A novel labeling technique reveals a function for histone H2A/H2B dimer tail domains in chromatin assembly in vivo

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During S phase in eukaryotes, assembly of chromatin on daughter strands is thought to be coupled to DNA replication. However, conflicting evidence exists concerning the role of the highly conserved core histone tail domains in this process. Here we present a novel in vivo labeling technique that was used to examine the role of the amino-terminal tails of the H2A/H2B dimer in replication-coupled assembly in live cells. Our results show that these domains are dispensable for nuclear import but at least one tail is required for replication-dependent, active assembly of H2A/H2B dimers into chromatin in vivo.

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In eukaryotes, assembly of chromatin on daughter strands is believed to be mechanistically coupled to concomitant replication of DNA during S phase (Almouzni and Mechal 1988; Smith and Stillman 1989). The assembly of chromatin is a multistep process in which the H3/H4 tetramer is first deposited onto the DNA, followed shortly by the deposition of H2A/H2B dimers and, in most eukaryotes, association of a linker histone to form the complete nucleosome (Wolffe 1998; Annunziato and Hansen 2000). Crystallographic studies have shown that all four histone proteins are composed of two distinct domains; a central histone-fold domain and a tail domain, both of which have distinct functions in chromatin [Arents and Moudrianakis 1993; Luger et al. 1997]. The histone-fold domains are assembled via multiple histone–histone interactions to form a central cylinder around which nucleosomal DNA is wrapped [Luger et al. 1997]. In contrast, the histone-tail domains are thought to mediate nucleosome stability and inter-nucleosome interaction and are required for formation of the compact chromatin fiber [García-Ramírez et al. 1992; Carruthers and Hansen 2000]. Moreover, the tail domains are key control elements in the regulation of the structural and functional state of chromatin [Wolffe and Hayes 1999]. Indeed, many chromatin-associated activities, such as transcription, DNA recombination, and repair have been intimately correlated with posttranslational modifications of the tails [Strahl and Allis 2000]. Early work suggested that, in addition to their role in postreplicative processes, the histone tails are critical for replication-coupled chromatin assembly during S phase [for review, see Annunziato and Hansen 2000]. For example, the amino-terminal tail of H4 in newly assembled chromatin was found to have a highly conserved pattern of acetylation on lysines 5 and 12 [Sobel et al. 1995]. Consistent with this idea, H3/H4 tails within biochemically fractionated replication complexes exhibit this conserved pattern of acetylation [Kaufmann et al. 1995; Chang et al. 1997; Tyler et al. 1999]. Simultaneous deletion of both H3/H4 tails in yeast is lethal and endogenous 2 µm plasmids isolated from these strains exhibit reduced superhelical density [Morgan et al. 1991; Ling et al. 1996]. However, the phenotypes of cells lacking H3/H4 tails suggest that these domains are required for essential functions throughout the cell cycle [Morgan et al. 1991]; deposition-related acetylation sites in these proteins can be mutated in yeast with little effect on cell viability or nucleosome assembly [Ma et al. 1998]. Moreover, microinjection of mRNAs encoding full-length and truncated H3 and H4 into Xenopus eggs indicates that only the central histone-fold domains of these proteins are required for replication-coupled chromatin assembly [Freeman et al. 1996]. In addition, both tails in the H3/H4 tetramer are dispensable for replication-coupled chromatin assembly by purified chromatin assembly factor-1 (CAF-1) in vitro [Shibahara et al. 2000].

The requirement for the amino-terminal tails of the H2A/H2B dimer for chromatin assembly is even less clear. Similar to H3 and H4, deletion of either the H2A or the H2B amino-terminal tail does not affect viability in yeast, but simultaneous deletion of both tails is lethal [Schuster et al. 1986]. However, the actual effects of these deletions and the importance of the tails in chromatin assembly remain undefined. Moreover, little is known about the requirement for H2A/H2B-specific chaperones during chromatin assembly in vivo. Indeed, in vitro experiments suggest that these histones may simply spontaneously assemble onto a preformed H3/H4 tetramer–DNA complex [Verreault et al. 1996; Shibahara et al. 2000]. However, the delayed kinetics of H2A/H2B deposition after the incorporation of H3/H4 during chromatin assembly suggests that the mere presence of the H3/H4 tetramer–DNA complex is not sufficient for H2A/H2B dimer incorporation [Smith and Stillman 1991; Almouzni and Wolffe 1993].

Here we present direct evidence that the H2A/H2B histone tail domains have a function in replication-coupled chromatin assembly within a living cell. Our method avoids complications from tail-dependent genetic effects not directly related to chromatin assembly by incorporating trace amounts of fluorescently tagged H2A/H2B dimers into cells of the naturally synchronous macromesodermal form of the slime mold Physarum polycephalum at the beginning of S phase. Examination of the location and fate of exogenous proteins within the cell reveals that the tails are not required for transport of H2A/H2B into nuclei but are required for efficient assembly into chromatin.

Results and Discussion

We wished to examine if the H2A/H2B amino-terminal tail domains are required for chromatin assembly in vivo...
by incorporating trace amounts of fluorescein-tagged exogenous proteins into Physarum macroplasmodia at the beginning of S phase (Thiriet and Hayes 1999, 2001). These H2A/H2B dimers were either full length or lacking one or both amino-terminal tails [Fig. 1A]. As a control, we first examined if dimers lacking tail domains compete efficiently with full-length proteins during nucleosome reconstitution in vitro. Standard salt-dialysis reconstitutions were performed with a 215-bp DNA fragment, the appropriate ratio of purified core histones, and trace amounts of the fluorescein-tagged H2A/H2B. Fluorographs of nucleoprotein gels showed that the efficiency of dimer assembly into nucleosomes in vitro was not significantly affected by the absence of the H2A/H2B amino-terminal-tail domains or by the position of the fluorescein tag within the dimer [Fig. 1B and results not shown]. This result is in agreement with previous work showing that histone octamers lacking tails can reconstitute nucleosomes in vitro (Ausio et al. 1989; Vitolo et al. 2000).

We then determined if exogenous H2A/H2B dimers could be introduced into live Physarum cells and we investigated the fate of the proteins after incorporation [Fig. 2]. We and others have previously established that Physarum macroploasma are capable of taking up exogenous proteins and using them in cellular metabolism (Bradbury et al. 1974; Thiriet and Hayes 1999, 2001). To exploit this feature, exogenous full-length H2A/H2B* dimer was incorporated into the cell by spreading a solution containing the proteins onto the upper plasmoidium surface and the fate of the proteins examined [Fig. 2]. After absorption, we found that the majority of the tagged protein remained full length after incorporation and >90% of the exogenous dimer was recovered in the nuclear fraction [Fig. 2A]. We then determined if the exogenous H2A/H2B* dimer was assembled into chromatin. Chromatin was purified from Physarum nuclei treated with micrococcal nuclease [MNase] and separated on nucleoprotein gels. The fluorescein-tagged H2A/H2B* comigrated precisely with the bulk nucleosomal ladder visualized by ethidium bromide staining [Fig. 2B]. To ensure that the fluorescent signal was due to the full-length peptide, proteins from mononucleosome and dinucleosome bands were prepared and analyzed in SDS-PAGE. The fluorograph of the gel revealed a single band corresponding to full-length H2B [Fig 2C]. The subcellular localization was confirmed by direct fluorescence microscopy, which shows an exact colocalization of Hoechst-stained nuclei and fluorescence due to exogenous H2A/H2B* [Fig. 2D]. Identical results were obtained with H2A*/H2B (data not shown).

In vivo, the majority of chromatin assembly takes place during S phase and is most likely coupled to DNA replication. To determine if the transport into nuclei or the assembly of the exogenous dimers into nucleosomes depended on DNA replication, we incorporated full-length dimer into macroplasmodial segments deposited onto either normal medium or medium containing the DNA synthesis inhibitor hydroxyurea [Fouquet et al. 1975] [Fig. 3A]. Analysis of cytoplasmic and nuclear fractions in SDS-PAGE revealed that the exogenous H2A/H2B* was located in nuclei regardless of the presence or absence of DNA replication, indicating that transport of...
the exogenous histones into *Physarum* nuclei is not dependent on this process [Fig. 3B].

In contrast, we find that assembly of exogenous H2A/H2B* into chromatin occurs in a DNA-replication dependent manner. We prepared chromatin from cells treated with hydroxyurea and analyzed the protein content of mononucleosome and dinucleosome bands by SDS-PAGE as described earlier. Again, we found the exogenous dimer was present in nucleosomes when cells were grown in normal medium [Fig. 3C, lanes 1,3 or 2 and 4, respectively] from cells treated or untreated with HU. (Fluo) Fluorograph. (C) SDS-PAGE of protein content in mononucleosomes and dinucleosomes from cells treated or untreated with HU (+/-HU). A fluorograph and a Coomassie-stained image of the gel are shown.

Previous studies have shown that, in *Physarum*, histones H2A and H2B are also synthesized during G2 phase (Loidl and Grobner 1987). We thus examined exogenous dimer uptake in G2 phase and found that assembly into chromatin was moderately (~30%) lower than the incorporation of full-length dimer containing both tails. In striking contrast, mononucleosomes and dinucleosomes from nuclei containing completely tailless H2A/H2B dimer did not reveal any detectable fluorescence in the protein gel [Fig. 4B, lane 4] or in the nucleoprotein gel [data not shown] despite the presence of this dimer in the nucleus [Fig. 4A]. We thus conclude that H2A/H2B dimers lacking both amino-ter-

Figure 3. Assembly of exogenous H2A/H2B dimers into *Physarum* chromatin requires DNA replication in S phase. (A) Plot of 32P-dTTP incorporation per unit of DNA in cells treated with or without hydroxyurea (HU) during S phase. (B) Nuclear localization of the exogenous histone dimer is not dependent on DNA replication. SDS-PAGE of cytoplasmic or nuclear fractions (lanes 1 and 3 or 2 and 4, respectively) from cells treated or untreated with HU. (Fluo) Fluorograph. (C) SDS-PAGE of protein content in mononucleosomes and dinucleosomes from cells treated or untreated with HU (+/-HU). A fluorograph and a Coomassie-stained image of the gel are shown.

Figure 4. Transport of histone H2A/H2B dimers into nuclei does not require the amino-terminal tails, but assembly into chromatin requires at least one tail domain. (Fluo) Fluorograph. (A) Cellular localization of exogenous histone H2A/H2B dimers. Cytoplasmic and nuclear fractions (lanes 1 and 2, respectively in each gel) were analyzed for each of the dimers shown at the top. The gels were stained and fluorographed, as indicated. (B) Assembly of exogenous H2A/H2B chromatin requires the amino-terminal-tail domains. Soluble chromatin was prepared from cells treated with each of the dimers listed at the top and analyzed as in Fig. 3C. The quantitation is the average of two independent experiments. ND, not determined.
minal tails are not assembled into chromatin, whereas the presence of either tail is sufficient to allow replica-
tion-coupled assembly but with reduced efficiency. Fur-
thermore, the absence of signal with completely tailless
dimers indicates that the exogenous dimers are not dis-
rupted on incorporation and do not exchange with en-
dogenous proteins. These results also indicate that in
Physarum, histone H2A and H2B amino-terminal tails
have redundant functions in chromatin assembly.

Genetic experiments in yeast showed that deletion of
both amino-terminal tails of the H2A/H2B dimer is le-
thal (Schuster et al. 1986). Interestingly, when we incor-
porated much higher amounts of completely tailless
dimer (>1 mg/mL) into plasmodia, we observed growth
defects and a detectable loss in the ability to produce
nucleosome ladders on nuclease digestion, similar to ob-
servations with H3/H4 tail-deletion mutants in yeast
(Ling et al. 1996). However, similar incorporation of full-
length proteins did not result in such phenotypes (data
not shown). Moreover, under conditions of high input of
exogenous proteins, we detected a reduction in the
amount of endogenous H2A/H2B from isolated nuclei.
These results further suggest that the exogenous pro-
teins compete for the same chromatin assembly path-
ways used by endogenous H2A/H2B dimer.

Yeast genetic experiments have also been used to dem-
onstrate that the H2A/H2B amino-terminal tail domains
have redundant functions essential for viability of the
cell (Schuster et al. 1986). Although it is possible that the
tails are required for S-phase-dependent chromatin as-
sembly in yeast, the exact growth-dependent function(s)
of these domains were not determined from these ge-
etic studies. Besides these studies, there is little evi-
dence concerning the role of the H2A/H2B amino-termi-
nal tails or dimer tail posttranslational modifications for
chromatin assembly in vivo [Annunziato and Hansen
2000]. Moreover, in vitro experiments suggest that as-
sembly of the H2A/H2B dimer into nascent chromatin
may occur passively, with the dimer spontaneously
binding the H3/H4 tetramer–DNA complex without the
assistance of specialized assembly factors [Verreault et
al. 1996]. CAF-1 has been shown to specifically associate
with the H3/H4 tetramer [Verreault et al. 1996; Chang et
al. 1997], and CAF-1 alone can mediate assembly of
nucleosomes in vitro with core histones and replicating
DNA [Verreault et al. 1996]. Thus nucleosome assembly
could depend only on CAF-1-dependent loading of the
H3/H4 tetramer onto DNA. However, our results dem-
onstrate that the H2A/H2B tail domains play an essen-
tial role in the assembly of these proteins into chromatin
in vivo and, moreover, that dimer assembly likely occurs
through an active mechanism.

Several “molecular chaperones” such as nucleoplas-
min and NAP-1 have been shown to be specifically as-
associated with a maternal pool of H2A/H2B dimers in
Xenopus and Drosophila, respectively, and to facilitate
nonspecific nucleosome assembly in vitro [Dilworth et
al. 1987; Ito et al. 1996]. Our findings suggest that H2A/
H2B chaperones are required in vivo and are likely to
interact with the amino-terminal tail domains. A recent
in vitro study showed that histone acetylation by p300
facilitates the transfer of H2A/H2B dimers from nucleo-
somes to the Drosophila molecular chaperone NAP-1 in
ATP-dependent remodeling processes related to tran-
scription [Ito et al. 2000]. Opposite mechanisms might
take place during chromatin assembly. However, the ac-
tual identity of such putative molecular chaperones and
the mechanism by which H2A/H2B is assembled onto
the tetramer–DNA complex in vivo remains to be deter-
mined.

In addition, our results also demonstrate that the
H2A/H2B tail domains are dispensable for nuclear im-
port. Nuclear import of H2A/H2B has recently been
shown to be mediated by several members of the karyopherin/importin family (Mosammaparast et al.
2001). Thus, facilitated nuclear import of these histones
requires elements of structure other than the tail do-
 mains, in agreement with previous work (Mosam-
maparast et al. 2001). Further, our results show that
chromatin assembly and nuclear import can be un-
coupled, suggesting that they occur by distinct molecu-
lar mechanisms. Perhaps histones are shuttled between
different chaperones or the histone chaperones respon-
sible for nuclear import require the addition of signals or
cofactors to assemble the proteins into nascent chroma-
tin.

We have also described a powerful strategy for follow-
ing the assembly of exogenous proteins into a living cell
in a cell-cycle dependent manner. This novel strategy
presents important advantages over genetic methods or
microinjection of RNAs [Freeman et al. 1996; Schuster et
al. 1986]. The incorporation of trace amounts of chemi-
cally tagged proteins avoids deleterious genetic effects
and allows the investigation of preassembled exogenous
complexes in live cells. Coupled with in vitro mutagen-
esis, this approach provides insights into the roles of pro-
tein domains when genetic analyses would lead to cell
lethality in which the underlying cause is often not
readily apparent [Schuster et al. 1986]. The natural syn-
chrony of millions of nuclei in a single Physarum plas-
modium makes it possible to incorporate at specific cell
cycle stages any exogenous protein or complex, given
sufficient similarity to the endogenous cellular ho-
mologs. Given the high degree of phylogenetic conserva-
tion in histone proteins, it is likely that the incorpora-
tion of trace amounts of exogenous histones we report
depends on interactions with endogenous histone chaper-
ones involved in nuclear import and chromatin assem-
bly. Indeed, incorporation of large amounts of exogenous
H2A/H2B appears to co-opt the assembly pathway used
by endogenous H2A/H2B. An important advantage of
this system is that if a mutant protein or complex is
incompatible with any interaction or process required
for a cellular function, introduction of trace amounts
into the cell is unlikely to cause cell lethality, because
the plasmodium will simply use endogenous homologs.

Materials and methods

Physarum culture

P. polycephalum, strain TU291, was cultured and cell cycle advance-
ment within macroplasmodia was followed by observation of mitosis by
phase contrast microscopy, as described [Thiriet and Hayes 1999]. Speci-
mens for direct fluorescence microscopy were fixed with ethanol,
mounted in glycerol/ethanol (1:1), and illuminated with the appropriate
wavelengths to visualize either Hoechst-stained DNA or the fluorescen-
tagged proteins. For experiments involving inhibition of DNA replica-
tion, the macroplasmodium fragments were cultured normally until the
desired cell cycle stage, 80 mM hydroxyurea was added to the media, and
protein solution was immediately deposited onto the cell surface as de-
scribed in the following sections.

Preparation of histone dimers

Genes coding for full-length and various tailless Xenopus laevis H2As
and H2Bs were modified by PCR to contain single cysteine substituitions
as outlined in Figure 1, and the proteins were overexpressed in bacteria and purified as described [Hayes and Lee 1997]. The purified proteins were modified with fluorescein-5-maleimide [-threefold excess, Pierce] for 30 min at room temperature. The reaction was stopped by addition of 10 mM dithiothreitol (DTT) and excess reagent removed by ion exchange chromatography on a Biorex 70 column. Following exhaustive washes with 0.6M NaCl, the modified dimers were then eluted with 1M NaCl in TE and dialyzed against 20 mM Tris-HCl (pH 7.2; Thiriet and Hayes 2001).

In vitro reconstitution of nucleosomes
A 215-bp DNA fragment containing a Xenopus borealis SSrRNA gene was obtained by EcoRI and DdeI digestion of plasmid pXP-10 [Vitolo et al. 2000]. Five micrograms of purified fragment was mixed with ∼3 μg purified core histones and ∼100 ng fluorescently tagged H2A/H2B dimers and nucleosomes reconstituted via a salt dialysis method as described [Hayes and Lee 1997]. Nucleosomes were analyzed by electrophoresis on 0.7% agarose, 0.5× TBE gels. The fluorescent proteins within the gel were imaged by fluorometry before staining the gel with ethidium bromide (Thiriet and Hayes 2001).

Incorporation of dimers into Physarum
Macroplasmodia were cut into halves and 400 μL H2A/H2B dimers at the concentrations indicated in the figure legends were deposited into the upper surface as described [Thiriet and Hayes 1999] with minor modifications. The incorporation was performed in the S phase of the cell cycle unless indicated in the text and treated plasmodia were kept in growth medium. Unless otherwise stated, the amounts of exogenous histones used in this study did not lead to detectable effects on growth of the cell or progression of the cell cycle [cf. Thiriet and Hayes 1999, 2001]. Controls were treated in the same way but with buffer only. Note that plasmodia cut into separated pieces remain synchronous for several cell cycles when cultured in normal conditions [Thiriet and Hayes 1999].

Isolation of nuclei and preparation of chromatin
Macroplasmodia segments on filter papers support were washed in 1 mM ethylenediamine tetraacetic acid (EDTA). The cells were then harvested and disrupted by Dounce homogenization in nuclei isolation buffer (15 mM MgCl2, 15 mM Tris-HCl at pH 8.0, 0.25 M hexylene glycol, 0.6% surfynol, 3 mM DTT). The nuclei were then pelleted by centrifugation at 400g for 5 min. The nuclear pellet was resuspended in 2 mL isolation buffer and carefully loaded onto a 1 M sucrose cushion in nuclei isolation buffer after centrifugation at 8000g for 20 min, and the nuclei were recovered from the bottom of the tubes. Nuclei were then washed with MNase buffer (60 mM KCl, 15 mM NaCl, 10 mM Tris-HCl at pH 7.5, 1 mM CaCl2, 0.5 mM MgCl2) and pelleted by centrifugation, and resuspended in the same buffer. Chromatin was prepared from freshly isolated nuclei by addition of one-twentieth volume MNase (0.3 U/μL) for the times indicated in the text. The digestion was stopped by addition of 5 mM EDTA and kept on ice for 10 min. Microfuge tubes were centrifuged for 5 min at maximal speed. The supernatant containing soluble chromatin was then dialyzed for ∼2 h against TE to remove salts.

Protein and chromatin analyses
Proteins in aliquots of cytoplasmic and nuclear fractions were analyzed in SDS-PAGE. The fluorescent proteins were detected by fluorometry of the gels before staining. Chromatin was analyzed by native 5% PAGE (acylamide/bisacrylamide: 40 : 1) in 0.5× TBE. Mononucleosomes and dinucleosome species were isolated from preparative-scale 1% agarose gels containing 0.5× TBE. One analytical lane was loaded next to the preparative, stained with ethidium bromide, and used for localizing the nucleosomal species to avoid destabilization of nucleoprotein complexes by ethidium bromide. Mononucleosomes and dinucleosomes were electroeluted from the gel and samples concentrated by evaporation in a speed-vac. Samples were normalized according to DNA content (500 ng per lane) and protein composition analyzed by SDS-PAGE.

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