MPD. The unit cell dimensions change as a function of MPD concentration. There is also a correlation between the MPD concentration and the quality of diffraction. The optimum concentration of MPD for the smallest unit cell parameters and best diffraction characteristics is about 22.5%, as adjusted by refractometry to verify the final value.

Acknowledgments

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[4] Creating Designer Histones by Native Chemical Ligation

By MICHAEL A. SHOGREN-KNAAK and CRAIG L. PETERSON

Introduction

Histones in all eukaryotic organisms are enzymatically altered to present a wide range of posttranslational modifications, including acetylation, phosphorylation, methylation, ubiquitination, and ribosylation. Moreover, these modifications play important roles in modulating nearly all DNA-associated processes, such as gene expression, epigenetic patterning, DNA repair, and replication. To probe how posttranslational modifications of histones influence both chromatin structure and the binding and function of chromatin-associated proteins, we describe a strategy that employs native chemical ligation[1,2] to generate designed histones. Specifically, we present the synthesis of wild-type *Xenopus laevis* histone H3 that can be modified at individual or multiple amino-terminal tail sites.[3]

[1] P. E. Dawson, T. W. Muir, I. Clark-Lewis, and S. B. Kent, *Science* **266,** 776 (1994).
[2] U. K. Blaschke, J. Silberstein, and T. W. Muir, *Methods Enzymol.* **328,** 478 (2000).
[3] M. A. Shogren-Knaak, C. J. Fry, and C. L. Peterson, *J. Biol. Chem.* **278,** 15744 (2003).

0076-6879/04 $35.00

This strategy, in principle, can be used to study virtually any histone modification and offers a number of advantages over alternative methods. Unlike enzymatically modified histones or those purified from cellular lysates, ligation provides homogeneously modified histones. Furthermore, because these synthetic histones can be readily assembled *in vitro* into chromatin, they are more physiologically relevant than peptide substrates. Thus, it is possible to study the effects of histone modifications on chromatin structure and function, where recognition or interaction between histone domains, octamer subunits, nucleosomes, or nucleosomal DNA is important.

In this chapter we discuss the criteria used to select the H3 ligation site, and the protocol used for the synthesis and purification of modified histone H3 tail thioester peptides, the generation of amino-terminal cysteine-containing histone H3 core protein, and the ligation of these constituents (shown schematically in Fig. 1).

Histone H3 Ligation Strategy and Design

Native chemical ligation is a technique for generating full-length proteins from unprotected synthetic peptides and/or expressed protein fragments, and it is well suited for including amino acids that are not directly incorporated into proteins via the genetic code.[1,2,4] Native chemical ligation requires an N-terminal polypeptide fragment ending in a C-terminal thioester, and a C-terminal polypeptide fragment beginning with an N-terminal cysteine (Fig. 1). When mixed together, these fragments can form a reversible covalent association via *trans*-thioesterification of the C-terminal thioester by the N-terminal cysteine thiol. This reaction intermediate can then rearrange irreversibly via an S- to N-acyl shift, resulting in a canonical amide peptide bond at the ligation site (Fig. 2). Furthermore, this method is compatible with proteins containing other cysteines, because thioester products with these cysteines cannot rearrange to form an amide bond and thioesterification is reversible.

For histone H3, the majority of amino acid side chains that are posttranslationally modified reside in the amino-terminal tail. To incorporate modified residues of interest into a ligated histone, we chose solid-phase peptide synthesis to generate the N-terminal thioester-containing fragment and recombinant expression of the C-terminal cysteine-containing protein fragment. However, it is also possible to study C-terminal modifications (e.g., in histone H2B) by recombinantly expressing N-terminal protein

[4] M. Huse, M. N. Holford, J. Kuriyan, and W. W. Muir, *J. Am. Chem. Soc.* **122,** 8337 (2000).

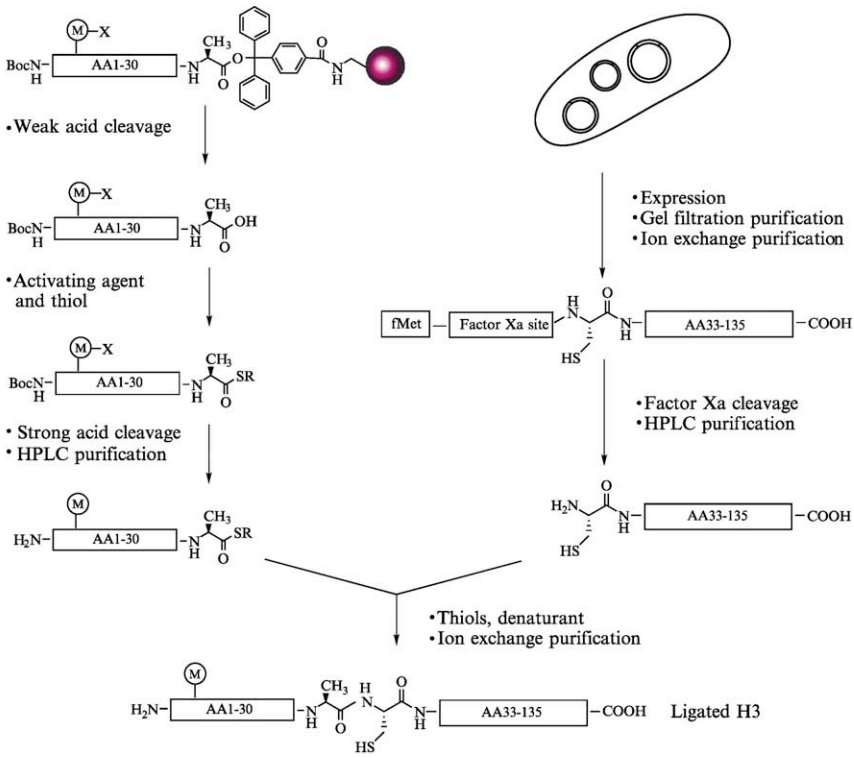

FIG. 1. Native chemical ligation strategy for generating histone H3 proteins containing specifically modified N-terminal residues. An N-terminal peptide fragment of histone H3 that contains specifically modified amino acid residues (in this example, a methylated lysine residue denoted by an encircled ''M''), and a C-terminal thioester moiety (COSR), is produced by standard solid-phase peptide synthesis on an acid-hypersensitive support (*left*). A C-terminal protein fragment of histone H3 containing an N-terminal cysteine residue is generated by proteolytic trimming of recombinant protein (*right*). Reaction of these two fragments in the presence of thiol reagents produces native full-length histone H3 containing the modifications of interest.

fragments that contain a C-terminal thioester and synthesizing C-terminal tail peptides containing an N-terminal cysteine.[2]

The bond between amino acid residues 31 and 32 was chosen as the histone H3 ligation site because it fulfills a number of criteria: first, a 31-amino acid peptide is synthetically tractable and allows many of the known H3 posttranslational histone modifications to be incorporated. Second, position 32 of histone H3 (threonine in wild-type *Xenopus* H3) is not highly

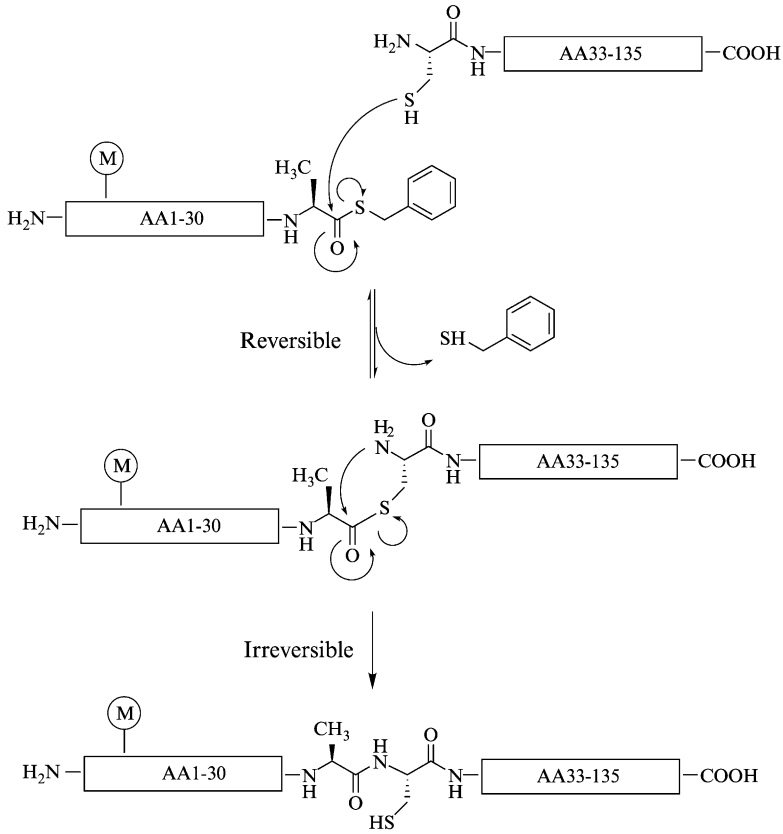

FIG. 2. Mechanism of histone H3 native chemical ligation. The C-terminal thioester of the N-terminal H3 peptide undergoes reversible *trans*-thioesterification with the cysteine thiol group of the C-terminal histone H3 fragment. This reaction intermediate rearranges irreversibly by an S- to N-acyl shift to generate native histone H3.

conserved among species,[5] suggesting it could be changed to cysteine without major structural or functional effects. Finally, positions 31 and 32 were expected to be compatible with ligation, as these residues are outside the structured core of the histone,[6] and position 31 (alanine) is not a proline, valine, or isoleucine residue, amino acids known to be detrimental to ligation.[2]

[5] S. Sullivan, D. W. Sink, K. L. Trout, I. Makalowska, P. M. Taylor, A. D. Baxevanis, and D. Landsman, *Nucleic Acids Res.* **30,** 341 (2002).

[6] K. Luger, A. W. Mader, R. K. Richmond, D. F. Sargent, and T. J. Richmond, *Nature* **389,** 251 (1997).

N-Terminal H3 Peptide Thioester Synthesis and Purification

Generation of peptides with a reactive C-terminal thioester group requires the selective thioesterification of the terminal peptide α-carboxylic acid group in the presence of other potentially reactive side-chain functional groups (Fig. 1, left side). To achieve this selectivity, peptides with a free α-carboxylic acid group, but protected side-chain groups, are generated by synthesizing the desired peptide on an acid-hypersensitive resin.[7] The synthesized peptides are then treated with a weak acid cleavage cocktail, which cleaves the peptide from the synthetic support to expose the free terminal α-carboxylic acid, while maintaining the side chain-protecting groups. The terminal α-carboxylic acid of the peptide is then thioesterified, using standard peptide-coupling reagents. Treatment of the peptide with a strong acid cleavage cocktail removes the side chain-protecting groups while maintaining the C-terminal thioester. Finally, high-pressure liquid chromatography (HPLC) is used to obtain the thioester ligation substrate in pure form.

Protected N-Terminal H3 Peptide Synthesis

Synthesis of the protected N-terminal H3 peptide from C to N terminus largely follows standard protocols for 9-fluorenylmethoxycarbonyl-(Fmoc)-based solid-phase peptide synthesis[8] and should be possible at many peptide synthesis facilities.

An acid-hypersensitive resin, NovaSyn-TGT resin (Calbiochem-Novabiochem; EMD Biosciences, San Diego, CA) preloaded with the *N*-α-Fmoc-protected residue 31 (alanine for wild-type *Xenopus* H3) is used as the synthetic support. This resin has a lower substitution (0.1–0.3 mmol/g) than many commonly used peptide resins, and thus reaction volumes may have to be adjusted accordingly to ensure proper resin solvation. However, in our experience this lower substitution resin provides better synthetic results than alternative higher substitution acid-hypersensitive peptide resins.

Standard side chain-protected Fmoc–amino acids are used to incorporate standard amino acids. Incorporation of acetylated lysine, phosphorylated serine or threonine, and mono-, di-, or tri-methylated lysine can be accomplished with *N*-α-Fmoc-*N*-ε-acetyl-L-lysine, *N*-α-Fmoc-*O*-benzyl-L-phosphoserine, *N*-α-Fmoc-*O*-benzyl-L-phosphothreonine (EMD Biosciences) *N*-α-Fmoc-*N*-ε-methyl-*N*-ε-Boc-L-lysine, *N*-α-Fmoc-*N*-ε-$(\text{methyl})_2$-L-lysine·HCl, and *N*-α-Fmoc-*N*-ε-$(\text{methyl})_3$-L-lysine chloride (Bachem Bioscience, King of

[7] S. Futaki, K. Sogawa, J. Maruyama, T. Asahara, M. Niwa, and H. Hoja, *Tetrahedron Lett.* **38,** 6237 (1997).
[8] G. B. Fields and R. L. Noble, *Int. J. Pept. Protein Res.* **35,** 161 (1990).

Prussia, PA), respectively. The N-terminal residue (alanine for *Xenopus* wild-type histone H3) should be incorporated as the *N*-α-butyloxycarbonyl (Boc)-protected residue (strong acid labile), because the amino terminus must be protected to prevent cross-reaction during thioesterification.

Fmoc deprotection is performed with standard bases, such as piperidine. Activation of the Fmoc–amino acid α-carboxylic acid can be accomplished with standard coupling reagents [e.g., 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU), and benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP)]. However, the coupling additive *N*-hydroxybenzotriazole(HOBt) should not be added, as it may cause loss of the peptide from the solid support during synthesis.[9] Double coupling of all residues and/or *N*-α-acetyl capping of uncoupled α-amines is suggested to improve the purity and yield of the N-terminal H3 peptide.[8]

1. Using an automated solid-phase peptide synthesizer, synthesize the desired peptides from residues 2–30 (RTKQTARKSTGGKAPRKQLATKAARKSAP, for wild-type *Xenopus* H3) on Fmoc-Ala-NovaSyn-TGT resin (EMD Biosciences) at a scale from 50 to 100 μmol.
2. Couple residue 1 (alanine for wild-type *Xenopus* H3) as the *N*-α-Boc-protected amino acid.
3. Cleave and analyze a small amount of the resin (50 mg) by reversed-phase HPLC and mass spectrometry, using standard protocols, to determine the identity and purity of the synthetic product.

Cleavage of Side Chain-Protected N-Terminal H3 Peptide

Side chain-protected N-terminal H3 peptide is cleaved from the acid-hypersensitive trityl-TentaGel support with a mild acid solution of acetic acid.[10] Because of its length and the high density of hydrophobic, protected basic residues, the protected N-terminal H3 peptide is relatively difficult to remove from the synthetic support using standard organic solvents, and requires the addition of trifluoroethanol, a reagent believed to disrupt highly insoluble β strand-aggregated peptides. Because both acetic acid and trifluoroethanol are potentially reactive in the subsequent thioesterification reaction, their complete removal by trituration and washing of the protected peptide is necessary.

[9] K. Barlos, O. Chatzi, D. Gatos, and G. Stavropoulos, *Int. J. Pept. Protein Res.* **37,** 513 (1991).
[10] H. Benz, *Synthesis* **4,** 337 (1993).

1. Prepare a 1:2:7 solution of acetic acid–trifluoroethanol–dichloromethane and add 1 ml to 50-mg portions of the dry peptide resin. Rock gently for 3 h at 25°.

2. Filter the resin from the protected peptide solution using a glass wool plug. Wash the resin twice, each time with 250 μl of the 1:2:7 acetic acid–trifluoroethanol–dichloromethane solution, and pool all eluate.

3. Reduce the volume of the pooled eluate to approximately one-fifth the original volume under a gentle stream of nitrogen or compressed air. Add the cloudy solution dropwise to 3 ml of 3:1 ethyl acetate–hexane and vortex the resulting white precipitate.

4. Centrifuge for 5 min at 5000*g* to pellet the peptide and decant the supernatant.

5. Resuspend the pellet in 1 ml of hexane and transfer to a weighed Eppendorf tube. Wash the pellet by vigorously vortexing. Centrifuge at 14,000 rpm in a microcentrifuge and decant the hexane from the pellet. Repeat the wash two additional times.

6. Dry the peptide overnight in a SpeedVac (Thermo Savant, Holbrook, NY). Yields for the side chain-protected, free carboxy-terminal acid peptide are typically about 15 mg. The peptide can be stored at −20° in a dry environment.

Thioesterification of N-Terminal H3 Peptide

The C-terminal thioester is generated from the side chain-protected, free carboxy-terminal acid peptide by activation of the C-terminal α-carboxylic acid with the activating reagent HBTU and coupling to benzyl mercaptan. The crude reaction mixture is dried and subjected to strong acid cleavage to remove the amino acid side chain-protecting groups. Crude thioester peptide is removed from the cleavage reagents and thioesterification reagents by trituration of the peptide. The crude thioester peptide is further purified by preparative reversed-phase HPLC to provide peptide thioester competent for native chemical ligation.

1. For each free carboxy-terminal acid peptide aliquot, grind the peptide pellet into a fine powder to improve its solubility. Add 1 ml of dimethylformamide (DMF) and divide the reaction into two portions. Add an additional 750 μl of DMF to each tube, as well as 5.6 μl of *N,N*-diisopropylethylamine. Heat the solution to 45° with vigorous mixing for 30 min, or until the peptide is dissolved.

2. Lower the temperature to 35° and add 9.79 mg of HBTU. Mix by vortexing and let sit for 1 min. Add 3.03 μl of benzyl mercaptan. Let the reaction proceed for 20 h at 35° with vigorous mixing. Benzyl mercaptan has an extremely strong odor and should be handled carefully.

3. Remove the liquid components of the reaction *in vacuo* until dry. Wash the pellet with 1 ml of water–0.1% trifluoroacetic acid. Remove the water solution *in vacuo* until dry. Because side-chain deprotection of peptides by strong acid reagents is adversely affected by DMF, thorough drying is necessary.

4. Remove the side chain-protecting groups of the peptide by addition of standard strong acid cleavage reagents.[11] For peptides that do not contain either tryptophan or methionine, this can be accomplished by adding 1 ml of 95:2.5:2.5 trifluoroacetic acid–water–triisopropylsilane and allowing the reaction to proceed for 2 h at 25° with gentle rocking.

5. Reduce the volume of the cleavage mixture to roughly one-fifth the original volume under a gentle stream of nitrogen or compressed air. Add 1 ml of 2:1 ether–hexane to triturate the peptide as a white precipitate. Vortex vigorously. Centrifuge at 14,000 rpm in a microcentrifuge for 2 min and decant the supernatant. Repeat the addition of 2:1 ether–hexane, vortexing, centrifugation, and decanting an additional three times.

6. Allow the peptide pellet to air dry for 20 min, and then resuspend in 1 ml of water–0.1% trifluoroacetic acid. Transfer to a weighed Eppendorf tube and dry in a SpeedVac. Typical yields should be roughly 6 mg.

7. Resuspend the peptide in water–0.1% trifluoroacetic acid to a concentration of 10 mg/ml. Purify the crude peptide by reversed-phase high-pressure liquid chromatography, using a semipreparative reversed-phase C_{18} column (ZORBAX 300SB-C_{18}, 9.4 × 250 mm, 5-μm particle size, 300-Å pore size; Agilent Technologies, Palo Alto, CA). Use a mixed solvent system of water–0.1% trifluoroacetic acid and acetonitrile–0.1% trifluoroacetic acid and monitor at 228 nm. Typically up to 2 mg of crude peptide can be purified over a shallow gradient from 18 to 22% organic solvent over 20 min at 2 ml/min. A number of UV-active peaks will be present, but the desired peptide should be the second of two closely eluting peaks (Fig. 3A), where the first peak is an isomeric form of the second peptide peak. Modified peptides do not tend to elute at drastically different times.

8. Dry and weigh the purified N-terminal H3 thioester peptide. Confirm the identity and purity of the product by mass spectrometry. Typical yields by weight fall between 30 and 50% of the initial crude weight. Redissolve the thioester peptide in water–0.1% trifluoroacetic acid, divide into individual 1.3-mg aliquots, and dry in a SpeedVac.

[11] NovaBiochem, "NovaBiochem Catalog and Peptide Synthesis Handbook." CB Bioscience, San Diego, CA, 1999.

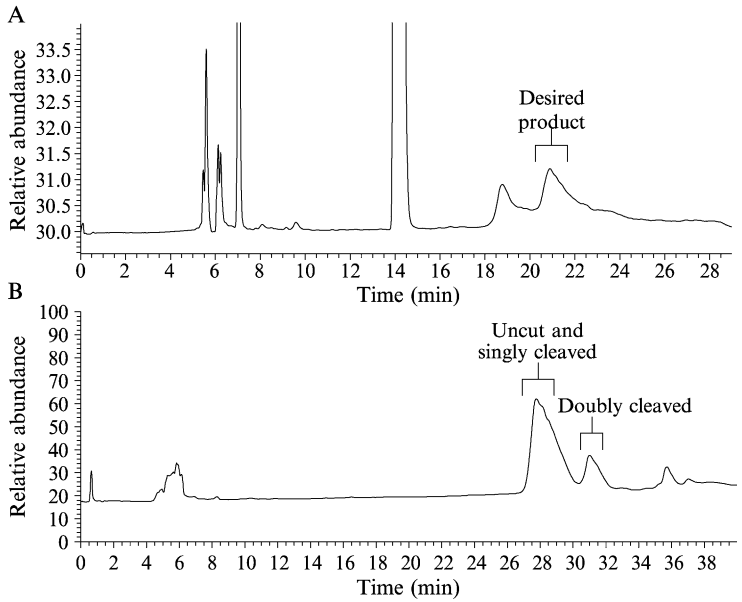

FIG. 3. High-pressure liquid chromatography purification of the N-terminal H3 thioester peptide and the C-terminal histone H3 fragment. (A) N-terminal H3 thioester peptide (20.75 min) is separated from other reaction products on a semipreparative C_{18} HPLC column. A representative trace of 0.5 mg of total protein is shown. Water–0.1% trifluoroacetic acid and acetonitrile–0.1% trifluoroacetic acid are used as the mobile phase, with protein eluted through a linear gradient of 18–22% organic solvent over 20 min at 2.0 ml/min and monitored at 228 nm. (B) Purification of histone H3 fragments after factor Xa protease treatment. Uncleaved and singly cleaved histone H3 digestion products (27.75 min) are separated from other reaction products on a semipreparative C_4 HPLC column. A representative trace of 2 mg of total protein is shown. Water–0.1% trifluoroacetic acid and acetonitrile–0.1% trifluoroacetic acid are used as the mobile phase, with protein eluted through a linear gradient of 37–48% organic solvent over 33 min at 2.5 ml/min and monitored at 235 nm.

C-Terminal Histone H3 Fragment Synthesis and Purification

Native chemical ligation requires a C-terminal peptide or protein fragment containing an N-terminal cysteine. Full-length *Xenopus* histone H3 can be expressed recombinantly in *Escherichia coli*.[12] Thus, it is possible to engineer the histone expression plasmid to contain an N-terminal cysteine residue at position 32. However, because the initiator formyl

[12] K. Luger, T. J. Rechsteiner, and T. J. Richmond, *Methods Enzymol.* **304,** 3 (1999).

methionine is not readily removed in *E. coli,* a protease site that can be cleaved C terminal to the recognition sequence (in this case, the factor Xa recognition site) is also engineered to allow production of a histone H3 fragment with an N-terminal cysteine.

Using this engineered histone H3 expression plasmid, the C-terminal histone H3 fragment is expressed in *E. coli,* inclusion bodies containing the expressed protein are isolated, and the C-terminal histone protein is purified by gel filtration and ion-exchange chromatography (Fig. 1, right side).[12] The protein is subjected to factor Xa proteolysis to remove both the N-terminal methionine and the factor Xa cleavage site and then purified away from overdigested histone by reversed-phase HPLC.

C-Terminal Histone H3 Fragment Plasmid Construction

DNA sequence corresponding to H3 amino acid residues 33 to 135 is amplified by polymerase chain reaction (PCR) from a wild-type *Xenopus* histone H3 expression plasmid, using an upstream primer containing an *Nde*I restriction site, a start codon, codons for a minimal factor Xa cleavage site (IEGR), and a cysteine codon for position 32 (5′-GCACTCGAGCCATATGATCGAAGGTCGTTGTGGCGGAGTCAAGAAACCTCACCGTTAC-3′) and a downstream primer containing a *Bgl*II restriction site (5′-AGCTCGCAATAGATCTAAGCCCTCTCGCCTCGGATTCT-3′). The resulting product is digested and ligated into an *Nde*I–*Bgl*II pET11c expression vector. The resulting plasmid is sequenced to confirm its identity and then transformed into a BL21(DE3) *E. coli* expression strain (Invitrogen, Carlsbad, CA).

C-Terminal Histone H3 Fragment Expression and Purification

Expression and purification of the C-terminal histone H3 fragment are performed largely as described by Luger and coworkers for recombinant wild-type *Xenopus* histone H3.[12] In our initial studies, we found yields and reproducibility of protein expression and purification, factor Xa cleavage, and the ligation steps were detrimentally affected by poor solubility of the expressed histone fragment. To address this issue, efforts were made to limit the exposure of the protein to conditions compatible with oxidation and to limit the frequency with which the histone was brought to dryness. In addition, we have found that mutation of Cys-111 to alanine appears to reduce aggregation and improve solubility.

Changes to the original histone purification protocol are as follows: after gel-filtration purification of the histone, fractions containing the histone are not dialyzed against a 2 m*M* 2-mercaptoethanol solution and dried, but are instead diluted into urea buffer [7 *M* urea, 20 m*M* sodium

acetate, 1 m*M* dithiothreitol (DTT), 1 m*M* EDTA, pH 5.2] to a final sodium chloride concentration of 200 m*M,* and then directly subjected to ion-exchange purification, with a gradient from 200 m to 600 m*M* sodium chloride. After ion-exchange chromatography, fractions containing the purified histone protein are pooled and dialyzed against three changes of a 5 m*M* solution of DTT at 4°. A small aliquot of the slightly cloudy mixture is diluted into unfolding buffer (7 *M* guanidine-HCl, 20 m*M* Tris, 10 m*M* DTT, pH 7.5) and quantified at 276 nm ($\varepsilon_{276} = 0.320$ ml·mg^{-1}·cm^{-1}). The histone is then subjected to factor Xa cleavage.

Factor Xa Cleavage and Purification of C-Terminal Histone H3 Fragment

Factor Xa protease cleaves C terminal to a preferred recognition site of Ile-Glu/Asp-Gly-Arg. However, factor Xa can cleave other basic sites, and with high enzyme concentration and lengthy exposure the histone H3 C-terminal fragment can become completely degraded. Under limited exposure to factor Xa, cleavage occurs primarily at the introduced recognition site with a secondary C-terminal site that removes the last seven amino acids of the histone, cleaving between residues corresponding to Arg-128 and Arg-129 of the wild-type H3 sequence. Under proper conditions it is possible to achieve a cleavage product distribution of about 20% uncleaved, 55% singly cleaved, and 25% cleaved both N and C terminally. Proteins that do not remove the N-terminal recognition sequence are unreactive in the native ligation step (i.e., they do not present an N-terminal cysteine) and can readily be separated from the full-length ligated histone. However, proteins cleaved at both the N and C termini can be ligated and are difficult to resolve from nontruncated ligation products. Thus, it is necessary to remove the double-cleaved factor Xa product before ligation, using reversed-phase high-pressure liquid chromatography.

1. Perform a set a of pilot factor Xa cleavages to determine the amount of enzyme that gives the best cleavage results. In three Eppendorf tubes dilute the C-terminal histone H3 protein solution to a final concentration of 0.5 mg/ml in factor Xa buffer (20 m*M* Tris, 100 m*M* NaCl, 2 m*M* $CaCl_2$). Add factor Xa protease (New England BioLabs, Beverly, MA) to a final concentration of 4, 2, and 1 μg/ml and incubate with rocking at 25° for 30 min.

2. Quench the reactions with phenylmethylsulfonyl fluoride (PMSF) protease inhibitor at a final concentration of 1 m*M*. Incubate with rocking at 25° for 30 min.

3. Resolve 4, 2, and 1 μg of total protein for each of the reaction trials on a sodium dodecyl sulfate (SDS)–18% polyacrylamide gel. Visualize the

proteins with Coomassie blue stain. The uncleaved, singly cleaved, and N- and C-terminally cleaved histones have a mass of 12.6, 12.0, and 11.2 kDa, respectively.

4. Scale up the cleavage reaction described in step 1, using an amount of factor Xa that will give the greatest amount of singly cleaved histone product according to the pilot reactions. Quench and analyze the reaction products as described in the preceding steps.

5. Dialyze the reaction mixture against three changes of 0.1% trifluoroacetic acid in water. Flash freeze and lyophilize to dryness.

6. Dissolve the protein to a concentration of about 10 mg/ml in unfolding buffer. Separate the doubly cleaved histone protein from the other histone products by reversed-phase HPLC with a semipreparative reversed-phase C_4 column (Vydac 214TP C_4, 10 × 250 mm, 5-μm particle size, 300-Å pore size; W. R. Grace & Co., Columbia, MD), using a mixed solvent system of water–0.1% trifluoroacetic acid and acetonitrile–0.1% trifluoroacetic acid, and monitor at 228 nm (longer wavelengths can be used if the signal is saturating). Typically, up to 2 mg of crude total protein can be purified over a shallow gradient from 37 to 48% organic solvent over 33 min at 2.5 ml/min. The desired singly cleaved histone protein elutes as a broad peak, while the double-cleaved product elutes directly afterward (Fig. 3B). Note that the uncleaved histone protein coelutes with the singly cleaved histone protein and is carried into the ligation reaction.

7. Pool all the desired HPLC samples. To determine the protein concentration, take a small volume of the protein solution, dry it in a SpeedVac, and quantify at 276 nm ($\varepsilon_{276} = 0.320\ \text{ml}\cdot\text{mg}^{-1}\cdot\text{cm}^{-1}$) in unfolding buffer. Divide the stock into individual aliquots corresponding to 1 mg of the singly cleaved product, flash freeze, and lyophilize.

Histone H3 Ligation

Ligation of polypeptides containing a C-terminal thioester and an N-terminal cysteine occurs spontaneously in aqueous solution. However, to drive the bimolecular reaction, an excess of peptide thioester over cysteine-containing protein is used. To promote ligation, guanidine is added to reduce secondary and tertiary structure. Benzyl mercaptan is added both to provide a reducing environment and to drive the reversibility of *trans*-thioesterification.[13] Benzene thiol is added to increase the rate of the reaction.[13] Both of these thiol additives have an extremely strong odor and should be handled carefully.

[13] P. E. Dawson, M. J. Churchill, M. R. Ghadiri, and S. B. H. Kent, *J. Am. Chem. Soc.* **119,** 4325 (1997).

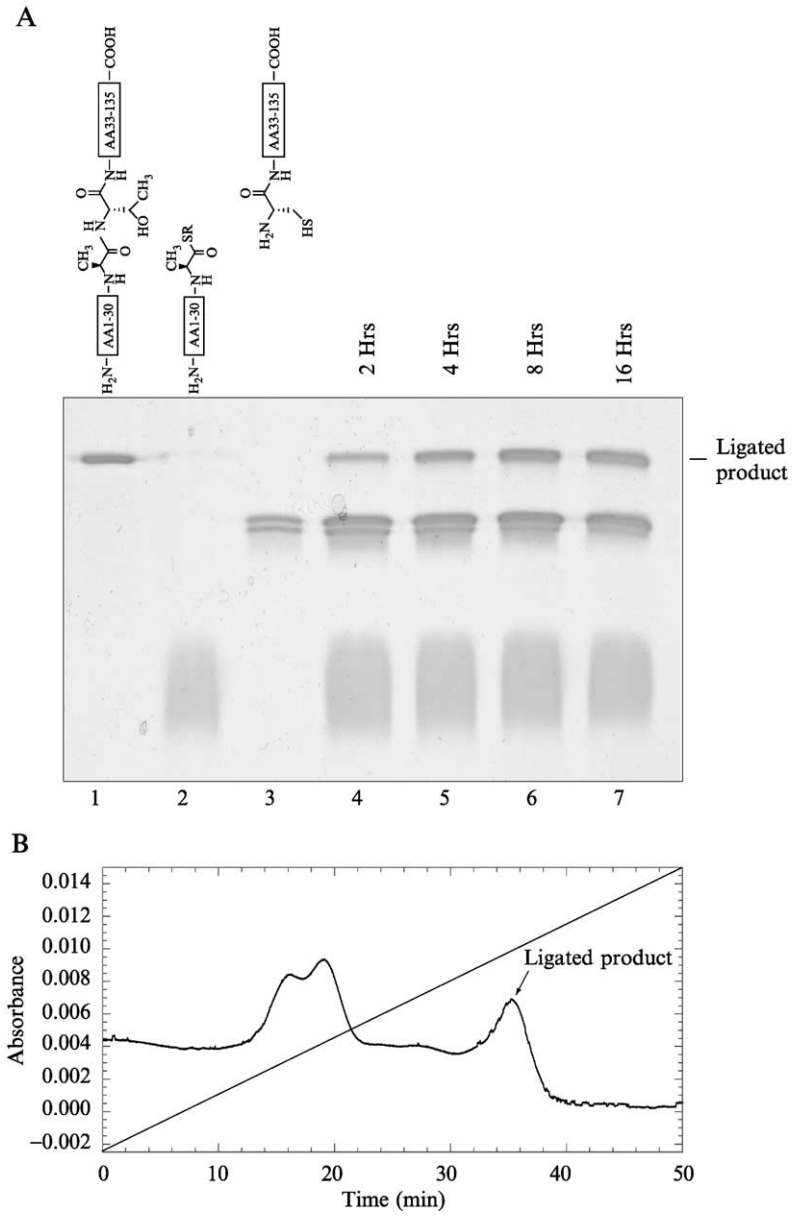

FIG. 4. Histone H3 ligation and purification. (A) The course of a histone H3 ligation reaction is analyzed on an SDS–18% polyacrylamide gel stained with Coomassie blue (lanes 4–7). For comparison, wild-type recombinant histone H3 (lane 1), synthetic N-terminal

Although almost all the singly cleaved C-terminal histone H3 fragment is converted into ligated product, some small fraction remains unreacted. Ion-exchange chromatography is used to remove the unreacted fraction as well as any uncleaved histone H3 and excess peptide. Because the C-terminal histone H3 fragment and the ligated product both have relatively poor solubility in the presence of the organic thiol additives, the reaction components are mixed with an acetonitrile–water solution before ion-exchange chromatography to aid solubility.

1. Perform a pilot ligation reaction by dissolving 1.00 mg of C-terminal histone H3 fragment (1×) and 1.3 mg of N-terminal H3 peptide (4× by weight) in 500 μl of ligation buffer (3 *M* guanidine-HCl, 100 m*M* potassium phosphate, pH 7.90). To the clear solution add 2.5 μl of both benzyl mercaptan and benzenethiol (0.5% each). Incubate the mixture at 25° with vigorous mixing.

2. Take 5-μl samples of reaction mixture (10 μg of total protein) over the course of 24 h. Add 20 μl of ligation buffer and then 75 μl of 25:75:0.1 acetonitrile–water–trifluoroethanol and desalt by dialysis. Separate 2 μg of protein on an SDS–18% polyacrylamide gel and visualize by Coomassie blue stain (Fig. 4A). Over the course of the reaction, the amount of ligated product should increase and then plateau. The amount of peptide thioester should decrease but not disappear.

3. Repeat step 1 with multiple 1-mg aliquots to that point in time at which the reaction does not proceed further as determined in step 2. Analyze the reaction on an SDS–18% polyacrylamide gel as described in step 2 to characterize the preparative reactions.

4. Prepare a stock of SAU-200 buffer (7 *M* urea, 20 m*M* sodium acetate, 200 m*M* sodium chloride, 1 m*M* DTT, 1 m*M* EDTA, pH 5.2), and SAU-600 buffer (7 *M* urea, 20 m*M* sodium acetate, 600 m*M* sodium chloride, 1 m*M* DTT, 1 m*M* EDTA, pH 5.2). Solubilize each reaction mixture by adding 2 ml of ligation buffer and then 22 ml of 25:75:0.1 acetonitrile–water–trifluoroethanol. Add 100 ml of SAU-200 buffer and load onto a Hi-Trap sulfopropyl HP ion-exchange column (Amersham Biosciences, Piscataway, NJ). Wash extensively with SAU-200. Elute the ligated histone with a linear gradient from 100% SAU-200 to 100% SAU-600 over 50 min (Fig. 4B).

histone H3 thioester peptide (lane 2), and undigested/singly cleaved C-terminal histone H3 proteins (lane 3) are included. (B) Ligation products are purified by ion-exchange chromatography. Shown is a representative trace from a 1-mg scale ligation reaction purified on a Hi-Trap sulfopropyl column. Over the course of 50 min, the sodium chloride concentration is increased linearly from 200 to 600 m*M* (7 *M* urea, 20 m*M* sodium acetate, 200–600 m*M* sodium chloride, 1 m*M* DTT, 1 m*M* EDTA, pH 5.2) and monitored at 280 nm. Full-length histone H3 elutes at 35 min.

5. Analyze fractions on an SDS–18% polyacrylamide gel and visualize by Coomassie blue stain. Pool the desired fractions. Dialyze against three changes of 5 m*M* DTT.

6. Quantify protein amounts by comparison with a wild-type histone H3 standard on an SDS–18% polyacrylamide gel. Characterize the identity of the histone H3 by mass spectrometry. Divide the samples into individual aliquots, flash freeze, and lyophilize.

Concluding Remarks

In our laboratory, we have generated histone octamers and nucleosomal arrays, using ligated wild-type and phosphorylated histone H3s, and have found that they display physical properties similar to those generated from recombinantly expressed histone H3.[3] In addition, as enzymatic substrates for remodeling and acetylation, these nucleosomal arrays display properties similar to those of wild-type, unligated arrays.[3] It should also be possible to incorporate ligated histones into other chromatin substrates, such as mononucleosomes.[12] In addition, with modification of the protocol described above, it should be possible to produce other histones with virtually any posttranslational modification and study them individually or in combination.

[5] Two-Dimensional Gel Analysis of Histones and Other H2AX-Related Methods

By DUANE R. PILCH, CHRISTOPHE REDON, OLGA A. SEDELNIKOVA, and WILLIAM M. BONNER

Background

Polyacrylamide was introduced by Ornstein[1] and Davis[2] as a more reproducible medium than starch for electrophoretic separations. At the same time, they also introduced a discontinuous buffer system that greatly increases sample resolution by electrically forcing proteins in dilute samples to concentrate into zones only microns thick. However, histone

[1] L. Ornstein, *Ann. N. Y. Acad. Sci.* **121,** 321 (1964).
[2] B. J. Davis, *Ann. N. Y. Acad. Sci.* **121,** 404 (1964).

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