The Histone Variant Macro-H2A Preferentially Forms “Hybrid Nucleosomes”

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The histone domain of macro-H2A, which constitutes the N-terminal one third of this histone variant, is only 64% identical to major H2A. We have shown previously that the main structural differences in a nucleosome in which both H2A moieties have been replaced by macro-H2A reside in the only point of contact between the two histone dimers, the L1-L1 interface of macro-H2A. Here we show that the L1 loop of macro-H2A is responsible for the increased salt-dependent stability of the histone octamer, with implications for the nucleosome assembly pathway. It is unknown whether only one or both of the H2A-H2B dimers within a nucleosome are replaced with H2A variant containing nucleosomes in vivo. We demonstrate that macro-H2A preferentially forms hybrid nucleosomes containing one chain each of major H2A and macro-HA in vitro. The 2.9-Å crystal structure of such a hybrid nucleosome shows significant structural differences in the L1-L1 interface when comparing with homotypic major H2A- and macro-H2A-containing nucleosomes. Both homotypic and hybrid macro-nucleosome core particles (NCPs) are resistant to chaperone-assisted H2A-H2B dimer exchange. Together, our findings suggest that the histone domain of macro-H2A modifies the dynamic properties of the nucleosome. We propose that the possibility of forming hybrid macro-NCP adds yet another level of complexity to variant nucleosome structure and function.

Histones are responsible for the packaging of eukaryotic DNA into a nucleoprotein complex termed chromatin, the fundamental unit of which is the nucleosome core particle (NCP). The NCP consists of an octamer of two copies each of four core histones, H2A, H2B, H3, and H4, that organizes 147 base pairs of DNA in a tight superhelix (1). The structure and function of chromatin and ultimately the accessibility of nucleosomal DNA is regulated either through covalent post-translational modification of histones, through interactions with non-histone proteins (for example, architectural proteins), and through the incorporation of non-allelic variants of histones. Histone variants exhibit sequence differences in strategic regions of the histone fold, resulting in functional differences in the variant nucleosome and in chromatin higher order structure (2–6).

Structural information on a variety of nucleosomes (either from different species or carrying histone variants or mutations) is now available (reviewed in Ref. 7; see also Refs. 3 and 8). One major conclusion from these studies is that the structure of the nucleosome is conserved across the evolutionary spectrum (Saccharomyces cerevisiae to Homo sapiens) and appears to be immune to significant changes in amino acid sequence (9). Major functional changes are caused by relatively subtle manipulation of very specific regions within the structured parts of the histones, resulting in subtly altered protein-protein or protein-DNA interactions and/or in changes in the molecular surface of the nucleosome.

The L1 loops of both H2A moieties in the nucleosome form the L1-L1 interface, the only region where sequence differences lead to a significant difference in structure (2, 3), suggesting that the L1 loop may at least in part be responsible for the functional characteristics exhibited by variant nucleosomes (11–14). Here we demonstrate that four amino acids in the L1 loop of macro-H2A stabilize the histone octamer in the absence of DNA and are thus solely responsible for altering the in vitro salt-dependent nucleosome assembly pathway.

Macro-H2A is implicated in the formation of transcriptionally silent heterochromatin (15–17). In addition to a histone H2A-like domain, it also has a C-terminal domain that has been shown to bind NAD metabolites (18) and an H1-like linker region. Although several studies have addressed the in vivo localization patterns of macro-H2A (16, 19–21), many aspects of targeting and replication-independent deposition of macro-H2A remain to be understood. For example, it is not known whether one or both of the H2A moieties within a single nucleosome are replaced by macro-H2A. We have devised an in vitro assay to test whether hybrid nucleosomes (nucleosomes containing one macro-H2A-H2B and one H2A-H2B dimer) are indeed formed and find that macro-H2A preferentially forms hybrid nucleosomes after salt-dependent reconstitution onto DNA.

The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1. This article was selected as a Paper of the Week. The abbreviations used are: NCP, nucleosome core particle; NAP-1, nucleosome assembly protein 1; yNAP-1, yeast NAP-1.

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defined-sequence DNA. To better understand the adaptability of the L1 loop in particular in forming this interface, we determined the 2.9-Å crystal structure of such a hybrid nucleosome.

Macro-H2A is found predominantly in transcriptionally inactive heterochromatic regions of the genome, such as the inactive X chromosome in female mammals (16). ATP-dependent chromatin remodeling factors and histone chaperones play important roles in promoting chromatin fluidity. It was found previously that macro-H2A-containing nucleosomes were refractory to remodeling by ATP-dependent chromatin remodeling factors in vitro (13). Here, we show that chaperone-assisted H2A-H2B dimer exchange is greatly inhibited in homotypic and hybrid macro-NCP. Together, our results demonstrate how relatively minor sequence and structural deviations in a variant histone result in altered nucleosome dynamics, with implications for gene regulation.

**EXPERIMENTAL PROCEDURES**

Expression and Purification of Histone Proteins; Reconstitution of Nucleosomes—The expression vector for the histone domain of macro-H2A was a kind gift from Dr. Saadi Khochbin. The coding region was subcloned into a pet3a vector. Expression plasmids for H2B and H2A from mouse (Mus musculus) were a kind gift from Dr. David Tremethick. The coding region for mouse H2A was subcloned into a pet15b vector, which adds a His6-tag at the N terminus of the protein. We also used previously described expression plasmids for histones H2A and H2B. 100 μl of nickel-nitriiotracetic acid beads (Sigma) were thoroughly washed in binding buffer (150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 5 mM imidazole). Nucleosomes set up with a variety of combinations of H2A-H2B dimers as indicated were loaded on to the beads and rocked for 2 h at 4 °C. The flow-through was collected followed by three washes with binding buffer. The beads were then rocked for 15 min in elution buffer (150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 1 mM imidazole). Fractions along with the output were analyzed for their histone content on an 18% SDS-PAGE gel.

Crystallographic Procedures—Hybrid macro-NCP (prepared and characterized as described above) was crystallized using salting in vapor diffusion at NCP concentrations between 8−12 mg/ml with salt concentrations of 34−37.5 mM KCl and 40−45 mM MnCl2. The crystals were soaked in 24% 2-methyl-2,4-pentanediol and 5% trehalose and frozen in liquid nitrogen as described previously (1). Data were collected at Advanced Light Source (Lawrence Berkeley National Laboratory) on beamline 5.0.2. Data from a single crystal were processed using Denzo and Scalepack (27). Protein Data Bank entry 1U35 was used as the search model in molecular replacement. Molecular replacement and subsequent rounds of refinement were performed using CNS (28). The program O was used for model building (29). The entire model was checked using simulated anneal omit maps for critical regions during various stages of refinement and a composite omit map at the end.

Crystallographic Coordinates and Structure Factors—The crystallographic coordinates and structure factors for the hybrid macro-NCP have been deposited under the ID number 2F8N.

**Macro-H2A Nucleosome Structure and Function**
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RESULTS

Macro-NCP and Major NCP Behave Differently during Salt-dependent Reconstitution Due to Differences in Histone Octamer Stability—We have previously shown that NCP preparations reconstituted from major-type histones and a 146-base pair DNA fragment are heterogeneous with respect to the position of the histone octamer on the DNA (Ref. 31 and references therein). These positional isoforms are distinguished by their different electrophoretic mobility and can usually be converted to a thermodynamically favorable position by incubation at 37 °C for 20–60 min (“heat shifting,” Fig. 1A, lanes 1 and 2). Macro-NCP also reconstitutes as two different species, but the heat-induced redistribution is reproducibly incomplete. A significant fraction of macro-NCP remains in the upper band after heating at 37 °C (Fig. 1A, lanes 3 and 4) and even higher temperatures (55 °C; data not shown). The histone composition of the bands was compared by SDS-PAGE. This analysis demonstrates that shifted macro-NCP contains a full complement of histones in stoichiometric amounts (Fig. 1B, compare lanes 5 and 6). However, the slower migrating species appears to be partially depleted for macro-H2A_HD and -H2B (Fig. 1B, compare lanes 3 and 4). We therefore hypothesize that the slower migrating band of macro-NCP obtained after salt gradient reconstitution consists of two species, one that can be fully shifted and thus represents nucleosomes with different translational positioning and one that cannot be converted to the thermodynamically more stable form by heating, due to a non-canonical histone composition.

We next investigated whether this is specific to assembly of macro-NCP by salt gradient dialysis. This method relies on the dissociation of the histone octamer (which is stable at 2 M NaCl in the absence of DNA but not under physiological conditions) into a (H3-H4)_2 tetramer and two H2A-H2B dimers at relatively high ionic strength, followed by the sequential deposition of (H3-H4)_2 tetramers and H2A-H2B dimers onto the DNA at consecutively lower salt concentrations. The delicate balance between the stability of the histone octamer on the one hand and the relative stability of the H2A-H2B dimer and (H3-H4)_2 tetramer interaction with DNA at critical ionic strength may be altered in macro-NCP. To address this possibility, we reconstituted macro-NCP using two different approaches. First, instead of mixing refolded macro-octamer with DNA, we used independently refolded macro-H2A_HD/H2B dimers, (H3-H4)_2 tetramer, and 146-bp DNA for salt gradient deposition. This approach has previously been shown to give identical results for Xla-NCP (14). Macro-NCP obtained with this method reproducibly exhibited complete heat-induced shifting, as observed for canonical Xla-NCP (Fig. 1C, lanes 1 and 2). Second, we reconstituted major and macro-NCP in a salt-independent way, using (γNAP-1, Fig. 1C, lanes 3–8). Again, macro-NCP obtained by this method was shifted completely upon incubation at 37 °C (lane 4).

Since macro-NCP did not assemble normally from refolded histone octamer using salt dialysis but only from individually purified macro-H2A-H2B dimers and (H3-H4)_2 tetramers using salt gradient deposition or NAP-1, we hypothesized that the macro-H2A_HD containing histone...
octamer may differ in its ability to dissociate into assembly-competent histone subcomplexes appropriately upon lowering the ionic strength, resulting in a compromised in vitro assembly pathway. To test this hypothesis, we monitored the ternary structure of the histone octamer by analytical gel filtration under varying ionic strengths. Histone octamer, (H3-H4)₂ tetramer, and H2A-H2B dimer exhibit distinct elution profiles on a size-exclusion column due to their 2-fold difference in molecular weight (14). Purified Xla- or macro-octamer, previously refolded at 2 M NaCl, was injected onto a column equilibrated with 1.7, 1.4, 1.1, 0.8, and 0.5 M NaCl, and the elution profiles were compared. Xla-octamer dissociates into H2A-H2B dimers and (H3-H4)₂ tetramer at 1.1 M NaCl, (Table 1 and Fig. 2, upper panel). In striking contrast, the macro-octamer remains intact down to a concentration of 0.5 M NaCl (Table 1 and Fig. 2, middle panel). Because histones (in particular the (H3-H4)₂ tetramer start to bind to DNA at a concentration of ~1.0 M NaCl during salt gradient reconstitution, this inability to dissociate properly may result in the deposition of an entire macro-octamer (or a hexamer, i.e. one H3-H4 tetramer and one macro-H2A-H2B dimer) on the DNA, resulting in the observed nucleosome species which is either non-canonical in its histone composition or its conformation, resulting in aberrant electrophoretic mobility.

Four Amino Acids in the L1 Loop Are Largely Responsible for the in Vitro Behavior of Macro-octamer and Macro-NCP—To determine which region within the histone-like domain of macro-H2A is responsible for this unusual behavior, we generated mutants of Xla-H2A in which parts of the amino acid sequence were exchanged for the corresponding regions from macro-H2A. We focused on regions in the macro-H2A sequence that were shown to be structurally significantly different compared with major H2A and/or that are implicated in interactions that contribute to stabilizing the nucleosome: the L1 loop and the docking domain.

We replaced the L1 loop of major H2A with that of macro-H2A by site-directed mutagenesis (N38YAE41 to H38PKY41) resulting in a mutant designated mL1-H2A (“macro L1 loop”). We used the same approach to replace the docking domain of major H2A with that of macro-H2A (L83I, Q84L, and R88A) to generate a mutant designated mDD-H2A (“macro docking domain”). Histone octamers were refolded from these mutant histones as described above, and NCPs in which major H2A was replaced with either mL1-H2A or mDD-H2A (mL1-NCPs and mDD-NCPs, respectively) were reconstituted by salt gradient dialysis. Both mutant NCPs were incubated at 37 °C for 1 h. While mL1-NCP showed incomplete heat induced shifting (Fig. 1A, lanes 5 and 6), and behaved essentially like macro-NCP, mDD-NCP behaved exactly like major NCP, i.e. heat-induced distribution to the shifted form was complete (Fig. 1A, lanes 7 and 8). This suggests that four amino acids in the L1 loop of macro-H2A are solely responsible for the changes in assembly that lead to the formation of the aberrant nucleosome species.

We next compared the ability of histone octamers reconstituted with this H2A/macro-H2A chimera to dissociate into histone subcomplexes. Consistent with the results described above, mL1-octamer behaved exactly like macro-octamer in that it dissociated at 0.5 M NaCl (instead of 1.1 M NaCl). Elution profiles at different salt concentrations were highly comparable (Table 1 and Fig. 2). Together, these results confirm our hypothesis that the increased stability of the macro-octamer, caused by sequence differences in the four-amino acid stretch of the L1 loop (H38PKY41), is the determining factor for the formation of aberrant nucleosomes.
Macro-H2A Nucleosome Structure and Function

Macro-H2A Preferentially Pairs Up with a Major-type H2A within a Single Nucleosome—The L1 loops of the two H2A moieties are involved in formation of an L1-L1 interface, which is the only site of interaction between the H2A-H2B dimers in the nucleosome (1). The loop is also responsible for the cooperative incorporation of the H2A-H2B dimers during nucleosome assembly. Almost all H2A variants exhibit significant sequence differences in the L1 loop. The likelihood of forming hybrid nucleosomes could therefore depend on the relative affinity between the L1 loops of different H2A variants and major H2A within the context of a nucleosome.

To determine the ability of different H2A variants to form hybrid nucleosomes in vitro, we reconstituted nucleosomes by salt gradient dialysis (24) using mixtures containing (H3-H4)2 tetramer, His-tagged major H2A-H2B dimer, macro-H2A-H2B dimer, and 146-bp α-satellite DNA in a molar ratio of 1:1:1:1 (Fig. 3A, lanes 5 and 6). For comparison, we performed this experiment with homotypic nucleosomes containing His-tagged major H2A (Fig. 3A, lanes 1 and 2) or homotypic nucleosomes containing macro-H2A (Fig. 3A, lanes 3 and 4) (molar ratio of tetramer:dimer:DNA = 1:2:1 in both cases) reconstituted in the same manner. Homotypic nucleosomes containing only variant H2A (no His-tag) and homotypic nucleosomes with only His-H2A show subtle differences in rates of migration on a 5% native PAGE gel (compare lanes 2 and 4 in Fig. 3A). Intriguingly, when macro-H2A-H2B dimers are present in equimolar ratios with His-tagged H2A-H2B dimers, we predominantly obtain nucleosomes that exhibit an intermediate electrophoretic mobility, indicative of hybrid nucleosomes (Fig. 3A, lanes 5 and 6).

To further analyze the histone content of the various nucleosome preparations, we performed batch nickel affinity chromatography to isolate nucleosomes that have the His-tagged H2A-H2B dimers (either homotypic nucleosomes containing only His-tagged H2A or putative hybrid nucleosomes with only one His-tagged H2A and one variant H2A) (Fig. 3D). Unbound and bound fractions were analyzed by SDS-PAGE which allows a separation of His-H2A, H2A, and macro-H2A. As expected, His-H2A-NCP binds with a high affinity to the nickel-nitrilotriacetic acid beads (Fig. 3B), whereas homotypic macro-H2A-NCP does not bind (Fig. 3C). In contrast, “hybrid NCPs” (Fig. 3A, lanes 5 and 6) exhibit bands of equal intensity on the gel representing both macro-H2A and His-tagged major H2A (Fig. 3D, elute). We conclude that macro-H2A preferentially forms hybrid nucleosomes in vitro. The L1 loop significantly contributes to the propensity of macro-H2A to form hybrid nucleosomes but is not sufficient (supplemental Fig. 1). Although mL1-H2A seems to make more hybrid nucleosomes than mDD-H2A (compare lane 5 in C with lane 5 in E and F of

| TABLE 2 |
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| Data collection and refinement statistics |
| Data collection |
| Space group | p212121 |
| Unit cell dimensions (Å) | a = 106.1, b = 109.3, c = 176.3; |
| Resolution range (Å) | 50–2.90 |
| Unique reflections | 44,768 |
| Completeness (%) | 97.96 |
| Resolution range (Å) | 50–2.9 |
| Root mean square deviation from ideality |
| Bonds (Å) | 0.007 |
| Angles (°) | 1.055 |
| Average B-factors (Å²) | 70.6 |
| Protein | 131.3 |
| DNA | 67.0 |

* Rmerge = ∑Ij − (Ij)/∑Ij, where Ij is the mean of the measurements for a single ikl.
* Residues included in each histone subunit: H3, 38-135; H4, 24-102; H4’, 20-102, macroH2A:14:119 (major H2A amino acid numbers); macro-H2A’, 14:19 (major H2A amino acid numbers); H2B, 30:122; H2B’, 27:122. The remaining histone tails were too disordered to be included in the final model.
supplemental Fig. 1), neither region drives the absolute preference for hybrid nucleosome formation seen in case of the histone domain of macro-H2A.

**Hybrid Macro-NCP Differs Significantly in the Structure of the L1L1 Interface Compared with Homotypic Macro-NCP**—We next wanted to investigate the structural rearrangements (if any) required to form this novel nucleosome species. To this end, we determined the crystal structure of a hybrid NCP. Samples were analyzed by native and SDS-PAGE prior to crystallization to verify the presence of a single species. Crystals diffracted to a highest resolution of 2.9 Å and exhibit highly similar crystallographic parameters compared with macro-NCP. A summary of the crystallographic statistics is presented in Table 2. We used the structure of the homotypic macro-NCP (Protein Data Bank ID 1U35) as a search model in molecular replacement for phase determination.

Since we do not see any crystal contacts in the structure of canonical nucleosomes that could potentially favor a particular orientation of the hybrid nucleosome in the crystal lattice, and since it was unlikely that the His-tag that is attached to the disordered N-terminal tail of H2A would contribute to crystal packing, we expected the density for the H2A moiety in the hybrid nucleosome to be a convolution between major H2A and macro-H2A (Fig. 4A). Surprisingly, detailed analysis of the
difference density ($F_o - F_c$) and simulated annealing-omit maps revealed a preference for one particular orientation that allowed us to clearly distinguish major H2A from macro-H2A. Fig. 4, B and C, show simulated annealing omit maps calculated excluding the relevant regions of the structure to eliminate the effect of model bias. These maps clearly demonstrate that the

FIGURE 5. The L1-L1 interface of hybrid macro-NCP is highly malleable. A, overall structure of hybrid macro-NCP, shown in a side view with parts of the DNA removed to reveal histone-histone contacts. H3 is blue, H4 is green, H2B is red, H2A is yellow, macro-H2A is gray, and DNA is off-white. The L1-L1 interface is indicated. B, a magnified view of the L1-L1 interface in the same orientation as in A. C, superposition of the main chain around the L1-L1 interface in hybrid macro-NCP homotypic H2A-NCP (X. laevis; Protein Data Bank ID 1AOI) and homotypic macro-NCP (Protein Data Bank ID 1U35) in the region encompassing the N-terminal end of the $\alpha_1$-helix, the L1 loop, and the C-terminal end of the $\alpha_2$-helix. D, stick representation of the boxed area in C showing a comparison between the L1-L1 interfaces of hybrid macro-NCP, homotypic H2A-NCP (Protein Data Bank ID 1AOI), and homotypic macro-NCP (Protein Data Bank ID 1U35).
stabilized by predominantly hydrophobic interactions between the aliphatic side chain of Lys$^{37}$ and Tyr$^{38}$ of one macro-H2A and Pro$^{39}$ of the other in homotypic macro-NCP (Fig. 5D, right). In hybrid macro-NCP, this 2-fold symmetry is lost in the L1-L1 interface, and solvent-mediated hydrogen bonds are formed between the L1 loops of H2A and macro-H2A (Fig. 5D, left). In addition, we also observe ionic interactions between macro-H2A and macro-H2B that are not found in homotypic macro-NCPs due to differences in side chain orientations. There is a solvent-mediated interaction between His$^{35}$ of macro-H2A and Glu$^{68}$ of H2B and a hydrogen bond between Tyr$^{38}$ of macro-H2A and His$^{35}$ of H2B (Fig. 4B).

**Chaperone-assisted H2A-H2B Dimer Exchange Is Inhibited by Both Homotypic and Hybrid Macro-NCPs—**Nucleosomes in transcriptionally active chromatin are often depleted of H2A-H2B dimers (32–34). Nucleosome assembly protein 1 (NAP-1) is one of several activities that is implicated in this phenomenon. NAP-1 mediates removal and exchange of H2A-H2B dimers from intact nucleosomes in vitro by yet unknown mechanisms (14). We have shown that macro-NCP and hybrid NCP may be more rigid due to structural changes in the L1-L1 interface (see above and Ref. 3, suggesting that NAP-1-mediated dimer exchange may be disfavored in macro-H2A containing nucleosomes).

To test this hypothesis, we incubated unlabeled nucleosomes (H2A-NCP, macro-NCP, and hybrid NCP) with a preassembled yNAP-1-H2A-H2B dimer complex in which H2B has been fluorescently labeled. Dimer exchange is identified by the appearance of a fluorescently labeled nucleosome band (Fig. 6A, lane 4). We find that exchange is severely impaired in macro-NCP and hybrid NCP compared with H2A-NCP under conditions where most, if not all, of the yNAP-1 in the reaction exists in a complex with a H2A-H2B dimer (Fig. 6A, lanes 2 and 6). However, if the experiment is repeated under conditions where excess yNAP-1 (in addition to the yNAP-1-H2A-H2B dimer complex) is present, exchange at levels comparable with that found for H2A-NCP was observed (Fig. 6C, compare lanes 2 and 6 with lane 4).

We next investigated whether the efficiency of H2A-H2B dimer exchange is affected by the type of histone that is exchanged into nucleosomes. We incubated all three nucleosomes with preassembled NAP-1-macro-H2A-H2B dimer complex. As in the experiment described above, H2B was fluorescently labeled. Efficient exchange is only observed for H2A-NCP and only if an excess

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**FIGURE 6. The dynamic properties of the nucleosome are altered by incorporation of one or two macro-H2A moieties.** A, macro-NCP, H2A-NCPs, and hybrid macro-NCPs incubated with preassembled NAP-1-H2A-H2B$^{*}$ complex at a NAP-1:H2A-H2B molar ratio of 1:1 assuming NAP-1 is a homodimer. H2B was fluorescently labeled with Alexa-488 at Cys$^{112}$. The 5% native gel was visualized at 302 nm without staining. B, same gel as in A after staining with ethidium bromide. C, comparison of levels of NAP-1-mediated dimer exchange in H2A-NCPs, macro-NCPs, and hybrid macro-NCPs at varying NAP-1:dimer ratio and varying dimer composition as indicated. F, NAP-1-mediated dimer exchange in mDD-NCPs, mDD + mL1-NCPs, and mL1-NCPs at a NAP-1:H2A-H2B ratio of 1:1. Other experimental conditions were identical to A. (mDD-NCPs = NCPs containing H2A with macro-H2A-docking domain, mL1-NCP = NCPs containing H2A with macro-H2A-L1 loop, mDD + mL1-NCPs = NCPs containing H2A with both the macro-H2A-docking domain and L1 loop).

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electrostatic density in these regions does indeed fit the sequence of macro-H2A and major H2A, respectively, and that little if any convolution between the two orientations exists.

As expected, we find that the overall structure of the hybrid NCP is highly similar to that of homotypic macro-NCPs (root mean square deviation of 0.6) and that of homotypic H2A-NCPs (Protein Data Bank ID 1A0I) (root mean square deviation of 0.8) (Fig. 5A). As seen in the structure of homotypic macro-NCP, sequence differences between macro-H2A and major H2A do not cause significant structural changes, the docking domain being an important example (3). The L1-L1 interface between the two H2A moieties is the only region that shows deviations from its homotypic counterparts (Fig. 5D). In homotypic H2A-NCP, this interface is stabilized by two symmetry related ionic interactions between Glu$^{35}$ of one H2A moiety and Asn$^{38}$ of the other (Fig. 5D, center), whereas it
of free yNAP-1 is added (Fig. 6E, compare lanes 8 and 12 with lane 4), whereas at lower yNAP-1-H2A-H2B dimer concentrations no exchange is observed for any of the nucleosomes (Fig. 6E, lanes 3, 7, and 11). It is noteworthy that we observe a higher level of exchange for hybrid NCP than for homotypic macro-NCP. Controls (Fig. 6E, lanes 1 and 2, 5 and 6, and 9 and 10) confirm results shown in Fig. 6, A–D, in an independent experiment. It is possible that the L1 loop (in light of its contribution to the stability of the histone octamer) may also play a role in inhibition of histone dimer exchange. To investigate this we incubated unlabeled nucleosomes containing mDD-H2A, mDD+3L1-H2A, or mL1-H2A with labeled yNAP1-H2A-H2B dimer complex. We find that even at the lower NAP1 concentrations, there is dimer exchange in all three nucleosomes comparable to that in the H2A-NCP (compare Fig. 6E with Fig. 6A, lane 4). We therefore conclude that while the L1-L1 interface might contribute significantly to the stability of the histone octamer, this alone is not sufficient to preclude chaperone assisted histone dimer exchange, even in combination with the docking domain which also shows significant sequence differences in macro-H2A.

Together, these data support the hypothesis that chaperone-mediated removal of macro-H2A-H2B dimers from a folded nucleosome is disfavored. Fig. 7 shows a model for chaperone-mediated histone exchange that best explains the current data. It assumes a lower affinity of yNAP-1 for the macro-H2A-H2B dimer compared with the canonical H2A-H2B dimer, both in solution and within the context of a nucleosome. This would account for the low to non-existent degree of exchange with macro-H2A-H2B dimer in all the nucleosomes at lower NAP-1 concentrations which can be overcome by excess of yNAP-1.

It is therefore possible that in addition to the conformational rigidity of different types of nucleosomes the relative affinity of chaperones for histone variants may also play a significant role in regulating the histone content of chromatin.

**DISCUSSION**

One important way of locally or globally altering chromatin structure and dynamics is the incorporation of histone variants into nucleosomes. Here we have demonstrated that the histone domain of macro-H2A has a significant effect on in vitro nucleosome assembly and dynamics due to amino acid differences in a four-amino acid stretch of macro-H2A that forms the only point of contact between the two H2A-H2B dimers. One unresolved question has been whether one or both of the H2A chains within the NCP are replaced with a histone variant. We have shown conclusively that macro-H2A preferably pairs up with a major-type H2A within the context of a single a nucleosome, forming what we have termed “hybrid nucleosomes.” We have determined the crystal structure of such a hybrid nucleosome and confirmed that the L1 loop adapts to accommodate the respective binding partner. Finally, we have found that both hybrid and macro-NCP appear to be refractory to chaperone-mediated histone exchange.

The histone octamer is not a physical entity and, in the absence of DNA, requires high ionic strength to maintain interactions between the H2A-H2B dimers and (H3-H4)2 tetramer (35). This requirement is changed dramatically for macro-H2A, where only 0.5 M NaCl (as opposed to 1.1 M NaCl for major-type H2A) is required to stabilize the histone octamer. Four amino acids within the L1 loop of macro-H2A are solely responsible for altering the salt dependent stability of protein-protein interactions in the histone octamer. The increased octamer stability may result in an anomalous in vitro nucleosome assembly pathway because salt-dependent nucleosome reconstitution is contingent upon timely dissociation of the histone octamer into the constituent (H3-H4)2 tetramer and H2A-H2B dimer. Indeed, macro-NCP prepared by standard salt gradient reconstitution exists in two different states, one of which may have either a non-canonical histone composition or an unusual nucleosome conformation. We confirmed this hypothesis by reconstituting macro-NCPs using macro-H2A-H2B dimers and (H3-H4)2 tetramer instead of prefolded octamer containing all four core histones and also by a salt-independent approach using the nucleosome assembly protein NAP-1. Both these alternative approaches resulted in macro-nucleosomes comparable to canonical nucleosomes.

Most in vitro analyses of chromatin containing histone variants have been done under the tacit assumption that both H2A chains within a single nucleosome are replaced by variant H2A. In fact, this is not postulated by replication-independent incorporation of histone variants. We have used biochemical and crystallographic approaches to demonstrate that the histone domain of macro-H2A prefers to form hybrid nucleosomes with major H2A in vitro. In contrast, H2A.Z and H2A.Bbd form stochastic mixtures of homotypic H2A-NCPs, variant-NCPs, and hybrid NCPs (10). Our structural studies confirm the prediction that the global structure of the nucleosome remains unaltered. The only region of significant structural difference is the L1-L1 interface, confirming the notion that the L1-L1 interface is an adaptable module that is, at least in part, responsible for governing the variant composition of nucleosomes. Previous attempts to demonstrate the existence of hybrid nucleosomes (in which a major-type H2A is paired with a H2A variant) in vivo have failed (36). Our results raise the possibility that both homotypic and heterotypic versions of macro-nucleosomes exist in varying contexts. It should be
noted that we have used the histone domain of macro-H2A, which constitutes only one-third of the full-length protein, in all our experiments. It is therefore possible that the presence of the large non-histone domain might tip the balance in favor of either homotypic or hybrid nucleosomes in vivo. Additionally, the relative amounts of major versus variant H2A and the presence or absence of variant-specific exchange factors at any given time will affect the ratios of homotypic versus heterotypic nucleosomes. Other H2A variants (H2A.Z and H2A.Bbd) form a random mixture containing varying amounts of hybrid and homotypic nucleosomes in vivo (10), which may also have significant functional implications in vivo.

Although the fate of histones during transcription is still controversial, it appears that at least the H2A-H2B dimer is removed as part of the nucleosome remodeling process (32, 33). Thus, subtle changes in the conformational flexibility of the L1-L1 interface may have major implications in the accessibility of local chromatin to cellular processes.

Yeast nucleosome assembly protein (yNAP-1) is capable of exchanging H2A-H2B dimers into intact nucleosomes (37). We expected nucleosomes containing macro-H2A to be refractory to chaperone-mediated dimer exchange because macro-H2A is found predominantly in transcriptionally repressed regions of the genome and because our earlier structural studies on macro-H2A-containing NCPS suggested an increased rigidity in the L1-L1 interface (3). We find that both homotypic and hybrid macro-NCPS are resistant to NAP-1-assisted H2A-H2B dimer exchange compared with non-variant nucleosomes.

We investigated the role of both the L1 loop and the docking domain independently and in combination by site-directed mutagenesis. Whereas the L1 loop is exclusively responsible for changing the stability of the histone octamer with respect to ionic strength, neither of these regions was sufficient to confer to the in vitro resistance to NAP-1-mediated H2A-H2B dimer exchange of macro-NCPS. This result suggests that differences in the overall affinity between the H2A (or macro-H2A)-H2B dimer and a histone chaperone has a significant effect on the dynamic behavior of the nucleosome. Similarly, the propensity to form hybrid nucleosomes does not exclusively rely on either of these two regions. Nevertheless, the in vitro preference of macro-H2A for forming hybrid nucleosomes suggests an additional level of structural and functional variability in macro-H2A function. In particular, our finding that hybrid and homotypic macro-NCPS exhibit differences in the efficiency with which histone dimers are exchanged into nucleosomes in a chaperone-dependent manner, and the finding that macro-NCPS is refractory to chromatin remodeling (13) suggests that an additional level of functional regulation is imparted on chromatin by the formation of hybrid structures.

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