structural differences that exist between these proteins in this region (*1*).

The other difference we observed was that the mixtures reconstituted with macroH2A1.2 and nucleosome core-particle DNA had a significant amount of material that sedimented more rapidly than mononucleosomes. Smaller amounts of rapidly sedimenting material were also present in our reconstitution mixtures with conventional H2A and core-particle DNA. This material was observed in previous studies of reconstituted nucleosome core particles, and it appears to involve an association between two mononucleosome cores that forms during reconstitution (*24*). It is not known how these structures are held together or whether they involve interactions that occur in intact chromatin. There are several ways that macroH2A could promote nucleosome−nucleosome interaction including core−core interactions involving the H2A region, protein−DNA interaction involving the basic region, and a self-association of the nonhistone region. Previously, we found that the nonhistone region of macroH2A1.1 can form dimers, although most of it behaves as a monomer (*31*). If macroH2A1.2 promotes nucleosome−nucleosome interactions in the context of chromatin fibers, it could stabilize higher-order structures.

Reconstitution of nucleosomes and chromatin is a valuable approach for examining nucleosome structure and function. A critical limitation of this approach for examining the effects of unusual core histones is the difficulty in obtaining a sufficient amount of protein in a highly purified form. Existing methods for expressing recombinant histones typically involve denaturation/renaturation procedures (*32*). While these procedures work well for core histones, we chose not to use them for macroH2A1.2 because we are currently unable to determine whether the nonhistone region has been correctly renatured. Here, we provide a method for expressing macroH2A1.2 in bacteria in a soluble form and for the purification of a full-length protein. This recombinant macroH2A1.2 can be reconstituted into nucleosomes with an efficiency that is comparable to conventional H2A. Studies of reconstituted macroH2A-containing nucleosomes and nucleosomal arrays should provide insights into the effects of macroH2A on chromatin structure and function.

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REFERENCES

1. Pehrson, J. R., and Fried, V. A. (1992) *Science 257*, 1398−1400.
2. Pehrson, J. R., Costanzi, C., and Dharia, C. (1997) *J. Cell. Biochem. 65*, 107−113.
3. Rasmussen, T. P., Huang, T., Mastrangelo, M. A., Loring, J., Panning, B., and Jaenisch, R. (1999) *Nucleic Acids Res. 27*, 3685−3689.
4. Chadwick, B. P., and Willard, H. F. (2001) *Hum. Mol. Genet. 10*, 1101−1113.
5. Costanzi, C., and Pehrson, J. R. (2001) *J. Biol. Chem. 276*, 21776−21784.
6. Pehrson, J. R., and Fuji, R. N. (1998) *Nucleic Acids Res. 26*, 2837−2842.
7. Costanzi, C., and Pehrson, J. R. (1998) *Nature 393*, 599−601.
8. Borsani, G., Tonlorenzi, R., Simmler, M. C., Dandolo, L., Arnaud, D., Capra, V., Grompe, M., Pizzuti, A., Muzny, D., Lawrence, C., and Ballabio, A. (1991) *Nature 351*, 325−329.
9. Brockdorff, N., Ashworth, A., Kay, G. F., Cooper, P., Smith, S., McCabe, V. M., Norris, D. P., Penny, G. D., Patel, D., and Rastan, S. (1991) *Nature 351*, 329−331.
10. Brockdorff, N., Ashworth, A., Kay, G. F., McCabe, V. M., Norris, D. P., Cooper, P. J., Swift, S., and Rastan, S. (1992) *Cell 71*, 515−527.
11. Brown, C. J., Ballabio, A., Rupert, J. L., Lafreniere, R. G., Grompe, M., Tonlorenzi, R., and Willard, H. F. (1991) *Nature 349*, 38−44.
12. Brown, C. J., Hendrich, B. D., Rupert, J. L., Lafreniere, R. G., Xing, Y., Lawrence, J., and Willard, H. F. (1992) *Cell 71*, 527−542.
13. Herzing, L. B. K., Romer, J. T., Horn, J. M., and Ashworth, A. (1997) *Nature 386*, 272−275.
14. Penny, G. D., Kay, G. F., Sheardown, S. A., Rastan, S., and Brockdorff, N. (1996) *Nature 379*, 131−137.
15. Lee, J. T., and Jaenisch, R. (1997) *Nature 386*, 275−279.
16. Marahrens, Y., Panning, B., Dausman, J., Strauss, W., and Jaenisch, R. (1997) *Genes Dev. 11*, 156−166.
17. Csankovszki, G., Panning, B., Bates, B., Pehrson, J. R., and Jaenisch, R. (1999) *Nat. Genet. 22*, 323−324.
18. Laemmli, U. K. (1970) *Nature 227*, 680−685.
19. Simon, R. H., and Felsenfeld, G. (1979) *Nucleic Acids Res. 6*, 689−696.
20. von Holt, C., and Brandt, W. F. (1977) *Methods Cell Biol. 16*, 205−225.
21. Rose, S. M., and Garrard, W. T. (1984) *J. Biol. Chem. 259*, 8534−8544.
22. Libertini, L. J., and Small, E. W. (1980) *Nucleic Acids Res. 8*, 3517−3534.
23. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Plainview, NY.
24. Tatchell, K., and Van Holde, K. E. (1977) *Biochemistry 16*, 5295−5303.
25. Hansen, J. C., Ausio, J., Stanik, V. H., and Van Holde, K. E. (1989) *Biochemistry 28*, 9129−9136.
26. Lutter, L. C. (1978) *J. Mol. Biol. 124*, 391−420.
27. Tatchell, K., and Van Holde, K. E. (1979) *Biochemistry 18*, 2871−2880.
28. Luger, K., Mader, A. W., Richmond, R. K., Sargent, D. F., and Richmond, T. J. (1997) *Nature 389*, 251−260.
29. Usachenko, S. I., Bavykin, S. G., Gavin, I. M., and Bradbury, E. M. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 6845−6849.
30. Belyavsky, A. V., Bavykin, S. G., Goguadze, E. G., and Mirazbekov, A. D. (1980) *J. Mol. Biol. 139*, 519−536.
31. Vijay-Kumar, S., Chandra, N., Dharia, C., and Pehrson, J. R. (1995) *Proteins: Struct., Funct., Genet. 22*, 290−292.
32. Luger, K., Rechsteiner, T. J., Flaus, A. J., Waye, M. M., and Richmond, T. J. (1997*) J. Mol. Biol. 272*, 301−311.

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