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Structures of human nucleosomes containing major histone H3 variants

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The nucleosome is the fundamental repeating unit of chromatin, *via* which genomic DNA is packaged into the nucleus in eukaryotes. In the nucleosome, two copies of each core histone, H2A, H2B, H3 and H4, form a histone octamer which wraps 146 base pairs of DNA around itself. All of the core histones except for histone H4 have nonallelic isoforms called histone variants. In humans, eight histone H3 variants, H3.1, H3.2, H3.3, H3T, H3.5, H3.X, H3.Y and CENP-A, have been reported to date. Previous studies have suggested that histone H3 variants possess distinct functions in the formation of specific chromosome regions and/or in the regulation of transcription and replication. H3.1, H3.2 and H3.3 are the most abundant H3 variants. Here, crystal structures of human nucleosomes containing either H3.2 or H3.3 have been solved. The structures were essentially the same as that of the H3.1 nucleosome. Since the amino-acid residues specific for H3.2 and H3.3 are located on the accessible surface of the H3/H4 tetramer, they may be potential interaction sites for H3.2- and H3.3-specific chaperones.

1. Introduction

Genomic DNA is highly compacted as chromatin and packaged into the nucleus. The fundamental repeating structure of chromatin is the nucleosome. Histones H2A, H2B, H3 and H4 are the protein components of the nucleosome. Two copies of each core histone, H2A, H2B, H3 and H4, form the histone octamer (Arents *et al.*, 1991), which is composed of a dimer of H3/H4 heterodimers (H3/H4 tetramer) and two H2A/H2B heterodimers. In the nucleosome, 146 base pairs of DNA are wrapped around the histone octamer (Luger *et al.*, 1997). The nucleosomes are connected by a short DNA segment called the linker DNA (Schalch *et al.*, 2005) and the nucleosome array is further folded into a higher ordered chromatin structure, probably through inter-nucleosome interactions.

The core histones share common structural features, consisting of N- and/or C-terminal tails and the histone-fold domain (Arents & Moudrianakis, 1995). The histone tails have flexible structures and are enriched with amino-acid residues targeted for post-translational modifications. The N-terminal tails protrude from the nucleosome surface (Luger *et al.*, 1997; Davey *et al.*, 2002) and function as target sites for *trans*-acting factors and/or as sites for inter-nucleosome interactions for higher ordered chromatin formation. In contrast, the histone-fold domains are well structured and consist of a long central α-helix (α2) bordered by two short α-helices (α1 and α3). The H2A/H2B and H3/H4 heterodimers are formed by interactions between the histone-fold domains.

Nonallelic isoforms of H2A, H2B and H3, called histone variants, exist in higher eukaryotes (Franklin & Zweidler,

1977). For H3, eight variants, H3.1, H3.2, H3.3, H3T, H3.5, H3.X, H3.Y and CENP-A, have been identified in humans (Palmer *et al.*, 1987; Albig *et al.*, 1996; Witt *et al.*, 1996; Ahmad & Henikoff, 2002; Malik & Henikoff, 2003; Henikoff *et al.*, 2004; Kamakaka & Biggins, 2005; Hake & Allis, 2006; Loyola & Almouzni, 2007; Ray-Gallet & Almouzni, 2010; Wiedemann *et al.*, 2010; Schenk *et al.*, 2011). These H3 variants are considered to have distinct functions in the organization of the chromatin structure. H3.1 and H3.2 are expressed in S phase and are incorporated into chromatin in a replication-dependent fashion with the specific histone chaperone CAF1 (Kaufman *et al.*, 1995). Unlike H3.1 and H3.2, H3.3 is constitutively expressed and is predominantly incorporated into transcriptionally active chromatin regions in a replication-independent fashion, probably by the specific histone chaperone HIRA (Ray-Gallet *et al.*, 2002). H3.3 is also incorporated at the telomeres of chromosomes with the help of specific histone chaperones, ATRX and DAXX, in an HIRA-independent manner (Goldberg *et al.*, 2010; Szenker *et al.*, 2011). H3T and H3.5 were found as testis-specific H3 variants in humans (Albig *et al.*, 1996; Witt *et al.*, 1996; Schenk *et al.*, 2011). Structural and biochemical analyses revealed that the H3T nucleosome may be assembled by the histone chaperone Nap2 (Tachiwana *et al.*, 2008) and is quite unstable compared with the H3.1 nucleosome (Tachiwana *et al.*, 2010). The instability of the H3T nucleosome may play an important role in the chromatin reorganization required for spermatogenesis. CENP-A is a centromere-specific H3 variant which functions as an epigenetic marker for kinetochore formation sites (Palmer *et al.*, 1987). Lastly, H3.X and H3.Y are novel histone variants that may be involved in the regulation of cellular responses to outside stimuli (Wiedemann *et al.*, 2010).

Among these H3 variants, H3.1, H3.2 and H3.3 are the most abundant and are considered to commonly exist in all types of tissues and cells. In the present study, we determined the crystal structures of human nucleosomes containing either the H3.2 or H3.3 variant and showed that their structures are essentially the same as that of the H3.1 nucleosome (Tsunaka *et al.*, 2005; Tachiwana *et al.*, 2010).

2. Experimental procedures

2.1. Purification of human histones

Human H2A, H2B, H3.1, H3.2, H3.3, H3T, CENP-A and H4 were overexpressed in *Escherichia coli* cells as described previously (Tanaka *et al.*, 2004). The cells producing the N-terminally His_6-tagged recombinant histones were collected and disrupted by sonication in 50 ml buffer *A* (50 m*M* Tris–HCl pH 8.0, 500 m*M* NaCl, 1 m*M* PMSF, 5% glycerol). The insoluble fraction containing the His_6-tagged histones was resuspended in 50 ml buffer *A* containing 7 *M* guanidine hydrochloride. The supernatants containing the His_6-tagged histones were combined with 4 ml (50% slurry) nickel–nitrilotriacetic acid (Ni–NTA) agarose resin (Qiagen) and mixed by rotation for 1 h at 277 K. The agarose beads were packed into an Econo-Column (Bio-Rad) and washed with 100 ml buffer *B* (50 m*M* Tris–HCl pH 8.0, 500 m*M* NaCl, 6 *M* urea, 5 m*M* imidazole, 5% glycerol). The His_6-tagged histones were eluted with a 100 ml linear gradient of imidazole from 5 to 500 m*M* in buffer *B* and the samples were dialyzed against buffer *C* (5 m*M* Tris–HCl pH 7.5, 2 m*M* β-mercaptoethanol). The His_6 tags were cleaved using thrombin protease (1 unit per milligram of histones; GE Healthcare) at room temperature for 3 h and each histone preparation was subjected to Mono-S column chromatography (GE Healthcare). The column was washed with buffer *D* (20 m*M* sodium acetate pH 5.2, 200 m*M* NaCl, 5 m*M* β-mercaptoethanol, 1 m*M* EDTA, 6 *M* urea) and the histones were eluted with a linear gradient of NaCl from 200 to 900 m*M* in buffer *D*. The purified histones were dialyzed against water, freeze-dried and stored at 277 K.

2.2. Preparation of the H3.2 and H3.3 nucleosomes

Histone octamers containing either H3.2 or H3.3 were prepared as described previously (Tachiwana *et al.*, 2010). The purified histone octamers (0.9 mg) were mixed with a 146-base-pair DNA (1 mg) in a solution containing 2 *M* KCl. The octamer–DNA mixtures were dialyzed against dialysis buffer (10 m*M* Tris–HCl pH 8.0, 1 m*M* EDTA, 1 m*M* dithiothreitol, 2 *M* KCl) at 277 K for 3 h. The KCl concentration in the dialysis buffer was then gradually decreased to 250 m*M* using a peristaltic pump (0.8 ml min^{-1} flow rate). The mixtures were finally dialyzed against 10 m*M* Tris–HCl buffer pH 8.0 containing 1 m*M* EDTA, 1 m*M* dithiothreitol and 250 m*M* KCl at 277 K for 3 h. The reconstituted nucleosomes were incubated at 328 K for 2 h and were separated from the free DNA and histones by nondenaturing polyacrylamide gel electrophoresis using a Prep Cell apparatus (Bio-Rad). The purified nucleosomes were concentrated and dialyzed against 20 m*M* potassium cacodylate buffer pH 6.0 containing 1 m*M* EDTA for crystallization or 20 m*M* Tris–HCl buffer pH 7.5 containing 1 m*M* EDTA and 1 m*M* dithiothreitol for biochemical analyses.

2.3. Crystallization and structure determination

Crystals of the purified H3.2 and H3.3 nucleosomes were obtained by the hanging-drop method. The drop included the H3.2 or H3.3 nucleosomes (1 µl) and a solution (1 µl) consisting of 20 m*M* potassium cacodylate buffer pH 6.5, 50–70 m*M* KCl and 70–120 m*M* $MnCl_2$. The reservoir solution consisted of 20 m*M* potassium cacodylate pH 6.5, 35–45 m*M* KCl and 55–80 m*M* $MnCl_2$. Crystals typically appeared within 7–10 d and grew to their full size over a period of 3–4 d. The H3.2 and H3.3 nucleosome crystals were soaked in a cryoprotectant solution consisting of 20 m*M* potassium cacodylate pH 6.5, 40 m*M* KCl, 55 m*M* $MnCl_2$, 28% 2-methyl-2,4-pentanediol and 2% trehalose for 5–10 s at room temperature and were flash-cooled in a stream of N_2 gas (100 K). The H3.2 and H3.3 nucleosome crystals both belonged to the orthorhombic space group $P2_12_12_1$ and contained one nucleosome per asymmetric unit. Data collection was performed on beamline BL41XU at the SPring-8 synchrotron-radiation source (Harima, Japan).

Diffraction data were integrated and scaled with *HKL*-2000 (Otwinowski & Minor, 1997). Subsequent data handling was performed using the *CCP*4 suite (Winn *et al.*, 2011). The phases of the H3.2 and H3.3 nucleosome structures were determined by the molecular-replacement method using *MOLREP* (Vagin & Teplyakov, 2010; Winn *et al.*, 2011). The coordinates of the H3.1 nucleosome structure (PDB entry 3afa; Tachiwana *et al.*, 2010) were used as a search model. The molecular-replacement solutions were subjected to rigid-body, energy-minimization and *B*-factor refinements using *CNS* (Brünger *et al.*, 1998). Further structural refinement consisted of iterative rounds of energy minimization and *B*-factor refinement using *CNS* (Brünger *et al.*, 1998) and model building using *Coot* (Emsley *et al.*, 2010). Data-collection and structure-refinement statistics are presented in Table 1. All structure figures were created using *PyMOL* (DeLano, 2002). The atomic coordinates of the H3.2 and H3.3 nucleosomes have been deposited with PDB codes 3av1 and 3av2, respectively.

2.4. Acid–urea–Triton (AUT) gel electrophoretic analysis

AUT gel electrophoretic analysis of the H3 histone variants was performed as reported previously (Zweidler, 1978) with modifications. HeLa cell histones were prepared as described previously (Simon & Felsenfeld, 1979). For the analysis, the histones were precipitated by trichloroacetic acid, washed with ice-cold acetone and then dissolved in AUT sample buffer (8 *M* urea, 1 *M* acetic acid, 60 m*M* ammonia, 0.004% methylene blue). The resolving gel contained 7 *M* urea, 15% acrylamide, 0.4% bisacrylamide, 1 *M* acetic acid, 8.3 m*M* thiourea, 60 m*M* ammonia and 0.375% Triton X-100 (polymerized with 0.06% hydrogen peroxide) and the stacking gel contained 7 *M* urea, 4.8% acrylamide, 0.16% bisacrylamide, 1 *M* acetic acid, 8.3 m*M* thiourea and 60 m*M* ammonia (polymerized with 0.075% hydrogen peroxide). The gel was pre-run for 1 h (0.42 W cm^{-1} for gels of 0.75 mm thickness) with the electrodes reversed in a running buffer consisting of 1 *M* acetic acid and 107 m*M* glycine. After the pre-run, the polarity of the electrodes was restored and the histones were fractionated through the AUT gel (0.42 W cm^{-1} for 4 h). The histones were visualized by Coomassie Brilliant Blue staining.

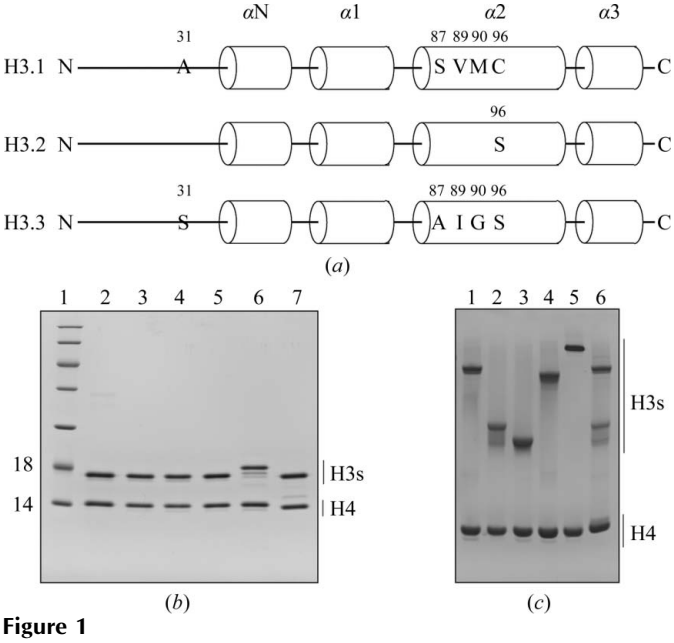


Figure 1
SDS–PAGE and AUT gel electrophoretic analyses of H3/H4 complexes. (*a*) Sequence comparison between human H3.1, H3.2 and H3.3. Amino acids that differ between H3.1 and H3.2 or H3.3 are indicated by capital letters. Cylinders indicate α-helices found in the crystal structure of the human nucleosomes. (*b*) SDS–PAGE analysis of H3/H4 complexes. Lane 1, size markers (kDa); lane 2, H3.1/H4; lane 3, H3.2/H4; lane 4, H3.3/H4; lane 5, H3T/H4; lane 6, CENP-A/H4; lane 7, HeLa H3/H4. (*c*) AUT gel electrophoretic analysis of H3/H4 complexes. Lane 1, H3.1/H4; lane 2, H3.2/H4; lane 3, H3.3/H4; lane 4, H3T/H4; lane 5, CENP-A/H4; lane 5, HeLa H3/H4.

Table 1
Crystallographic statistics.

Values in parentheses are for the highest resolution shell.

	H3.2 nucleosome	H3.3 nucleosome
Data collection		
Space group	$P2_12_12_1$	$P2_12_12_1$
Unit-cell parameters (Å)	*a* = 106.47, *b* = 109.63, *c* = 181.93	*a* = 105.84, *b* = 109.69, *c* = 181.18
Resolution (Å)	50–2.5 (2.59–2.50)	50–2.8 (2.90–2.80)
Reflections (measured/unique)	1390849/74215	1175309/52981
$R_{\rm merge}$† (%)	8.0 (68.9)	7.3 (51.2)
$\langle I/\sigma(I)\rangle$	11.3 (3.0)	14.2 (4.0)
Completeness (%)	99.9 (100)	99.8 (99.8)
Multiplicity	7.3 (6.6)	5.2 (4.9)
Overall *B* factor from Wilson plot (Å^2)	60.1	70.1
Refinement		
Resolution (Å)	50–2.5	50–2.8
R factor/free *R* factor‡ (%)	24.5/29.0	23.0/27.2
R.m.s. deviations		
Bond lengths (Å)	0.007	0.007
Bond angles (°)	1.12	1.17
Ramachandran plot, residues in		
Most favourable regions (%)	94.6	92.0
Additionally allowed regions (%)	5.0	7.7
Generously allowed regions (%)	0.5	0.3

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of an observation and $\langle I(hkl)\rangle$ is the mean value for that reflection. ‡ *R* factor = $\sum_{hkl} ||F_{\rm obs}| - |F_{\rm calc}|| / \sum_{hkl} |F_{\rm obs}|$, where $F_{\rm obs}$ and $F_{\rm calc}$ are the observed and calculated structure-factor amplitudes, respectively. The free *R* factor was calculated using 5% of the data that were excluded from the refinement.

3. Results

3.1. Histones H3.1, H3.2 and H3.3 are major H3 variants in HeLa cells

Human histones H3.1, H3.2 and H3.3 have the same length and their amino-acid sequences are highly conserved. As shown in Fig. 1(*a*), H3.1 and H3.3 differ by five amino acids while H3.1 and H3.2 differ by only one. Consequently, they exhibit indistinguishable migration distances on SDS–PAGE (Fig. 1*b*). However, on an acid–urea–Triton (AUT) gel H3.2 and H3.3 show markedly faster migration compared with that of H3.1 (Fig. 1*c*, lanes 1–3). This is probably caused by the cysteine–serine difference at amino-acid position 96 (Fig. 1*a*).

To confirm that the migration differences observed for the recombinant H3 variants are also visible in H3 variants purified from cells, we purified the H3/H4 complexes from nuclear pellets of HeLa cells. AUT gel electrophoresis of the H3/H4 complex revealed that the single band of H3 observed on SDS–PAGE (Fig. 1*b*, lane 7) was composed of three major bands with migration distances similar to those of recombinant histones H3.1, H3.2 and H3.3 (Fig. 1*c*, lane 6). These results demonstrated that the recombinant and native H3.1, H3.2 and H3.3 variants migrate similarly when fractionated by AUT gel electrophoresis. Moreover, the AUT gel electrophoresis data are consistent with the fact that H3.1, H3.2 and H3.3 are the major H3 variants and are present at an approximate ratio of 7:2:1 in HeLa cells.

3.2. Crystal structures of the human H3.2 and H3.3 nucleosomes

We previously determined the crystal structure of the nucleosome core particle containing H3.1 at 2.5 Å resolution (Tachiwana *et al.*, 2010). In the present study, histone octamers and nucleosome core particles containing H3.2 or H3.3 were reconstituted using the methods that were employed for those containing H3.1. These methods are essentially the same as those described previously (Luger *et al.*, 1999). We found that the reconstitution yields of histone octamers and nucleosome core particles containing H3.1, H3.2 and H3.3 were nearly the same. The nucleosome core particles containing H3.2 and H3.3 were crystallized with potassium chloride and manganese chloride in a similar manner to the H3.1 nucleosome. The crystal structures of the nucleosome core particles containing H3.2 and H3.3 were solved at 2.5 and 2.8 Å resolution, respectively (Table 1), by the molecular-replacement method using the H3.1 nucleosome as the search model. The overall structures of the H3.2 and H3.3 nucleosomes were essentially similar to that of the H3.1 nucleosome (Fig. 2), as also indicated by the root-mean-square deviations (r.m.s.d.s) of the histone octamers. The r.m.s.d. values were 0.120 Å for the H3.1 and H3.2 histone octamers, 0.149 Å for the H3.1 and H3.3 histone ocatmers and 0.144 Å for the H3.2 and H3.3 histone octamers.

H3.1 and H3.2 differ by one amino-acid residue at position 96, where H3.1 has a cysteine residue and H3.2 has a serine residue. H3.3 also has a serine residue at this position, in addition to four further amino-acid differences from H3.1 at amino-acid positions 31, 87, 89 and 90. Except for the amino-acid residue at position 31, which is located in the flexible N-terminal tail, all of the other residues are buried inside the nucleosome. These residues are located in the N-terminal region of the α2 central helix in the histone-fold domain, which directly interacts with H4 (Fig. 3). Thus, although the present nucleosome structures show that the H3.1/H4, H3.2/H4 and H3.3/H4 dimers have nearly identical structures in their respective nucleosomes, the variant-specific amino-acid residues of H3.1, H3.2 and H3.3 may affect H3/H4 dimer structures that are not incorporated into the nucleosome. Furthermore, the variant-specific amino-acid residues are apparently accessible to other factors if the H3/H4 dimer is not incorporated into the nucleosome. This suggests that these residues may function as binding sites for their specific chaperones, such as CAF1 (Kaufman *et al.*, 1995) and HIRA (Ray-Gallet *et al.*, 2002), which directly bind to H3/H4 as part of the histone-deposition complexes for nucleosome assembly *in vivo* (Tagami *et al.*, 2004).

4. Discussion

H3.1, H3.2 and H3.3 are the major H3 variants in mammals. Our AUT gel electrophoretic analysis revealed that the major bands of H3 variants purified from HeLa nuclear extracts correspond to H3.1, H3.2 and H3.3. In contrast, bands corresponding to the testis-specific H3 variant H3T and the centromere-specific H3 variant CENP-A were not visible in the purified HeLa H3/H4 fraction, indicating the much lower abundance of these H3 variants in HeLa cells.

In the present study, we determined the crystal structures of nucleosomes containing H3.2 and H3.3 and found that the structures of the H3.1, H3.2 and H3.3 nucleosomes are

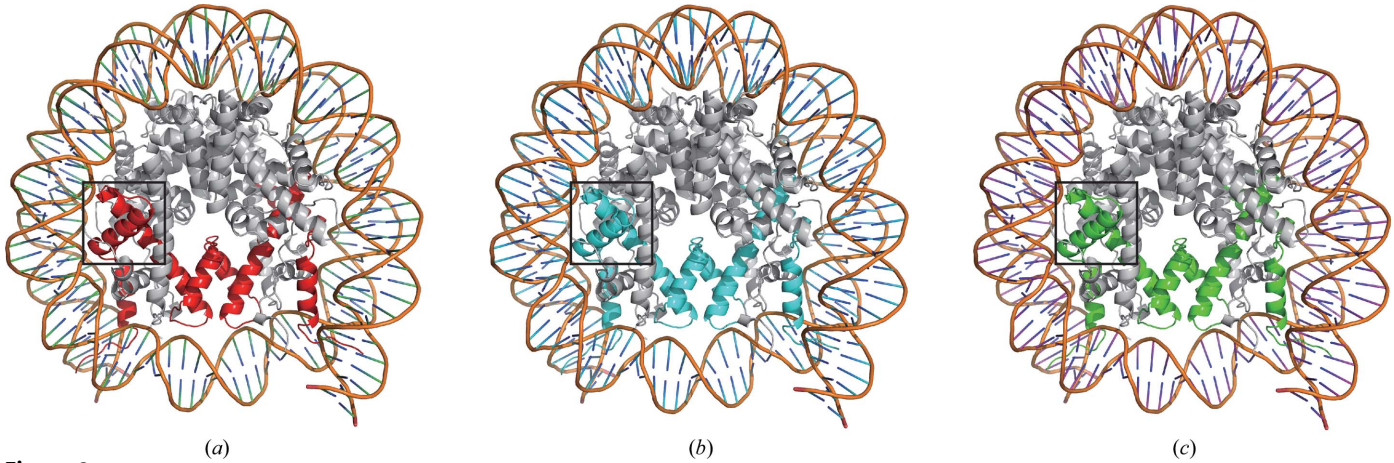
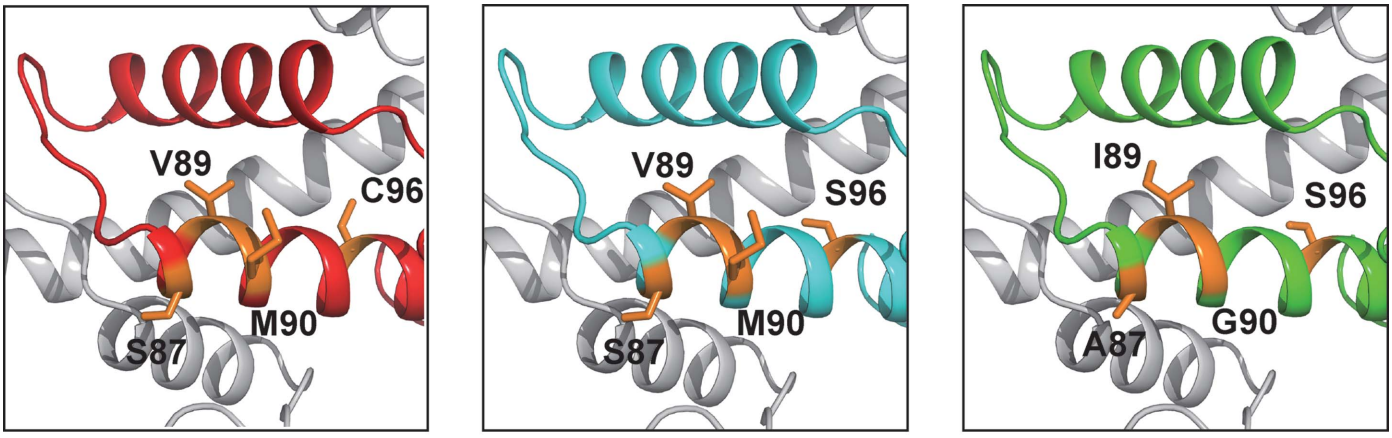


Figure 2
Crystal structures of the H3.1, H3.2 and H3.3 nucleosomes. The H3.1, H3.2 and H3.3 variants are shown in red, cyan and green, respectively. The amino-acid residues that differ between the variants are located in the boxed regions. (*a*) H3.1 nucleosome. (*b*) H3.2 nucleosome. (*c*) H3.3 nucleosome.



(*a*) (*b*) (*c*)

Figure 3
Enlarged views of the regions containing the amino-acid residues that differ between the H3.1, H3.2 and H3.3 variants. The amino-acid residues that are not conserved among H3.1, H3.2 and H3.3 are coloured orange. (*a*) H3.1 nucleosome. (*b*) H3.2 nucleosome. (*c*) H3.3 nucleosome.

essentially the same. H3.1 (and/or H3.2) and H3.3 are considered to form distinct pre-nucleosome assembly complexes in the cytoplasm (Tagami *et al.*, 2004) and may be assembled into specific regions of chromosomes. There are five amino-acid differences between human H3.1 and H3.3, four of which are located on the N-terminal segment of the α2 helix. These residues are located at or near the surface of the H3/H4 dimer and may be suitable for chaperones to bind during the formation of pre-nucleosome assembly complexes. Notably, this region is distal from the tetrameric interface of H3 (the α3 helix), which has been shown to interact with the H3/H4 chaperone Asf1 (English *et al.*, 2006; Natsume *et al.*, 2007). Specific H3.1 and H3.3 chaperones, such as CAF1 and HIRA (Tagami *et al.*, 2004), respectively, may recognize the variant-specific amino-acid residues, possibly through different interaction mechanisms.

We previously reported that the universal histone chaperones Nap1 and sNASP bind to H3.1, H3.2 and H3.3 and efficiently promote the formation of the H3.1, H3.2 and H3.3 nucleosomes (Osakabe *et al.*, 2010). In the present study, we have shown that the H3.2 and H3.3 nucleosomes can be reconstituted by the salt-dialysis method with efficiencies equal to that for the H3.1 nucleosome and that the structures of the three nucleosomes are similar. Therefore, the specific functions of the H3.1, H3.2 and H3.3 nucleosomes are probably not dependent on the core structure visible by X-ray crystallography, but may be attributable to their interacting factors. Enzymes that promote post-translational modifications of histones may be candidates for such histone-binding proteins. The post-translational modification patterns of H3.1, H3.2 and H3.3 are reportedly different in chromatin (Hake & Allis, 2006; Loyola *et al.*, 2006) and H3.1-specific (and/or H3.2-specific) or H3.3-specific pre-nucleosome assembly complexes may be responsible for their distinct post-translational modifications (Tagami *et al.*, 2004; Loyola *et al.*, 2006). Chromatin-remodelling factors and components of heterochromatin may also interact with the specific amino-acid residues of the H3 variants during nucleosome-reorganization processes such as transcription and DNA replication.

In conclusion, we have determined the crystal structures of the human H3.2 and H3.3 nucleosomes and found that they were essentially the same as that of the H3.1 nucleosome. The structures are expected to provide a structural basis for future studies aimed at understanding the functional differences between these H3 variants.

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