

Replication-independent core histone dynamics at transcriptionally active loci in vivo

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We used a novel labeling technique in the naturally synchronous organism *Physarum polycephalum* to examine the fate of core histones in G2 phase. We find rapid exchange of H2A/H2B dimers with free pools that is greatly diminished by treatment of the cells with α -amanitin. This exchange is enhanced in pol II-coding sequences compared with extragenic regions or inactive loci. In contrast, H3/H4 tetramers exhibit far lower levels of exchange in the pol II-transcribed genes tested, suggesting that tetramer exchange occurs via a distinct mechanism. However, we find that transcribed regions of the ribosomal RNA gene loci exhibit rapid exchange of H3/H4 tetramers. Thus, our data show that the majority of the pol II transcription-dependent histone exchange is due to elongation in vivo rather than promoter remodeling or other pol II-dependent alterations in promoter structure and, in contrast to pol I, pol II transcription through nucleosomes in vivo causes facile exchange of both H2A/H2B dimers while allowing conservation of epigenetic "marks" and other post-translational modifications on H3 and H4.

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It has been known for several decades that activation of transcription by RNA polymerase II is accompanied by modulations in promoter chromatin structure, resulting in drastic increases in accessibility to endonucleases (Wu et al. 1979; Wu 1980; Keene et al. 1981). Multiple processes contribute to generating the open state of promoter regions, including disruption of canonical nucleosomes and higher order chromatin structures upon specific post-translational modifications of core histones (Roth et al. 2001; Zhang and Reinberg 2001) and the action of ATP-dependent chromatin remodeling complexes (Narlikar et al. 2002; Lusser and Kadonaga 2003). These processes likely work in concert at many loci (Cosma 2002; Boeger et al. 2003; Reinke and Horz 2003).

In addition to the structural alterations in chromatin associated with transcription initiation, some disruption

of nucleosome structure likely accompanies RNA pol II elongation in vivo. Early in vitro experiments suggested that H2A/H2B dimers can be displaced by RNA polymerase II binding to nucleosome core particles (Baer and Rhodes 1983). Likewise, pulse-chase experiments in cultured mammalian cells indicated that exchange of H2A/H2B dimers in chromatin with newly synthesized histone pools occurs in a replication-independent (RI) fashion, while relatively little turnover of H3/H4 tetramers was detected outside of S phase (Jackson and Chalkley 1985). Interestingly, RI incorporation of nascent H2A/H2B occurs preferentially in nucleosomes containing acetylated H4 (Perry et al. 1993) and appears to be localized to transcriptionally active chromosomal regions (Hendzel and Davie 1990). Indeed, cytological analyses of GFP-histone fusions indicates that transcription-dependent exchange of H2A/H2B, but not H3/H4, occurs in mammalian cells (Kimura and Cook 2001). However, Henikoff and colleagues have shown that the histone variant H3.3 accumulates in a RI manner on rDNA repeats in *Drosophila* cells and quantification of H3.3 in nuclei of a *Drosophila* cell line suggests that exchange is widespread and includes many, if not all pol II-transcribed genes (Ahmad and Henikoff 2002; McKittrick et al. 2004).

A possible mechanism for the observed RI incorporation of H2A/H2B was suggested by Studitsky and collaborators, who found that transcription of RNA pol II (but not RNA pol III or SP6 polymerase) through single nucleosomes in vitro led to the generation of histone hexamer-DNA complexes composed of a tetramer of H3/H4 and a single H2A/H2B dimer (Kireeva et al. 2002). Moreover, FACT, a complex that facilitates transcription elongation through nucleosomal templates by RNA pol II (Orphanides et al. 1999) and colocalizes on *Drosophila* polytene chromosomes with RNA pol II (Saunders et al. 2003), stimulates Pol II-dependent formation of hexamer-DNA complexes in vitro (Belotserkovskaya et al. 2003). However, while there is biochemical evidence supporting the hypothesis that RNA pol II activity induces exchange of H2A/H2B dimers from chromatin, it is unclear whether the majority of exchange occurs during transcription initiation or elongation in vivo and whether pol II induces comparative levels exchange of H3/H4 tetramers.

Results and Discussion

To investigate RI histone octamer dynamics in vivo, we used a novel labeling approach that exploits two remarkable properties of the slime mold *Physarum polycephalum*, its ability to grow as a single macropolymodal cell with $\sim 10^8$ nuclei that proceed with complete synchrony through a 9-h cell cycle, and its ability to internalize exogenous proteins and complexes (Bradbury et al. 1974; Prior et al. 1980; Thiriet and Hayes 2001). While the majority of histone synthesis in *Physarum* occurs during S phase, when replication of chromatin takes place, extrachromosomal elements containing the rDNA loci are known to replicate throughout the cell cycle (Benard et al. 1995), indicating that pools of histone remain available during G2 phase (note *Physarum* lacks a distinct G1 phase). In addition, some H2A/H2B dimer synthesis is known to occur during G2 phase (Loidl and

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Grobner 1987). To study the fate of exogenous H2A/H2B dimers incorporated into *Physarum* cells during G2 phase, we prepared H2A/Flag-H2B dimers in which nine amino acid residues containing the Flag epitope were added to the N terminus of H2B (Thiriet 2004). The anti-Flag antibody did not cross-react with any *Physarum* epitopes or hinder the ability of the Flag dimer to reconstitute into nucleosomes in vitro (Fig. 1B; Supplementary Fig. S2; data not shown). H2A/Flag-H2B dimers introduced into *Physarum* cells during G2 phase almost exclusively localized to nuclei (Fig. 1A,B). We estimated that the exogenous proteins represent $\leq 5\%$ of the total H2A/H2B in the cell (see Materials and Methods). Experiments carried out in the absence or presence of α -amanitin, which inhibits RNA polymerase II transcription in a wide range of organisms including *Physarum* (Pieron and Sauer 1980; Bushnell et al. 2002), showed that the nuclear localization of the exogenous H2A/Flag-H2B was independent of transcription (Fig. 1A,B).

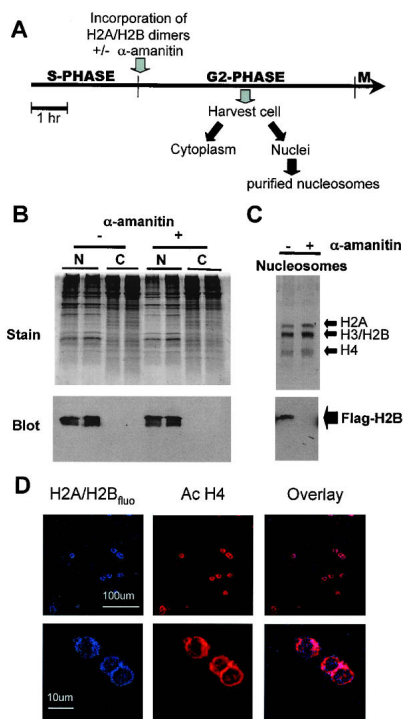


Figure 1. Exogenous core histones H2A/H2B localize to *Physarum* nuclei and are assembled into nucleosomes when introduced during G2 phase. (A) Experimental strategy for incorporation of exogenous histones into *Physarum* cells. Solutions containing H2A/Flag-H2B were applied to the upper surface of macroplasmidia at the beginning of G2 phase, then cells were grown for 3 h prior to analysis. (B) Exogenous H2A/H2B localize to nuclei independent of transcription. Proteins were introduced at the beginning of G2 phase in the absence or presence of α -amanitin and subcellular distribution in nuclear (N) and cytoplasmic (C) fractions analyzed by SDS-PAGE and Western blots with anti-Flag antibodies. Duplicate experiments are shown. (C) Exogenous H2A/Flag-H2B is assembled into nucleosomes via an α -amanitin sensitive process. Nucleosomes were purified from macroplasmidia treated with exogenous H2A/H2B in the absence or presence of α -amanitin and protein content analyzed. (Top) Coomassie-stained gel. (Bottom) Western blot of the gel. (D) Exogenous H2A/H2B dimers colocalize with transcriptionally active chromatin. Incorporation of fluorescein-labeled dimers was carried out in G2 phase for 3 h, cell explants were squashed, fixed, and stained for acetylated H4 with antipeptide-acetylated H4 peptide antibodies. Nuclei were imaged by confocal microscopy.

We previously had found that H2A/H2B localized within nuclei could remain unassembled into chromatin during S phase, strongly suggesting the existence of a pool of free nuclear histones (Thiriet and Hayes 2001). Thus, the accumulation of H2A/H2B dimers in G2 nuclei did not necessarily indicate assembly into chromatin. Therefore, we examined whether the exogenous proteins were assembled into nucleosomes, and whether this incorporation was dependent upon RNA polymerase II activity. Nuclei were prepared from α -amanitin treated and untreated plasmidia and mononucleosomes isolated by MNase digestion, followed by sucrose gradient fractionation. Western blots of proteins from the mononucleosome fractions clearly showed that Flag-tagged H2A/H2B dimers were present in nucleosomes isolated from control cells not treated with α -amanitin, but were greatly diminished in nucleosomes from cells treated with the drug (Fig. 1C). These results indicate that although exogenous H2A/H2B dimers localize into *Physarum* nuclei regardless of transcriptional activity, assembly of dimers into nucleosomes during G2 phase is dependent upon transcription by RNA polymerase II.

Numerous studies have shown that histone acetylation is correlated with RNA polymerase II activity. Therefore, to substantiate the above observations, we compared the intranuclear localization of histone H4 acetylation, previously shown to colocalize with transcriptional activity in *Physarum* nuclei (Waterborg and Matthews 1983) and exogenous H2A/H2B modified with fluorescein. Confocal analyses of squashes stained with antibodies to acetylated H4 and direct fluorescence showed that labeled dimers colocalized in transcriptionally active regions located near the nuclear periphery (Fig. 1D; Waterborg and Matthews 1983). This pattern contrasted with exogenous H2A/H2B dimers incorporated during S phase, which stained chromatin homogeneously throughout the nucleus (Thiriet and Hayes 2001) and suggests a global relationship between transcription activity and incorporation of H2A/H2B dimers into chromatin during G2 phase.

To exclude the possibility of indirect effects of α -amanitin and further investigate the RI incorporation of H2A/H2B into chromatin, we examined specific loci by chromatin immunoprecipitation (ChIP) analysis of macroplasmidia treated with H2A/Flag-H2B dimers. Two developmentally regulated *Physarum* profilin genes, one inactive and the other actively transcribed, (Fig. 2A) were chosen as targets for ChIP (Maric et al. 2003). Chromatin immunoprecipitations from cells prepared in the absence of exogenous dimers showed that neither of the targeted sequences bound nonspecifically to the anti-Flag antibody-coated beads under our experimental conditions (Fig. 2B, lane 2). ChIP experiments with cells in which H2A/Flag-H2B dimers were incorporated during G2 phase indicated that the exogenous dimer was assembled into chromatin at the active *ProP* locus to a significantly greater extent than the silent *ProA* locus (Fig. 2B, lane 4). We noted some exogenous dimer was detectable at the silent locus, perhaps due to a lower level of DNA repair-related "background" incorporation of dimers (Mello and Almouzni 2001).

Differences in H2A/H2B dimer exchange between the active and inactive profilin genes may be due to a greater locus-wide passive exchange of dimers, promoter remodeling, or the direct action of the elongating polymerase. In order to address this issue, we examined H2A/H2B

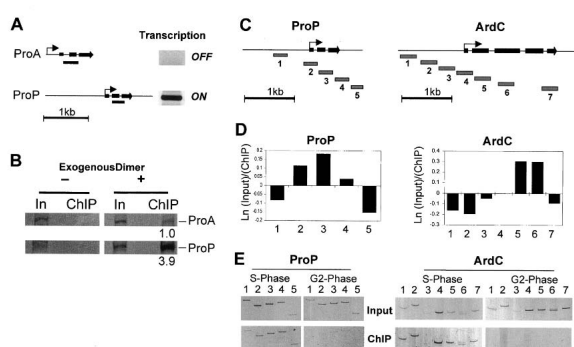


Figure 2. Exogenous H2A/H2B dimers are preferentially assembled into nucleosomes on transcribed sequences of active genes. (A) Diagram of profilin *ProA* and *ProP* loci, which are transcriptionally inactive and active, respectively, in *Physarum* macroplasmidia. Lines below the loci correspond to the fragments analyzed in B. (Right) The transcription state of the genes was confirmed by RT-PCR. (B) Incorporation of exogenous H2A/H2B into *Physarum* chromatin. Extract from an untreated control cell (-) or a single cell treated with exogenous H2A/H2B (+) as described in Figure 1A was analyzed by ChIP. The enrichment of fragments after the immunoprecipitation from the active *ProP* locus relative to the *ProA* locus is listed below the lanes (average of two experiments). PCR reaction products using the input extract (In) and the immunoprecipitate (ChIP) are shown. (C–E) Incorporation of exogenous histone complexes into *Physarum* chromatin throughout the *ProP* and *ArdC* loci. (C) DNA regions amplified by PCR in the ChIP assays (bars below lines). (D) Extent of incorporation of exogenous H2A/Flag-H2B into chromatin during G2 phase. Histones were introduced at the beginning of G2 phase, and cells harvested for ChIP 3 h later. Numbers correspond to individually amplified regions shown in C. The intensities of amplified bands were normalized to internal controls and the $\ln(\text{ChIP band}/\text{input band})$ shown in the bar graph. (E) Incorporation of exogenous H3/H4 into chromatin throughout the *ProP* and *ArdC* loci during S and G2 phase. Exogenous (H3/H4)₂ tetramers were introduced into *Physarum* cells at the beginning of S or G2 phase, and the extent of incorporation into chromatin throughout each locus was analyzed 3 h later as in D.

dimer exchange in extragenic versus coding regions of the active *ProP* locus and a second locus, *ArdC*, which includes a constitutively active actin gene (Fig. 2C). The quantification of ChIP results in the *ProP* locus reveals that the exchange of exogenous dimers into chromatin was low in the promoter region, increased in the coding region, and decreased at the end of the coding region (Fig. 2C,D, left). However, because of a poly A/T track in the *ProP* promoter region, we could not examine the extent of assembly in this region in greater detail. Analysis of the *ArdC* locus revealed the same general pattern as on the *ProP* locus, where the promoter region exhibited a lower exchange, while the coding region exhibited a higher level of assembly with a decrease at the end of the coding region (Fig. 2C,D, right). We therefore conclude that histone dimers exchange with a significantly higher efficiency within actively transcribed compared with extragenic sequences.

The above results show that RNA pol II transcription induces H2A/H2B exchange with free pools and that this exchange is highly localized to transcribed sequences. We next determined whether incorporation into chromatin and differential exchange also occurs for the H3/H4 tetramer. We first incorporated Flag-tagged H3/H4 tetramers into macroplasmidia during S phase to determine whether these proteins assembled into native

Physarum chromatin in vivo (Thiriet and Hayes 2001). The exogenous H3/H4 localized to *Physarum* nuclei and are assembled into nucleosomes (Supplementary Fig. S2), constituting <5% of total endogenous H3/H4 estimated as above for H2A/H2B (data not shown). ChIP experiments with the same sequences examined for the H2A/H2B experiments showed that when incorporated during S phase, exogenous H3/H4 tetramers were associated with all sequences tested throughout the *ProP* and *ArdC* loci (Fig. 2E, S-Phase).

To examine the RI assembly of H3/H4 into chromatin during G2 phase, exogenous proteins were incorporated at the onset of G2 phase under identical conditions to those used for H2A/H2B and assembly into chromatin analyzed by the ChIP assay. The results of the PCR amplification revealed that the exogenous tetramers were not efficiently incorporated into chromatin at any position throughout these loci (Fig. 2E, G2-Phase). Note the extremely low incorporation of exogenous tetramers in G2 phase did not allow for accurate determination of relative amounts of incorporation across the locus. We estimate that the incorporation of the H3/H4 in G2 phase to be at least 10-fold lower than the incorporation in S phase. These results are consistent with the examination of global incorporation of exogenous tetramer in G2 phase (data not shown). Therefore, we concluded that although the exogenous H3/H4 tetramers were efficiently assembled into native chromatin in a replication-dependent manner during S phase, in contrast to H2A/H2B, these proteins did not exhibit appreciable transcription-dependent incorporation into chromatin during G2 phase.

The experiments above monitored the incorporation of Flag-tagged histones into chromatin at specific loci. However, it is possible that exchange during transcription, especially exchange of H3/H4 tetramers, is limited to a specific histone variant, such as tetramers containing H3.3, since some major H3s appear to not contain critical sequences required for RI exchange (Ahmad and Henikoff 2002). Thus, to examine the possibility that pol II-dependent exchange requires protein sequences and/or post-translational modifications not present within our exogenous proteins, and to determine the relative rate of exchange of core histones at specific loci, we incorporated trace amounts of exogenous Flag-tagged histones in S phase, washed the cells, then monitored the replacement of the tagged histones at specific loci during G2 phase. Note that this procedure would detect exchange of any endogenous histones including RI-specific histone variants. Consistent with previous results, we found that only a very small fraction of the total H2A/H2B dimer on the silent *ProA* locus underwent exchange with endogenous free histones during G2, while, in contrast, the active *ArdC* locus exhibited a striking pattern of exchange with the free pool of endogenous histones. Moreover, exchange of H2A/H2B was significantly more rapid for the coding region than the promoter region of the *ArdC* locus (Fig. 3).

In contrast, analyses of the kinetics of exchange of H3/H4 tetramers during G2 phase revealed that the exogenous histone tetramers did not undergo significant exchange from chromatin in either active or inactive loci. Indeed, we found that maximally only ~5% of the total H3/H4 tetramers exchanged in transcribed regions, in contrast to ~90% for H2A/H2B dimers. Therefore, our data show that RNA Pol II elongation does not induce

exchange of the vast majority of H3/H4 tetramers on these genes, excluding the possibility that significant amounts of tetramer exchange was not detected in previous experiments due to a requirement for a specific histone variant.

We next considered the possibility that since transcription and replication overlap in the selected active loci, significant levels of a transcription-related histone variant may have been assembled during S phase, perhaps to the exclusion of the exogenous proteins. In this case, little turnover of labeled proteins incorporated during S phase might be detected. To address this possibility, we repeated the analysis on another locus that is known to be transcriptionally silent during S phase, but is induced during G2 phase. The α -tubulin B gene locus (*AltB*) is actively transcribed only at the end of G2 phase and replicates in early S phase, with no overlap between the two processes (Carrino and Laffler 1986). We incorporated histones in S phase, washed the cells, and analyzed the histone content at the *AltB* locus before and after activation of the gene during G2 phase. Despite the small size of this locus, the extent and pattern of exchange is consistent with the other loci. We find little difference in the extent of exchange of H3/H4 between cells harvested before or after transcription of the gene is initiated (Fig. 4, black bars). However, after 1 h of transcriptional activation, H2A/H2B histone dimers exhibit low levels of exchange in extragenic regions and higher levels of exchange in the intragenic regions (Fig. 4, gray bars). In contrast, exchange of histone H3/H4 tetramers

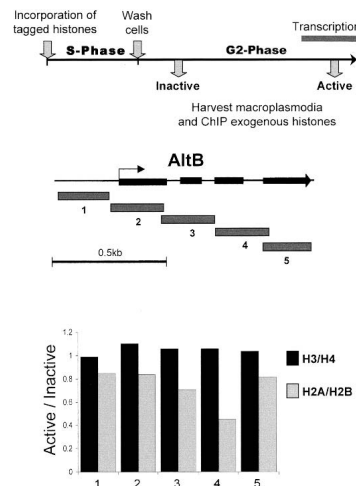


Figure 4. Exchange of core histone proteins throughout the *AltB* locus before and after activation of transcription. Core histones on endogenous sequences were labeled *in vivo* as in Figure 3; then, the relative amount of H2A/Flag-H2B and H3/Flag-H4 associated with each *AltB* sequence was determined before (Inactive) and after (Active) transcription activation of the gene. The ratio of Active/Inactive ChIP was plotted for each of the indicated sequences. Results are the average of two independent experiments with $\leq 5\%$ average variation.

is minimal throughout the locus, regardless of when the ChIP experiment is carried out, consistent with the results obtained with the *ProA* and *ArdC* loci. These results indicate that accumulation of specialized transcription-related histone variants during S phase did not affect our determination of histone turnover on these loci.

Our results indicate that little or no transcription-dependent exchange of H3/H4 occurs on the active pol II loci examined. Since RI independent incorporation of H3.3-GFP/H4 has been observed on pol I-transcribed rDNA loci in *Drosophila* cells (Ahmad and Henikoff 2002), we examined whether exchange of H3/H4 occurs on *Physarum* rDNA loci using our "ChIP-decay" assay. Importantly, we found efficient exchange of H3/H4 could be detected in pol I-transcribed regions compared with the nontranscribed ribosomal gene spacer elements (Supplementary Fig. S3). Importantly, both the spacer elements and the transcribed region appear to be assembled into nucleosomes to an extent similar to bulk chromatin (Supplementary Fig. S3). These data strongly suggest the presence of an H3.3-like activity in *Physarum* and are in agreement with results from other systems (Ahmad and Henikoff 2002; Malik and Henikoff 2003; see below).

Our results support a model whereby RNA polymerase II elongation through nucleosomes induces displacement of H2A/H2B dimers followed by nucleosome reassembly *in vivo*. We find that H2A/H2B are incorporated into nucleosomes *in vivo* in a replication-independent manner that requires the activity of RNA polymerase II, and that this exchange is confined to regions transcribed by the polymerase. Exchange of H3/H4 occurs with a rate that is at least 20 times slower than exchange of H2A/H2B, and does not appear to be localized to transcribed regions in the pol II loci tested. This model is consistent with the observations that in a purified model system, RNA polymerase II elongation through nucleosomes *in vitro* generates a histone hexamer-DNA com-

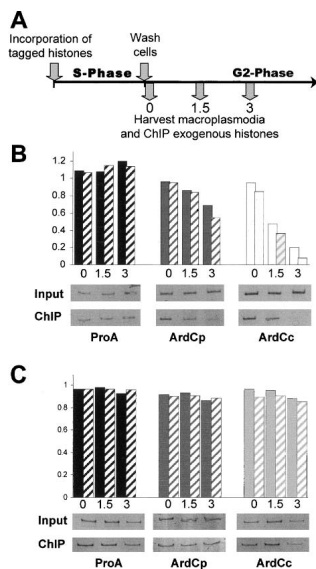


Figure 3. Kinetics of exchange of core histone proteins on active loci. (A) Experimental strategy. Nucleosomes were labeled *in vivo* by incorporation of Flag-tagged histones at the beginning of S phase for 3 h, the cells were then washed, and the loss of labeled proteins from the chromatin of the indicated loci monitored by ChIP immediately (0) and 1.5 and 3 h after washing. (B) Exchange of H2A/H2B on sequences within the inactive *ProA* gene (ProA) and the active *ArdC* promoter (ArdCp) and coding region (ArdCc). Primers used were, ProA, ArdC 2, and ArdC 6 from Figure 2. The enrichment of the target sequence relative to the 0.5% of the input is plotted with results from two independent experiments shown. (C) Exchange of H3/H4 was monitored as in B, except that cells were treated with H3/Flag-H4.

plex lacking a single H2A/H2B dimer (Kireeva et al. 2002), and that the chromatin-specific transcription elongation complex FACT stimulates formation of hexamers in this system (Belotserkovskaya et al. 2003). These complexes are perhaps related to the partial nucleosomes previously observed after pol II transcription of chromatin templates *in vivo* (Sathyanarayana et al. 1999), and may represent intermediates during the facile exchange of histone dimers with free pools *in vivo* observed in our experiments. Importantly, our data indicate that both H2A/H2B dimers are eventually displaced by passage of the polymerase. Finally, based on quantification of immunoreactivity and total proteins, we estimate the fraction of nucleosomes in which exogenous H2A/H2B has been incorporated in an α -amanitin-sensitive manner to be ~1%, suggesting that the exchange is widespread and involves many pol II genes.

Initiation of transcription involves the opening of chromatin structure at the promoter region to provide access for transcription factors to bind DNA sequences (Wolffe 1998). In most cases, promoter chromatin disruption involves histone post-translational modification and nucleosome remodeling, and recent analysis indicates that ATP-dependent chromatin remodeling complexes can catalyze the exchange of H2A/H2B dimers between chromatin fragments *in vitro* (Bruno et al. 2003). However, our results indicate that the majority of H2A/H2B exchange is confined to the structural gene itself; thus, promoter remodeling accounts for little of the overall exchange observed in previous *in vivo* experiments (Hendzel and Davie 1990; Kimura and Cook 2001). Importantly, our results demonstrate that this exchange is likely due to RNA polymerase II elongation through the chromatinized structural gene rather than just greater levels of passive exchange in transcriptionally active, highly acetylated, open chromatin domains. Nonetheless, it is possible that remodeling activities facilitate dimer exchange during elongation.

The far lower levels of RI exchange we and others (Kimura and Cook 2001) detect for H3/H4 compared with H2A/H2B suggest that these two phenomena are not directly mechanistically linked *in vivo*. This is in contrast to replication during S phase, where both H2A/H2B dimers and H3/H4 tetramers are incorporated into *Physarum* chromatin to approximately equal extents (Thiriet and Hayes 2001; Fig. S2). Thus, replacement of tetramers containing major H3 with H3.3 (Ahmad and Henikoff 2002) does not appear to occur to a significant extent on any of the polymerase II genes we investigated. While it is unclear whether *Physarum* contains a specialized H3.3-like variant, biochemical analyses indicate the presence of at least two independently synthesized H3 variants (J. Waterborg, pers. comm.). Nonetheless, it is important to note that in all organisms examined to date, H3.3-like RI exchange activity is supplied by either a specialized H3 variant or is inherent within a dual-purpose H3.3, as in yeast (Malik and Henikoff 2003). Thus, our data raise the possibility that pol II-dependent incorporation of H3.3/H4 tetramers is a relatively infrequent process, such that significant accumulation occurs only on highly transcribed loci and/or that such an exchange is specific to pol I-transcribed regions (Ahmad and Henikoff 2002; Supplementary Fig. S3). Indeed, recent comparisons of the level of RNA polymerase II activity and the ability to ChIP histones H3/H4 on specific sequences in yeast cells suggest that transient displace-

ment of nucleosomes can occur on highly transcribed sequences in this organism (Lee et al. 2004; Schwabish and Struhl 2004). Finally, it will be interesting to examine whether processes such as nucleotide excision repair account for the "background" level of exchange we observed on silent loci (Mello and Almouzni 2001).

Materials and methods

Physarum cultures

Physarum polycephalum strain TU291 was maintained in liquid culture. Naturally synchronous macroplasmidia were cultured as described (Thiriet and Hayes 1999b) and remain completely synchronous through several 9-h cell cycles. The onset of mitosis was determined by phase contrast microscopy of tiny explants (Thiriet and Hayes 1999b). The 15-min mitosis is directly followed by 3 h of S phase (determined by ³H thymidine incorporation; data not shown), then a 6-h G2 phase. There is no G1 phase.

Preparation of exogenous proteins and incorporation into macroplasmidia

Flag-tagged histones H2B and H4 were generated by addition of the Flag-coding sequence to the wild-type *Xenopus* genes expressed in bacterial cells and purified in complexes with *Xenopus* H2A or H3, respectively (Perry et al. 1985; Thiriet 2004) (oligonucleotide primer sequences in Supplementary Fig. S1). Flag-tagged dimers and tetramers reconstituted into nucleosomes *in vitro* in a manner identical to untagged proteins (data not shown). Fluorescently labeled histones were prepared as described (Thiriet and Hayes 2001). Incorporation of exogenous histone complexes were carried out essentially as described (Thiriet and Hayes 2001). Briefly, 400 μ L of solutions containing 10 mM Tris, HCl at pH 7.2, 5 mM ATP, and ~5 μ g/mL of histone complexes were spread onto the upper surface of macroplasmidia at the desired cell cycle stage for the times noted in the figure legends. Following the incubation, the plasmidia are washed with 5 mM EDTA and then harvested for further analysis (Thiriet and Hayes 1999a; Thiriet 2004).

Confocal analyses

Following incorporation of fluorescent dimers in G2 phase, macroplasmidium explants were smeared, fixed with ethanol, and permeabilized as described (Thiriet and Hayes 1999a). Cells were stained with anti-penta-acetyl H4 tail serum (Upstate Biotechnologies), and rhodamine-coupled goat anti-rabbit secondary antibodies were diluted at 1/500. After exhaustive washings with PBS, the slides were observed with a Leica confocal microscope.

Isolation of nuclei and preparation of chromatin

Cytosolic extracts and nuclear fractions were prepared by Dounce homogenization as described (Thiriet and Hayes 2001). Nuclei were isolated by percoll gradients as described by Nothacker and Hildebrandt (1988), modified as described (Thiriet 2004). The amount of exogenous proteins in nuclei after incorporation was determined by titrations with standards using anti-Flag Western blots and determination of the total amount of endogenous histones by SDS-PAGE and Coomassie staining against standards. The exogenous histones represent ~5% of the total histones in the cell in S phase and ~2.5% in G2 phase. Experiments using α -amanitin were carried out according to Pierron and Sauer (1980). Nucleosomes were prepared by MNase digestion of nuclei as described (Thiriet and Hayes 2001), followed by fractionation of 5%–20% sucrose gradients.

ChIP analyses

Following incubations, macroplasmidia were washed in 5-mM EDTA as described above, then the cells and filter paper support were incubated for 8 min in 1% formaldehyde to allow cross-linking. Nuclear fractions were prepared by homogenization, followed by a low-speed centrifugation. Nuclei were sonicated six successive times for 5 sec with output level 2 (Branson sonifier). Chromatin was then separated from the insoluble fraction by centrifugation for 10 min, maximum speed in a standard microfuge (14 kG). Soluble fractions were incubated overnight with anti-flag antibody coupled to agarose (Sigma). The beads were then washed as

recommended by the manufacturer prior to treatment with proteinase K, and cross-links were reversed by heating the samples at 65°C–70°C overnight, followed by phenolchloroform extraction and ethanol precipitation (Thiriet 2004). Amplification of immunoprecipitated DNA was carried as described by Hecht and Grunstein (1999) with the primers listed in Supplementary Figure S1. ChIP responses were all normalized to internal controls from unrelated loci and were shown to be in the range of linear response (data not shown).

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