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The Histone Variant H3.3 Marks Active Chromatin by Replication-Independent Nucleosome Assembly

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Summary

Two very similar H3 histones—differing at only four amino acid positions—are produced in *Drosophila* cells. Here we describe a mechanism of chromatin regulation whereby the variant H3.3 is deposited at particular loci, including active rDNA arrays. While the major H3 is incorporated strictly during DNA replication, amino acid changes toward H3.3 allow replication-independent (RI) deposition. In contrast to replication-coupled (RC) deposition, RI deposition does not require the N-terminal tail. H3.3 is the exclusive substrate for RI deposition, and its counterpart is the only substrate retained in yeast. RI substitution of H3.3 provides a mechanism for the immediate activation of genes that are silenced by histone modification. Inheritance of newly deposited nucleosomes may then mark sites as active loci.

Introduction

Histone octamers package the DNA of eukaryotic genomes into arrays of nucleosomes. Local modifications of chromatin are important for gene activity and are thought to be accomplished by targeting histone-modifying enzymes to particular segments (Jenuwein and Allis, 2001). Acetylation, phosphorylation, and methylation of histones can alter the conformation of nucleosomes or can function as specific binding sites for enzymes that alter chromatin structure (Wolffe, 1998; Marmorstein, 2001). The use of alternate histones provides another way of modifying chromatin. For example, the *Drosophila* genome encodes three variants of histone H3. The major H3 and replacement H3.3 histones (Fretzin et al., 1991; Akhmanova et al., 1995) are canonical in that they are phylogenetically conserved throughout the histone fold and N-terminal tail domains, while the third variant is the highly diverged centromeric histone Cid (Henikoff et al., 2000). The inclusion of any variant histone in a nucleosome is expected to alter the functional properties of chromatin.

The bulk of nucleosome assembly occurs as DNA is replicated, and assembly factors that can accomplish deposition of histone H3 have been extensively characterized (Mello and Almouzni, 2001). However, some histone deposition occurs outside of S phase. The replacement histone H3.3 slowly replaces H3 after differentiating cells have exited the cell cycle (Lennox and Cohen, 1988; Pina and Suau, 1987) and during spermatogenesis before DNA becomes repackaged with protamines (Akhmanova et al., 1997). The mechanics of histone replacement are not clear. A study in *Tetrahymena* concluded that no protein difference between histone H3 variants was required for replacement histone deposition and that expression of either variant outside of S phase appeared to be sufficient (Yu and Gorovsky, 1997). In contrast, by examining the dynamics of histone proteins in *Drosophila* nuclei we show that the major histone H3 and the replacement histone H3.3 proteins have distinct properties during in vivo chromatin assembly. Histone H3.3 participates in replication-independent (RI) nucleosome assembly and is targeted to transcriptionally active loci throughout the cell cycle. Transcription-coupled deposition of H3.3-containing nucleosomes may be a general mechanism for rapidly replacing permanently modified nucleosomes and for heritably activating genes.

Results

H3.3 Is Deposited by a Replication-Independent Pathway

To monitor histone dynamics in vivo, we constructed fusion genes encoding various histones and the green fluorescent protein (GFP) under the control of heat shock-inducible promoters. These constructs were transfected into exponentially growing *Kc* cells and induced as described (Henikoff et al., 2000; Ahmad and Henikoff, 2001). We have previously reported that the deposition of histone H3-GFP in the nucleus parallels that of nucleotide analog incorporation into DNA (Ahmad and Henikoff, 2001). Localization of histone H3-GFP was completely blocked by pretreatment of cells with the DNA replication inhibitor aphidicolin, demonstrating that the deposition of histone H3 is strictly replication dependent. Detection of a component of the DNA replication machinery, PCNA (Ng et al., 1990; Henderson et al., 2000), also confirms that deposition of histone H3-GFP is coupled to DNA replication: PCNA, BrdU, and H3-GFP give similar labeling patterns both in early S phase (when euchromatic DNA is replicating; Figure 1A) and in late S phase (when heterochromatic DNA is replicating; Figure 1B). BrdU and H3-GFP closely overlap because both are present for the entire 2 hr labeling period. PCNA labeling does not precisely overlap, as it provides a "snapshot" of replication only at the time of fixation (Leonhardt et al., 2000). In subsequent labeling experiments, we use PCNA to indicate the cell cycle stage.

Expression of H4-GFP gave qualitatively similar replication patterns to those of H3-GFP in 60% of labeled nuclei (Figure 2A, left and middle). These results are consistent with the assembly of histone H4 and histone H3 into nucleosomes during DNA replication. However, the remaining 40% of cells with H4-GFP expression showed five to nine discrete labeled foci in gap phase nuclei (Figure 2A, right). These foci were typically found in or near heterochromatin and nucleoli. Pretreatment of cells with aphidicolin completely abolished H4-GFP, PCNA, and deoxynucleotide analog replication patterns, but did not prevent the localization of H4-GFP to discrete

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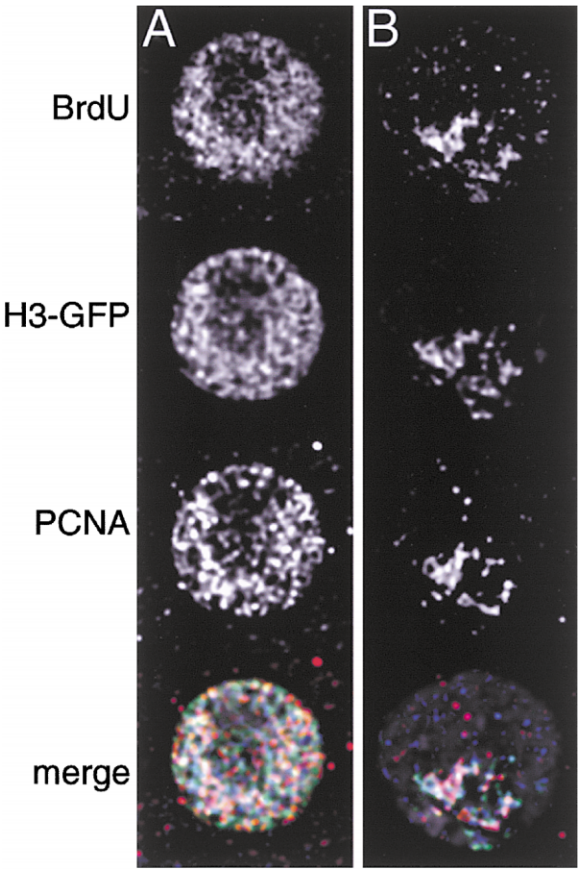

Figure 1. Histone H3-GFP Deposition Coincides with DNA Synthesis

Drosophila nuclei contain a chromocenter of heterochromatic DNA. Euchromatic DNA replicates early in S phase and is largely nonoverlapping with late replication in heterochromatin. Cells were induced to produce H3-GFP, and BrdU was added to the culture media for a period of 2 hr. Detection of PCNA in fixed cells gives a snapshot of replication foci at that time.

(A) A nucleus in early S phase shows similar patterns of labeling with anti-BrdU antibody, histone-GFP fluorescence, and anti-PCNA antibody.

(B) A nucleus in late S phase shows overlapping patterns of BrdU and histone-GFP, and a similar but not identical pattern of anti-PCNA staining due to the progress of DNA replication over the time course. In the merged images, BrdU is blue, H3-GFP is green, PCNA is red, and DNA staining (DAPI) is in gray.

foci (Figure 2B). These must be sites in the genome at which RI deposition of histone H4 occurs.

Since histone H3 deposition is strictly replication dependent, we reasoned that RI deposition of histone H4 might be accompanied by the deposition of H3 variants to form variant nucleosomes. Centromeric histones are thought to be included in nucleosomes at centromeres, and we have previously demonstrated that the *Drosophila* centromeric H3 variant Cid localizes to centromeres by a RI pathway (Ahmad and Henikoff, 2001). Thus, we expected that some sites showing H4 RI deposition would be centromeres. Detection of centromeres in *H4GFP*-transfected cells demonstrates that four to six of the H4 RI foci were indeed centromeres (Figure 2D), consistent with the assembly of nucleosomes containing Cid and H4 at these sites. We reasoned that the remaining H4 sites must be incorporating the final histone H3 variant, H3.3. Indeed, expression of H3.3-GFP in cells demonstrated that this variant does undergo both replication-coupled (RC) and RI deposition (Figures 2C and 2E). None of the H3.3-GFP foci coincided with centromeres, showing that centromeres exclusively use the Cid histone.

We confirmed that the H3.3-GFP is tightly bound to chromatin by extracting cells with 1.5 M salt before fixation. After this treatment, nuclei retain 48% of the H3.3-GFP but only 22% of the H2B-GFP ($p = 0.001$). Such differential extraction is expected from the biochemical properties of these histones (Wolffe, 1998), and the proper behavior of GFP-tagged histones has been extensively documented (Kimura and Cook, 2001).

Replication-Independent Deposition at rDNA Arrays

To map the locations of the sites in the nucleus where RI deposition of histone H3.3 and H4 occurs, we examined mitotic figures from cells transfected with histone-GFP constructs. The G2 phase in *Kc* cells is 4–6 hr long (Dolfini et al., 1970); thus, mitotic figures with H3-GFP labeling first appear 4–6 hr after heat-shock induction (Figure 3A) and show patterns consistent with histone-GFP production in late S phase, when heterochromatin was replicating (Ahmad and Henikoff, 2001). In contrast, labeled mitotic figures with H3.3-GFP and H4-GFP appear within 2 hr of induction (Figures 3B and 3C). H4-GFP showed prominent labeling at a single extended site near the middle of an X chromosome (Figure 3B). The pattern of H3.3-GFP was very similar to that of H4-GFP, showing the greatest labeling over an extended site on the X chromosome and at low levels specifically in euchromatin (Figures 3C and 3D). These cells must have been in the G2 phase of the cell cycle when histone-GFP was produced. This was confirmed by the presence of H3.3 labeling on mitotic chromosomes that showed no incorporation of pulse-labeled nucleotides (Figure 3E) and by observing mitotic figures from aphidicolin-treated cultures that nevertheless displayed H3.3-GFP labeling (Figure 3F). Thus, these mitotic labeling patterns with H3.3-GFP and H4-GFP must have resulted from RI deposition.

The extended appearance and proximal location of the prominent H3.3 and H4 site on the labeled X chromosome suggested that it coincides with the large rDNA gene repeat array on this chromosome. In situ hybridization with probes to the 28S rDNA gene confirmed that this is so (Figure 3G). Quantitative measurements of GFP signal over the rDNA array and over all of the chromosomes indicated that ~40% of all histone H3.3 in the cell is deposited at the rDNA locus ($n = 5$ spreads). In *Tetrahymena*, a histone H3 replacement variant is enriched in the transcriptionally active macronucleus, suggesting that this *Tetrahymena* variant potentiates active chromatin (Allis and Wiggins, 1984). We presume that the high intensity of histone H3.3-GFP staining at the rDNA locus in *Drosophila* is due to the combination of its densely repeated genes with high transcriptional activity.

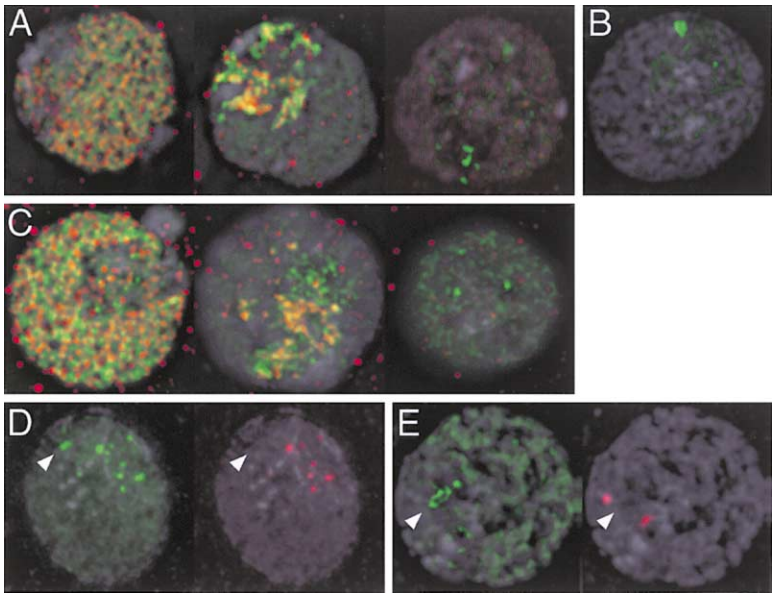

Figure 2. RC and RI Deposition of Histone-GFP Fusion Proteins

(A) Histone H4-GFP (green) deposits at replicating DNA, indicated by localization of PCNA (red) in both early (left) and late S phase (middle) cells. Some gap phase cells that lack detectable PCNA (right) show localized RI deposition of histone H4-GFP.

(B) Blocking DNA synthesis by aphidicolin treatment before producing H4-GFP eliminates replication patterns, but nuclei continue to display foci of RI deposition.

(C) Histone H3.3-GFP (green) localizes to replicating DNA, indicated by localization of PCNA (red), as well as to foci in gap phase cells that lack detectable PCNA (right).

(D) In gap phase cells, many H4-GFP foci (green), but not all, correspond to centromeres, which are marked by anti-Cid antibody (red). The arrowhead indicates a spot of H4-GFP deposition that does not coincide to a centromere.

(E) In gap phase cells, H3.3-GFP deposits at sites in the nucleolus (arrowhead), but not at centromeres (red). DNA staining (DAPI) is in gray.

H3.3 Incorporates De Novo into Growth-Induced rDNA Arrays

Notably, we often observed labeling with H3.3-GFP and H4-GFP of only one X chromosome. This is not due to absence of rDNA from other X chromosomes in these cells because the detection of 28S rDNA by in situ hybridization confirmed rDNA arrays are present on each of the three X chromosomes (Figure 3G). Other studies have pointed out that many *Drosophila* cell lines (including *Kc*) carry two distinguishable kinds of X chromosomes: a short one (XS) that resembles the normal X of flies, and a longer X (XL) (Privitera, 1980; Echalier, 1997). The origin of XL has been attributed to an expansion of the rDNA locus on this chromosome, presumably as these cells adapted to culture conditions. We observed that the rDNA array on XL was always labeled by H3.3-GFP (Figure 4A), consistent with this locus being active in all cells. However, in some experiments, variable numbers of cells had additional labeling on XS chromosomes (Figure 4B). To test whether some of this variability between experiments was due to differences in growth conditions, we transfected cells with the histone H3.3-GFP construct and then induced expression in samples of this culture 16 or 24 hr later. We found that many

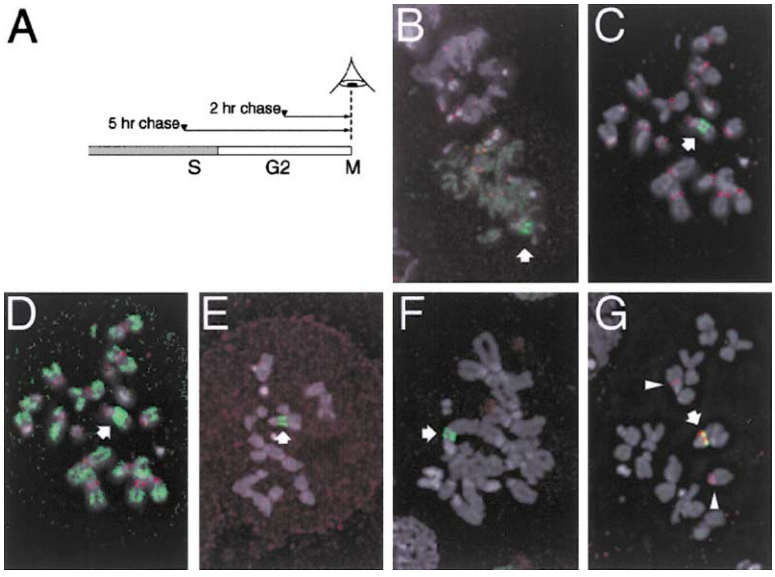

Figure 3. RI Deposition Occurs at the rDNA Locus and in Euchromatin

(A) Scheme for examining mitotic spreads from cells induced in the G2 phase of the cell cycle. G2 in *Kc* cells is 4–6 hr long; thus mitotics observed 2 hr after induction were in G2 when induced. Many mitotic spreads observed 5 hr after induction were in late S phase when induced.

(B) Mitotic figures labeled with histone H4-GFP (green) appear within 2 hr of induction. GFP signal localizes to a large site on an X chromosome (arrow). Centromeres are detected with anti-Cid (red).

(C) RI deposition of H3.3-GFP (green) resembles that of H4-GFP.

(D) Increased gain of the green channel from (C) shows that H3.3-GFP labels the euchromatic arms of all chromosomes at a low level.

(E) Lack of nucleotide labeling (red) after pulsing cells with nucleotide analog immediately before induction of H3.3-GFP (green) confirms that this mitotic spread is from a cell that was in G2 and that deposition is replication independent.

(F) Mitotic figures labeled with H3.3-GFP (green) continue to appear even when DNA replication is blocked with aphidicolin shortly before induction, indicating that these cells had completed S phase before induction.

(G) In situ hybridization detects a large rDNA array (28S probe, red) that corresponds to the intense site of H3.3-GFP RI deposition on the XL chromosome (arrow). Additional rDNA genes are present on XS chromosomes (arrowheads).

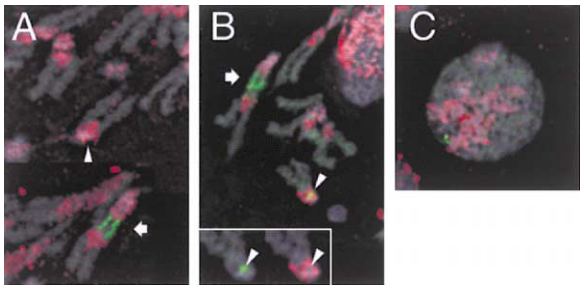

Figure 4. H3.3 Marks Activated rDNA Arrays

Some mitotic spreads (A) show intense H3.3-GFP labeling (green) only on the large rDNA array on the elongated XL chromosome (arrow), but not on XS chromosomes (arrowhead), which also carry rDNA genes. Another spread (B) shows H3.3-GFP labeling on both XL and XS chromosomes. The inset shows an enlargement of the proximal part of the XS chromosome. Wherever H3.3-GFP is present, there is a gap in heterochromatin (antibody to $H3^{di\text{-}MethylK9}$, red). (C) RI H3.3-GFP labeling shows little overlap with heterochromatin in interphase nuclei. DNA staining (DAPI) is in gray.

cells from exponentially growing cultures showed RI labeling on both XL and XS chromosomes (mean number of labeled loci/metaphase spread = x = 1.66, SD = 0.63), while metaphase spreads from the later time point, when culture growth had slowed, showed labeling on only the one XL (x = 1, SD = 0, p = 0.004). This change in frequency suggests that the smaller rDNA arrays on XS chromosomes are maintained in a transcriptionally silent state but can be activated.

We considered that the silencing of XS rDNA arrays might be due to heterochromatin-mediated silencing. Indeed, staining of metaphase spreads from cells expressing histone H3.3-GFP for the heterochromatin marker $H3^{di\text{-}MethylK9}$ ($H3^{Me}$) revealed that rDNA arrays labeled by RI deposition of H3.3-GFP are depleted for $H3^{Me}$, in spite of being flanked on both sides by heterochromatin (Figures 4A and 4B). In every XS chromosome where the proximal region was labeled with H3.3-GFP, a corresponding gap in the $H3^{Me}$ pattern was found (Figure 4B, inset). That sites heavily labeled with H3.3-GFP were largely unlabeled with $H3^{Me}$ was confirmed in interphase nuclei (Figure 4C). We conclude that the chromatin state of rDNA arrays can be reversed in response to changes in growth conditions, and H3.3 accumulates de novo at activated genes.

Amino Acid Changes toward H3.3 Allow Replication-Independent Deposition

Characteristic amino acid substitutions distinguish major histone H3 proteins from their replacement histone H3 paralogs (Figure 5A), suggesting that some or all of these substitutions are responsible for differences in deposition (Waterborg and Robertson, 1996). However, the RI deposition of replacement histone H3 variants has been attributed entirely to its availability in the gap phases of the cell cycle (Yu and Gorovsky, 1997). Instead, our results producing H3-GFP and H3.3-GFP from the same inducible promoter argue that at least some of the characteristic substitutions specify which assembly pathway is used. It is clear that H3-GFP fails to undergo RI deposition, because when the protein is produced

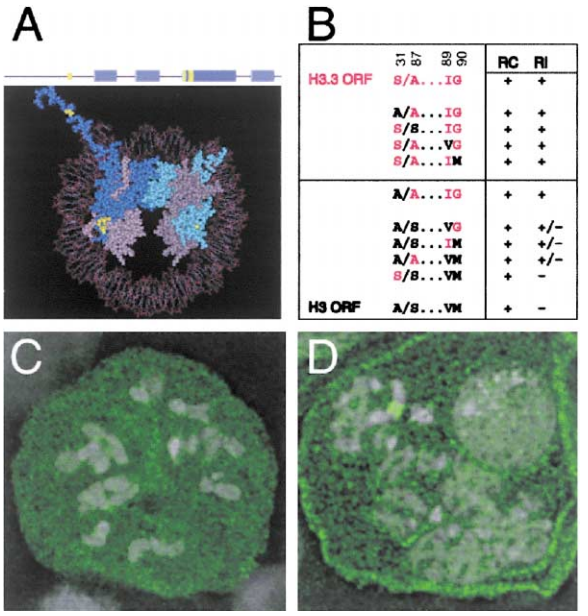

Figure 5. Amino Acid Changes in H3.3 Determine Assembly Pathways

(A) Structure of histone H3 and the $(H3{\bullet}H4)_2$ tetramer. A schematic of the H3 protein is shown (top), with α helices of the protein indicated with blue cylinders. The $(H3{\bullet}H4)_2$ tetramer (bottom) is drawn with Cn3D (NCBI). The two H3 chains are in shades of blue, and H4 chains are in shades of gray. Only one N-terminal tail of H3, starting at residue 20, is shown. The four positions that distinguish major histone H3 from H3.3 are highlighted in yellow.

(B) Site-directed mutations in the H3 and H3.3 ORFs and their activity when expressed in cells. RC deposition was scored in interphase cells, and RI deposition by the labeling of the rDNA locus on metaphase spreads 2 hr after induction (efficient [+], weak [+/−], or none [−]). The efficiency of RI deposition (the ratio of GFP intensity at labeled rDNA arrays to the background intensity) for mutants scored as weak was <5 (H3.3 gave a ratio of 29). Red residues indicate identities in H3.3, and black indicates identities found in H3.

(C) H3-GFP protein (green) does not localize to chromatin when induced in gap phase cells. H3-GFP protein is distributed throughout the cytoplasm.

(D) The $H3^{A/S...IM}$-GFP protein (green) localizes poorly to the rDNA locus. DNA staining (DAPI) is in gray.

after S phase, it does not deposit onto DNA (Figure 5C). To identify which of the four differences between *Drosophila* H3 and H3.3 are responsible for differential deposition, we used site-directed mutagenesis to alter the histone-GFP fusion genes (Figure 5B). Single mutations were introduced into the H3.3-GFP fusion gene (with amino acid residues S31 A87 I89 G90; abbreviated "S/A...IG") to match each of the H3 identities (A31 S87 V89 M90; "A/S...VM"). Each of these permutated templates was transfected and expressed in cells, and the ability of the resultant fusion proteins to participate in RC and RI deposition examined. All mutant proteins were efficiently deposited onto replicating DNA, demonstrating that these changes did not interfere with chromatin assembly. However, none of the mutations prevented deposition at the rDNA array, and thus we conclude that no single identity is necessary for the RI pathway. We also introduced the converse mutations into the H3-GFP fusion gene to match each of the H3.3

identities. Strikingly, each of three mutations was sufficient by itself to confer partial RI activity (Figures 5B and 5D). All three of these positions lie in the core of the histone (Figure 5A). To further confirm that these residues specify assembly pathways, we converted all three positions in H3 to the H3.3 identities (A/A...IG). As expected, this mutant undergoes both RC and RI deposition. Since any one change at these positions in H3 allows some RI deposition, it appears that this is a default ability of H3 variants. We conclude that the identities at these positions specify assembly pathways and that the combination of residues in the major H3 histone actively prevents RI assembly.

Replication-Coupled Deposition Requires the Conserved N-Terminal Tail

The above analysis demonstrates the existence of RC and RI deposition pathways that use different histone H3 variants. These pathways may be mediated by different nucleosome assembly machines, raising the possibility that the conserved portions of canonical H3 variants are important in one pathway but not the other. The histone-fold domain of histone H3 is essential for correct folding of the protein in the nucleosome (Arents and Moudrianakis, 1995); thus it is unlikely to be dispensable in any histone H3 variant. A role for the extended N-terminal tail of H3 in nucleosome assembly has been examined both in vivo (Ling et al., 1996; Freeman et al., 1996) and in vitro (Shibahara et al., 2000; Quintini et al., 1996) and has been found to be dispensable.

We examined whether the N-terminal tail regions of histone H3 and H3.3 are required for either nucleosome assembly pathway in *Drosophila* cells. A series of deletions was generated that removed portions of the histone tail from GFP fusion constructs (Figure 6A). We transfected these constructs into cells and induced expression as before. The distribution of histone-GFP was compared to the PCNA pattern in individual nuclei to determine whether RC deposition with the deleted protein would still occur. In this experimental system, we find that the N-terminal tail of histone H3 is essential for in vivo RC nucleosome assembly. Histone H3 proteins deleted for this region localize poorly to replicating DNA or remain diffuse throughout the nucleus (Figures 6B and 6C). Deletion of the N-terminal tail does not inhibit tetramer formation with histone H4 in vitro (Shibahara et al., 2000), and H3 continues to be imported into the nucleus (Figure 6), implying that these truncated proteins are defective for a later step in RC nucleosome assembly.

Replication-Independent Deposition Does Not Require the N-Terminal Tail

Uncovering a requirement for a region in the N-terminal tail of histone H3 for RC deposition prompted us to examine whether this region is also required for RI deposition of histone H3.3. Most truncated histone H3.3-GFP proteins were efficiently used for RI deposition (Figure 6A) and were resistant to salt extraction (data not shown), although larger deletions produced aberrant protein aggregates in some nuclei (Figure 6D). Only the most proximal deletion (deleting 40 of the 44 residues from the N-terminal tail) showed a reduced intensity of rDNA labeling (Figure 6E). This deletion extends into a critical region of histone H3 that passes through the DNA gyres in the nucleosome (Luger et al., 1997). A similar deletion is lethal in yeast (Mann and Grunstein, 1992), suggesting that the region is required to form a proper nucleosomal particle. We conclude that RI nucleosome assembly machinery can deposit a truncated histone H3.3 protein.

Because the N-terminal tail is essential for RC but not for RI deposition, deletion constructs of histone H3.3-GFP separate these two pathways of nucleosome assembly. Staining for PCNA in cells producing a truncated histone H3.3-GFP revealed that nucleosome assembly even in S phase cells is not limited to replicating DNA: some RI deposition of histone H3.3 occurs in euchromatin and in the nucleolus (Figures 6F and 6G). Thus, although the bulk of nucleosome assembly uses the vastly more abundant histone H3 and is coupled to DNA replication, at some sites nucleosomes are assembled continually throughout the cell cycle.

Discussion

Differences between H3 and H3.3 Specify the Nucleosome Assembly Pathway

Two very similar forms of histone H3 are produced in *Drosophila* cells. The major histone H3 genes are greatly upregulated during S phase for the assembly of newly replicated chromatin (Osley, 1991), and the two orphan genes encoding the variant histone H3.3 are expressed at constitutive levels throughout the cell cycle (Akhmanova et al., 1995). We show that there are two pathways for chromatin assembly in *Drosophila* cells: one that assembles nucleosomes during DNA replication, and a second that assembles them only at particular loci by a RI mechanism. The existence of an RI pathway is demonstrated by multiple lines of evidence: (1) tagged histones deposit in interphase cells that lack replication foci; (2) they deposit when DNA replication is blocked; (3) they label mitotic chromosomes when produced during G2; and (4) histone H3.3, but not H3, is the substrate for RI deposition.

Before nucleosome assembly, histones form stable $(H3{\bullet}H4)_2$ tetramers and H2A•H2B dimers (Krude and Keller, 2001). Our results indicate that both H3- and H3.3-containing tetramers can be deposited during DNA replication. Since H3 and H3.3 are identical across the tetramer protein interaction surface (Luger et al., 1997), and if assembly is unbiased, both homotypic and heterotypic tetramers will be produced every cell cycle.

Extensive biochemical studies have led to a stepwise model for the assembly of nucleosomes (Krude and Keller, 2001). The chromatin assembly factor (CAF) includes proteins that promote the folding and assembly of $(H3{\bullet}H4)_2$ tetramers and that subsequently deposit histones onto newly replicated DNA (Stillman, 1986). Components of CAF are recruited to sites of DNA replication in vivo by a specific interaction with PCNA (Shibahara and Stillman, 1999). However, alternative assembly activities must also exist because CAF is nonessential in both *Saccharomyces* (Enomoto et al., 1997; Kaufman et al., 1997) and *Arabidopsis* (Kaya et al., 2001). At least one factor that stimulates CAF activity also has nucleosome assembly activity on its own (Tyler et al., 1999).

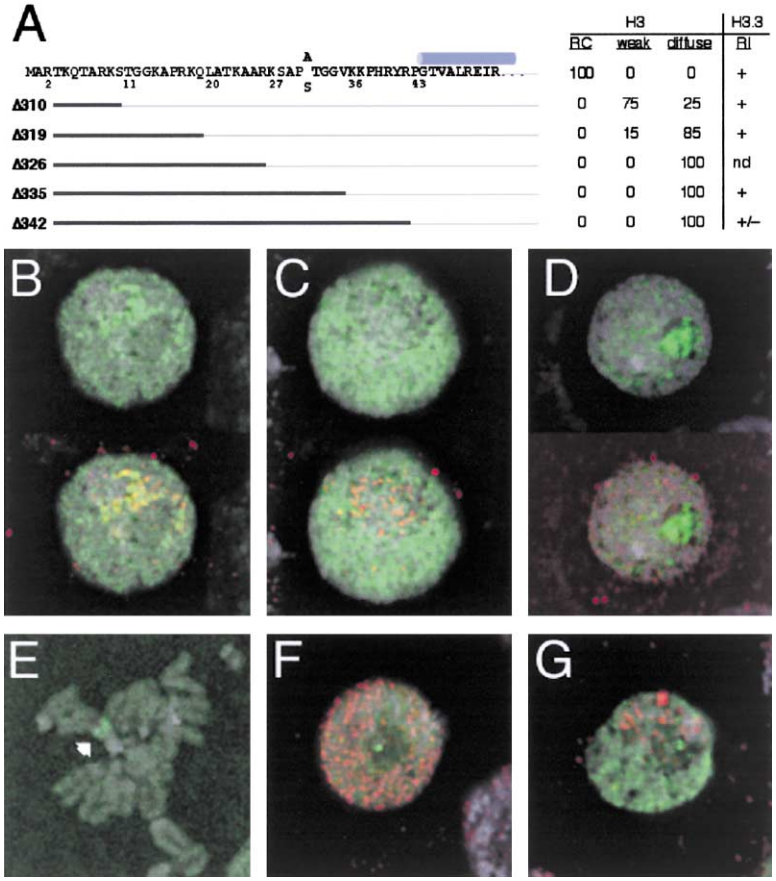

Figure 6. The N-Terminal Tail Is Required for RC but Not RI Deposition

(A) Schematic of deletions that remove portions of the tail from histone-GFP fusion proteins. Amino acid residues that are unique to H3 are indicated above the consensus sequence, and those unique to H3.3 are below it. The blue cylinder marks the beginning of the core region. Thick horizontal lines delimit the deletions made in histone H3 and H3.3 constructs. To score RC deposition, at least 20 GFP^{+} nuclei with late S phase PCNA patterns were scored for overlap between GFP and PCNA. The percentage of nuclei with overlap (indicating efficient *RC* deposition), *weak* overlap with general nuclear fluorescence, and only *diffuse* fluorescence is given. The RI deposition of truncated H3.3-GFP proteins was scored on mitotic spreads.

(B) $H3^{\Delta 319}$-GFP weakly localizes to replicating DNA (PCNA, red).

(C) $H3^{\Delta 326}$-GFP is diffuse throughout a late S phase nucleus.

(D) Occasional nuclei show aggregates of $H3.3^{\Delta 335}$-GFP protein.

(E) $H3.3^{\Delta 342}$-GFP shows reduced localization (arrow) to the rDNA locus in mitotic spreads, with $<$5-fold enrichment over background.

(F and G) $H3.3^{\Delta 335}$-GFP localizes by RI deposition to euchromatin and the nucleolus in early (F) and late (G) S phase cells (PCNA, red).

Chromatin remodeling factors are additional candidates for alternative activities since some can transfer nucleosomes to DNA in vitro (Ito et al., 1997; Lorch et al., 1999). Indeed, two remodeling complexes, RSF and ACF both promote transcription and mediate nucleosome assembly (LeRoy et al., 1998; Loyola et al., 2001; Levenstein and Kadonaga, 2002). From our work, it is clear that RC and RI deposition actually use different histone H3 variants, and we suggest that histone H3.3 is the correct substrate for chromatin assembly factors that are transcriptionally linked. It will be interesting to see if known assembly factors prefer this variant.

Histone H3 and H3.3 differ at only four amino acid positions, and mutational analysis reveals that a single change at any one of the three positions in the histone core allow some RI deposition. Thus, we interpret RI deposition as a default feature of canonical histone H3 variants, and only the three residues found in H3 will preclude RI deposition. Such specificity could be understood if these positions in histone H3 make contacts that only fit with replication-specific assembly factors, or bind an accessory protein that alters the activity of a more general nucleosome assembly factor (Figure 7). The three positions in H3 are in solvent-accessible regions of the nucleosome particle (Arents et al., 1991; Luger et al., 1997) (Figure 5A) and thus contacts with these positions before assembly is plausible. It is simplest to imagine that replication-specific assembly proteins recognize tetramers that contain H3 and localize them to newly replicated DNA. All remaining tetramers might then associate with RI assembly factors and be recruited to active loci.

The idea that distinct complexes mediate the two kinds of nucleosome assembly is supported by our finding that the extreme N-terminal tail of histone H3 variants is required for RC deposition but not RI deposition. Studies with histones H2A and H2B in *Physarum* (Thiriet and Hayes, 2001) reveal clear deficiencies in the RC deposition of tailless histones. However, in in vitro experiments where tailless histones are the only ones available, chromatin can be assembled (Shibahara et al., 2000; Quintini et al., 1996). In our experiments and those in *Physarum*, tailless histones must deposit as efficiently as endogenous full-length histones or they will be out-competed and will not appear in chromatin. Assembly may also be affected by predeposition modifications (Sobel et al., 1995) or cell type differences. For example, chromatin assembly in embryos with rapid nuclear division may be less stringent than assembly in somatic cells, as we have examined here. Indeed, in *Xenopus* early embryonic nuclei, truncated H3 can be deposited into chromatin, albeit inefficiently (Freeman et al., 1996).

There appear to be multiple mechanisms that ensure the utilization of different histone H3 variants by nucleosome assembly pathways. In *Tetrahymena*, only the replacement H3 variant gene *hv2* is expressed in gap phase cells, and this histone undergoes RI deposition. Deletion of *hv2* is viable but is accompanied by the constitutive expression of a major H3 gene (Yu and Gor-

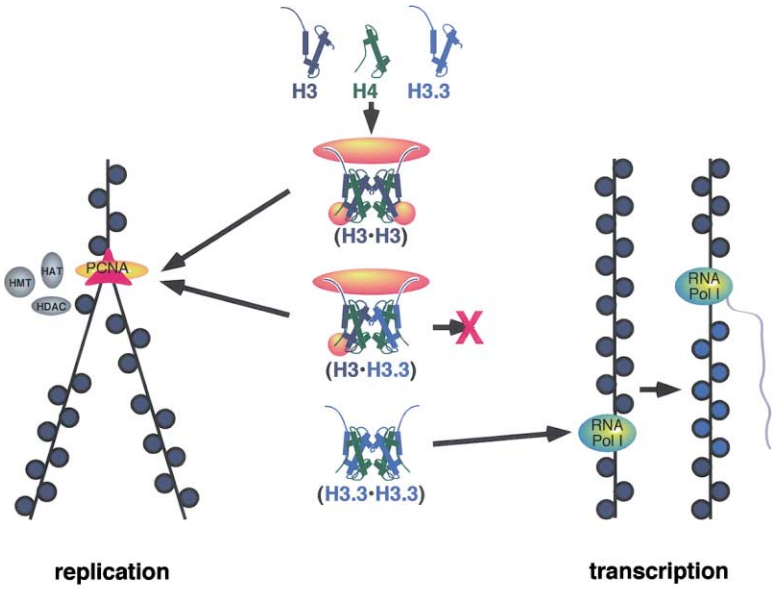

Figure 7. Model for the Specification of Nucleosome Assembly Pathways by Histone H3 Variants

Histone proteins (blue and green) form homotypic and heterotypic tetramers. Replicative chaperones or assembly factors (yellow) make contacts with the N-terminal tails and with a region in the core of the major H3 chain (including S87 and VM89, 90). Contact with both tails and with at least one core region is required for RC deposition. The complex is committed to assembly on newly replicated DNA (left) by recruitment to replication forks, and chromatin modifications are maintained by enzymes (gray) that accompany DNA polymerase. Heterotypic tetramers can bind the core region replicative factor through one H3 chain; thus H3.3 expressed during S phase undergoes RC deposition as heterotetramers. Homotypic H3.3 tetramers lack the core recognition sites and fail to bind the replicative factor. Targeting of homotypic H3.3 tetramers to active loci may be mediated by an interaction between RI assembly factors and transcription factors or may simply fill in gaps in chromatin left after RNA polymerase passage and nucleosome displacement (right).

ovsky, 1997). Thus, the differential production of histone variants in *Tetrahymena* appears to direct their use by RC and RI pathways. It is likely that the differential production of the animal histone H3 and H3.3 also contributes to their use by different assembly pathways because production of the major H3 protein is down regulated in gap phase cells (Harris et al., 1991). At least one transcriptional activator sequence that directs S phase expression of the major H3 coincides with the codons that distinguish it from H3.3 (Bowman and Hurt, 1995). However, in addition to differences in production, we find that nucleosome assembly pathways distinguish between histone H3 variant proteins in *Drosophila*. Indeed, deletion of even one of the two histone H3.3 genes in mice is semilethal, implying that animal H3.3 is not redundant with H3 (Couldrey et al., 1999). This difference between *Tetrahymena* and animals may be due to the evolutionary history of histone H3 variants: the ciliate replacement H3 variant *hv2* appears to have had a separate origin from that of animal and that of plant replacement H3 histones (Thatcher et al., 1994; Waterborg and Robertson, 1996). Regardless of whether differential production or substrate specificity direct their utilization, distinctive replacement H3 histones are the normal substrate for RI pathways.

Yeast Have Retained Only the H3.3 Counterpart

Alternate interpretations of the phylogenetic history of the histone H3 family have been proposed. One analysis suggested that a replacement histone H3 variant was the common ancestor (Wells et al., 1986), but other interpretations have proposed that replacement histones have multiple independent origins (Thatcher et al., 1994; Waterborg and Robertson, 1996). We believe that the presence of paralogous histone H3 genes in many organisms may preclude delineation of which sequence is ancestral. However, our findings suggest that a replication-independent nucleosome assembly pathway is essential in all cells. This implies that, functionally, a replacement histone H3 has always been extant. In organisms that encode only one kind of canonical histone H3 protein that is used throughout chromatin (Thatcher et al., 1994; Waterborg and Robertson, 1996; Waterborg et al., 1995), we expect that this H3 variant must undergo both RC and RI deposition. Fungal lineages are particularly intriguing in this regard because all ascomycetes, including laboratory yeasts and molds, carry only one canonical histone H3. Each of these is identical to animal H3.3 at positions 89 and 90, and often identical at position 31 (Baxevanis and Landsman, 1998; and data not shown). Thus, by this criterion, we propose that the solitary histone H3 proteins in ascomycetes are equivalent to histone H3.3. Indeed, nucleosome assembly activity in the cell cycle gap phases has been detected in *Saccharomyces* (Altheim and Schultz, 1999). These fungi appear to have lost their ancestral H3, as we find that genomes from the Basidiomycota sister clade have both H3 and H3.3 (The Institute for Genomic Research, Cryptococcus neoformans genome project at http://www.tigr.org; and DOE White Rot Genome Project at http://www.jgi.doe.gov/programs/whiterot.htm). Histone H2A in *Saccharomyces* may have an analogous evolutionary history, since it now performs the functions of the H2A and the H2A.X variants in other organisms (Downs et al., 2000). Thus, both histone H3 and H2A in *Saccharomyces* appear to be evolutionary derivatives of replacement genes.

The lack of an H3 counterpart in yeasts and molds may provide insight into differences between simple fungi and complex multicellular eukaryotes in maintaining silent chromatin. Much of the *Saccharomyces* genome is continually in a transcriptionally competent state (Sherman, 1997), similar to H3.3-containing regions in complex genomes. Perhaps this relative lack

of silent chromatin allowed the loss of the strictly RC histone substrate. Heterochromatic silencing in yeast may be needed only at special sites, such as silent mating type loci and telomeres, where SIR-based silencing has evolved. In multicellular eukaryotes, the need for maintaining most of the genome in a continuously silent state in differentiated cells may favor maintaining two distinct H3 histones.

Replication-Independent Deposition of H3.3 Marks Active Chromatin

What essential function might replication-independent nucleosome assembly serve? Targeting of histone H3.3 may be due to transcriptional activity at these sites. Passage of RNA polymerase has been shown to displace nucleosomes (Pfaffle et al., 1990; Clark and Felsenfeld, 1992), although transcription without nucleosome dissociation has also been reported (Studitsky et al., 1994). Our results indicate that in a natural context, nucleosomes are indeed displaced, because newly synthesized histones take their place. This has two consequences: first, the rapid switching of histone modifications; and second, the establishment of a heritable distinction between active and bulk chromatin.

While histone modifications such as phospho and acetyl groups are catalytically added and removed and may be maintained by enzymes that accompany DNA replication machinery (Rountree et al., 2000), it appears that lysine methylation is irreversible (Jenuwein, 2001). Gene silencing is associated with H3 methylation at residue K9, and this would be long lived if dilution of the methylated histone through DNA replication and nucleosome segregation were the only method for its elimination. However, new nucleosome assembly by an RI pathway is a logical method for rapid gene activation, as all modified histones can be replaced within one cell generation (Figure 7). The rapid turnover of a replacement histone H3 variant in alfalfa (Waterborg, 1993) supports this view.

The regeneration of nucleosomes by RI assembly also alters chromatin, because a variant histone is incorporated. Histone H3.3 includes a serine at residue 31, and modifications of this site would provide unique regulation of active chromatin. Modification of $(H3.3{\bullet}H4)_2$ tetramers before deposition could also effectively target modifications to active regions. Finally, the inheritance of variant nucleosomes through cell division might predispose regions to be transcriptionally active again. It is striking that histone H3.3 mostly localizes to an rDNA array, and numerous cases are known where the activity of one rDNA array is heritable (nucleolar dominance) (Reeder, 1985; Pikaard, 2000). Nucleolar dominance has been observed in cell lines and in interspecific hybrids and may be an example of regulation when rDNA gene copy number varies. Since some RNA polymerase I components remain associated with active rDNA arrays through mitosis, the inheritance of these proteins has been suggested as the basis for nucleolar dominance (Roussel et al., 1996). Similarly, the inheritance of variant nucleosomes provides an obvious mechanism for epigenetic inheritance at rDNA arrays and at euchromatic genes. Thus, transcription would allow RI deposition of H3.3, which would in turn maintain the active state.

Experimental Procedures

Constructs

We used the heat shock-inducible *HS-H3-GFP* and *HS-H2B-GFP* plasmids previously described (Henikoff et al., 2000) and constructed similar fusions for the *Drosophila H3.3A* and *H4* genes, with a six amino acid linker (SRPVAT) between GFP and the last residue in these ORFs. These constructs are designated *HS-H3.3A-GFP* and *HS-H4-GFP*, respectively. To generate N-terminal deletions of the H3 and H3.3 tails, primers to the internal segments of the ORFs specified in the text that included an XbaI site and the first three codons of the ORF (MAR) at their 5′ end were used in PCR with a primer to the GFP ORF with Ampli-Taq Gold (Perkin-Elmer, Foster City, CA) or Platinum Taq (GIBCO-BRL, Grand Island, NY) enzymes to generate truncated ORFs from *HS-H3-GFP* and *HS-H3.3A-GFP* templates. These products were digested with XbaI and EagI and cloned into the XbaI, EagI-digested *HS-H2B-GFP* vector. For site-directed mutagenesis of *H3-GFP* and of *H3.3A-GFP*, 2.9 kb fragments containing the complete genes were each subcloned into pUC19, and the QuikChange kit (Stratagene, La Jolla, CA) was used with primers including the codon changes specified in the text. All deletion and mutation constructs were confirmed by BigDye sequencing (ABI, Foster City, CA). These plasmids were then used for transfections into *Kc* cells.

Immunostaining and DNA FISH

Culture, transfection, fixation, and image collection methods have been previously described (Henikoff et al., 2000; Ahmad and Henikoff, 2001). Transfected constructs were induced for 1 hr at 37°C and returned to 25°C for recovery for 2 hr before fixation. The G2 phase of the cell cycle is 4–6 hr long in this cell line (Dolfini et al., 1970; Ahmad and Henikoff, 2001); thus all mitotic figures in these preparations are from cells that were induced after S phase was complete. To mark centromeres, DNA replication forks, and heterochromatin, we used rabbit antisera to the Cid histone (Henikoff et al., 2000), PCNA (Henderson et al., 2000), and histone $H3^{di\text{-}MethylK9}$ (UpState Biotech, Lake Placid, NY), respectively, followed by anti-rabbit IgG goat antibodies conjugated with either Texas-Red or with Cy5 fluorochromes (Jackson ImmunoResearch, West Grove, PA). For triple labeling of nucleoside incorporation, histone-GFP deposition, and PCNA distribution, cells transfected with the *HS-H3-GFP* plasmid were induced, and BrdU and deoxycytidine were added to the culture media for a final concentration of 100 μg/ml at the start of the recovery period. Cells were fixed and immunostained for GFP and PCNA using mouse monoclonal anti-GFP antibody (Molecular Probes, Eugene, OR) and rabbit anti-PCNA antibody, respectively, followed by anti-mouse Rhodamine-Red-conjugated monovalent Fab fragments (Jackson ImmunoResearch) and anti-rabbit Cy5-conjugated antibody. Slides were then refixed with Carnoy's fixative and processed as described (Van Hooser and Brinkley, 1999), except that DNA was denatured with 0.07 N NaOH for 30 min at 25°C. Incorporated BrdU was detected using mouse FITC-conjugated antibody (Roche, Pleasanton, CA).

For DNA FISH and GFP detection, we immunostained for GFP using a mouse monoclonal anti-GFP antibody (Molecular Probes), followed by anti-mouse FITC-conjugated antibodies (Jackson ImmunoResearch). Slides were then refixed and denatured as above. A probe to the 28S rDNA gene was prepared using the primers CGAAAGACCAATCGAACCATCTAG and GAACCGTATTCCCTTTC GTTCAA. These were used to amplify a 1 kb fragment in PCR, and this product was used with the BioPrime DNA labeling kit (GIBCO-BRL). Hybridization was performed overnight at 25°C, and bound probe was detected using Texas-Red-conjugated streptavidin (Pierce Chemical Co., Rockford, IL).

Construct Evaluation and Image Quantitation

Images were analyzed using DeltaVision software (Applied Precision, Issaquah, WA). Transfection efficiencies were estimated as the fraction of interphase cells with GFP fluorescence and were typically ~70%. Each construct was tested at least four times. RC deposition was evaluated in transfected and induced cells with a late S phase (heterochromatic) PCNA pattern, which allows a close assessment of the GFP and PCNA patterns. RI deposition was as-

sayed by counting the fraction of metaphase spreads 2 hr after induction with GFP labeling at the X chromosome rDNA locus. The frequency of GFP fluorescence in interphase cells served as the expected frequency for labeled metaphase figures if the histone-GFP protein could undergo RI deposition. At least 20 metaphase figures were examined for each sample. For quantitative measurements of histone H3.3-GFP deposition, we used DeltaVision object-building software to define DAPI-stained chromosomes in metaphase spreads from cultures transfected with *HS-H3.3A-GFP*. The integrated pixel intensities in the GFP channel over these chromosomes was then measured, as was deposition specifically at the rDNA locus, defined as the intensely labeled contiguous segment of the XL chromosome. Background intensity was subtracted from each of these, and the ratio of the two measures estimates the fraction of histone H3.3 that is targeted to the rDNA locus. The ratio between the peak pixel intensity at a labeled rDNA locus and the mean background intensity was used as an estimate of the efficiency at which mutated H3.3-GFP proteins underwent RI deposition. The unmutated H3.3A-GFP construct gave an efficiency ratio of 29 (n = 7). Mutations that were scored as defective for RI deposition gave ratios less than five.

In Situ Salt Extraction

Cells were transfected with *HS-H2B-GFP*, and *HS-H3.3-GFP* constructs were grown on coverslips, induced, and allowed to recover for 2 hr. H3.3-GFP histone in nuclear preparations was undetectable by Western analysis with anti-H3 antibodies (data not shown). Extraction of nuclear proteins was performed as described (Balajee and Geard, 2001), except that we used 1.5 M NaCl in order to extract H2B, but not H3 and H4. Parallel sets of cells were mock treated with extraction buffer with only 130 mM NaCl. Cells were then fixed as above, and we measured the GFP fluorescence intensities in seven to eight random fields of nuclei to quantitate the amount of histone-GFP proteins in mock-treated cells and the amount retained after extraction.

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