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Dynamic Changes in Chromatin Structure Through Post-Translational Modifications of Histone H3 During Replication Origin Activation

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ABSTRACT

Genome duplication relies on the timely activation of multiple replication origins throughout the genome during S phase. Each origin is marked by the assembly of a multiprotein pre-replication complex (pre-RC) and the recruitment of the replicative machinery, which can gain access to replication origins on the DNA through the barrier of specific chromatin structures. Inheritance of the genetic information is further accompanied by maintenance and inheritance of the epigenetic marks, which are accomplished by the activity of histone and DNA modifying enzymes traveling with the replisome. Here, we studied the changes in the chromatin structure at the loci of three replication origins, the early activated human lamin B2 (LB2) and monkey Ors8 (mOrs8) origins and the late-activated human homologue of the latter (hOrs8), during their activation, by measuring the abundance of post-translationally modified histone H3. The data show that dynamic changes in the levels of acetylated, methylated and phosphorylated histone H3 occur during the initiation of DNA replication at these three origin loci, which differ between early- and late-firing origins as well as between human- and monkey-derived cell lines. These results suggest that specific histone modifications are associated with origin firing, temporal activation and replication fork progression and underscore the importance of species specificity. J. Cell. Biochem. 108: 400–407, 2009.

KEY WORDS: CELL CYCLE; CHROMATIN STRUCTURE; DNA REPLICATION; HISTONE MODIFICATIONS; REPLICATION TIMING

Replication of both the eukaryotic genome and epigenome rely on the initiation of DNA replication from specific chromosomal regions, termed origins, at specific times during S phase. Replication origins are marked by the presence of DNA consensus sequences and the binding of the Origin Recognition Complex proteins (ORC1-6), which leads to the assembly of a pre-RC [DePamphilis et al., 2006; Sclafani and Holzen, 2007; Rampakakis et al., 2009]. Although DNA replication in lower eukaryotes initiates from well characterized consensus sequences, metazoan origins exhibit a certain degree of degeneracy. As a result, both origin selection and temporal activation are characterized by plasticity; for example, during the development of *Xenopus* and *Drosophila* embryos there is a dramatic change in origin usage from widespread random initiations to specific initiation sites, which correlates with the onset of zygotic transcription and global chromatin remodeling [Shinomiya and Ina, 1991; Hyrien and Mechali, 1993; Hyrien et al., 1995; Maric et al., 1999; Sasaki et al., 1999]; while the human β-globin locus replicates early in S phase in pre-erythroid cells that express globin, but late in non-erythroid cells which do not express it. This origin plasticity is more likely due to reversible changes in chromatin structure as opposed to the rigidity of the genetic code [Aladjem, 2007; Jorgensen et al., 2007a].

Recent studies showed that chromatin structure could indeed affect origin selection, activation and temporal program. Thus, treatment of HeLa cells with the histone deacetylase (HDAC) inhibitor trichostatin A (TSA), led to a more dispersive pattern of initiation site selection as well as an earlier activation of the late-firing β-globin origin [Kemp et al., 2005]. SIR2, a histone deacetylase specific for acetyl-lysines K9 and K14 of histone H3 and K16 of histone H4, inhibits activation of some origins, but not others [Pasero et al., 2002; Pappas et al., 2004], by promoting an unfavorable chromatin structure for pre-RC assembly [Crampton et al., 2008]. Deletion of the Rpd3 HDAC in budding yeast promoted an earlier activation of many late-firing replication origins [Vogelauer et al., 2002; Aparicio et al., 2004], while deletion of the silent chromatin proteins Sir3 and Ku advanced the replication timing of certain origins [Stevenson and Gottschling, 1999; Cosgrove et al., 2002].

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Overall, these data suggested a role for epigenetic control of origin firing. To date, however, the chromatin events regulating the initiation of DNA replication are not well understood. Histone acetylation appears to be an important regulator of origin activation in some cases, but it is neither the sole nor necessary determinant of origin firing [Prioleau et al., 2003]. The methylation status of histone H3 also seems to be an important molecular event during the initiation of DNA replication [Stedman et al., 2004; Zhou et al., 2005], but it alone is not sufficient to dictate the spatial and temporal regulation of chromosomal domains [Wu et al., 2005]. It is, therefore, likely that multiple histone modifications collaborate to specify the replication program observed in vivo. This hypothesis is supported by evidence demonstrating that a number of chromatin modifying enzymes associate with the DNA polymerase processivity factor PCNA [Groth et al., 2007; Moldovan et al., 2007], which are rapidly accumulating [Esteve et al., 2006; Jorgensen et al., 2007b].

In the present study we examined the chromatin structure of the chromosomal loci of three well-characterized replication origins, the early firing human LB2 and monkey mOrs8 origins, and the late-firing human hOrs8 origin, during their activation, as evidenced by post-translational modifications (PTMs) of histone H3. Previous studies regarding the epigenetic control of replication origins have provided significant data [Prioleau et al., 2003; Stedman et al., 2004; Zhou et al., 2005], but were limited because of their static nature, looking at origin chromatin structure in cells that were asynchronous or synchronized at the G_1 and G_2/M phases of the cell cycle. Here, we examined real-time chromatin changes, which permit the identification of dynamic, reversible histone modifications within initiation sites. The data show that late-firing origins have a compact chromatin structure during S phase, which opens only transiently during their activation and reverts rapidly to its initial status. The origin-specific increase in acetylated histone H3 at K9 and K14 (H3K9/K14ac) as well as methylated histone H3 at K4 (H3K4me3), but not in adjacent non-origin containing chromosomal regions during origin firing, suggests the spatiotemporal-specific targeting of H3 acetylases and H3 K4 methylases in late-firing origins. In contrast, early firing origins display histone modifications that are associated with open chromatin structure, which is only passively affected during their activation by passage of the replication fork. Finally, transient H3 PTM fluctuations in the levels of phosphorylated H3 at serine 10 (H3S10ph) (monkey replication origin), methylated H3 at K9 (H3K9me3) and acetylated H3 at K9 and K14 (H3K9/K14ac) (early firing origins) were observed, which were associated with both origin- and non-origin containing regions, suggesting that they are related to histone maturation during fork progression. Overall, the data indicate that dynamic changes in the chromatin structure occur during origin activation and underscore the need for a finer identification of chromatin modifiers that transiently interact with replication origins in order to elucidate their regulation.

MATERIALS AND METHODS

CELL CULTURE, CELL CYCLE ANALYSIS, AND DRUG TREATMENTS

For synchronization to late G_1 phase, asynchronous HeLa and CV-1 cells, cultured in alpha minimum essential medium (α-MEM) supplemented with 10% fetal bovine serum, penicillin (100 μg/ml), streptomycin (100 μg/ml) and 1 mmol/L L-glutamine (complete medium) at 37°C and 5% CO_2, were subjected to a double thymidine/mimosine block as previously described [Sibani et al., 2005]. In brief, cells were cultured in complete medium in the presence of 2 mM thymidine (Sigma) for 12 h (h), released for 10 h in pre-warmed complete medium without thymidine, and then incubated for 12–14 h in complete medium containing 400 μM mimosine (Sigma). For S phase synchronization, the cells were released from the G_1/S block into pre-warmed complete medium and harvested every 2 h in order to be used for downstream applications. Cell-cycle progression was monitored by FACS analysis; cells were fixed in 70% ice-cold ethanol, washed twice with ice-cold phosphate buffered saline (PBS) and resuspended in Vindelov's solution [Vindelov, 1977] (3.4 mM Tris, 75 μM Propidium Iodide, 0.1% NP-40, 700 U/L RNase A (Roche Diagnostics), 0.01 M NaCl) overnight (o/n) at 4°C. Analysis was performed using a Beckman flow cytometer and the WinMDI program.

EXTRACT PREPARATION, IMMUNOBLOT ANALYSIS, AND QUANTIFICATION

Nuclear cell extracts (NEs) were prepared as previously described [Dignam et al., 1983] and the protein concentration of each extract preparation was determined using the Bradford Protein Assay. Western blot analysis was carried out according to standard protocols [Sambrook et al., 1989]. For immunostaining the following antibodies were used: anti-H3K9/K14ac (Upstate; 06-599), anti-H3K9me3 (Upstate; 07-442), anti-H3K4me3 (Upstate; 07-473), anti-H3S10ph (Upstate; 05-817), anti-actin (A 2066; Sigma). Proteins were visualized using the enhanced chemiluminescence (ECL) kit according to the manufacturer's instructions (Amersham Biosciences) and the signals were quantified using the ImageJ program.

ISOLATION OF GENOMIC AND NASCENT STRAND DNA

Isolation of nascent strand DNA was performed using the λ exonuclease method as previously described [Giacca et al., 1997; Tao et al., 2000]. Briefly, at the indicated time points following release from late G_1 synchronization, the cells were washed twice with PBS and lysed in Hirt's Lysis buffer [Hirt, 1967] (50 mM Tris-HCl, pH 8.0, 0.6 M NaCl, 1 mM EDTA and 0.5% SDS). Following a 10 min incubation at room temperature (rt), the lysate was digested overnight with 0.1 mg/ml Proteinase K at 65°C, while nucleic acids were extracted by the standard phenol/chloroform method and sheared by passage through a 26G3/8 needle. 20 μg of DNA were denatured at 100°C for 5 min, phosphorylated with 10 U of T4-polynucleotide kinase (New England Biolabs) for 30 min at 37°C and digested with λ exonuclease (NEB) o/n at 37°C. In order to separate the nascent DNA from Okazaki fragments, the samples were subjected to electrophoresis on a 2% agarose gel, the DNA was visualized by staining with 0.01% (w/v) methylene blue (Sigma), and the origin-containing nascent DNA ranging between 350 and 1,000 bp in size, was excised from the gel and purified using the QiaExII extraction kit (QIAGEN), as per the manufacturer's

instructions. DNA was eluted with dH_2O and quantified by Real-Time PCR, using the LightCycler FastStart DNA Master SYBR Green I kit (Roche Diagnostics), as per the manufacturer's instructions. The sequences and amplification conditions for all primer sets are listed in Table I. Non-replicating genomic DNA from serum-starved HeLa cells was included in each reaction set to create a standard curve necessary for the quantification of the PCR products. A negative control without template DNA was also included with each set of reactions. The PCR products were resolved by electrophoresis on a 2% agarose gel, stained with ethidium bromide and visualized with an Eagle Eye apparatus (Speed Light/BT Sciencetech-LT1000).

CHROMATIN IMMUNOPRECIPITATION ASSAY (ChIP)

For the ChIPs, sheared chromatin lysates from 2×10^7 cells were pre-cleared by incubation with 50 μl of Protein G or Protein A agarose (Roche Diagnostics) to reduce background DNA precipitation caused by nonspecific binding to the beads, as previously described [Novac et al., 2001; Sibani et al., 2005]. Pre-cleared lysates were incubated overnight with either 10 μg of anti-H3K9/K14ac (Upstate; 06–599), or anti-H3K9me3 (Upstate; 07–442), H3K4me3 (Upstate; 07–473), anti-H3S10ph (Upstate; 05–817) or pre-immune serum with constant shaking. Protein G or A was added and incubated at 4°C for 1 h. The pelleted beads were washed successively with 1 ml Lysis Buffer for 15 min at 4°C, followed by 1 ml of WB1 (50 mM Tris-HCl pH 7.5, 500 mM NaCl, 0.1% NP40, 0.05% sodium deoxycholate, complete protease inhibitors tablet (Roche Diagnostics)), 1 ml of WB2 (50 mM Tris-HCl pH 7.5, 0.1% NP40, 0.05% sodium deoxycholate, complete protease inhibitors tablet) and 1 ml sterile TE lacking any protease inhibitors. The beads were subsequently resuspended in 200 μl TE/1% SDS, incubated at rt for 15 min and centrifuged at 1,000 g for 1 min. Half of the supernatant was then incubated overnight at 65°C to reverse the crosslinks, followed by digestion with 100 μg of Proteinase K at 55°C for 2 h. The DNA was purified using QIAquick PCR purification kit (Qiagen), and eluted in 100 μl 10 mM Tris-HCl (pH 8.0). The remaining half of the supernatant was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and Western blot analysis.

TABLE I. Sequences and Amplification Conditions of Primers Used for the Real-Time PCR Quantification of DNA With the LightCycler

Primer name	Sequence (5′–3′)	Amplicon size (bp)	$T_{Annealing}$ (°C)
LB2-F	GGCTGGCATGGACTTTCATTTCAG	232	66
LB2-R	GTGGAGGGATCTTTCTTAGACATC		
LB2C-F	GTTACCAGTCAGGCGCATGGGCC	240	66
LB2C-R	CCATCAGGGTCACCTCTGGTTCC		
Ors8-F	TTGCACTTCACAGAGCAGTCAT	320	66
Ors8-R	GACCCACAAAGGCAAAAGTACC		
Ors8 + 2kb F	CCCTGAGGCAGGAGTGTTTGCC	520	65
Ors8 + 2kb R	GTATGCTCAATCTGCCCAACGG		

Names and sequences of primers used for real-time quantification of DNA with the LightCycler (Roche Diagnostics). "F" and "R" designate the forward and reverse primers respectively. The size of the PCR products in base pairs (bp) and the annealing temperature (TAnnealing) used in the PRC cycling conditions in °C is also indicated.

RESULTS

REPLICATION TIMING OF THE LAMIN B2, hOrs8, AND mOrs8 REPLICATION ORIGINS

To evaluate the chromatin structure during origin activation two different cell lines were used, the human cervical cancer HeLa cell line and the Green African Monkey kidney fibroblast CV-1 cell line. The cells were synchronized to G_1/S by treatment of the cells with thymidine and mimosine and subsequent released into S phase by addition of complete media, as shown in Figure 1A. Mimosine is an inhibitor of the serine hydroxymethyltransferase (SHMT) expression, which is involved in the conversion of serine to glycine and 5,6,7,8-tetrahydrofolate, used in purine production during synthesis of RNA primers in DNA replication [Lin et al., 1996]. Following this treatment both the HeLa and CV-1 cells were synchronized at the G_1/S border and upon release they traversed through S phase in 8 h, entering G_2/M in 10 h (Fig. 1C). The bell-shaped distribution of the cells in S-phase indicates a good cell cycle progression of both cell lines upon release from G_1/S (Fig. 1C –■–).

The Ors8 replication origin, originally isolated from early S-phase monkey kidney (CV-1) cells (mOrs8), has been shown to replicate early in those cells [Kaufmann et al., 1985; Zannis-Hadjopoulos et al., 1988], but its human homologue (hOrs8) replicates late in S phase [Callejo et al., 2006], while the lamin B2 (LB2) origin is early firing in human cells [Biamonti et al., 1992]. To verify the temporal activation of these origins in our system we measured the nascent DNA strand abundance at these chromosomal regions as a function of S phase progression. In agreement with previous studies the LB2 origin was found to be activated early (within the first 2 h upon S phase entry; Fig. 2A), while the human Ors8 (hOrs8) fired late in S phase (6 h upon S phase entry; Fig. 2B), by comparison to its monkey counterpart (mOrs8), whose maximal activation occurred within the first half of S phase (within 4 h upon S phase entry; Fig. 2C).

CHROMATIN STRUCTURE OF HUMAN REPLICATION ORIGINS DURING ACTIVATION

Origin activation involves loading of the pre-RC proteins during late-M and G_1 phases followed by those of the replication machinery during S phase. Histone modifications can affect the chromatin accessibility to various DNA-binding factors, hence representing an important barrier to the initiation of replication. The role of chromatin structure with regards to pre-RC assembly has been extensively studied [Aggarwal and Calvi, 2004; Stedman et al., 2004; Zhou et al., 2005; Crampton et al., 2008]. To investigate the chromatin dynamics that regulate the recruitment of the replicative polymerases during S phase, we used a chromatin immunoprecipitation (ChIP) assay, using antibodies targeted against H3K9/K14ac, H3K4me3, H3K9me3, and H3S10ph. Chromatin was immunoprecipitated and the levels of origin-DNA were determined by real-time PCR, using specific primers. In order to also identify reversible histone modifications we looked at real-time chromatin changes by performing ChIP at three different time points per origin, before (BA), during (A) and after their activation (AA). The results show that the early firing LB2 origin is associated with high levels of the "activating" PTMs, H3K9/K14ac (Fig. 3A, bar set LB2 BA, white

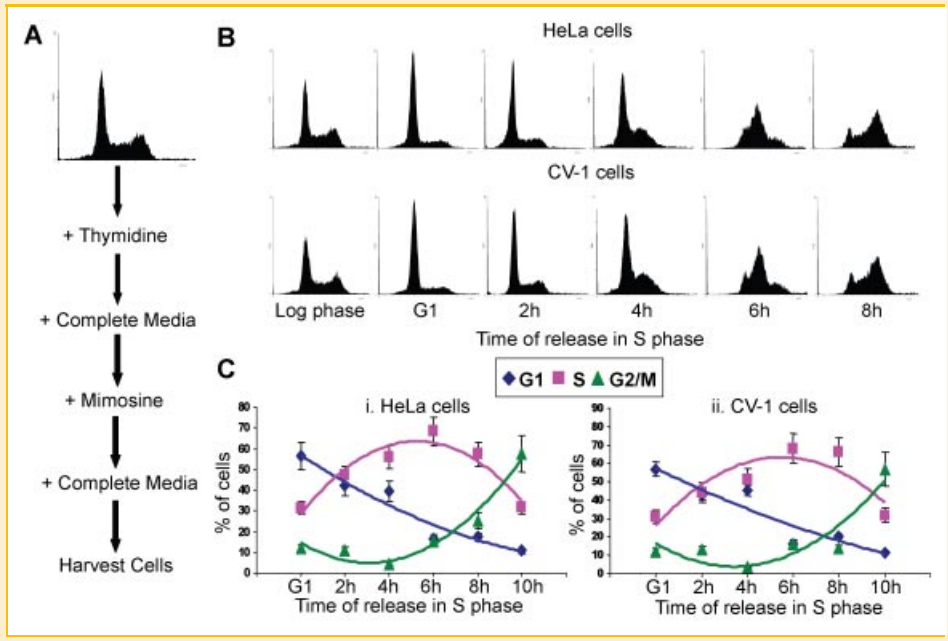

Fig. 1. Cell cycle synchronization and release in S phase. A: Double thymidine/mimosine protocol used for G_1/S blockage. B: Cell cycle progression of HeLa and CV-1 cells following G_1/S release. Representative FACS analyses of asynchronous (log phase), late G_1 and S phase cells at the time-points indicated following release from the double thymidine/mimosine block. Both cell lines traverse through S phase with the same kinetics. C: Quantification of the cell cycle distribution of HeLa and CV-1 cells at G1 (–◆–), S (–■–) and G2/M (–▲–) phases. Values are expressed as percentages of cells in each phase and represent the average of three experiments. The error bars are equivalent to 1 standard deviation (SD).

bar) and H3K4me3 (Fig. 3A, bar set LB2 BA, black bar), but low levels of the "silencing" H3K9me3 (Fig. 3A, bar set LB2 BA, dark gray bar) before activation, indicating an overall open chromatin structure. The late-firing hOrs8 origin, on the other hand, retained a more compact conformation until its activation, as evidenced by low levels of H3K9/K14ac (Fig. 3B, bar set hOrs8 BA, white bar) and H3K4me3 (Fig. 3B, bar set hOrs8 BA, black bar) and increased H3K9me3 levels (Fig. 3B, bar set hOrs8 BA, dark gray bar). Upon firing, both origins were subject to transient chromatin remodeling. Thus, during its activation, LB2 became enriched in H3K9/K14ac and H3K4me3 (Fig. 3A, bar set LB2 A) and began reverting to its initial status 2 h later, with an associated increase in H3K9me3 (Fig. 3A, bar set LB2 AA). A similar oscillation in the H3K9/K14ac and H3K4me3 levels was observed during hOrs8 firing, which was also accompanied by a temporary decrease in H3K9me3 (Fig. 3B, bar sets hOrs8 A and AA). Notably, none of the two origins were found to be associated with H3S10ph (Fig. 3A,B, light gray bars), suggesting that H3 phosphorylation is not a critical determinant of the initiation of DNA replication.

The chromatin structure at two non-origin containing chromosomal regions, the first located 4 kb upstream of the LB2 origin (LB2C) and the second 2 kb downstream of the hOrs8 origin (hOrs8 + 2kb), was also examined to analyze whether the observed dynamic epigenetic changes were origin-specific or not, rather than being due to propagation of the chromatin organization during replication fork passage [Groth et al., 2007]. As shown in Figure 3A, LB2C undergoes similar epigenetic alterations as the LB2 origin during origin activation, namely a transient increase in H3K9/K14ac and H3K4me3 levels (bar set LB2C A) followed by an increase in H3K9me3 post-activation (bar set LB2C AA). The resemblance of the chromatin dynamics at the LB2 and LB2C regions can be explained by the maturation mechanism that takes place during epigenetic maintenance, which occurs at the replication fork (see Discussion Section). In contrast, the non-origin-containing hOrs8 + 2 kb region resembles the hOrs8 origin only in the H3K9me3 levels, while the "activating" PTMs do not follow the same pattern, remaining constant during the course of origin firing (Fig. 3B, A,AA). These findings suggest first, that the "activating" histone marks associated with H3 maturation at the LB2 origin are locus-specific and are established after nucleosome assembly, since they are not observed at the hOrs8 + 2 kb locus; and second, that late-firing origins maintain a compact chromatin structure during S phase, which transiently opens up during activation, possibly due to the timely and targeted recruitment of chromatin modifiers in order for the replicative machinery to access the DNA for replication. Following origin firing they rapidly revert to their initial status, obtaining again a closed chromatin structure.

COMPARISON BETWEEN THE CHROMATIN DYNAMICS OF THE hOrs8 AND THE mOrs8

The Ors8 locus has been identified as a replication initiation site in both human [Callejo et al., 2006] and monkey [Kaufmann et al., 1985] cells. Interestingly, although the mOrs8 replicates in the first half of S phase in CV-1 cells (Fig. 2C), the hOrs8 is late-firing in human cells (Fig. 2B). We examined whether this phenomenon is associated with differential epigenetic marks imprinted on these

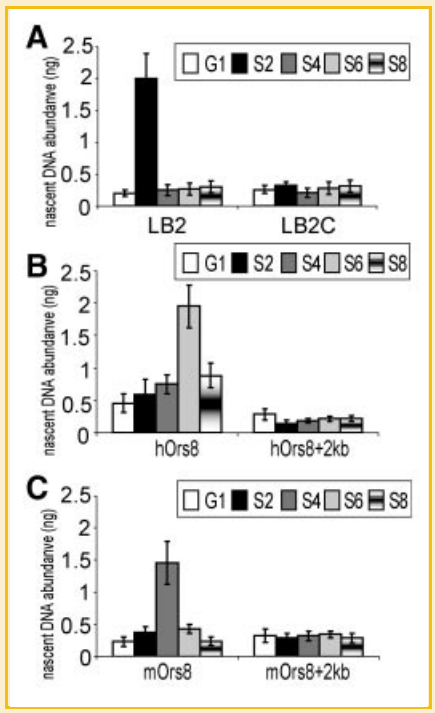

Fig. 2. Origin activation as a function of S phase progression. Histogram plots of the LB2 (A), hOrs8 (B) and mOrs8 (C) origin activities at G1 and through S phase at 2, 4, 6, and 8 h post-release from the double thymidine/mimosine block, as measured by nascent-DNA-strand abundance. The LB2, hOrs8 and mOrs8 regions comprise the respective origins, while the LB2C, hOrs8 + 2 kb and mOrs8 + 2 kb are distal origin-lacking regions. Values are expressed as ng of nascent DNA at each locus and represent three experiments ±1 SD.

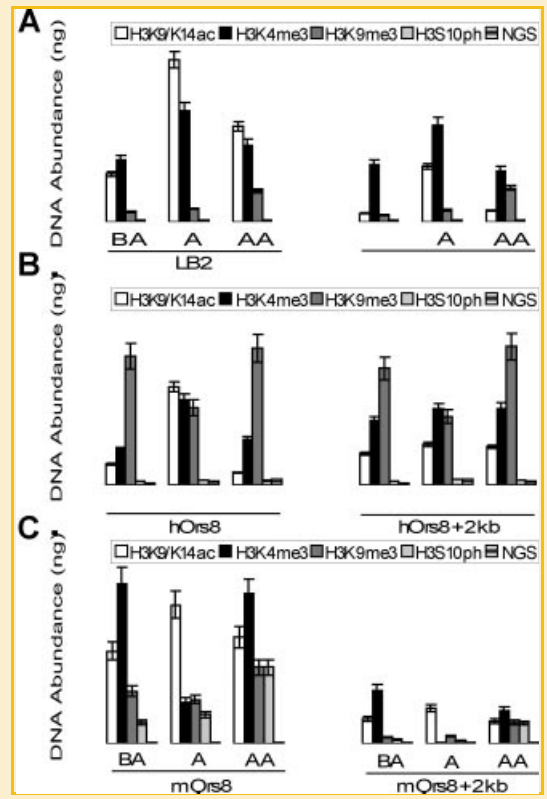

Fig. 3. Chromatin immunoprecipitation assay of post-translationally modified histone H3 during origin activation. The key to the various modifications is indicated at the top of the figure. Abundance of DNA immunoprecipitated with anti-H3K9/K14ac (white bars), anti-H3K4me3 (black bars), anti-H3K9me3 (dark gray bars), anti-H3S10ph (light gray bars) and pre-immune serum (shaded bars) at the LB2 (A), hOrs8 (B) and mOrs8 (C) in HeLa and CV-1 cells is shown. For each immunoprecipitate, the abundance of the origin-containing (LB2, hOrs8, and mOrs8) and origin-lacking (LB2C, hOrs8 + 2 kb and mOrs8 + 2 kb) regions before (BA), during (A), and after (AA) origin activation was determined. Normal goat serum (NGS) was used as a negative control. Each bar represents the average of at least three experiments and 1 SD.

respective chromosomal regions in human and monkey cells. Indeed, the results show that the mOrs8 behaves as an early firing origin (Fig. 3C, left panel), being subject to a transient opening (enrichment in H3K9/K14ac) during its activation (Fig. 3C, A) and compacting thereafter (decrease in H3K9/K14ac and increase in H3K9me3) (Fig. 3C, AA), due to replication fork progression, as indicated by the same pattern of imprinting at its associated control (non-origin-containing) region (Fig. 3C, right panel, BA, A, and AA). Surprisingly, unlike LB2, the mOrs8 locus is also subject to a temporary decrease in H3K4me3 (Fig. 3C, left panel, BA, A, and AA) indicating that newly synthesized histones in monkeys are poor in the activating H3K4me3, as well as a post-activation increase in H3S10ph at both the origin and the control regions (Fig. 3C, left and right panels, AA), indicating the presence of a H3 kinase activity at the monkey replisome.

Altogether, the data indicate that different epigenetic events take place in early- and late-firing origins, which differ between humans and monkeys. The latter is also evident in the epigenetic programs of HeLa and CV-1 cells during S phase (Fig. 4). With the exception of histone H3 acetylation which remained relatively constant, the levels of all the other histone H3 PTMs fluctuated during S phase, exhibiting different patterns in HeLa (Fig. 4A,C) and CV-1 cells (Fig. 4B,D). These data indicate that PTMs are highly regulated throughout S phase and suggest that different chromatin modifying activities take place at different timepoints.

DISCUSSION

The chromatin environment is known to influence both origin selection and replication timing, but the precise underlying molecular mechanisms in mammals have not been well characterized. In this study we have examined the histone H3 tail modifications at the chromosomal loci of three replication origins, the early firing human LB2, the late-firing human hOrs8 and the early firing monkey mOrs8, during their activation. The data show that the DNA region corresponding to the early firing LB2 origin normally retains an open chromatin configuration, as indicated by the high levels of the "activating" PTMs H3K9/K14ac and H3K4me3

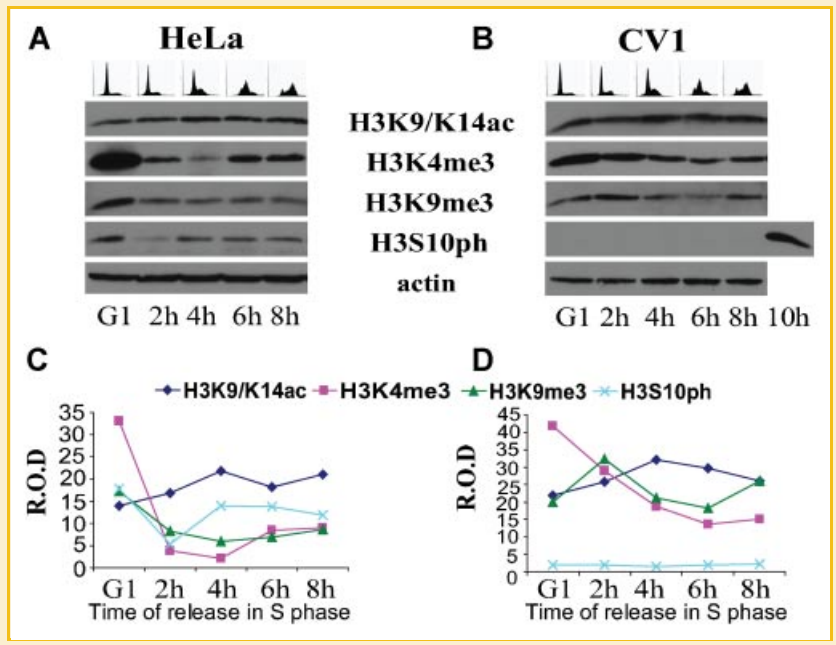

Fig. 4. Western blot analysis and quantification of post-translationally modified histone H3 during S phase. Representative Western blot analyses of H3K9/K14ac, H3K9me3, H3K4me3, and H3S10ph in nuclear extracts prepared from G_1/S synchronized or S phase (S2-S8) Hela (A) and CV-1 (B) cells. Actin was used as a loading control. C,D: Relative optical densities (ROD) of histone H3K9/K14ac (–◆–), H3K4me3 (–■–), H3K9me3 (–▲–), and H3S10ph (–×–) immunoreactivities. Values were normalized with the ROD value of the actin band within the actin lane. The experiment was done in duplicate and average values were plotted. For H3S10ph, extracts prepared 10 h after release from G_1/S were included as positive controls. At this timepoint cells have entered G_2/M and therefore the mitosis-specific H3S10ph [Paulson and Taylor, 1982] is enhanced.

and low levels of the "silencing" H3K9me3. This is in agreement with previous studies in *S. cerevisiae* showing that histone hyperacetylation correlates with early activation of replication origins [Vogelauer et al., 2002]. During origin firing, the LB2 locus becomes further enriched in H3K9/K14ac and H3K4me3, suggesting a transient opening in chromatin structure, which reverts to its initial status within 2 h. Furthermore, the origin-lacking region (LB2C), located 5 kb upstream of the lamin B2 origin (LB2), undergoes similar structural modifications during origin firing, indicating that this phenomenon is not origin-specific. Similar observations were also evident at the early firing mOrs8 replication origin and its associated origin-lacking downstream chromosomal region (mOrs8 + 2 kb). The resemblance of the chromatin dynamics at the origin-containing (LB2 and mOrs8, respectively) and origin-lacking (LB2C and mOrs8 + 2 kb, respectively) regions can be explained by the maturation mechanism that takes place during epigenetic maintenance, which occurs at the replication fork; progression of the replisome through the high-order chromosomal structure involves: (i) the transient disruption of nucleosomes ahead of the replication fork and their transfer onto nascent DNA (parental histone segregation), and (ii) the transfer of newly synthesized histones that participate in the assembly of new nucleosomes (de novo histone deposition). Previous studies suggest that newly replicated chromatin contains a range of H3 and H4 post-translational modifications, indicative of transcriptional activity [Jackson et al., 1976; Allis et al., 1985; Benson et al., 2006], which are removed during chromatin maturation [Annunziato and Seale, 1983; Taddei et al., 1999], but lack modifications that correlate with heterochromatic silencing [Benson et al., 2006]. In agreement, our results indicate that newly replicated DNA is packaged with histones that are enriched in H3K9/K14ac and MeK4, but poor in H3K9me3. Considering that the mean speed of replication fork progression is approximately 1 kb/min [Daboussi et al., 2008], it would require 5 min to transmit the nascent-chromatin epigenetic marks from the LB2 origin to the non-origin-containing LB2C region, which would explain the reason why these marks are similar at both regions.

On the other hand, the late-firing hOrs8 origin possesses a tightly packaged chromatin configuration before its activation, as evidenced by the low levels of H3K9/K14ac and H3K4me3, but increased H3K9me3 levels. This is in agreement with previous findings showing that origins with closed chromatin structure, located at the methylated CpG islands of the transcriptionally silenced allelle of the inactive X chromosome, replicate later than their active unmethylated counterparts [Gomez and Brockdorff, 2004]. These methylated CpGs are known to be recognized by methylated DNA binding proteins, which in turn recruit histone deacetylases and chromatin silencers [Hendrich and Tweedie, 2003]. During hOrs8 firing the levels of H3K9/K14ac and H3K4me3 levels oscillated, which was also accompanied by a temporary decrease in H3K9me3, in a manner reminiscent of the LB2 locus. However, the transient opening of the hOrs8 locus was origin-specific, since the downstream origin-lacking hOrs8 + 2 kb did not undergo similar chromatin opening. We are in favor of a model that involves synthesizing and/or targeting different chromatin regulators at specific time-points during S phase to designate origin activation and temporal regulation. Such a model would explain why most

heterochromatin is duplicated late in S phase, with certain exceptions, such as centromeres in *S. pombe* and the chicken beta-globin locus [Kim et al., 2003; Prioleau et al., 2003], while euchromatin is early replicating [Woodfine et al., 2004; Jeon et al., 2005].

Temporal regulation of both the expression and targeting of specific chromatin modifiers to origins might be responsible for the differential timing of origin activation as well as for the dynamic changes in the chromatin structure during S phase. In support of this, our data indicate that the chromatin environment in the nucleus is subject to drastic reorganization during S phase (Fig. 4), which may explain why DNA injected into nuclei of cells in early S phase assumes an active hyperacetylated chromatin conformation, whereas DNA injected in late S phase nuclei are packaged into condensed hypoacetylated chromatin [Zhang et al., 2002]. Identification of the chromatin modifying enzymes that are implicated in origin firing as well as replication fork progression will require further rigorous and fine analyses that will enable detection of transient interactions and enzymatic activities during the cell cycle.

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