Structure of a Covalently Cross-Linked Form of Core Histones Present in the Starfish Sperm†

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Received April 21, 1997; Revised Manuscript Received July 24, 1997⊗

ABSTRACT: The post-translational modification of core histones plays an essential role in chromatin remodeling processes. We recently reported the occurrence of a novel histone modification, involving a ϵ-(γ-glutamyl)lysine cross-link between a glutamine residue of histone H2B and a lysine residue of histone H4 in the testis of the starfish, *Asterina pectinifera* [Shimizu, T., Hozumi, K., Horiike, S., Nunomura, K., Ikegami, S., Takao, T., and Shimonishi, Y. (1996) *Nature 380*, 32]. In order to determine the complete structure of the modified histone heterodimer, p28 from both testis and sperm was purified. p28 was digested with *Achromobacter lyticus* protease I or *Staphylococcus aureus* V8 protease to give proteolytic fragments that were separated by HPLC. Amino acid analysis, sequencing, and mass spectrometric analysis of the fragments showed that the amino acid sequences of these fragments are identical to those of both histones H2B and H4, except for two NH_2-terminal peptides obtained by digestion with *A. lyticus* protease I. One of the peptides, K8, was identical to that reported previously, and the other was a here-to-fore unidentified peptide, which was designated K10. Amino acid and positive-ion FAB-MS/MS analyses of K10 showed that it to be a Gly-Glu-Lys Gly-Leu-Gly-Lys-Gly-Gly-Ala-Lys fragment, derived from Gly^8-Lys^{10} of histone H2B and Gly^9-Lys^{16} of histone H4. The yields of K8 and K10 were calculated to be 47 and 42%, respectively, expressed as the percent of the total amount of p28 used in the experiment. Based on these data, the structure of p28 was determined to be a heterodimer, composed of histones H2B and H4, formed through a transglutaminase-catalyzed acyl transfer reaction between Gln^9 of histone H2B and Lys^5 or Lys^{12} of histone H4.

The most abundant proteins associated with eukaryotic DNA are histones, a family of basic proteins found in all eukaryotic nuclei (*1*). The amino acid sequences of four core histones (H2A, H2B, H3 and H4) from distantly related species are remarkably similar (*2*). However, minor histone variants, which are encoded by genes that differ from the highly conserved major types, are also known to exist, particularly in gametes and early embryos of multicellular animals (*3*). In addition to primary sequence variation, histones undergo a variety of post-translational modifications, including acetylation, phosphorylation, methylation, ubiquitination, and ADP-ribosylation, that in turn alter the charge and the conformation of the molecules (*3*). The majority of these modifications occur at the NH_2-terminal regions of core histones and, most likely, modulate the affinity of the histones for DNA (*4, 5*).

Some animals, such as sea urchins and starfish, retain histones in the male spermatogenetic process, and as a result, these histones are present in mature spermatozoa (*3, 6*). The structures of sperm or testicular histones of these animals have been investigated in considerable detail in order to understand the effect and possible functions of histone variants on the fundamental remodeling of chromatin, a highly compacted complex of histones and DNA (*3*). In terms of the spermatogenic histones of starfish, the origin of several histone variants that migrate anomalously on polyacrylamide gel electrophoresis (*6, 7*) has not been fully resolved.

We have investigated the structure of p28, one of the histone molecules of the starfish (*Asterina pectinifera*) testis and found that p28 is a heterodimer composed of histones H2B and H4 produced via a transglutaminase-catalyzed acyl transfer reaction between Gln^9 of histone H2B and Lys^5 of histone H4 (*8*). However, lysine residues of histone H4 are located not only at the 5th position but also at the 8th, 12th, and 16th positions. The ϵ-amino groups of lysine residues located at positions other than the 5th position could also serve as an amine donor in the transglutaminase reaction. Detailed studies of the structure of p28 have shown that the 12th lysine residue also participates in the cross-linking of histones H2B and H4. In this paper, we report the complete structure of p28 as determined by biochemical and mass spectrometric analyses.

† This work was supported, in part, by a grant-in-aid for scientific research from the Ministry of Education, Science, Sports and Culture, Japan.

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⊗ Abstract published in *Advance ACS Abstracts*, September 15, 1997.

S0006-2960(97)00922-7 CCC: $14.00

MATERIALS AND METHODS

Materials. Specimens of *Asterins pectinifera* were collected from coastal waters off Japan during their breeding season and maintained in artificial seawater in laboratory aquaria. Testes were removed from the males, washed with calcium-free seawater (CFSW[1]), and stored at −80 °C until use. Sperm were released from cut testicular fragments and collected by brief centrifugation. CFSW was obtained from Jamarin Laboratory (Osaka, Japan). *Achromobacter lyticus* protease I and *Staphylococcus aureus* V8 protease were purchased from Wako (Tokyo, Japan) and Promega (Madison, WI), respectively. Subtilisin and carboxypetidase Y were obtained from Boehringer Mannheim, and leucine aminopeptidase and γ-glutamyl-ϵ-lysine were from Sigma (St. Louis, MO). Thermolysin and Pro-specific endopeptidase were purchased from Seikagaku Kogyo (Tokyo, Japan). μBondasphere C4-300A, Cosmosil 5Ph-AR-300, Shodex Asahipak GS-520 HQ, and Inertsil ODS HPLC columns were obtained from Waters (Milford, MA), Nakarai Tesque (Kyoto, Japan), Showa Denko (Tokyo, Japan), and GL Sciences (Tokyo, Japan), respectively. Bio Gel P-60 and P-10 were purchased from Bio-Rad (Richmond, CA). $H_2{}^{18}O$ (97 atom %) was purchased from the Commissariat a l'Energie Atomique, France.

Isolation of p28 and Histones H2B and H4 from Testes. Total histone fraction was obtained by extracting testes (3 g, wet weight) with 0.4 N H_2SO_4 as described previously (*9*). This fraction (33 mg of BSA equivalent) was dissolved in 1.5 mL of 8 M urea containing 1% (v/v) 2-mercaptoethanol. The solution was then held for 1 h at room temperature, HCl was added to a final concentration of 20 mM, and the mixture was centrifuged at 15000*g* for 10 min. The supernatant was fractionated by chromatography on a Bio Gel P-60 column (1.6 × 60 cm) equilibrated and eluted with an aqueous solution that was 20 mM in HCl and 50 mM in NaCl. Aliquots of each fraction were subjected to SDS−PAGE by the method of Laemmli (*10*). Fractions rich in p28 and histones H2B and H4, as determined by Coomassie stain on a 15% SDS gel, were recovered separately by precipitation with 10% (w/v) TCA, washed with acetone, and then dried *in vacuo*. The fractions were dissolved in 0.1% TFA, and the solutions were separately chromatographed by a RP-HPLC (μBondasphere C4-300A) column (3.9 × 150 mm). Histones were separated using a linear gradient of 30−70% acetonitrile (1%/min) in 0.1% TFA at a flow rate of 0.5 mL/min. Under these conditions, p28 and histones H2B and H4 were eluted at 58, 54, and 57% acetonitrile, respectively. All fractions were then dried *in vacuo*. p28 was further gel-filtered by HPLC on a Shodex GS-520 column (7.6 × 300 mm), equilibrated, and eluted with 10 mM aqueous HCl. The p28 fraction was dried *in vacuo* and further purified by HPLC using RP C4 and phenyl (Cosmosil 5Ph-AR-300) columns (4.6 × 150 mm) using similar gradient conditions as used for the first RP-HPLC. The amounts of p28 and histone H2B were determined by extracting the Coomassie-stained proteins on each band with formamide, followed by measurement of the absorbance of the dye in the extracts at 595 nm as described by Makino et al. (*11*).

To prepare large amounts of p28 and histone H2B, total testicular histones (330 mg of BSA equivalent) were fractionated by chromatography on a Bio Gel P-60 column (5.0 × 100 cm). Fractions rich in p28 and histone H2B were recovered by TCA precipitation, dissolved in SDS−sample buffer and heated at 60 °C for 1 h. These materials were separated by SDS−PAGE using an 18% gel with a wide sample well (11 cm in length). The Coomassie-positive bands, corresponding to p28 and histone H2B, were excised, and the proteins in the gel were separately extracted with 70% formic acid, followed by gel filtration using a Bio Gel P-10 column (1.0 × 20 cm) and 70% formic acid as an eluant. This fraction, which eluted with the void volume, was dried *in vacuo* and dissolved in distilled water. The amounts of p28 and histone H2B recovered were 0.6 and 10 mg (BSA equivalent), respectively.

Proteolytic Digestions. Purified histones were *S*-pyridylethylated (*12*) and digested at 37 °C for 16 h with *A. lyticus* protease I at an enzyme/substrate molar ratio of 1/100 in 20 mM Tris-HCl (pH 9.0) containing 2 M urea or with *S. aureus* V8 protease at an enzyme/substrate molar ratio of 1/50 in 25 mM sodium phosphate (pH 7.8).

The NH_2-terminal fragment (residues 1−31) of histone H2B produced by treatment with *S. aureus* V8 protease was separated by HPLC as described below. Half of the proteolytic fragments was dissolved in 20 mM Tris-HCl (pH 7.4) containing 2 mM $CaCl_2$ and digested at 37 °C for 16 h with thermolysin at an enzyme/substrate molar ratio of 1/50. The other half was dissolved in 0.1 M ammonium hydrogen carbonate (pH 8.0) and digested at 37 °C for 6 h with Pro-specific endoprotease at an enzyme/substrate molar ratio of 1/30.

Separation and Characterization of Proteolytic Fragments. Proteolytic digests were separately loaded onto a RP-HPLC (Inertsil ODS) column (4.6 × 250 mm). Fragments were eluted with 5% acetonitrile−0.1% TFA at a flow rate of 1 mL/min using a linear gradient of 5−60% acetonitrile (1%/min) in 0.1% TFA, after a 10-min hold. The isolated fragments were dried *in vacuo*, and their amino acid composition, mass values, and NH_2-terminal sequences determined.

Amino Acid and NH_2-Terminal Sequence Analyses. Samples were hydrolyzed in 6 N HCl containing 3% (w/v) phenol at 110 °C for 24 h. The hydrolysates were concentrated *in vacuo*, dissolved in 10 mM HCl, and analyzed with a Hitachi Model 835 amino acid analyzer (Tokyo, Japan). In some experiments, a purified proteolytic fragment was sequentially digested with subtilisin, leucine aminopeptidase, and carboxypeptidase Y according to the method of Cariello et al. (*13*). The digests were analyzed as described above.

Amino acid sequences of peptides were analyzed using an Applied Biosystems Model 477A sequencer equipped with a 120A PTH-amino acid analyzer (Palo Alto, CA). Histone H4 was chemically deblocked using TFA according to the method of Sobel et al. (*14*) prior to microsequencing.

ESI-MS, MALDI-MS, and Positive-Ion FAB-MS/MS. The ESI-MS spectra were obtained on a JMS-HX/HX110A double-focusing mass spectrometer (JEOL, Tokyo, Japan) equipped with an ESI ion source (Analytica of Branford,

[1] Abbreviations: CFSW, calcium-free seawater; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; RP, reversed phase; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; ESI, electrospray ionization; MS, mass spectrometry; FAB, fast atom bombardment; MS/MS, tandem mass spectrometry; MALDI, matrix-assisted laser desorption ionization; NMR, nuclear magnetic resonance.

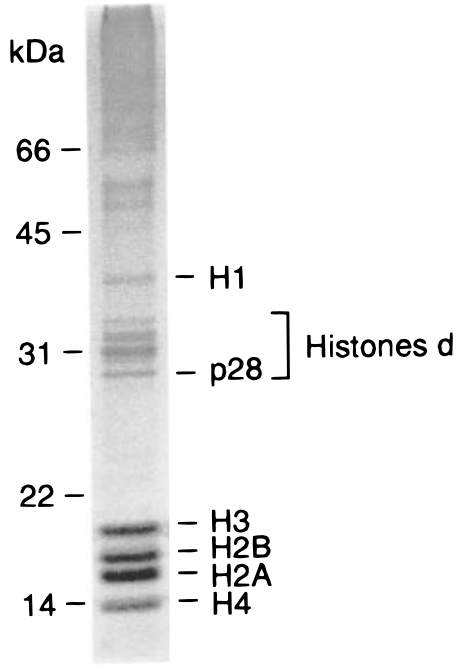

FIGURE 1: Separations of the total histone fraction prepared from *Asterina pectinifera* testes by SDS−PAGE on a 15% gel. The positions of histones H1, H2A, H2B, H3, H4, and p28 together with histones d are shown at the right. The mobility of the molecular weight standards are indicated (in kDa) at the left.

Branford, CT) and acquired in the *m/z* 300−2500 range by scanning a magnetic field in 4.0 s with a mass resolution of 1000. The mass calibration was performed using a mixture of KI and CsI (1:2, w/w) at a 7-kV accelerating voltage in the positive-ion FAB mode prior to installing the ESI source. Heated nitrogen at 170 °C was used as the drying gas and was introduced into the capillary region at a flow rate of 25 L/min. Samples (300 pmol) were dissolved in 10 μL of a solvent mixture of 0.2% TFA acetonitrile, water, 2-propanol, and 2-methoxyethanol (1/1/1/1, v/v) and infused into the ESI source at a flow rate of 1 μL/min with a Harvard syringe pump (Harvard Apparatus, South Natick, MA). Experimental details of this procedure have been described previously (*15*).

MALDI-MS was performed using a Voyager-RP time-of-flight mass spectrometer (PerSeptive Biosystems, Flamingham, MA). The spectra were obtained using a linear-mode measurement, wherein the ions, accelerated at 25 kV, were passed through a 1.3-m flight tube to the detector. Peptides (10−50 pmol/μL) were dissolved in 50% acetonitrile−0.1% TFA. Sample solutions (1μL) were placed on flat surfaces (2.7 mm diameter) of a stainless steel plate; mixed with the matrix solution, which was the supernatant of a 50% acetonitrile−0.1% TFA solution; saturated with α-cyano-4-hydroxycinnamic acid; and air-dried. The ions were generated by irradiating the sample area with the output of a nitrogen laser (337 nm). The signal was obtained by modulating the position of the spot area of an incident laser light with a stepper motor.

Positive-ion FAB-MS/MS spectra were obtained with the same instrument as was used for ESI-MS spectra, except that a FAB ion gun, a collision cell, and a photodiode array detector were used. Peptides (0.5 μg) were placed on the tip of a stainless steel probe, mixed with a liquid matrix [a mixture of dithiothreitol and dithioerythritol, 5:1 (w/w)], and then ionized by bombardment with xenon atoms accelerated at a 6-kV potential. The precursor ion, accelerated at 10 kV, was selected with a mass-resolving power by MS1 such that only the monoisotopic ion was transmitted into the collision cell, operated at 8 kV potential. The amount of argon gas introduced into the cell was adjusted to reduce the intensity of the precursor ions by 50%. The MS/MS product ion spectra were then acquired on an array detector with 10% mass dispersion by stepping up the electric and magnetic fields on MS2, where the magnetic field (B2) was

Table 1: Amino Acid Composition of p28, Histone H2B, and Histone H4

	p28 (residues/molecule)		H2B (residues/molecule)		H4 (residues/molecule)	
amino acid	analysis[a]	sequence[b]	analysis[a]	sequence[b]	analysis[a]	sequence[b]
Asp	11.5	5	6.3	2	5.0	3
Asn		6		4		2
Thr	12.4	15	8.0	9	5.0	6
Ser	12.3	14	10.3	12	1.8	2
Glu	15.8	10	9.6	6	6.1	4
Gln		5		3		2
Pro	6.3	6	5.8	5	1.1	1
Di-Me-Pro[c]	ND[f]	1	ND	1	ND	0
Gly	26.3	25	9.2	8	16.4	17
Ala	19.0	19	12.0	12	7.0	7
Val	14.3	16	6.3	7	7.1	9
Met	4.1	4	2.9	3	1.0	1
Ile	11.2	13	6.3	7	5.2	6
Leu	13.0	13	5.2	5	8.0	8
Tyr	9.6	9	4.7	5	4.0	4
Phe	3.8	4	1.7	2	1.9	2
Lys	28.0	30	19.2	20	9.6	10
Di-Me-Lys[d]	ND	1	ND	0	ND	1
His	4.7	5	2.8	3	2.0	2
Trp	ND	0	ND	0	ND	0
Pe-Cys[e]	0.7	1	0.0	0	0.8	1
Arg	18.2	21	6.5	7	12.8	14
total		223		121		102

[a] Values are based on the theoretical number of Ala residues/molecule of p28, histone H2B, and histone H4 being 19.0, 12.0, and 7.0, respectively. [b] Values are deduced from the sequence data. [c] Di-Me-Pro, *N*,*N*-dimethylproline. [d] Di-Me-Lys, *N*,*N*-dimethyllysine. [e] Pe-Cys, *S*-pyridylethylcysteine. [f] ND, not determined.

calibrated using a 20% acetonitrile solution containing LiI, NaI, KI, and CsI (1.5/4.5/0.01/1.0, mol/mol).

In some experiments, the purified proteolytic fragment (1 μg) was dissolved in 50 μL of 50 mM ammonium hydrogen carbonate (pH 7.8) containing 40 atom % $H_2{}^{18}O$ and incubated at 37 °C for 16 h with 0.1 μg of *A. lyticus* protease I, which has a high affinity for the COOH-terminal Lys residue of the peptide. The resulting peptide, partially labeled with ^{18}O at the COOH-terminus, was then directly subjected to FAB-MS/MS (*16*).

NMR Spectroscopy. Histone H2B (10 mg of BSA equivalent) was digested with *A. lyticus* protease I, and the fragments were separated on an Inertsil ODS column (10 × 250 mm). Fractions that contained the NH_2-terminal fragment were pooled, dried, and dissolved in ^2H_2O. The 500-MHz ^{1}H-NMR spectrum was obtained on a JNM-A500 spectrometer (JEOL, Tokyo, Japan), operated in the Fourier transform mode with the probe at room temperature, employing a broad decoupling method (*17*).

RESULTS

Separation of Sperm Histones. Total histone fraction of starfish testes contained slow-moving core histone dimers, denoted histones d, in addition to histones H2A, H2B, H3, and H4 with ordinary molecular masses (Figure 1). Of the seven histones d, p28 was easily separated from the others and isolated in pure form, as described below. The ratio of the amount of p28 to histone H2B was 0.1, as determined by Coomassie-binding capacity of the proteins. The total histone fraction was gel-filtered to separate p28 and histones H2B and H4. p28 was purified to homogeneity from the p28 fraction by HPLC using RP and gel filtration columns. Histones H2B and H4 from testes were purified to homogeneity by RP-HPLC. In a separate experiment, p28 and histones H2B and H4 were also isolated from sperm, which had been removed from the testis and freed from other testicular cells.

Amino acid analysis of p28 showed that the amino acid profile was nearly identical to that of the sum of sperm histones H2B and H4 (Table 1). Furthermore, ESI-MS gave a molecular mass for p28 of 24 654.0, which is 16.9 mass units less than the sum of the observed molecular masses of histones H2B (13 362.4) and H4 (11 308.5) (Figure 2).

Primary Structure of Sperm Histones H2B and H4. Chromatographically pure histone H2B (10 nmol) was digested with *A. lyticus* protease I or *S. aureus* V8 protease, and each digest was separated by HPLC using a RP column (data not shown). Amino acid composition and mass values of the separated peptides are shown in Table 2. NH_2-terminal sequences of the separated peptides were determined by conventional microsequencing, except for fragments AP1 and V3 (Table 2). Fragment AP1 (residues 1−7), with blocked NH_2-terminal, was subjected to FAB-MS/MS analysis. As shown in Figure 4, the NH_2-terminal amino acid residue appears to be either *N*,*N*-dimethylproline or *N*-formylproline, and the sequence of 2nd−7th amino acid residues is Pro-Lys-Pro-Ser-Gly-Lys. ^{1}H-NMR analysis of fragment AP1 indicated the presence of two methyl signals at δ 3.24 (3 H, s) and 3.25 (3 H, s), which are assigned to the *N*-methyl groups (Figure 5), indicating that the NH_2-terminal amino acid residue of AP1 is *N*,*N*-dimethylproline.

In further experiments, fragment V3 (residues 1−31), produced by *S. aureus* V8 protease treatment, was further digested with thermolysin or Pro-specific endopeptidase to give the peptides (dimethyl-Pro[1]-Pro-Lys-Pro-Ser-Gly-Lys-Gly-Gln-Lys-Lys[11] and Gly[17]-Ala-Pro-Ser-Thr-Asn-Lys-Lys-Arg-Lys-Arg-Lys-Arg-Lys-Glu[31], or Lys[11]-Ala-Gly-Lys-Ala-Lys-Gly-Ala-Pro[19], respectively) that were separated by RP-HPLC (data not shown). The NH_2-terminal sequences of the separated peptides were determined by FAB-MS/MS or by the automated Edman degradation reaction. The sequence thus determined (upper sequence in Figure 3) shows that the starfish sperm histone H2B is unique in that the 9th residue is Gln in contrast to the sequence of histone H2B of other organisms in which no Gln residues are present among the NH_2-terminal 20 amino acid residues.

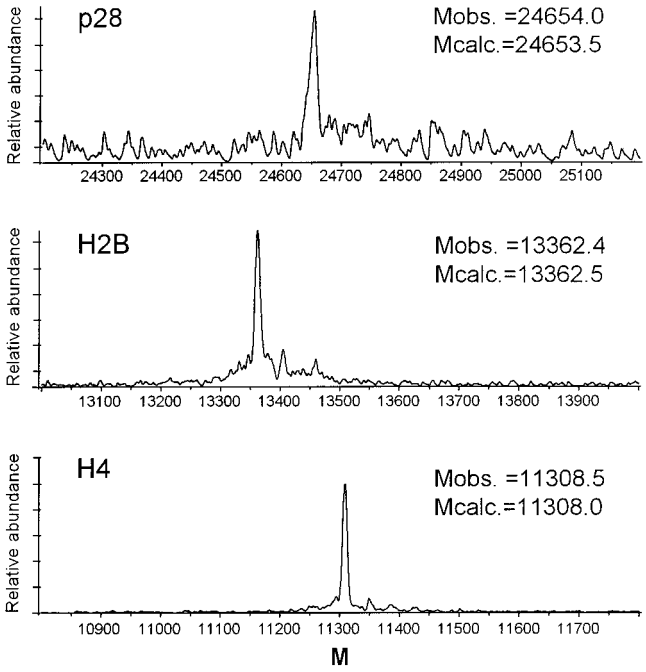

FIGURE 2: Deconvoluted ESI-MS spectra of p28 and histones H2B and H4. The theoretical masses were calculated from the primary structures corresponding to p28 and histones H2B and H4, respectively, as shown in Figure 3.

Chromatographically pure histone H4 (10 nmol) was digested with *A. lyticus* protease I or *S. aureus* V8 protease, and the resulting peptides were separated by HPLC using an RP column (data not shown). Amino acid composition, mass values, and NH_2-terminal sequences of separated peptides were determined, and the results of these data are summarized in Figure 3 (lower sequence). The structure of the fragment (residues 17−31), produced by *A. lyticus* protease I treatment of histone H4, was determined by FAB-MS/MS analysis, showing that the 4th amino acid residue is ϵ-*N*,*N*-dimethyllysine (Figure 6). Histone H4, which was chemically deblocked using TFA prior to microsequencing, gave the sequence of the 18 NH_2-terminal amino acid residues (Figure 3). The sequence of histone H4 is identical to that of sea urchin (*Psammechinus miliaris*) gonad histone H4 (*18*).

These results show that none of the ϵ-amino groups of Lys[5], Lys[8], Lys[12], and Lys[16] of sperm histone H4 are acetylated whereas those of histone H4 obtained from *A. pectinifera* embryos are acetylated to varying degrees (data not shown). Apart from the NH_2-terminal Pro, none of the amino acid residues of sperm histone H2B are modified. The theoretical values calculated from the sequence of histone

Table 2: Amino Acid Composition and Mass Values of Peptides Produced by Digestion of Histone H2B with *A. lyticus* Protease I or *S. aureus* Protease

	peptide produced by *A. lyticus* protease I (residues/molecule)									
amino acid	AP1	AP2	AP3	AP4	AP5	AP6	AP7	AP8	AP9	AP10
Asx			1.00(1)				1.06(1)			3.86(4)
Thr		0.88(1)	0.95(1)		1.62(2)	0.83(1)	1.06(1)		2.69(3)	
Ser	1.00(1)[c]		0.86(1)		1.00(1)	1.00(1)	1.95(2)	0.91(1)	1.85(2)	2.70(3)
Glx						1.22(1)	1.09(1)	1.14(1)	3.01(3)	2.12(2)
Pro	1.69(2)		0.99(1)				0.67(1)		0.89(1)	
Gly	1.08(1)		1.11(1)		0.37(0)	0.92(1)	1.07(1)	1.19(1)	1.00(1)	
Ala		1.14(1)	1.12(1)			0.92(1)			1.95(2)	4.76(5)
Val		1.00(1)		0.96(1)		1.09(1)	0.90(1)		1.67(2)	0.98(1)
Met				1.00(1)						1.75(2)
Ile							1.00(1)	1.85(2)	0.87(1)	2.64(3)
Leu									3.85(4)	0.98(1)
Tyr					1.02(1)			2.89(3)		0.95(1)
Phe										2.10(2)
Lys	1.86(2)	1.06(1)	1.28(1)	1.31(1)	1.07(1)	0.98(1)	1.11(1)	1.00(1)	1.00(1)	1.00(1)
His						1.04(1)	0.85(1)			0.96(1)
Arg									1.75(2)	1.81(2)
total	7[d]	4	7	3	5	8	11	9	22	28
position	1−7	113−116	17−23	40−42	117−121	105−112	43−53	31−39	83−104	54−81
m/z										
observed[a]	738.4[e]	418.4	674.7	377.4	599.5	828.7	1169.1	1135.7	2383.0	3200.0
calculated[b]	738.8	418.5	674.7	377.5	599.7	828.9	1169.3	1136.3	2383.8	3200.7

	peptide produced by *S. aureus* protease (residues/molecule)								
amino acid	V1	V2	V3	V4	V5	V6	V7	V8	V9
Asx			1.16(1)		1.02(1)		0.84(1)	1.13(1)	2.91(3)
Thr			1.09(1)	3.51(4)	1.83(2)				1.01(1)
Ser		0.85(1)	2.00(2)	1.00(1)	2.64(3)		1.95(2)	1.00(1)	3.41(4)
Glx	1.04(1)	1.12(1)	2.20(2)		1.21(1)	1.07(1)	0.91(1)	1.20(1)	
Pro			2.90(3)				1.06(1)	1.08(1)	
Gly			4.15(4)	1.08(1)			1.00(1)	1.09(1)	1.15(1)
Ala	2.00(2)	2.14(2)	3.14(3)	1.04(1)	2.04(2)		1.74(2)		1.08(1)
Val		1.00(1)		0.96(1)				1.94(2)	1.03(1)
Met								1.11(1)	1.91(2)
Ile	0.89(1)				1.00(1)	0.95(1)		2.05(2)	2.01(2)
Leu		1.08(1)			1.04(1)		2.67(3)		
Tyr				1.10(1)	0.88(1)			2.94(3)	
Phe						1.00(1)			1.00(1)
Lys		1.09(1)	11.90(11)	3.03(3)	2.13(2)			2.23(2)	1.10(1)
His		1.03(1)			1.03(1)			0.85(1)	
Arg	0.95(1)		3.02(3)		1.99(2)		1.08(1)		
total	5	8	31[d]	12	17	3	12	16	17
position	68−72	102−109	1−31	110−121	73−89	65−67	90−101	32−47	48−64
m/z									
observed[a]	559.6	854.8	3402.2	1285.6	1963.7	408.3	1296.5	1942.3	1802.7
calculated[b]	559.6	855.0	3402.1	1285.5	1963.2	408.5	1296.6	1942.3	1803.1

[a] The data represent the average mass value of MALDI-MS analysis. [b] Values were calculated based on the amino acid sequence data. [c] Values in parentheses are derived from the sequence data. [d] Dimethylproline, one residue of which is present, is not detected upon amino acid analysis of the acid hydrolysate. [e] This value was deduced from a monoisotopic mass value observed by FAB-MS/MS, as shown in Figure 4.

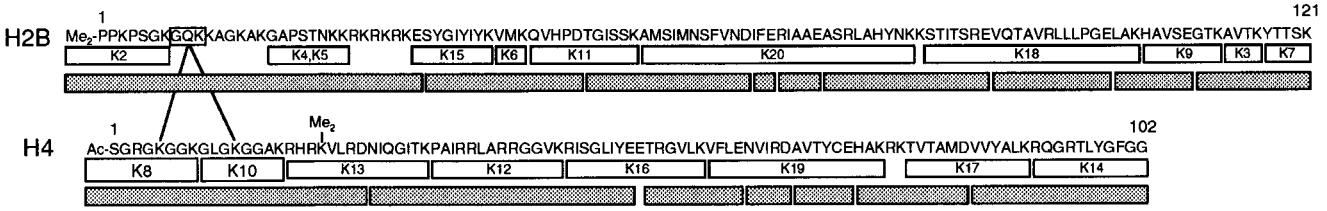

FIGURE 3: Primary structure of p28. Open and stippled bars below the sequence (single-letter code) of histones H2B and H4 indicate the fragments produced by treatment of p28 with *A. lyticus* protease I and *S. aureus* V8 protease, respectively. The NH_2-terminal fragment produced by *S. aureus* V8 protease digestion of histones H2B and H4 was determined as a cross-linked form by ESI-MS analysis (data not shown).

H2B (13 362.5) and histone H4 (11 308.0) are consistent with their experimentally observed masses, 13 362.4 and 11 308.5 (Figure 2), respectively.

Structure of p28. The structure of p28 was previously shown to be a modified histone, which contains an ϵ-(γ-glutamyl)lysine cross-link between histones H2B and H4. This conclusion is based on the fact that K8, obtained by digestion of p28 with *A. lyticus* protease I, was determined to be the peptide that is cross-linked between Gln^9 of histone H2B and Lys^5 of histone H4 (*8*). The yield of K8, however,

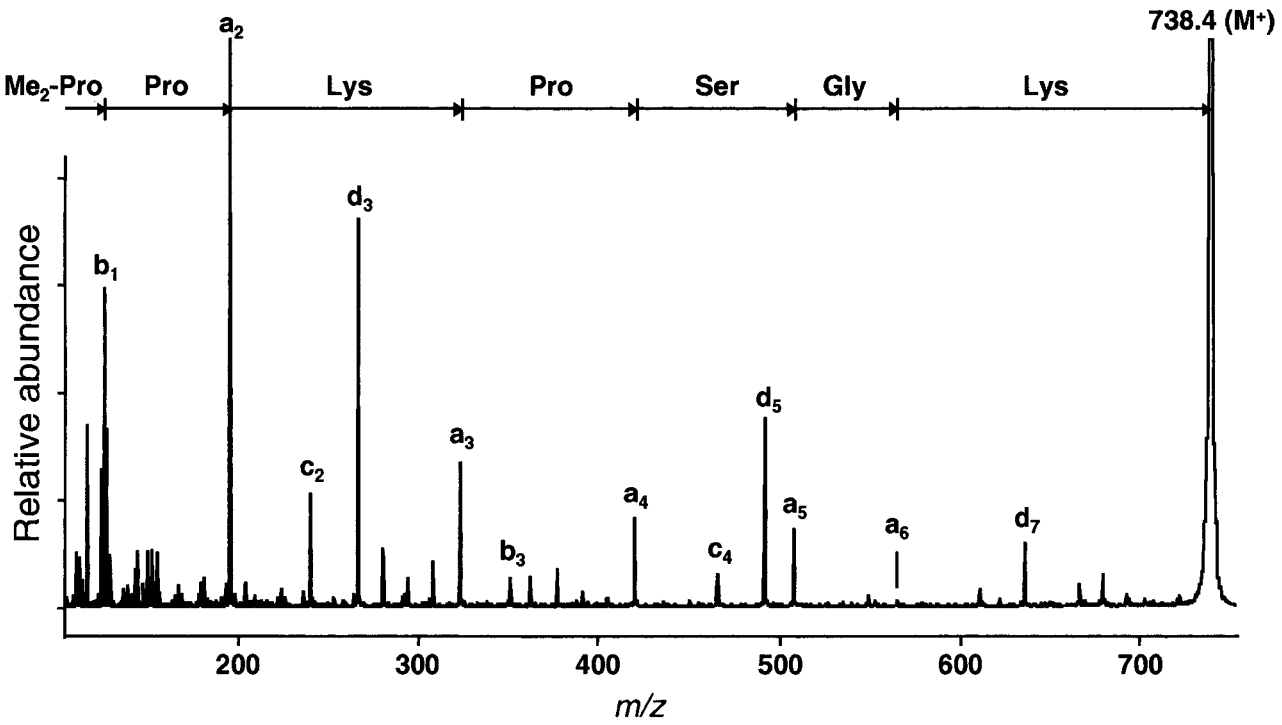

FIGURE 4: Positive-ion FAB-MS/MS spectrum of fragment AP1. The nomenclature of sequence ions for the peptide is as described elsewhere (*33*).

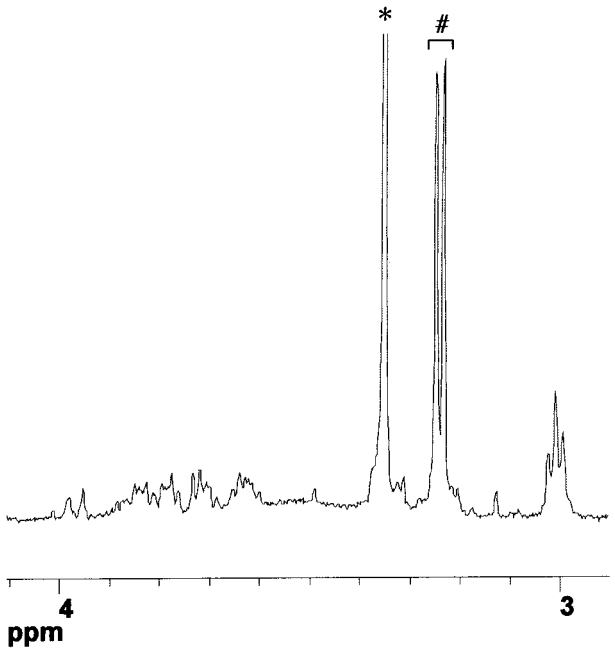

FIGURE 5: 500-MHz ^{1}H-NMR spectrum of fragment AP1. Chemical shifts are expressed in parts per million downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate but were actually measure by reference to internal methanol ($\delta = 3.35$ ppm in ^2H_2O at 27 °C), and the signal is indicated by the symbol (*). A doublet peak (#) is assigned to the *N*-methyl group on *N*, *N*-dimethylproline.

was less than 50% of the total amount of p28 that was subjected to proteolytic hydrolysis. We have now been able to identify another fragment, K10, that was separated by RP-HPLC of an *A. lyticus* protease I digest (Figure 7) and report its structure based on analysis by positive-ion FAB-MS/MS (Figure 8). The value of 1001.4 observed for the molecular ion signal is 17 mass units less than the sum of the theoretical molecular masses of Gly^8-Lys^{10} of histone H2B and Gly^9-Lys^{16} of histone H4. The difference of 17 mass units is consistent with that observed for intact p28. Upon collision-induced dissociation, the precursor ion gave the COOH-terminal product ions as the major fragments due to charge retention on the Lys residues in the molecule. The product ions could be assigned to those arising from cleavage of the peptide backbone and on the cross-linking component, which indicated the structure shown in Figure 8. In addition, an exhaustive digestion of K10 with subtilisin, leucine aminopeptidase, and carboxypeptidase Y (*13*) gave 1 mol of ϵ-(γ-glutamyl)lysine on amino acid analysis (Table 3). These data show that the histone H2B and H4 molecules are cross-linked between Gln^9 of histone H2B and Lys^{12} of histone H4. In the amino acid analysis, fragments K8 (1.50 nmol) and K10 (1.34 nmol) were both recovered from the RP column (Figure 7). The yields of K8 and K10 were calculated to be 47% and 42%, respectively, expressed as a percent of the total amount of p28 used in this experiment (3.2 nmol). The results indicate that the histone H2B and H4 molecules are cross-linked between Gln^9 of histone H2B and Lys^5 or Lys^{12} of histone H4 (Figure 3) and that these two Lys residues may serve as nearly equivalent substrates for transglutaminase, which catalyzes an acyl transfer reaction between the γ-carboxamide group of Gln residues and the ϵ-amino group of Lys residues (*19*).

DISCUSSION

During spermatogenesis, spermatogonia give rise to mature spermatozoa whose cellular and nuclear morphologies are usually distinctly different from those of their precursor cells (*3*). Typically, the final product of this developmental sequence is a streamlined cell with little cytoplasm and a condensed nucleus. In most species, major changes occur in the types of structural proteins associated with the nuclear DNA of spermatids, and these are often assumed to function in chromatin compaction. The remodeling of chromatin resulting in the mature starfish sperm nucleus is not as extreme as for the well-documented cases of protamine-containing sperm of rodents or trout. In mature starfish

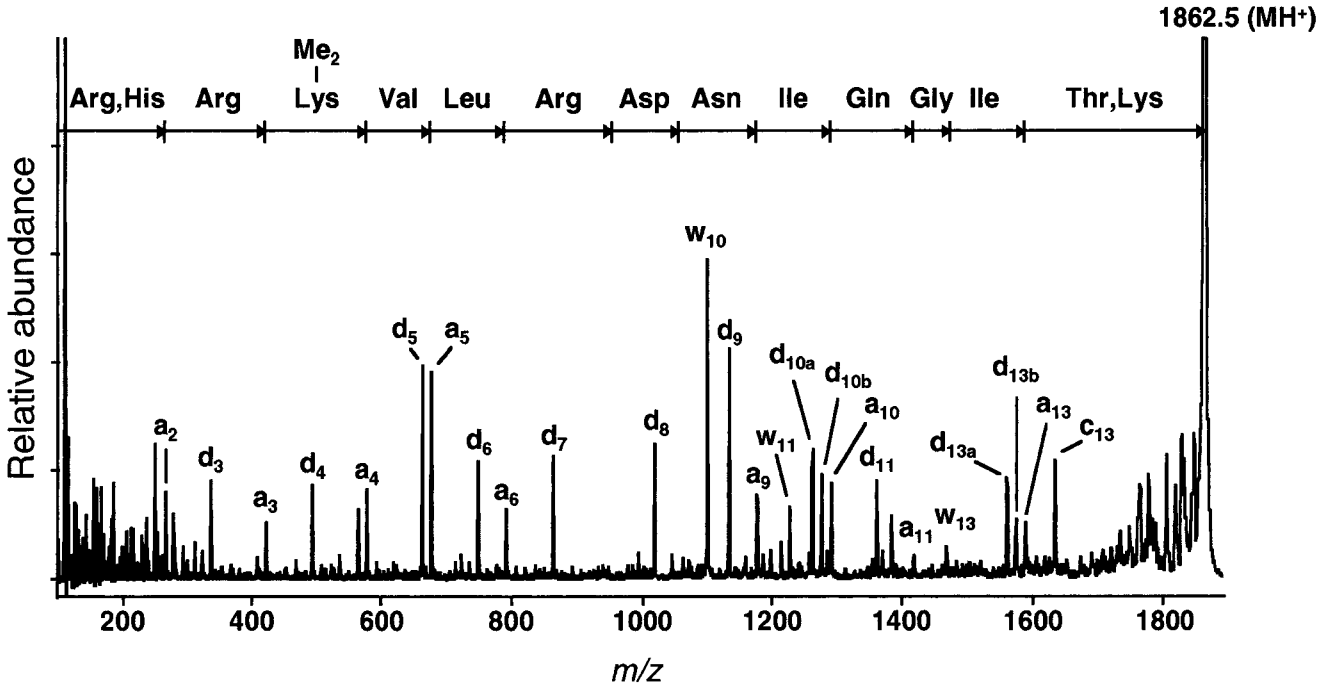

FIGURE 6: Positive-ion FAB-MS/MS spectrum of the fragment (residues 17−31) produced by *A. lyticus* protease I treatment of histone H4.

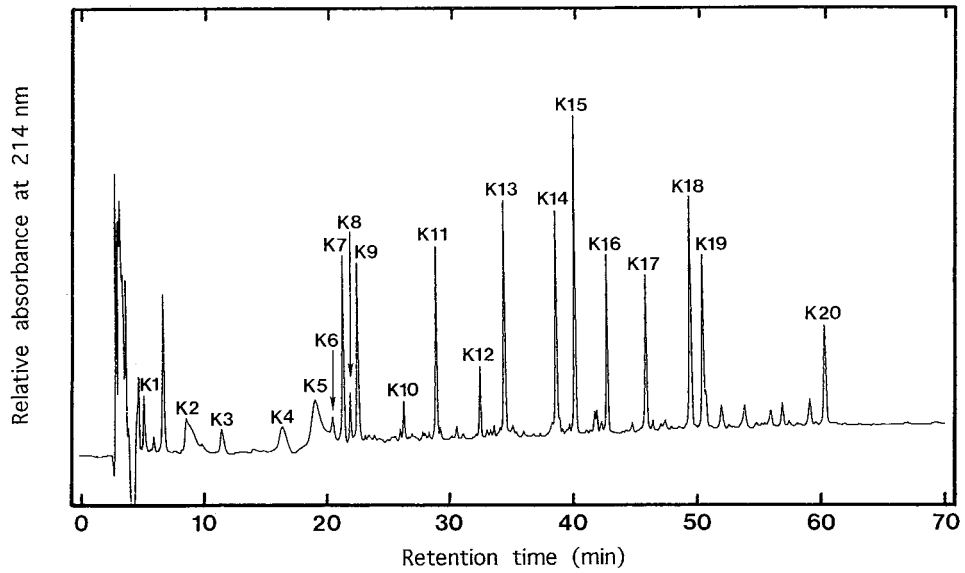

FIGURE 7: HPLC profile of *A. lyticus* protease I peptides derived from *S*-pyridylethylated p28. Purified p28 was *S*-pyridylethylated and digested with *A. lyticus* protease I. The digest was loaded onto an Inertsil ODS column (4.6 × 250 mm). Fragments were eluted at a flow rate of 1 mL/min with a linear gradient of 5−60% acetonitrile (1%/min) in 0.1% TFA after a 10-min hold in 5% acetonitrile-0.1% TFA.

sperm, no protamines are present, and linker and core histones are retained (*6*, *7*). The sperm chromatin is organized into essentially typical core nucleosomes (*20*).

Core histones undergo several post-translational modifications such as acetylation, phosphorylation, methylation, ADP-ribosylation, ubiqutination, etc. (*21*−*24*). The results described in this work provide evidence for the additional modification of core histones. This modification, which is cross-linking by an ϵ-(γ-glutamyl)lysine bridge between two core histone molecules, is considered to be produced by a reaction catalyzed by transglutaminase. However, production of such isopeptide cross-links could be accounted for by several other mechanisms such as activation of a γ-carbonyl of one histone molecule by esterification followed by a nucleophilic attack from an ϵ-amino groups of the appropriate Lys residue of another histone molecule (*25*). Recent findings that transglutaminase activity has been detected in the nucleus of rabbit liver (*26*) and starfish embryos (unpublished results) favor the idea that the formation of this type of histone dimers is catalyzed by transglutaminase.

The acetylation of histone H4 competes with the transglutaminase-catalyzed histone dimerization reaction involving a cross-link between a Gln residue of histone H2B and a Lys residue of histone H4, because an acetylated lysine residue is not a functional amine donor substrate for transglutaminase. Preliminary work in our laboratories have indicated that a significant amount of p28 is produced in *A. pectinifera* embryos at the midblastula stage but not in embryos at earlier stages. The treatment of an *A. pectinifera* embryo with trichostatin A, a potent inhibitor of histone deacetylase (*27*), induces hyperacetylation of histone H4 and causes developmental arrest at the early gastrula stage (*9*).

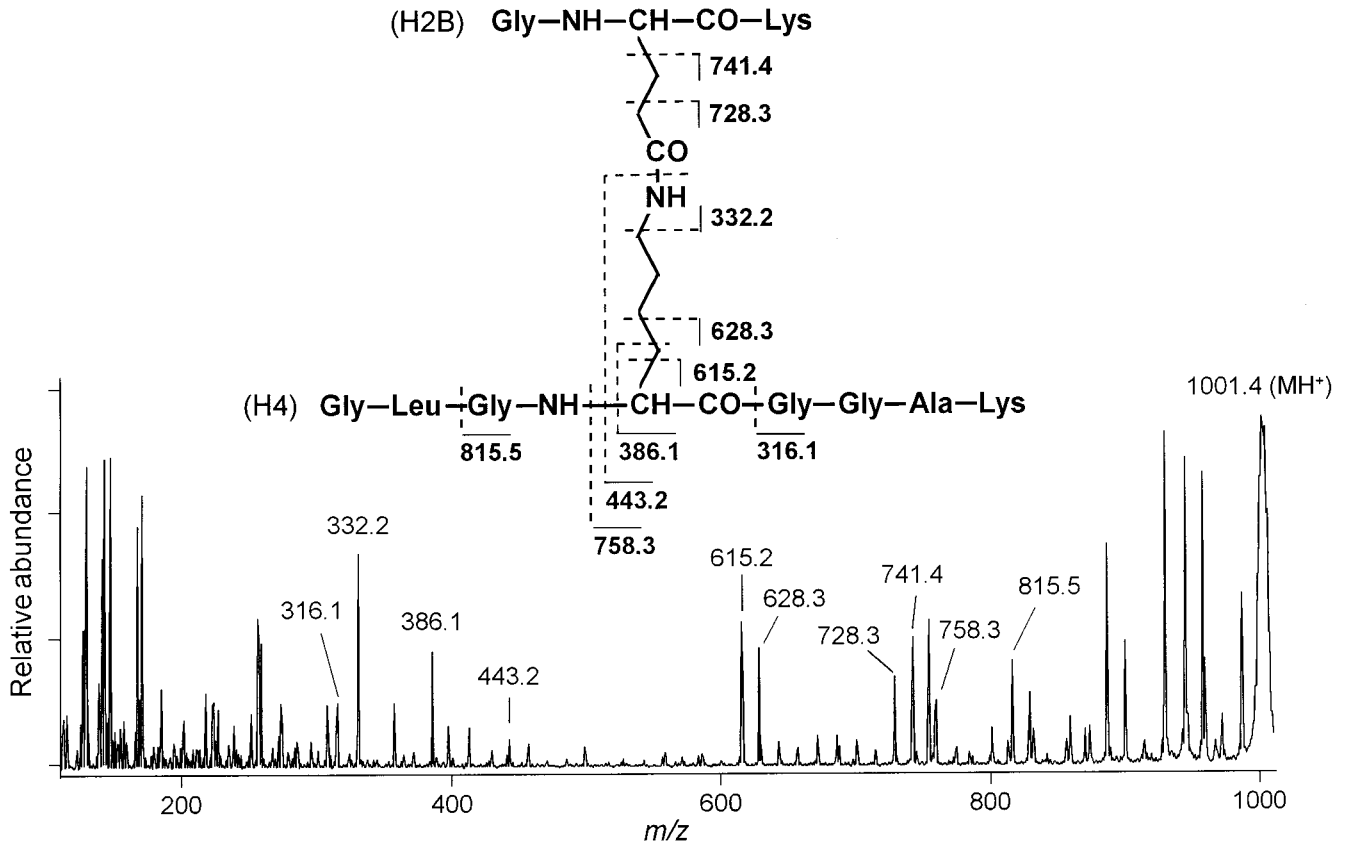

FIGURE 8: Positive-ion FAB-MS/MS spectrum of fragment K10. Mass values and the proposed assignment of structure are shown for the key product ions in the spectrum. The signals at 815.5, 758.3, and 316.1 are *z*-type ions around the peptide bond (*33*). The remaining values are for ions arising from cleavage at the linkage between the side chains of Gln^9 of histone H2B and Lys^{12} of histone H4. The assignments of product ions were aided by comparing two FAB-MS/MS spectra derived from the precursor ions with and without ^{18}O atoms incorporated at the two COOH-termini of the partially ^{18}O-labeled fragment K10, as described under Materials and Methods.

Table 3: Amino Acid Composition of Fragment K10 (Residues/Molecule)

amino acid	reagent of hydrolysis	
	HCl	protease[a]
Glx	1.2 (1)[b]	ND[c] (0)
Gly	5.2 (5)	4.8 (5)
Ala	1.1 (1)	1.1 (1)
Leu	1.1 (1)	1.0 (1)
Lys	3.0 (3)	2.0 (2)
ϵ-(γ-glutamyl)lysine		1.0 (1)

[a] Subtilisin, leucine aminopeptidase, and carboxypeptidase Y. [b] Values in parentheses are derived from sequence data. [c] ND, not detected.

Trichostatin A treatment causes suppression of the appearance of p28 in embryonic cells (unpublished results). When Lys^5 and Lys^{12} of histone H4 are acetylated, they may be incapable of functioning as an amine donor substrate in the transglutaminase-mediated cross-linking reaction, and this may explain the mechanism by which trichostatin A suppresses the formation of p28 during the midblastula stage.

Ballestar et al. (*28*) have recently shown that core histones are remarkably good glutaminyl substrates for transglutaminase 2, a member of the transglutaminase family that is a ubiquitiously distributed, soluble enzyme (*29*−*32*). They incorporate monodansylcadaverine more rapidly than many physiological transglutaminase substrates. When the reaction is carried out with native nucleosomes prepared from chicken erythrocyte nuclei, Gln^5 and Gln^{19} of histone H3 and Gln^{22} of histone H2B, which are not labeled in free histones, are specifically modified (*28*). Such basal reactivity changes may reflect structural features of nucleosomal organization. The results described in this paper indicate that starfish histone H2B is a good glutaminyl substrate for nuclear transglutaminase and could equally incorporates an ϵ-amino group of Lys^5 or Lys^{12} of histone H4 as an amine donor, which might be spatially close to Gln^9 of histone H2B in the nucleosome of starfish sperm. Such cross-linking core histone might be involved in structural changes of nucleosome, which leads to chromatin condensation.

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BI970922N