Facile synthesis of site-specifically acetylated and methylated histone proteins: Reagents for evaluation of the histone code hypothesis

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Facile synthesis of site-specifically acetylated and methylated histone proteins: Reagents for evaluation of the histone code hypothesis

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The functional capacity of genetically encoded histone proteins can be powerfully expanded by posttranslational modification. A growing body of biochemical and genetic evidence clearly links the unique combinatorial patterning of side chain acetylation, methylation, and phosphorylation mainly within the highly conserved N termini of histones H2A, H2B, H3, and H4 with the regulation of gene expression and chromatin assembly and remodeling, in effect constituting a "histone code" for epigenetic signaling. Deconvoluting this code has proved challenging given the inherent posttranslational heterogeneity of histone proteins isolated from biological sources. Here we describe the application of native chemical ligation to the preparation of full-length histone proteins containing site-specific acetylation and methylation modifications. Peptide thioesters corresponding to histone N termini were prepared by solid phase peptide synthesis using an acid labile Boc/HF assembly strategy, then subsequently ligated to recombinantly produced histone C-terminal globular domains containing an engineered N-terminal cysteine residue. The ligation site is then rendered traceless by hydrogenolytic desulfurization, generating a native histone protein sequence. Synthetic histones generated by this method are fully functional, as evidenced by their self-assembly into a higher order H3/H4 heterotetramer, their deposition into nucleosomes by human ISWI-containing (Imitation of Switch) factor RSF (Remodeling and Spacing Factor), and by enzymatic modification by human Sirt1 deacetylase and G9a methyltransferase. Site-specifically modified histone proteins generated by this method will prove invaluable as novel reagents for the evaluation of the histone code hypothesis and analysis of epigenetic signaling mechanisms.

The functional capacity of genetically encoded proteins can be powerfully expanded by posttranslational modification. Protein side chain covalent modifications such as acetylation and methylation can potentiate local and global protein architecture and generate novel functional groups for binding, catalysis, or signaling. Such modifications are intimately integrated into many aspects of modulation of biological recognition and cellular proliferation. This is exemplified by the growing number of proteins whose activities are regulated by these modifications, in particular histones H2A, H2B, H3, and H4 (1, 2).

Allfrey (3) first linked histone acetylation and methylation to control of RNA synthesis in 1964. Since that time, the site-specific acetylation and methylation patterning of conserved histone N termini has been intimately linked to chromatin remodeling, transcriptional regulation, cancer proliferation, and epigenetic signaling (4, 5). Reversible histone acetylation facilitates access of transcriptional machinery to DNA by disruption of nucleosome–nucleosome and nucleosome–DNA interactions, and provides novel recognition sites for the recruitment of other accessory regulatory factors (4, 6). Likewise, histone methylation is an important mediator of both transcriptional silencing and activation (5, 7, 8).

Allis has recently formulated a "histone code" hypothesis that suggests that the timing, type, placement, and sequence of histone posttranslational modifications comprises a code for protein recruitment, substrate specificity and chromatin remodeling in service of transcriptional control (4, 9, 10). A growing body of evidence supports this hypothesis (4–10). Although the scope of a single uniform histone code is the subject of ongoing debate (1, 11), clearly the dynamic balance of coordinate histone posttranslational modifications is a prime factor in facilitating protein–protein interactions that help orchestrate the highly complex process of gene-specific transcription.

For years, several formidable barriers have made decoding the molecular logic of histone posttranslational modification experimentally challenging. Histones isolated from natural sources are highly heterogeneous with respect to posttranslational modification state, type, and position. In addition, enzymatic synthesis of full-length histones containing complex patterns of sequence-specific modifications has been largely precluded because the enzymes that modify histones exhibit high sequence specificity and positional redundancy. The lack of site-specifically modified full-length histones negatively impacts not only the analysis of chromatin assembly and remodeling, nucleosome–nucleosome interactions, and gene-specific transcription, but also hampers substrate specificity studies of histone modification enzymes.

We report here a facile method for the production of native-sequence site-specifically acetylated and methylated histones by using the combination of native chemical ligation (NCL) and hydrogenolytic desulfurization. To illustrate the general applicability of this method, we have synthesized site-specifically acetylated and methylated *Xenopus laevis* histones H3, H4, and H3–H4 hybrid proteins. Histones generated by this method are fully functional, as evidenced by their self-assembly into a higher order H3/H4 heterotetramer, their deposition into regular nucleosome arrays, and utilization as substrates for histone modifying enzymes.

Experimental Methods

Synthesis of Histone N-Terminal Peptide Thioesters. Site-specifically acetylated or methylated histone H3- and H4-derived N-

A preliminary account of this research was presented at the 17th American Peptide Symposium, San Diego, CA, July, 2001.

Abbreviations: NCL, native chemical ligation; SPPS, solid-phase peptide synthesis; Gdn·HCl, guanidine hydrochloride; FT-ESI, Fourier transform electrospray ionization; MS, mass spectrometry.

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terminal peptides were assembled by manual solid-phase peptide synthesis (SPPS) using either Boc/trifluoroacetic acid (TFA) or Fmoc/piperidine strategies. For the Fmoc synthesis of histone H4-derived peptide benzyl thioester **1**, 2-chlorotrityl resin was used as an acid-labile support. After linear assembly, the peptides were cleaved from the solid support with MeOH/AcOH/TFE (1:1:8 vol/vol/vol), then purified by flash chromatography. The protected peptide acids were esterified with benzyl mercaptan, side chain deprotected with TFA, precipitated by trituration with ice-cold Et_2O, and purified by HPLC using linear gradients of Buffer A (99.9:0.1 H_2O/CF_3COOH) and Buffer B (90:10:0.1 $CH_3CN/H_2O/CF_3COOH$) to yield peptide thioester **1**. Histone H3- and H4-derived peptide thioesters **2**, **3**, **8**–**10** were synthesized by using the Boc/HF strategy with *in situ* neutralization (12) on methylbenzyhydrylamine resin containing a 3-mercaptopropionic acid linker (13) (see Fig. 2). Acetyl and trimethylated lysine residues were introduced as Boc-Lys(Ac)-OH or Boc-Lys$(Me)_3$-OH, respectively. Coupling efficiencies were monitored by Kaiser analysis (14). After HF/*p*-cresol cleavage, the peptides were purified by HPLC (Vydac C_{18}). Composition of the peptides was confirmed by matrix-assisted laser desorption ionization time-of-flight MS.

Production of *X. laevis* Histones H3 and H4 C Termini Containing N-Terminal Cys Residues. We used Luger's plasmids encoding for *X. laevis* histones H3 and H4 as the templates (15) to prepare H3 (A25C) and H4 (A15C) point mutants by using the QuikChange site-directed mutagenesis method (Stratagene). Next, the N-terminal sequences of histones H3 (ΔN24) and H4 (ΔN14) were deleted by PCR amplification with primers encoding for an N-terminal Met initiation codon adjacent to the Ala-to-Cys mutation. After PCR, the insert was digested with *Nde*I and *Hin*dIII and ligated into a pET 30b vector previously digested with *Nde*I and *Hin*dIII. The TAA stop codon was removed for histone H4 during PCR amplification to introduce a hexahistidine tag (KLAAALEHHHHHH) at the C terminus. In contrast, the C terminus of histone H3 contained a TAG stop codon. *Escherichia coli* BL21-Codon Plus (DE3)-RIL cells (Novagen) harboring expression plasmids for histone H3(ΔN24) A25C (**11,** see Fig. 2) and H4(ΔN14) A15C (**4**, see Fig. 2) C-terminal domains were grown in LB media (50 μg/μl kanamycin) at 37°C. Cells were induced at OD_{600} = 0.6 with IPTG (1 mM) and harvested after 3 h by centrifugation. The following steps were performed at 4°C. Cell pellets were resuspended in cold 50 mM Tris·HCl buffer (pH 8, 50 ml) with EDTA (1 mM, Buffer C). The cell suspension was lysed with a French press then clarified by ultracentrifugation. The pellet was suspended in Buffer C (50 ml), centrifuged for 15 min, suspended in 50 ml of Buffer C containing 2 M urea, centrifuged, suspended in 50 ml of Buffer C containing 1% Triton X-100, and ultracentrifuged for 30 min at 4°C (26,000 × *g*). The pellet was suspended in Buffer D [6 M guanidine hydrochloride (Gnd·HCl)/100 mM sodium phosphate buffer pH 7.5, 10 ml] and stirred overnight at room temperature. After ultracentrifugation, the supernatant was lowered to pH 5.5 with 1 M NaH_2PO_4 then applied to a C_{18} HPLC column for purification. Purified proteins were confirmed for composition by high-resolution Fourier transform electrospray mass spectrometry (FT-ESI MS).

NCL Reaction and Desulfurization. NCL reactions were performed by a modification of the method of Dawson (16). Recombinant **4** or **11** was added to an appropriate thioester (e.g., **1**-**3**, **8**–**10**), premixed with thiophenol (2%, vol/vol) in phosphate buffer solution (pH 7.5) containing 6 M Gnd·HCl (2 mM final concentration of peptides). Ligation reactions were performed with 1.5-fold molar excess of thioester, and the reaction was monitored by HPLC. Ligation reactions with H4 (15–102)-His_6 A15C (**4**) were performed at room temperature. Ligations involving histone H3 (25–133) A25C (**11**) were performed at 60°C. Ligation yields with thiols **4** or **11** ranged from 50% to 60%.

Native-like full-length H3 and H4 proteins were generated by desulfurization of the ligation products (17). Histone proteins (20 mg) were dissolved in argon-degassed phosphate buffer solution (pH 5.8) containing 6 M Gnd·HCl to a final concentration of 0.5–2 mg/ml. Freshly prepared Raney nickel was added (10-fold excess wt/wt), and the reaction was placed under an atmosphere of hydrogen. Yields after ligation, desulfurization, and reversed-phase HPLC purification ranged from 10% to 35%.

High-Resolution MS. Lyophilized protein samples were resuspended in ESI buffer (50% H_2O/ACN/HCOOH 50:49:1, vol/vol/vol), centrifuged at 20,000 × *g* in an Eppendorf microcentrifuge for 4 min, then the supernatant was loaded into a microelectrospray assembly terminating in an ESI tip (50 μm inner diameter; New Objective, Cambridge, MA). A syringe pump provided a flow rate of 0.5 μl/min, and the typical ESI voltage was 2.2–2.7 kV. An 8.5 Tesla FT-ESI mass spectrometer was used for mass analysis. Construction and operation of this instrument is similar to that of a 9.4 Tesla FT MS that has been described (18). Transients were stored with a MIDAS data station as 512,000 data sets, and a hanning or hamming apodization is applied before fast Fourier transformation. Spectra were calibrated externally using bovine ubiquitin, 8,564.64–5 Da. The italicized number after a M_r value indicates the mass difference (in units of 1.00235 Da) between that peak and the monoisotopic peak.

Assembly and Purification of the cH3/rH4 Tetramer. Recombinant *X. laevis* histone H4 was expressed in *E. coli* and purified according to the method of Luger (ref. 15; see supporting information, which is published on the PNAS web site, www.pnas.org). Histone tetramers were obtained by mixing equimolar amounts of synthetic or recombinant histone H3 with recombinant histone H4 in 6 M urea followed by dialysis against refolding buffer (2 M NaCl/10 mM Tris·HCl, pH 7.5/1 mM EDTA/5 mM 2-mercaptoethanol). The sample was concentrated in a Millipore microconcentrator and then loaded into a HiLoad Superdex 200 HR 10/30 (AP Biotech) previously equilibrated with refolding buffer. Fractions containing the desired tetramer were identified by using SDS/18% PAGE and compared with a standard of HeLa cell core histone octamers.

Assembly of Native Histone H2A/H2B Dimers. Native H2A/H2B dimers were purified from HeLa cell core histone octamers as described (19).

Chromatin Assembly. RSF-mediated chromatin assembly and micrococcal nuclease digestion was performed as described (8).

Histone Methylation and Deacetylation Experiments. Histone methyltransferase assays were performed as described using G9a (20, 21). Reaction mixtures without *S*-adenosylmethionine (SAM) were preincubated in the presence or absence of recombinant human Sirt1 and 100 μM NAD^+ for 10 min, then [^{3}H]SAM was added to initiate methyl transfer. The reactions were stopped at 5 min by addition of 5× SDS sample buffer, separated by SDS/PAGE, and visualized by autoradiography.

Results and Discussion

A NCL Strategy for Synthesis of Histone Proteins. NCL is a methodology by which two unprotected peptides, bearing a reactive group, either a C-terminal thioester or N-terminal cysteine, can be chemoselectively condensed in aqueous solution at neutral pH to form a peptide bond with a Cys residue at the ligation site (13, 22–25). Because the majority of histone posttranslational modifications are found on residues within the first 30 aa of their

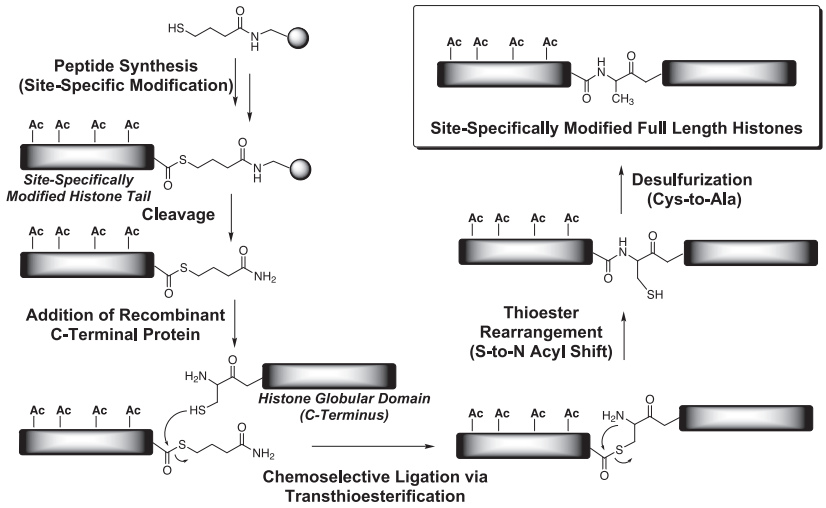

Fig. 1. General scheme for the synthesis of site-specifically modified histones by using native chemical ligation combined with desulfurization.

N termini, we devised a modular NCL strategy for histone synthesis whereby peptides corresponding to this region were synthesized by SPPS and then ligated to a recombinant protein fragment corresponding to the histone C-terminal globular domain (Fig. 1). Because the Xaa–Cys NCL junction site can be made effectively traceless by desulfurization with H_2/Raney nickel (17), we judiciously positioned the site of ligation to be adjacent to alanine residues found in the native histone sequences.

Synthesis of Thioesters of N-Terminal Histone Tails. To prepare thioesters of N-terminal histone tails, we chose to use a Boc/HF SPPS strategy on a methylbenzydrylamine resin containing a 3-mercaptopropionic acid linker. Histone H3 and

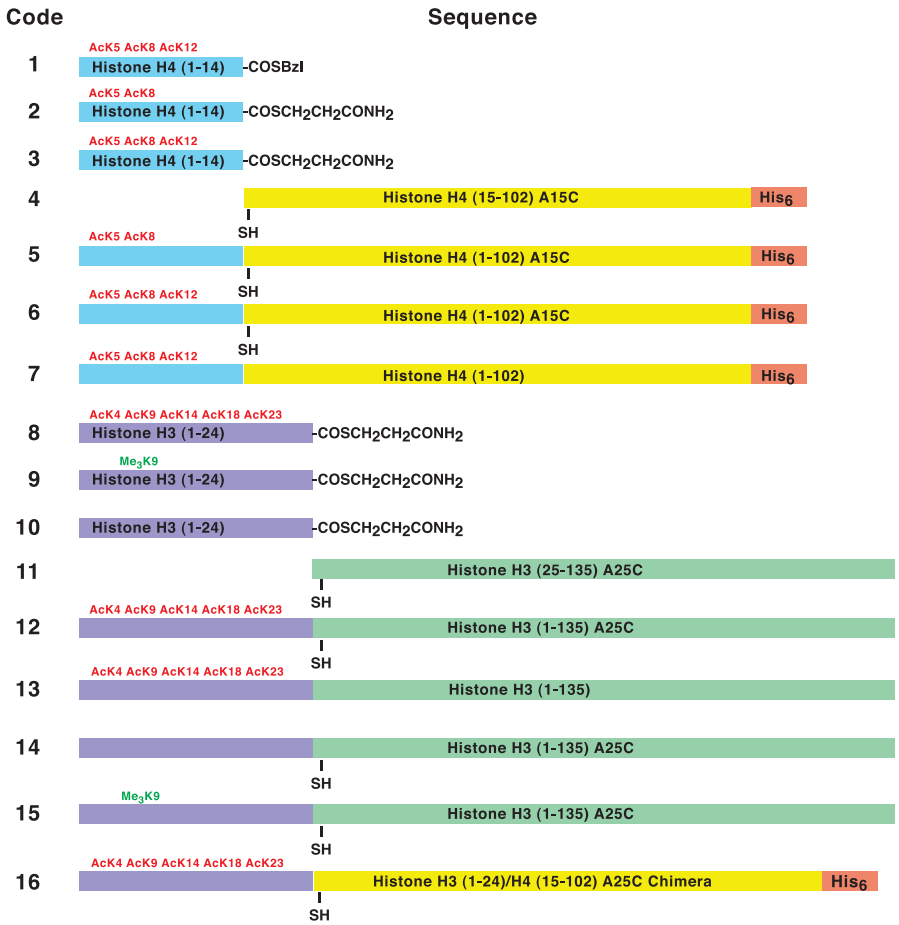

Fig. 2. Sequences of histone derived proteins and peptides used in this study.

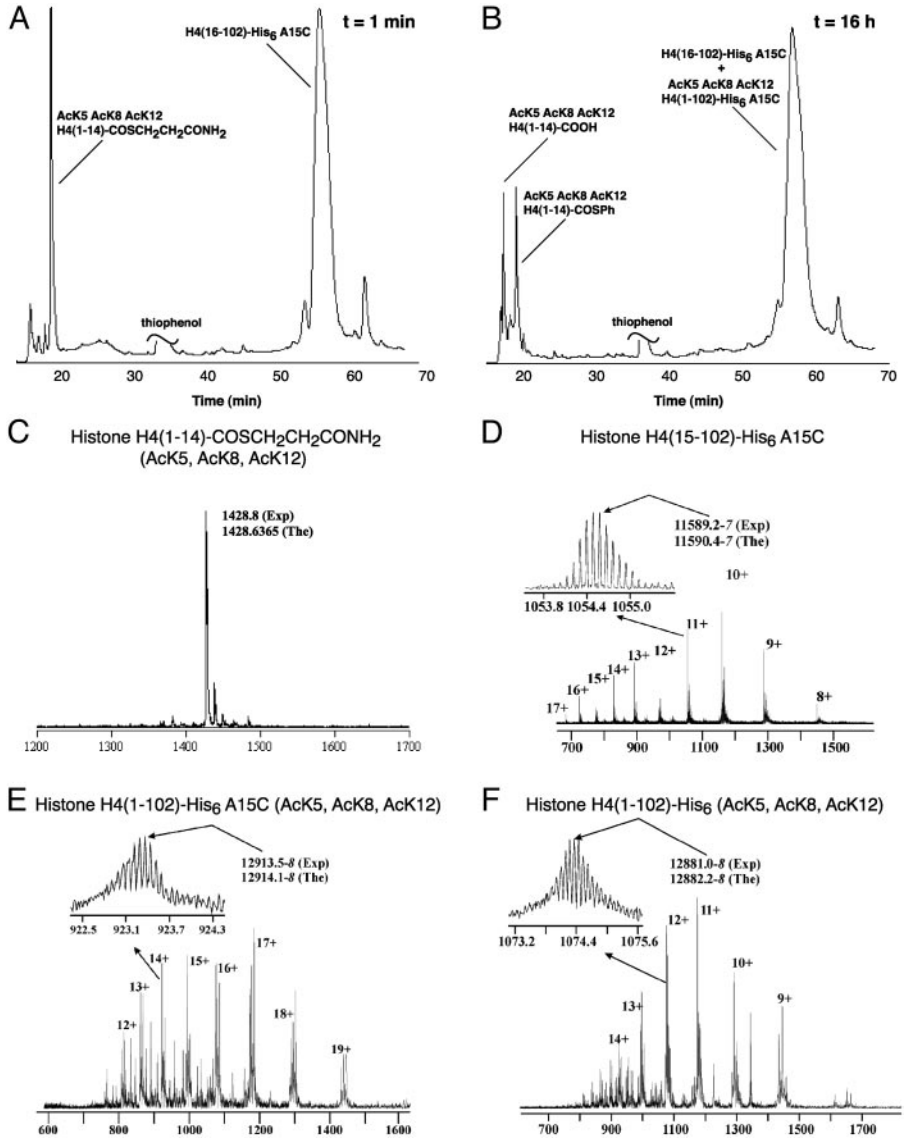

Fig. 3. (*A*) Time course studies of native chemical ligation of H4 thioester **3** (2 mM) with H4 thiol **4** (2 mM) in phosphate buffer solution (10 mM, pH 7.5) containing 6 M Gnd·HCl and 2% thiophenol (vol/vol) at room temperature (left trace). Under these HPLC conditions, thiol **4** and ligation product **6** coeluted at 57 min. (*B*) Data after 16 h (right trace) indicated near complete consumption of the starting thioester **3** as well as partial hydrolysis of the unreacted thiophenol adduct (peaks at 18 and 19.5 min). The reaction proceeded to 60% completion in 16 h as judged by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) MS analysis. Thiol **4** and the ligation product **6** were subsequently separated by an additional preparative HPLC step under different gradient conditions. (*C*) Mass analysis of the reactants and products of the native chemical ligation synthesis and desulfurization of acetylated *X. laevis* histone H4. Experimentally determined and actual average masses are indicated on each figure panel. The italicized number after a M_r value indicates the mass difference (in units of 1.00235 Da) between that peak and the monoisotopic peak. This MALDI-TOF MS spectrum depicts the average mass of the purified thioester **3**. (*D*) FT-ESI MS spectrum of H4 C-terminal recombinant protein thiol **4**. (*E*) FT-ESI MS spectrum of the native chemical ligation product **6**. (*F*) FT-ESI MS spectrum of the desulfurization reaction product **7** containing a native histone H4 primary sequence.

H4-derived peptides **2**, **3**, and **8**–**10** were synthesized by manual SPPS using the rapid *in situ* neutralization protocol of Kent and coworkers (12) and confirmed for composition by MS (Fig. 2). To complement the Boc approach, we used an Fmoc/TFA strategy for the synthesis of acetylated histone H4 thioester peptide **1** (Fig. 2). Although both thioesters **1** and **2** exhibited similar reactivity during NCL ligation, the Boc/HF synthesis strategy is a preferred method over previous methods because it consistently provided material in high yield, with few synthetic steps, and with high purity and chiral integrity (26).

Production of Histone H3 and H4 C-Terminal Globular Domains. We prepared truncated histone H4 (**4**) and H3 (**11**) globular C-terminal domains containing an N-terminal cysteine residue recombinantly. In the constructs, the N-terminal Cys residues were preceded by the Met initiator. *E. coli* cells harboring either $H4_{\Delta N15}$ A15C or $H3_{\Delta N24}$ A25C expression plasmids overproduced the desired proteins efficiently as inclusion bodies. Isolation and purification of these proteins required the use of reducing agents to prevent disulfide dimerization. Fortuitously, FT-ESI MS analysis of proteins **4** and **11** confirmed that the N-terminal methionine of each protein had been >90% removed during the expression. Yield of purified recombinant histones averaged 4 mg/liter of cell culture.

Native Chemical Ligation and Desulfurization. Full-length acetylated, methylated or unmodified H4 and H3 histones and an

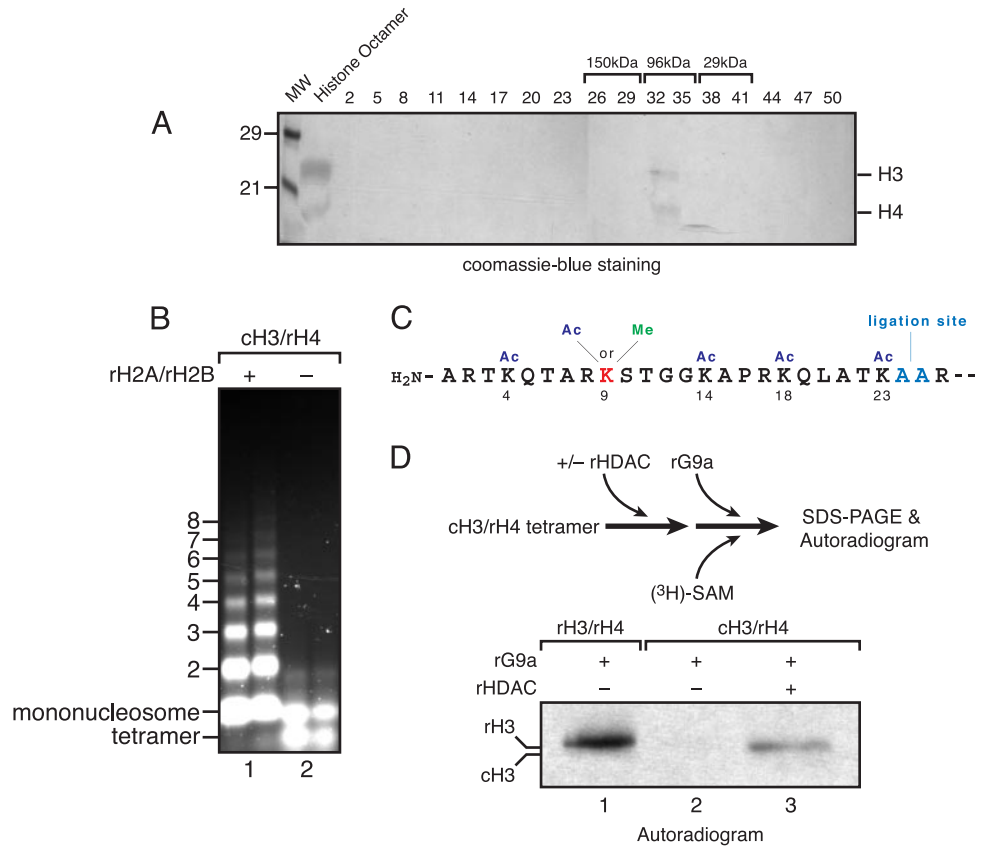

Fig. 4. (*A*) Assembly of histone tetramers from synthetic histone H3 (cH3, prepared by NCL) and recombinant histone H4 (rH4) as monitored by size exclusion chromatography. Lane 1, molecular mass marker proteins; lane 2, standard of HeLa cell core histone octamers. Numbers above the gel indicate elution fraction number and the approximate protein mass range for each fraction. (*B*) Micrococcal nuclease digestion analysis of nucleosome formation catalyzed by RSF. Lanes 1 and 2 show the formation of nucleosomes, an indicator of successful chromatin assembly with reaction mixtures containing RSF, a DNA plasmid template, the cH3/rH4 tetramer, and two different concentrations of HeLa histones H2A and H2B. In contrast, control lanes 3 and 4 do not form chromatin as histones H2A and H2B were omitted from these reaction mixtures. (*C*) Synthetic histone H3 sequence indicating original acetylation modification sites, the NCL ligation junction, and the H3 K9 preferred site of methylation by the G9a methyltransferase. (*D*) Enzymatic methylation and deacetylation of a cH3/rH4 tetramer. Autoradiographic analysis of cH3/rH4 methylation by G9a with and without prior treatment with human histone deacetylase Sirt1. Lane 1, control experiment depicting methylation of unacetylated recombinant H3/H4 tetramer with G9a; lane 2, failure of G9a to methylate hyperacetylated cH3/rH4 tetramer because K9 is acetylated; lane 3, brief treatment of acetylated cH3/rH4 tetramer with Sirt1 followed by incubation with G9a results in the removal of AcK9 and the subsequent G9a-catalyzed methyl transfer to the newly liberated K9 side chain amine.

H3/H4 chimera protein were assembled by native chemical ligation of N-terminal peptide thioesters with the C-terminal protein thiols (Figs. 1 and 2). Histone H4 proteins containing a C-terminal hexahistidine affinity tag were prepared with two (K5 and K8) or three (K5, K8, and K12) acetyl lysines. Similarly, three different H3 proteins containing either no modification, five acetyl lysines (K4, K9, K14, K18, and K23), or a single trimethylated lysine (K9) were prepared. To demonstrate the modularity of the NCL assembly, we also prepared a penta-acetylated chimera containing the tail of H3 fused to the globular domain of H4. Ligation reactions were catalyzed by the addition of 2% thiophenol (16). Because of the limited solubility of truncated H3 in the ligation buffer, these reactions were conducted at 60°C, whereas H4 ligations proceeded smoothly at 25°C.

Depicted in Fig. 3 *A* and *B* is the results of a typical NCL reaction as monitored by analytical HPLC. In this example, a triacetylated histone H4 thioester (**3**) was ligated to the H4 globular domain protein thiol (**4**). For H4 and H3 NCL reactions, all starting materials, intermediates, ligation, and desulfurization products were fully characterized by MS methods (Fig. 3 *C–F*). Histones **6** and **12** created by NCL methods were converted back into their respective native primary sequences by cysteine desulfurization with H_2/Raney nickel, generating proteins **7** and **13** (17). Isolated yields for unoptimized desulfurizations ranged from 10% to 30%. This low recovery was attributed to adsorption of the product to the Raney nickel catalyst. In addition to native primary sequence histones, we prepared diacetylated H4 (**5**), an unacetylated H3 (**14**), a trimethylated H3 (**15**), and a pentaacetylated H3/H4 chimera (**16**) (Fig. 2). Assembly of such modified proteins, as shown below, demonstrates the widespread applicability of this improved technique for histone synthesis.

H3/H4 Tetramer Assembly. To demonstrate the capacity of synthetic histones to fold into higher ordered assemblies, we prepared a synthetic H3/recombinant H4 heterotetramer (abbreviated cH3/rH4 for chemically synthesized H3, recombinantly produced H4) (Fig. 4). The H3/H4 tetramer is the biologically active folded form of H3 and H4 required for nucleosome deposition and chromatin assembly. Equimolar pentaacetylated synthetic histone H3 (cH3, **13**) and recombinant histone H4 (rH4) (15) were mixed together under denaturing conditions, simultaneously refolded and self-assembled into the heterotetramer by removal of the denaturant with dialysis, then lastly purified by size exclusion chromatography (Fig. 4*A*). Both cH3/rH4 tetramer and a rH3/rH4 tetramer control (19) exhib-

ited identical size exclusion chromatography elution profiles, strongly suggesting that the heterotetramer was properly folded. MS analysis of the cH3/rH4 tetramers purified by gel filtration confirmed the presence of each histone (data not shown). Furthermore, in addition to **13**, histones **12**, **14**, and **15** were also found to easily self-assemble into a heterotetramer with recombinant histone H4 (data not shown). These results suggest that neither N-terminal modifications nor the presence of an internal cysteine residue significantly impacted the ability of synthetic histones to fold into tetrameric assemblies.

Incorporation of Synthetic Histones into Chromatin. Reinberg and coworkers recently discovered that purified human ISWI-containing (Imitation of Switch) factor RSF (Remodeling and Spacing Factor), an ATP-dependent nucleosome deposition complex, is capable of *in vitro* reconstitution of chromatin without a requirement for additional chaperone proteins (8, 27, 28). In the presence of ATP, a DNA template, a recombinant H3/H4 tetramer, and histones H2A and H2B, RSF assembles regularly spaced nucleosome arrays (8, 28). RSF-reconstituted chromatin has also been shown to be an effective template for the analysis of transcription, confirming that histones in these complexes are properly folded and functional (8, 27, 28). To prepare chromatin by using synthetic histones, we challenged RSF with the synthetic cH3/rH4 tetramer (histone **13** plus recombinant H4), a DNA template, ATP, and HeLa cell core histones H2A and H2B. Indeed, as depicted in Fig. 4*B*, RSF efficiently assembled chromatin containing the synthetic H3. Similarly, tetramers made from H3 analogues **12** and **14** were assembled into chromatin by RSF (data not shown). These data confirm that synthetic histones are capable of being incorporated into native-like chromatin assemblies.

Folded Synthetic Histones as Substrates for Histone Modification Enzymes. Lastly, we evaluated the ability of full-length folded synthetic histones to serve as substrates for histone modification enzymes. We tested the ability of the human histone methyltransferase G9a to transfer [^{3}H]methyl groups from its [^{3}H]*S*-adenosylmethionine (SAM) cofactor to the K9 position of the folded cH3/rH4 tetramer (Fig. 4). Because the K9 site of cH3 (**13**) was effectively blocked by the acetyl group introduced during SPPS, no transfer of [^{3}H]methyls was observed, as was anticipated (Fig. 4*B*). In contrast, a control of recombinantly produced rH3/rH4 tetramers containing a free H3–K9 side chain amine exhibited robust methylation by G9a. However, methylation at K9 was observed to take place after the cH3/rH4 tetramer was briefly treated with human histone deacetylase Sir1 and NAD^+ to liberate K9 before the addition of G9a and [^{3}H]SAM (Fig. 4*C*). These data show that folded synthetic histone proteins can be properly posttranslationally modified by histone modification enzymes. These unique substrates can be used to probe the substrate specificity, processivity, and kinetic and chemical mechanisms of histone modification enzymes.

Conclusions

Here we described a preparation of site-specifically acetylated and methylated full-length histones containing a native primary sequence by using a combination of chemical synthesis, native chemical ligation, and postassembly desulfurization. The successful assembly of histone H3 and H4 variants containing a significant ensemble of acetylated, methylated, or chimeric structures reflects the ease and generality to which these protein reagents can be constructed. Furthermore, site-specifically modified synthetic histones were shown to properly fold and self-assemble, to be efficiently used by RSF for chromatin reconstitution, and to be appropriately used as substrates for histone modification proteins. Collectively, these results highlight the potential of these new protein reagents for use in decoding the molecular logic of the histone code hypothesis, epigenetic signaling mechanisms, and other histone-related biological phenomena.

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