

Duplication and Maintenance of Heterochromatin Domains

Angela Taddei,* Danièle Roche,* Jean-Baptiste Sibarita,* Bryan M. Turner,[‡] and Geneviève Almouzni*

*Institut Curie, Research section, UMR 144 et 218 du Centre National de la Recherche Scientifique (CNRS), 75248 Paris cedex 05, France; and [‡]Anatomy Department, University of Birmingham Medical School, Edgbaston, Birmingham, B152TT United Kingdom

Abstract. To investigate the mechanisms that assure the maintenance of heterochromatin regions, we took advantage of the fact that clusters of heterochromatin DNA replicate late in S phase and are processed in discrete foci with a characteristic nuclear distribution. At the light microscopy level, within these entities, we followed DNA synthesis, histone H4 acetylation, heterochromatin protein 1 (Hp1 α and - β), and chromatin assembly factor 1 (CAF-1). During replication, Hp1 α and - β domains of concentration are stably maintained, whereas heterochromatin regions are enriched in both CAF-1 and replication-specific acetylated isoforms of histone H4 (H4Ac 5 and 12). We defined a time window of 20 min for the maintenance of this state. Furthermore, treatment with Trichostatin A (TSA), during and

after replication, sustains the H4Ac 5 and 12 state in heterochromatin excluding H4Ac 8 and 16. In comparison, early replication foci, at the same level, did not display any specific enrichment in H4Ac 5 and 12. These data emphasize the specific importance for heterochromatin of the replication-associated H4 isoforms. We propose that perpetuation of heterochromatin involves self-maintenance factors, including local concentration of Hp1 α and - β , and that a degree of plasticity is provided by the cycle of H4 acetylation/deacetylation assisted by CAF-1.

Key words: heterochromatin • nuclear organization • histone H4 acetylation • chromatin assembly factor 1 • heterochromatin protein 1

IN eucaryotic cells, the genome is packaged into large scale chromatin structures giving rise to distinct functional domains (Gasser and Laemmli, 1987; Cook, 1995; Strouboulis and Wolffe, 1996; Marshall et al., 1997; Bridger and Bickmore, 1998; Lamond and Earnshaw, 1998). The way in which these domains are established and then propagated at each cell division is still an open question (Lewin, 1998). To gain insight into this important issue, domains of heterochromatin make an attractive model due to their defined properties. Historically, based on cytological observations, a distinction was made between euchromatin and heterochromatin (Heitz, 1928). By definition, heterochromatin corresponds to regions of the genome that remain intensely stained throughout the cell cycle and appear condensed. During interphase, heterochromatin domains are mainly located at the nuclear periphery or surrounding the nucleoli. The largest regions of heterochromatin are found on the inactivated X chromosome (in female mammalian cells) and near centromeres and telomeres. They generally replicate late in S phase (Goldman et al., 1984; Hatton et al., 1988) and are involved in various nuclear functions, including nuclear ar-

chitecture, chromosome segregation, and gene silencing (Wallrath, 1998).

Among heterochromatin markers, the best known protein to date, heterochromatin protein 1 (HP1)¹ was identified in *Drosophila* as an antigen localized to pericentric heterochromatin (James and Elgin, 1986; James et al., 1989). Genetic studies in *Drosophila* involved HP1 in a specific gene silencing process called position effect variegation (PEV; Henikoff, 1990; Elgin, 1996; Wakimoto, 1998). This effect is thought to be driven by heterochromatin proteins which, by propagating the organization of densely packed chromatin fibers, would help to form transcriptionally inert domains (Henikoff, 1990; Elgin, 1996; Cavalli and Paro, 1998). Several homologues of HP1 have now been identified in vertebrates: HP1 α , M31, and M32 in mice; and HP1 α , - β , and - γ in humans and *Xenopus* (Singh et al., 1991; Saunders et al., 1993; Le Douarin et al., 1996; Ye and Worman, 1996; Pak et al., 1997). HP1 β (M31, also called MOD1; Singh et al., 1991) is generally considered as the true homologue of the *Drosophila* protein

Address correspondence to G. Almouzni, Institut Curie, Research section, UMR 144 et 218 du CNRS, 75248 Paris cedex 05, France. Tel.: +33 (0)1 42 34 64 10. Fax: +33 (0)1 42 34 64 21. E-mail: almouzni@curie.fr

1. *Abbreviations used in this paper:* BiodU, Biotin-16-deoxyuridine; BrdU, 5-bromo-2'-deoxyuridine; CAF-1, chromatin assembly factor 1; H4Ac n, histone H4 acetylated at lysine n; HAT, histone acetyl transferase; HDAC, histone deacetylase; HP1, heterochromatin protein 1; pAb, polyclonal antibody; TSA, Trichostatin A.

(Aagaard et al., 1999), however, both Hp1 α and - β share common properties and are associated with heterochromatin (Horsley et al., 1996; Wallrath, 1998).

Another standard marker of heterochromatin domains from mammals to yeast is the presence of under-acetylated histone H4 isoforms (Jeppesen and Turner 1993; O'Neill and Turner, 1995; Belyaev et al., 1996; Braunstein et al., 1996). This highly conserved protein, together with the other histones H3, H2A, and H2B, form a histone octamer around which DNA can wrap to achieve the first level of chromatin organization, the core nucleosome (van Holde, 1988; Wolffe, 1995). The NH₂-terminal tail of all core histones can be acetylated *in vivo* and steady-state levels of acetylation depend on the balance between two enzymatic activities, the histone acetyltransferases (HATs) and deacetylases (HDACs; Tsukiyama and Wu, 1997; Kuo and Allis, 1998). H4 can be acetylated *in vivo* on four lysine residues (K5, K8, K12, and K16). Newly synthesized histone H4 is acetylated at lysines 5 and 12 (H4Ac 5 and 12), a pattern of acetylation that is highly conserved among eucaryotes (Sobel et al., 1995). Thus, it is generally assumed that histone H4 is incorporated in such a form during DNA replication (Annunziato, 1995), although neither of these acetylated sites is strictly required for chromatin assembly associated with replication *in vivo* and *in vitro* (Ma et al., 1998b; Zhang et al., 1998). Therefore, a crucial question with respect to heterochromatin is how its underacetylated state is established and controlled (Grunstein, 1998) since this is a critical parameter for both the maintenance of a transcriptional repressed state and for chromosome segregation in yeast (Ekwall et al., 1997; Grewal et al., 1998). In principle, one could imagine that underacetylated forms of histone H4 might be used for chromatin assembly in late S phase, thus ensuring a secure maintenance of the underacetylated state of heterochromatin. Alternatively, the deposition of acetylated forms could be followed by their rapid deacetylation, thereby providing a window of opportunity during which alternative states could be established.

Newly synthesized histones have been proposed to require cellular factors for their regulated deposition (Annunziato, 1995; Sogo and Laskey, 1995). To date, one of the best candidates for facilitating histone deposition during DNA synthesis is chromatin assembly factor 1 (CAF-1). This protein complex contains three polypeptides, p150, p60, and p48, and is associated with newly synthesized and acetylated histones, H3 and H4, in nuclear extracts (Kaufman et al., 1995; Verreault et al., 1996). CAF-1 colocalizes, in general, with sites of DNA replication (Krude, 1995). Functional homologues have been identified in different species, including *Xenopus*, *Drosophila*, and yeast (Gailard et al., 1996; Kamakaka et al., 1996; Enomoto et al., 1997; Kaufman et al., 1997). Surprisingly, in yeast cells none of the genes corresponding to the individual CAF-1 subunits (CAC1, 2, and 3), are essential. However, CAC mutants were found to be moderately sensitive to UV irradiation and exhibited gene silencing defects (Enomoto et al., 1997; Kaufman et al., 1997; Monson et al., 1997; Enomoto and Berman, 1998). Thus, whether CAF-1 is involved in assembling appropriately acetylated histones at heterochromatin and elsewhere remains to be determined (Grunstein, 1998).

The spatio-temporal organization of the S phase in higher eucaryotes provides an opportunity to address these issues in defined regions of the nucleus. Indeed, in higher eucaryotes, DNA synthesis takes place in discrete granular sites that correspond to several replication forks clustered in foci. These foci, with an average size of 500 nm (Nakamura et al., 1986; Nakayasu and Berezney, 1989), vary in number, location, and size at different times during S phase (Nakayasu and Berezney, 1989; van Dierendonck et al., 1989; Fox et al., 1991; O'Keefe et al., 1992; Hozak et al., 1993). The largest foci are observed in late S phase, at which time a characteristic pattern for replication has been defined (O'Keefe et al., 1992).

These properties of heterochromatin in vertebrates make them particularly amenable to direct studies using immunofluorescence. We have used specific antibodies to follow and relate the dynamics of DNA synthesis, histone H4 acetylation, and the presence of HP1 α , HP1 β , and CAF-1 within heterochromatin domains. This was combined with a novel method for spatio-temporal analysis. Local concentrations of HP1 (α and β) were stably maintained in these domains during replication. During that time, acetylated H4Ac 5 and 12 (replication-associated forms) and CAF-1 were found transiently in heterochromatin domains. Furthermore, treatment with a histone deacetylase inhibitor showed that the dynamics of acetylation of histone H4 in heterochromatin is restricted to K5 and K12, and to the time of DNA replication. Significantly, CAF-1 was also found at early replication foci, although no enrichment of specific H4 Ac isoforms was detected. We integrate these data into a model for duplication of heterochromatin, highlighting the importance of replication-associated acetylation dynamics.

Materials and Methods

Cell Culture, Synchronization, and Specific Treatments

HeLa and L929 cells were grown in 9-cm diam glass petri dishes (Falcon) in DME supplemented with 10% FCS, 10 μ g/ml antibiotics (penicillin and streptomycin), and 2 mM L-glutamine (GIBCO BRL) at 37°C in an atmosphere of 5% CO₂. For synchronization, HeLa cells grown to ~50% confluence were arrested in early S phase with 2 mM hydroxyurea (Sigma Chemical Co.) for 16 h, then released by several washes in PBS before a final wash in fresh medium. FACS analysis showed that these cells remained in S phase for 10 h before entering G2 (3 h), and finally mitosis (1 h). For late S phase samples, cells were routinely collected 7.5–8 h after release. For immunofluorescence, cells were grown on glass coverslips in culture dishes. For Trichostatin A (TSA; Sigma Chemical Co.) treatment, exponentially growing cells were incubated in the presence of TSA (50 ng/ml, a dose compatible with cell growth) in complete medium for a chosen time.

In Situ Replication Assays

5-bromo-2'-deoxyuridine (BrdU; Sigma Chemical Co.) incorporation was performed by incubating exponentially growing cells in the presence of 40 μ M BrdU for 10 min. Cells were then washed in PBS and fixed with 2% paraformaldehyde in PBS for 15 min at room temperature. For pulse-chase experiments, cells were washed twice with prewarmed PBS and once in prewarmed medium after the BrdU pulse, and then cultured for different time periods. The cells were further processed for isolation of nuclei or fixed with 2% paraformaldehyde in PBS.

Biotin-16-deoxyuridine (BiodU) incorporation on isolated nuclei was performed essentially as described by Krude et al. (1997). In brief, after nuclei isolation *in vitro* run-on was initiated by adjusting the nuclear preparation to final concentrations: 40 mM K-Hepes, pH 7.8; 7 mM MgCl₂; 3 mM

ATP; 0.1 mM each of GTP, CTP, and UTP; 0.1 mM each of dATP, dGTP, and dCTP; 40 μ M bio-dUTP; 20 mM creatine phosphate; 0.5 mM DTT; and 2.5 μ g phosphocreatine kinase (all Boehringer Mannheim Corp.). After a 10-min incubation at 37°C, reactions were stopped by a 25 \times dilution in PBS and immediately fixed in 2% paraformaldehyde. Nuclei were then deposited onto polylysine-coated coverslips by gentle centrifugation through a 30% cushion of glycerol in PBS, rinsed twice with PBS, and permeabilized in 0.2% Triton X-100 in PBS for 5 min. For the BiodU detection FITC-conjugated streptavidin (Enzo) was used, and fluorescence analysis carried out as described.

Immunolabeling

Paraformaldehyde-fixed cells or nuclei were washed twice in PBS and then incubated in 0.2% Triton X-100 in PBS for 5 min and blocked in 5% BSA, 0.1% Tween 20 in PBS (blocking buffer) for 10 min. Specific antibodies were then added at the appropriate dilution in blocking buffer. Incubation was carried out for 45 min at room temperature. To visualize the staining, FITC or Texas red-conjugated goat anti-rabbit or anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc.) were used. During the final washing steps with 0.1% Tween 20 in PBS, 0.25 μ g/ml, 4,6-diamino-2-phenylindole (DAPI; Sigma Chemical Co.) was added for DNA staining.

In experiments involving BrdU immunodetection, a denaturation step in 4 N HCl for 10 min at room temperature was incorporated, after the permeabilization treatment, to render BrdU incorporated into DNA accessible. Neutralization was achieved by washes in PBS, and the slides were further processed as described above. Since detection of the nuclear proteins studied here is sensitive to HCl extraction, a specific protocol was used for double staining with BrdU. Proteins were first revealed (first and second antibodies) on paraformaldehyde-fixed cells as described. After 3 washes in PBS and 0.1% Tween 20, a second fixation step was included using either 2% paraformaldehyde in PBS for 15 min or 5 mM ethylene glycol-bis(succinimidylsuccinate; Pierce Chemical Co.) in PBS for 30 min at 37°C, followed by several washes in 0.3 M glycine in PBS. After this step, DNA was denatured with 4 N HCl, before BrdU revelation. The coverslips were mounted in Vectashield (Vector Laboratories Inc.). Despite postfixation, some loss of signal could still occur. Incorporation of BiodU (on isolated nuclei) into DNA was used instead of BrdU as an alternative (not shown). This allowed us to avoid the HCl denaturation since the biotinyl residue is accessible in the double helix of DNA.

Antibodies

Rabbit polyclonal antibodies (pAbs) against the different acetylated isoforms of histones H4 and H2A have been characterized in detail (Turner et al., 1989). They recognize H4 isoforms acetylated on one of the four lysines (K) acetylated *in vivo* or H2A acetylated at lysine 5 (H2A Ac 5). They were used at final dilutions of 1:500 for R101/12 (K12), R14/16 (K16), and R123 (H2A Ac 5), and 1:1,000 for R232/8 (K8) and R41/5 (K5). mAbs against HP1 α (2HP1H5) and HP1 β (IMOD-1A97), kindly provided by Dr. R. Losson and Dr. P. Chambon (CNRS, Strasbourg, France), were used at final dilutions of 1/400. The mAb against human CAF-1 p150 (mAb1) was kindly provided by Dr. B. Stillman (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY; Smith and Stillman, 1991). The rabbit pAb1, directed against the human CAF-1 p60, was kindly provided by Dr. Marheineke (Ecole Normale Supérieure, Ulm, France; Marheineke and Krude, 1998; Martini et al., 1998). The specificities of the antibodies were tested by Western analysis on all sources of material studied. Monoclonal rat anti-BrdU was purchased from Harlan; Sera-Labo, Texas red goat anti-rabbit immunoglobulin (Ig), FITC goat anti-rabbit Ig, and Texas red goat anti-rabbit Ig antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc.

Image Acquisition and Quantitative Analysis

Image acquisition was performed using a Leica TCS-4D confocal scanning microscope, equipped with an Acousto-Optical Tuneable Filter (AOTF), with a 100 \times NA 1.4 plan-apochromat oil immersion objective. Single optical sections are presented. FITC and Texas red were excited by the argon-krypton laser at 488 and 568 nm, respectively. Red and green fluorescence were separated by a 580-nm long-pass dichroic beam-splitter. A 520-nm band-pass filter and a 590-nm long-pass filter were used to minimize cross-talk and stop laser scattered light. DAPI staining, shown in Fig. 1, was acquired with the UV laser. Before acquiring a double staining *z* series, the intensity of excitation wavelengths and the power of photodetectors were

adjusted to avoid cross-talk. The fluorescence signals from both fluorochromes were recorded simultaneously in one scan, and saved separately on two channels to be processed independently.

Three-dimensional, two color images of doubly stained nuclei were recorded using the same equipment. The voxel dimensions (*x*, *y*, *z*) of each recorded stack was adjusted to 0.05 \times 0.05 \times 0.25 μ m to verify the Shannon sampling criteria (Goodman, 1968). The averaging parameter was adjusted to have the best signal/noise ratio without bleaching the sample during the acquisition process. For H4Ac and H2AAc staining we could not reach this criteria without bleaching, leading us to perform the quantitative analysis on single optical sections (2D analysis). The same setup was used for all sample acquisition to facilitate comparison between data. Numerical data were stored in 8-bit tiff format series for each color and transferred for processing and analysis. Noise reduction and image quantification was developed in house. Metamorph software (Universal Imaging) was used as a support for executing our own Dynamic Link Libraries (DLL) written in Visual Basic and C languages for displaying results and basic treatments. A nonlinear rank-adaptive denoising filter (Vila and Bolon, 1993) was applied to better distinguish fluorescent structures from background fluorescence without loss of resolution in transition regions. Red and green volumes were thresholded separately to avoid background contributions in the quantitative analysis. To make the measurements reproducible and comparable, red and green thresholds, designated Tr and Tg, respectively, remained unchanged for each volume corresponding to each series.

Voxels are sorted in four categories: C, colocalized; R, red only; G, green only; and N, noise. In N, voxels displaying green and red signal intensities below both thresholds Tr and Tg were counted. In C, voxels displaying green and red signal intensities above both thresholds Tr and Tg were counted. In G, voxels displaying green signal intensity above Tg and red signal intensity below Tr were counted. In R, voxels displaying red signal intensity above Tr and green signal intensity below Tg were counted. Data presented in Figs. 2 and 6 were computed similarly in 2D, using pixels instead of voxels.

A control experiment, in which replication sites pulse-labeled with BrdU were revealed by a balanced mix of Texas red and FITC secondary antibodies, gave 90% of colocalizing BrdU within the total population of BrdU [(C) / (C+R) ratio], using the same parameters as in Fig. 4. This 90% approaches the 100% normally anticipated. The difference is due to the definition of our thresholds, which eliminates noise, but also leads to systematic underestimation of our values. In addition, we find that these values are dependent on the shape of the objects revealed in each color and this has to be taken into account for interpretation of the data. In all the graphs, for each point several experimental measurements were collected and the average value has been plotted in arbitrary units. For each couple of markers, the plots represent the red signals colocalizing with the green signals in arbitrary units [(C) / (C + R) ratio] normalized to the maximal value obtained in all the experiments involving this couple of markers. For comparison of the relative enrichment of the various acetylated isoforms of histones at either CAF-1 (see Fig. 3) or BrdU (see Figs. 2 and 6) sites, all histone signals (in green, which were similar in shape) were considered as one marker. Normalization was thus achieved using the common maximal value obtained for each set of experiments involving either CAF-1 or BrdU independently.

Results

There Is No Visible Disruption in HP1 α and - β Domains at Late Replication Foci

To investigate possible disorganization on a large scale that could occur during replication of heterochromatin domains, we followed the distribution of HP1 α and HP1 β , two heterochromatin variants of HP1 (Le Douarin et al., 1996; Singh et al., 1991). Using an mAb, we examined the relationship between HP1 α or - β and replication foci by immunofluorescence in mouse (L929) and human (HeLa) cells. The fluorescence signals from both fluorochromes were recorded simultaneously in a single scan under conditions that ensure that the illumination and detection were free of lateral shifts, a precondition for secure recognition of colocalization in the specimen. Different stages

of S phase were identified by the distinct BrdU incorporation patterns characteristic of early, mid, or late replicating foci (Nakayasu and Berezney, 1989; van Dierendonck et al., 1989; Fox et al., 1991; O'Keefe et al., 1992). Typical results obtained with early or late replication profiles are presented in Fig. 1. Our confocal analysis revealed, for both HP1 α and HP1 β , intensely labeled areas in dots corresponding to large blocks of pericentric heterochromatin identified by dense DAPI staining in mouse cells at all stages of S phase. In addition, we reproducibly observed a general diffuse nuclear staining, the significance of which remains to be established. Hp1 α and - β dots were never associated with replication foci in early S phase. Remarkably, in cells characterized by a late replication pattern, a significant proportion of BrdU incorporation sites could be found within HP1 (α or β) dots. We further confirmed these observations in HeLa cells (Fig. 1). We conclude that, for two different species, the existence of Hp1 α and - β domains can coincide at this scale, with regions of DNA undergoing replication.

Enrichment of H4Ac 5 and 12 and Exclusion of H4Ac 8 and 16 Replication Foci Is Visible only in Late S Phase

From mammals to yeast, it has been demonstrated that H4 underacetylation is a characteristic feature of heterochromatin (Jeppesen and Turner, 1993; O'Neill and Turner, 1995; Belyaev et al., 1996). To examine the state of H4 acetylation at both early and late replication foci in HeLa cells, we combined pulse labeling with BrdU and immunodetection using antibodies specific for H4 acetylated at lys 5, 8, 12, or 16 (Turner et al., 1989). An antibody to acetylated H2A was used as a control for an acetylation group unrelated to de novo chromatin assembly. Confocal analy-

sis showed that the different H4 and H2A antibodies displayed a general punctate nuclear staining (enriched in some regions and excluded from the nucleolus). In early S cells, replication foci appeared equivalently associated with the four acetylated isoforms of H4. In contrast, in late S cells, we distinguished two different staining patterns (Fig. 2 A): H4 isoforms acetylated at lys 5 or 12 were specifically enriched at late replication foci, whereas isoforms acetylated at lys 8 or 16 were excluded from these particular regions as is acetylated H2A. Similar results were obtained using mouse L929 or *Xenopus* A6 cells (not shown). This was further confirmed using a less destructive method (not shown, see Materials and Methods) that reveals the replication foci by in vitro run-on in the presence of Bio-16-dUTP (Krude, 1995). To quantify these observations, we developed a program to estimate the degree of colocalization between two markers in sets of confocal optical sections. This program and experimental controls to validate its application are described in Materials and Methods. Thus, we could analyze the average behavior of several hundred foci, providing a more accurate estimation of the statistical significance of our observations (Fig. 2 B). From these data, we conclude that H4 acetylated at lys 5 or 12 is specifically enriched at late replicating foci, whereas forms acetylated at lys 8 or 16 are excluded. Since acetylation at lys 5 and 12 is specific to newly synthesized histone H4, this enrichment probably corresponds to the deposition of H4 on newly replicated heterochromatin regions. It is remarkable, however, that this specific enrichment could only be detected at late replication foci. The absence of specific enrichment of H4Ac 5 and 12 at early replication foci could be due to the multiple acetylation/deacetylation events occurring concurrently in euchromatin regions that may mask changes associated specifically

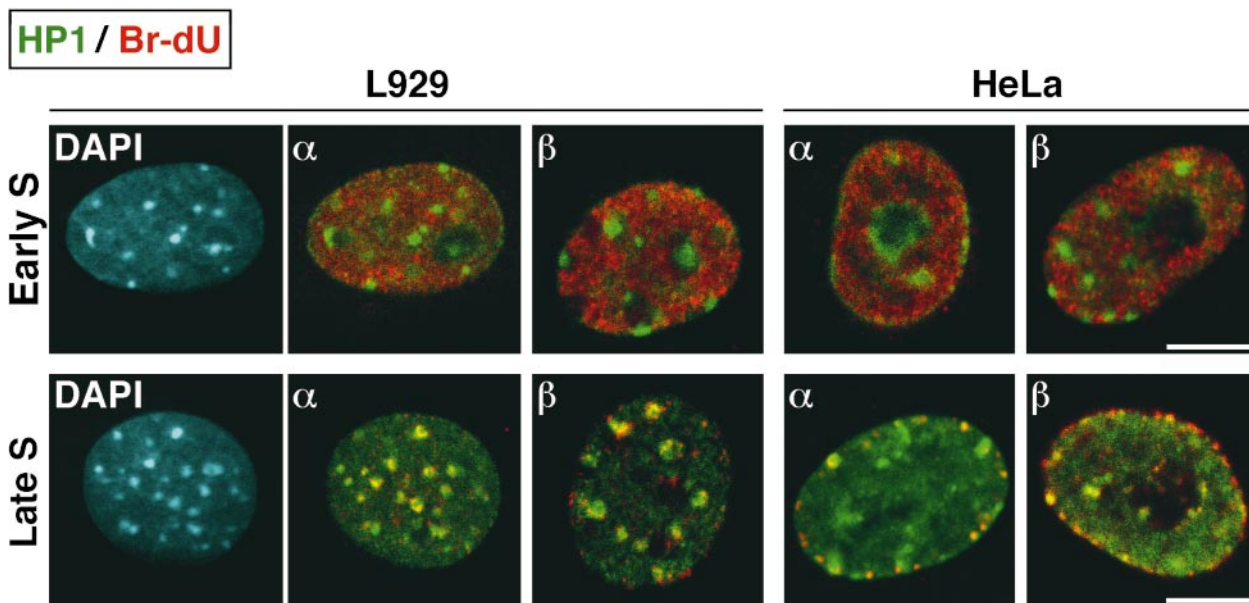
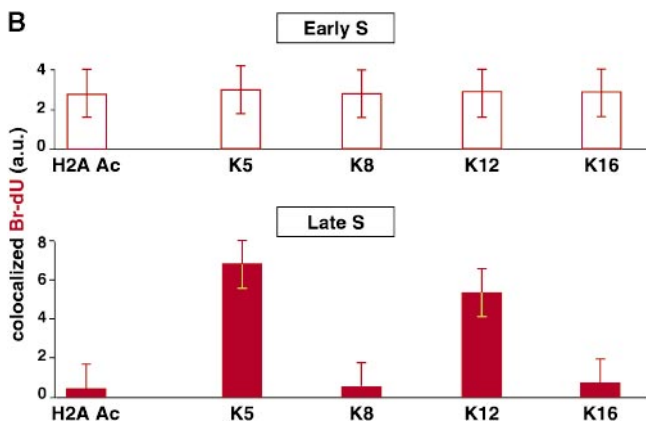
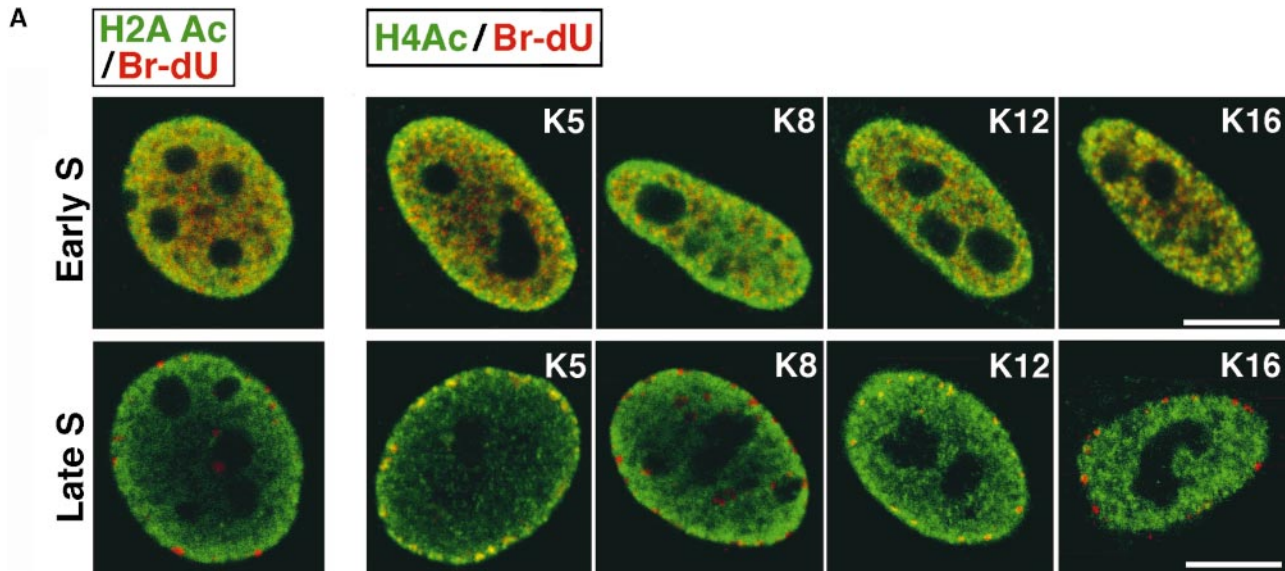


Figure 1. Domains rich in Hp1 α and - β can be found at late replicating foci. Visualization of DNA replication sites in asynchronous populations of mouse (L929) and human (HeLa) cells was performed by in vivo BrdU pulse labeling and detected by immunofluorescence (red). Hp1 α and - β were localized using specific mAbs (green). DNA was visualized by DAPI counterstaining (blue). Images of cells harboring replication foci patterns characteristic of either early (top) or late (bottom) S phase are shown. Bars, 10 μ m.



the acetylated isoforms in arbitrary units [(C) / (C + R), ratio normalized as described in Materials and Methods]. Error bars correspond to the maximum variance experimentally obtained.

with replication. Alternatively, the difference between the early and late replication profile could reflect a different mechanism for H4 deposition.

Although Equally Associated at Early and Late Replication Foci, CAF-1 Colocalizes with H4Ac 5 and 12 While Excluding H4Ac 8 and 16 only at Late Replication Foci

CAF-1 previously had been reported to be generally located at replication foci (Krude, 1995). However, a strict comparison between early and late replication foci was not available. Using an mAb directed against p150-CAF-1 (Smith and Stillman, 1989), a general localization of this subunit at both late and early replication foci was observed by confocal analysis (Fig. 3 A). Quantification of these data in sets of confocal sections, demonstrated that newly replicated regions are equally associated with CAF-1 in either early or late replication foci (Fig. 3 B). Importantly, we observed that not all the p150-CAF-1 foci were associated with replication foci (green spots), prompting us to analyze the temporal association of CAF-1 with

Figure 2. Specific enrichment of H4 acetylated at lys 5 and 12 can be detected by immunofluorescence at late, but not at early replication foci in HeLa cells. A, DNA replication sites in an asynchronous population of HeLa cells were labeled in vivo by BrdU pulse (red). Different acetylated isoforms of H4 and H2AAc 5 were localized using pAbs specific for each acetylated residue (K5, K8, K12, and K16, green). BrdU incorporation site detection (red) was achieved after a second fixation and DNA denaturation by HCl (Materials and Methods). Early or late replicating cells were identified by their BrdU staining pattern. Digital merge of both signals, corresponding to single optical sections, are shown in each case. Bars, 10 μ m. B, Confocal sections from experiments similar to those presented in A were collected for a series of nuclei. For each of these sections, colocalization was determined according to the procedure described in Materials and Methods. The plot represents the BrdU signals colocalizing with H2AAc 5 and each of

DNA synthesis. In early S phase cells (Fig. 3 A), double staining patterns revealing each specific acetylated isoform of H4, together with the p150-CAF-1 subunit, were similar. In contrast, in late S phase p150-CAF-1 could be found associated with H4Ac 5 or 12, but never with H4Ac 16 or 8. Since H4Ac 8, in addition to H4Ac 5 and 12, were found in a complex with CAF-1 in human nuclear extracts (Verreault et al., 1996), these findings suggest that the CAF-1 complex including H4Ac 8 is not involved at late replication foci. The association of CAF-1 with early and late replication foci argues for a general chromatin assembly mechanism throughout S phase. Remarkably, H4Ac 5 and 12 association with CAF-1 reproduces the pattern of H4 acetylation associated with DNA replication in heterochromatin regions (Fig. 2).

The Temporal Relationships between Two DNA Synthesis Events Can Be Reflected by their Spatial Overlap

We used the BrdU/BiodU double labeling approach to relate a temporal link between two DNA synthesis events to

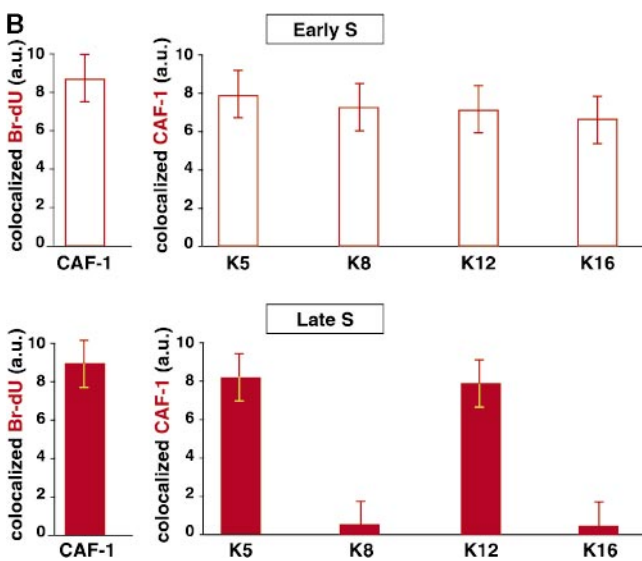
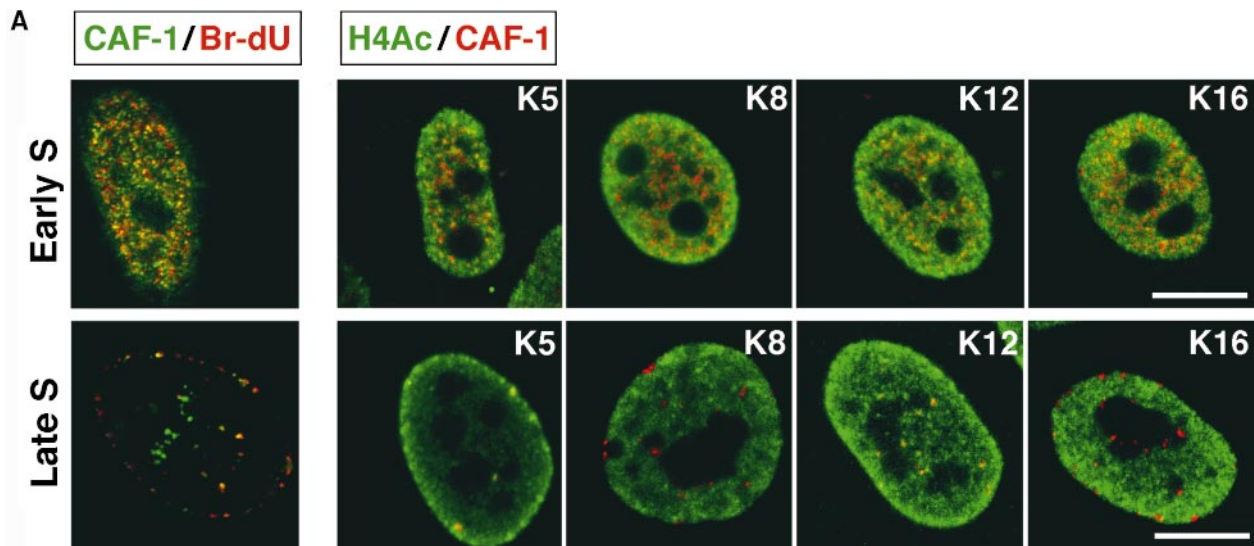


Figure 3. CAF-1 is associated with early and late replication foci, but it is specifically associated with H4 Ac 5 and 12 only at late replication foci. **A**, Left, CAF-1/BrdU. DNA replication sites in an asynchronous population of HeLa cells were labeled in vivo by BrdU pulse (red). CAF-1 p150 subunit was localized using a specific mAb (green) in either early or late replicating cells, identified by their BrdU staining pattern. Right, H4Ac/CAF-1. Different acetylated isoforms of H4 were localized using pAbs specific for each acetylated residue (K5, K8, K12, and K16, green), and CAF-1 p150 subunit, using a specific mAb (red). Early or late replicating cells were identified by their CAF-1 staining pattern. Digital merge of both signals, corresponding to single optical sections, are shown in each case. Bars, 10 μ m. **B**, Stacks of confocal acquisitions from experiments similar to those presented in **A** were collected for series of nuclei and processed as in Fig. 4 **C**. Each measure corresponds to the average behavior of at least 100 foci from one nucleus. The plot presented on the left represents the BrdU signal colocalizing with CAF-1 in arbitrary units [(C) / (C + R), ratio normalized as described in Materials and Methods]. The plot presented on the right represents the CAF-1 signal colocalizing with each acetylated isoform of H4 in arbitrary units [(C) / (C + R), ratio normalized as described in Materials and Methods]. Note that the independent processing of these two sets of data only allow to compare separately CAF-1/BrdU on the one hand, and H4 Ac/CAF-1 on the other hand. Error bars correspond to the maximum variance experimentally obtained.

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the spatial overlap of their corresponding signals. Synchronized cells were allowed to progress to late S phase, pulsed first with BrdU (see Materials and Methods) and chased for various times in vivo before a second pulse labeling was performed on isolated nuclei by in vitro run-on in the presence of Bio-16-dUTP. We compared the localization of the BrdU-labeled patches with sites of DNA synthesis after various times, by merging both signals acquired by confocal microscopy (Fig. 4). In nuclei labeled immediately after the first pulse, both in vivo and in vitro replication sites clearly overlapped. Indeed, individual foci appeared mainly yellow, as shown in Fig. 4 **B**, even though the colocalization was not perfect, as expected for two consecutive events. Within 15 min, a partial separation of the two signals could already be observed. In the same domain we could visualize red (in vivo pulse with BrdU), green (in vitro pulse with BiodU), and yellow signals (both

pulses). Although we cannot exclude that some red, green, and yellow domains could correspond to closely linked clusters of separate smaller foci, the frequency of this arrangement diminishing with time is in favor of a vast majority being the same foci. Foci in which only BiodU (green) or BrdU (red) was incorporated were likely to be those that had been switched on or off (respectively) during the chase. Consistent with this interpretation, we observed that the number of the latter increased with time, reflecting the progressive extinction of foci, whereas only a few new foci appeared during the chase, presumably due to the ending of S phase. In the same way, within double-labeled domains, separation between both signals increased with time and the respective BiodU and BrdU signals could be perfectly distinguished after 30 min. One hour after BrdU pulse, only a few BrdU foci were still associated with BiodU foci. In summary, the overlap be-

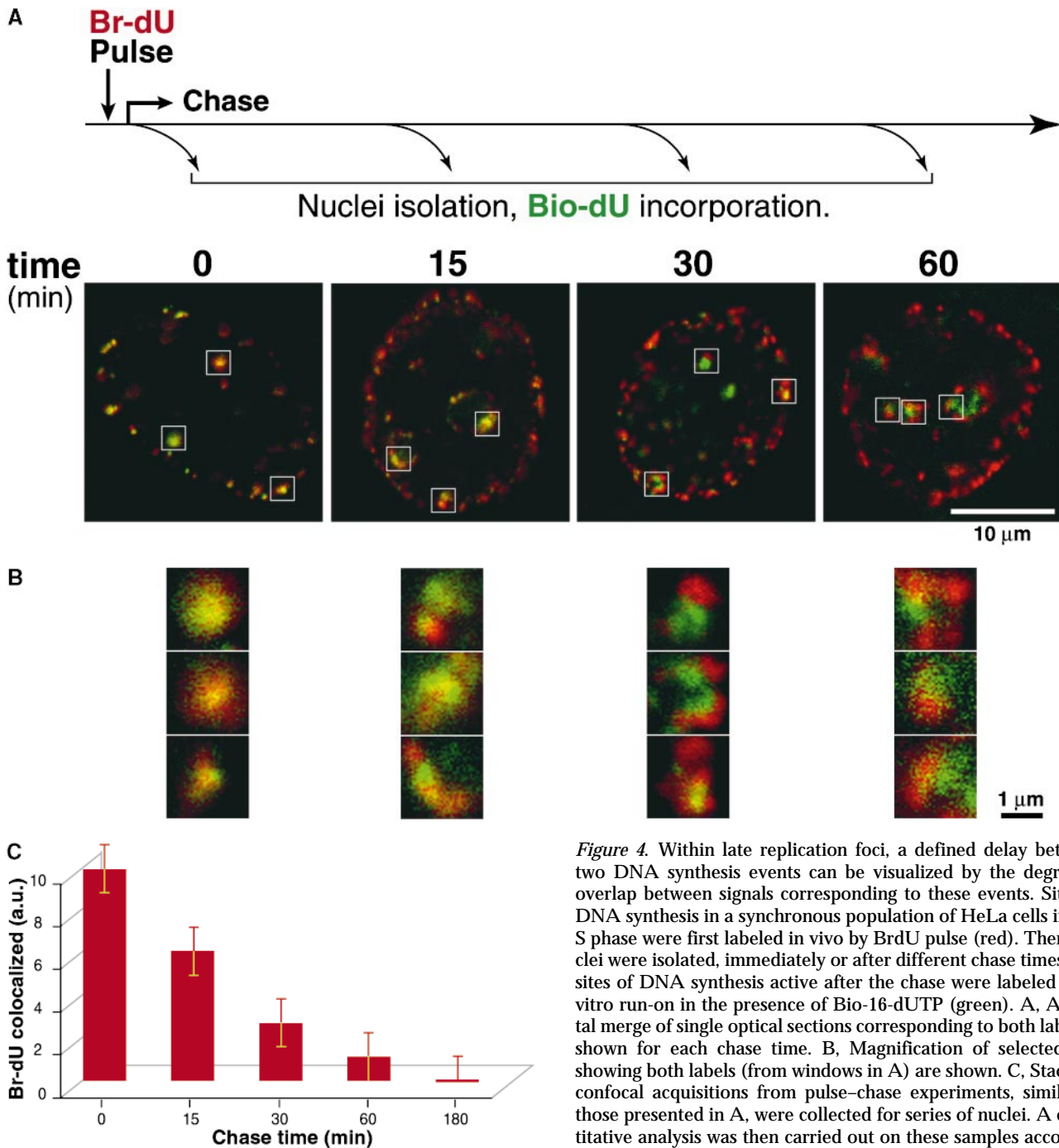


Figure 4. Within late replication foci, a defined delay between two DNA synthesis events can be visualized by the degree of overlap between signals corresponding to these events. Sites of DNA synthesis in a synchronous population of HeLa cells in late S phase were first labeled in vivo by BrdU pulse (red). Then, nuclei were isolated, immediately or after different chase times, and sites of DNA synthesis active after the chase were labeled by in vitro run-on in the presence of Bio-16-dUTP (green). **A**, A digital merge of single optical sections corresponding to both labels is shown for each chase time. **B**, Magnification of selected foci showing both labels (from windows in **A**) are shown. **C**, Stacks of confocal acquisitions from pulse-chase experiments, similar to those presented in **A**, were collected for series of nuclei. A quantitative analysis was then carried out on these samples according to the procedure described in Materials and Methods (3D analysis).

Each measurement corresponds to the average behavior of at least 100 foci from one nucleus. The plot represents the BrdU signals colocalizing with BiodU in arbitrary units [$(C) / (C + R)$, ratio normalized as described in Materials and Methods] as a function of chase time. Error bars correspond to the maximum variance experimentally obtained.

tween the two signals decreased as a function of time, due to both extinction and firing of replicating foci during the chase and to the progressive separation of clusters corresponding to DNA synthesized during the two pulses within one focus.

Quantification of BrdU-labeled regions associated with BiodU-labeled regions in sets of confocal sections showed a decrease in their spatial association, consistent with the

visual observation (Fig. 4 C). These results are in agreement with previous in vivo studies in rodent cells using double pulse labeling in vivo and different methods of quantification (Manders et al., 1996; Ma et al., 1998a). The consistency of these data further supports the validity of our method. In this analysis, patches of synthesis occurring at 15 min intervals (corresponding approximately to the synthesis of 50 Kb between both pulses) can be distin-

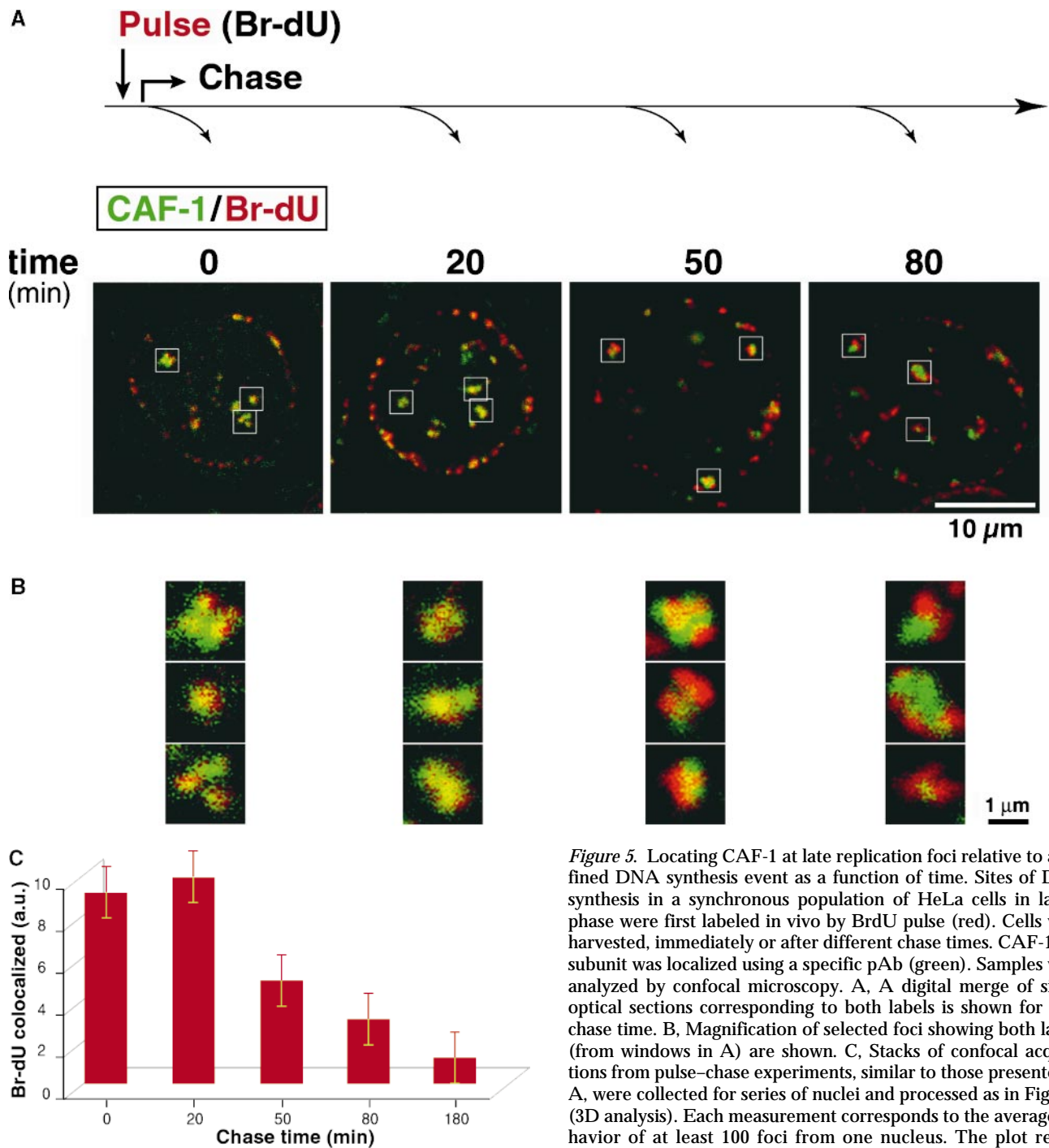


Figure 5. Locating CAF-1 at late replication foci relative to a defined DNA synthesis event as a function of time. Sites of DNA synthesis in a synchronous population of HeLa cells in late S phase were first labeled in vivo by BrdU pulse (red). Cells were harvested, immediately or after different chase times. CAF-1 p60 subunit was localized using a specific pAb (green). Samples were analyzed by confocal microscopy. **A**, A digital merge of single optical sections corresponding to both labels is shown for each chase time. **B**, Magnification of selected foci showing both labels (from windows in **A**) are shown. **C**, Stacks of confocal acquisitions from pulse-chase experiments, similar to those presented in **A**, were collected for series of nuclei and processed as in Fig. 4 C (3D analysis). Each measurement corresponds to the average behavior of at least 100 foci from one nucleus. The plot represents the BrdU signals colocalizing with CAF-1 in arbitrary units

[(C) / (C + R), ratio normalized as described in Materials and Methods] as a function of chase time. Error bars correspond to the maximum variance experimentally obtained.

guished. Thus, this analysis could be used as a reference to study the large scale relationships between factors of interest and DNA synthesis.

CAF-1 Localization at Heterochromatin Domains Is Temporally Linked to, but Not Superimposable with DNA Synthesis

We investigated how the association of recently replicated

DNA with CAF-1 evolved as a function of time in a pulse-chase experiment. Synchronized cells were allowed to progress to late S phase, pulsed with BrdU (see Materials and Methods), and chased for various times in vivo. After the chase, CAF-1 and sites of BrdU incorporation were revealed, and their localization compared by merging both signals acquired by confocal microscopy. Immediately after the pulse, BrdU incorporation sites widely overlapped with CAF-1 sites (Fig. 5), although labeled sites were not

exclusively yellow, as shown in Fig. 5 B. The signals corresponding to CAF-1 and BrdU had not separated within 20 min. This contrasts with the signals corresponding to two successive DNA synthesis events, which were separated at 15 min after BrdU pulse (Fig. 4). After 50 min, CAF-1 and BrdU signals had begun to separate, and reached complete separation at 80 min after pulse. As in the previous experiment, few foci with a single color appeared as chase time increased. Hence, CAF-1 association with BrdU incorporation sites appears related to DNA synthesis, but does not strictly follow the same dynamics.

Considering the association of BrdU signal with CAF-1, our quantitative analysis showed no significant change within the first 20 min after BrdU pulse (Fig. 5 C). After this time, following the extinction of replication foci, the percentage of colocalization decreased as a function of time. Furthermore, comparing the quantitative analysis of BrdU/CAF-1 and BrdU/BrdU pulse-chase experiments, a time difference of 20 min could be seen between release of CAF-1 and cessation of DNA synthesis. It is remarkable that after 80 min post-BrdU pulse, whereas replication had stopped within foci labeled at the time of the pulse, CAF-1 could still be found associated with BrdU incorporation sites. Further supporting these findings, we found that the two largest subunits of CAF-1, p60 and p150, detected respectively using a pAb and an mAb, behaved similarly in this assay. A similar analysis performed on early replicating foci gave comparable results (not shown), further suggesting a general behavior of CAF-1 throughout S phase. This behavior of CAF-1 is consistent with the rapid association of this assembly factor with newly replicated regions of DNA and its retention for at least 20 min at these locations.

The Association of H4Ac 5 and 12 With Heterochromatin Domains Is Transient

We then investigated how the association of H4Ac 5 with recently replicated regions of the genome evolved as a function of time in a pulse-chase experiment carried out as described above. Considering data in Figs. 2 and 3 showing that replication specific acetylated isoform of H4 (H4Ac 5 and 12) could not be detected enriched at early replication foci, we concentrated our study on late replication foci. Immediately after the pulse, BrdU incorporation sites were widely stained with H4Ac 5 antibody (Fig. 6), although labeled sites were not exclusively yellow, as shown in Fig. 6. The signals corresponding to BrdU were still associated with H4Ac 5 after a 20-min chase. Thus, clusters of newly synthesized DNA can be found associated with H4Ac 5 for at least 20 min. After 50 min, BrdU incorporation sites appeared partially dissociated from H4Ac 5 and both signals were completely distinct in G2 phase (after a 3-h chase). BrdU signal associated with H4Ac 5 was determined after different chase times on several optical sections from different nuclei to establish the statistical significance of our observations (Fig. 6 C). Similar data were obtained for H4Ac 12 in both human (Fig. 7) and *Xenopus* (not shown). Thus, H4 acetylation at lys 5 and 12 appeared to be transiently associated with late replicated sequences in both mammals and amphibians. This result contrasts with the maintenance of lys 12 acetylation

reported for heterochromatin from both yeast and *Drosophila* (Turner et al., 1992; Braunstein et al., 1996). The loss of H4Ac 5 and 12 enrichment at late replicated sequences is likely to result from an enzyme catalyzed deacetylation or a loss of epitope accessibility. Preliminary immunolabeling experiments have failed to detect any specific enrichment of deacetylase catalytic subunits at late replication foci (not shown). This may be because the specific enzymes involved still have to be identified, though we cannot exclude the possibility that the deacetylase activity at replication foci is locally regulated by a cofactor or a modification, rather than by varying the amount of enzyme present. Alternatively, without any specific enrichment of HDAC, the lack of HAT in these regions could be sufficient to account for H4 deacetylation following replication as supported by Fig. 7.

Most importantly, these data strongly support the existence of a time window of ~ 20 min, during which heterochromatin regions are enriched in acetylated H4, followed by a deacetylation event.

The Association of Heterochromatin Regions with H4 Acetylation Is Stabilized upon Addition of TSA during their Replication

An asynchronous population of HeLa cells was pulsed with BrdU and chased for 90 min either in the presence or absence of TSA, a histone deacetylase inhibitor (Yoshida et al., 1995). After a 90-min chase in the absence of TSA, all four acetylated isoforms of H4 were reproducibly excluded from late replicated regions (Fig. 7 A). This is consistent with data in Fig. 6 showing that H4Ac 5 was no longer associated with late replicated foci after 80 min chase. In the presence of TSA, however, association of H4Ac 5 and 12 was detected, whereas H4Ac 8 and 16 staining were excluded from BrdU-stained regions. These data demonstrate that the loss of H4Ac 5 and 12 after 90 min chase is due to a deacetylation event. Under these conditions, an enhancement of H4Ac 5, 8, and 12 staining at the nuclear periphery was observed in all cells of an asynchronous population, indicating that it is unrelated to the replication process (Fig. 7). Although the significance of this observation is unclear, it provided a useful marker for the TSA effect. We next investigated the dynamics of H4 acetylation in heterochromatin regions outside S phase. To ensure that cells were out of S phase, they were cultured 14 h after the BrdU pulse, allowing late replicated cells to exit mitosis. At this time, cells were either mock-treated or treated with TSA (90 min, 50 ng/ml). In mock-treated cells, BrdU staining was excluded from staining with any of the four acetylated isoforms, consistent with the general underacetylated state of H4 in heterochromatin regions. In TSA-treated cells, despite an increase in H4Ac 5, 8, and 12 at the nuclear periphery, the BrdU staining remained excluded from all acetylated isoforms of H4 (Fig. 7 B). Thus, none of the acetylated forms of H4 could be stabilized by TSA treatment under these conditions. Taken together, these results indicate that the dynamics of histone H4 acetylation in heterochromatin regions is strictly limited to lys 5 and 12 and restricted to the time of DNA replication.

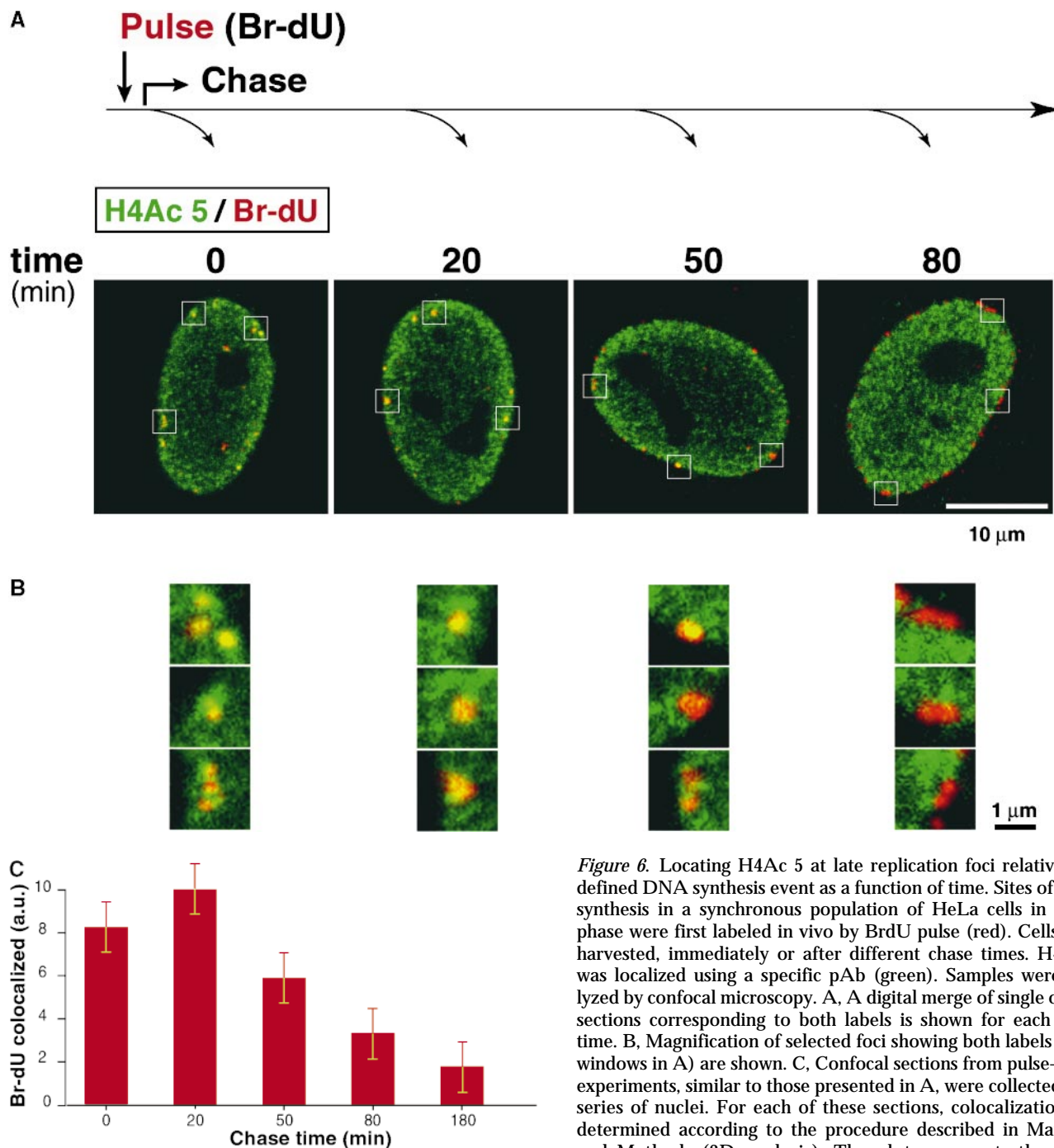


Figure 6. Locating H4Ac 5 at late replication foci relative to a defined DNA synthesis event as a function of time. Sites of DNA synthesis in a synchronous population of HeLa cells in late S phase were first labeled in vivo by BrdU pulse (red). Cells were harvested, immediately or after different chase times. H4Ac 5 was localized using a specific pAb (green). Samples were analyzed by confocal microscopy. **A**, A digital merge of single optical sections corresponding to both labels is shown for each chase time. **B**, Magnification of selected foci showing both labels (from windows in **A**) are shown. **C**, Confocal sections from pulse-chase experiments, similar to those presented in **A**, were collected for a series of nuclei. For each of these sections, colocalization was determined according to the procedure described in Materials and Methods (2D analysis). The plot represents the BrdU signals colocalizing with H4Ac 5 in arbitrary units $[(C) / (C + R)]$, ratio normalized as described in Materials and Methods] as a function of chase time. Error bars correspond to the maximum variance experimentally obtained.

R), ratio normalized as described in Materials and Methods] as a function of chase time. Error bars correspond to the maximum variance experimentally obtained.

Discussion

Immunofluorescence analysis of heterochromatin domains at late replicating foci shows that these domains contain specific acetylated forms of histone H4, together with the largest subunit of CAF-1. The enrichment of the histone H4 acetylated at lys 5 and/or 12 was in striking contrast with the exclusion of H4Ac 8 and 16. We followed the large scale dynamics of this domain through the tem-

poral analysis of DNA synthesis, the presence of CAF-1, acetylated H4, Hp1 α , and Hp1 β . Thus, we can distinguish properties of heterochromatin domains that are either stable (Hp1 α and - β concentration) or transient (H4Ac 5 and 12, and CAF-1). Comparison with early replicating foci/euchromatin regions shows that CAF-1 is also associated with DNA synthesis in these regions. In contrast, we could not reveal any specific enrichment in H4Ac 5 and 12, compared with 8 and 16.

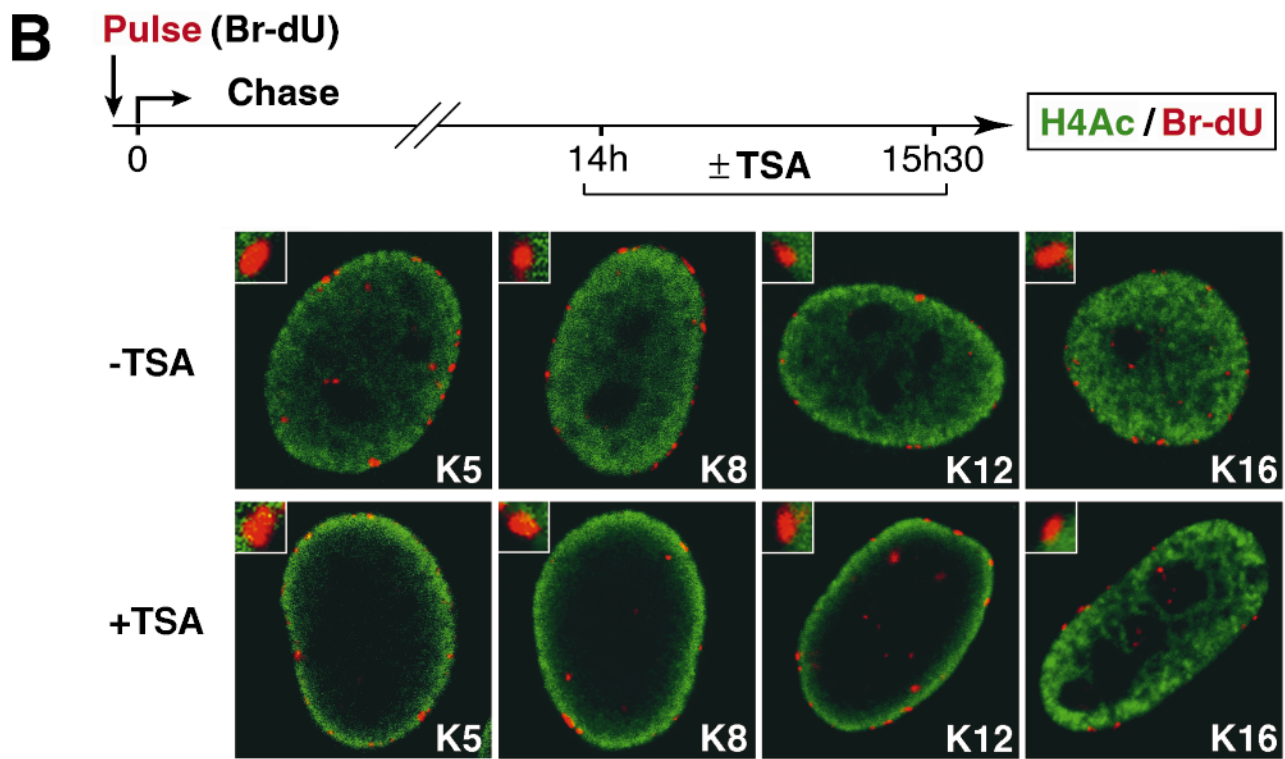
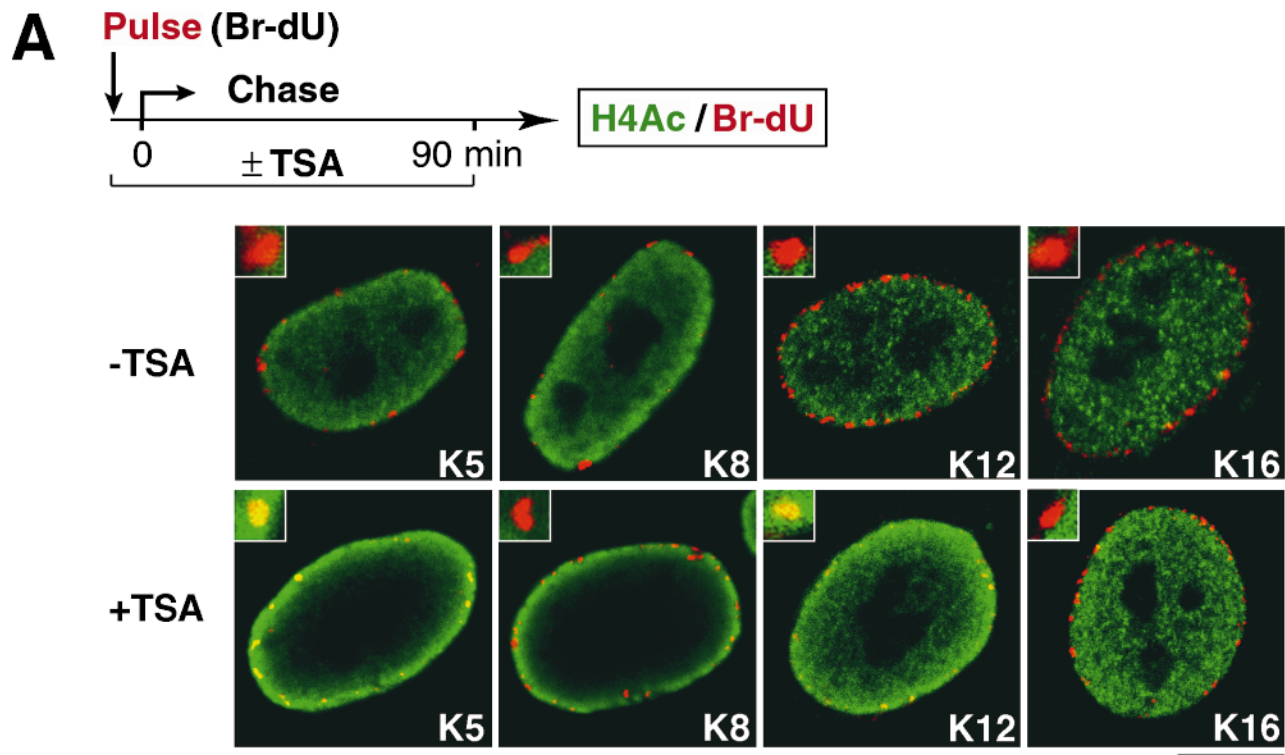


Figure 7. Dynamics of H4 acetylation in heterochromatin regions is restricted to the time of their DNA replication. A, Sites of DNA synthesis in an asynchronous population of HeLa cells were first labeled in vivo by BrdU pulse (red), and chased for 90 min either in the presence or absence of TSA (50 ng/ml). Different acetylated isoforms of H4 were localized using specific pAbs (K5, K8, K12, and K16, green). B, Sites of DNA synthesis in an asynchronous population of HeLa cells were first labeled in vivo by BrdU pulse (red), and chased for 14 h to allow late replicating cells, labeled during the pulse, to exit mitosis, before prolonged incubation in the absence or presence of TSA (50 ng/ml) for 90 min. Different acetylated isoforms of H4 were localized using specific pAbs (green). Digital merge of both signals, corresponding to single optical sections, are shown in each case. Bars, 10 μ m. Note that these images were processed differently to permit visualization, because the intensities recorded were very different + or - TSA. Enlarged inserts (top left of each images) correspond to selected foci.

We discuss how the replication-associated cycle of acetylation/deacetylation may have a specific importance in heterochromatin regions.

Hp1 α and - β Are Maintained through DNA Replication

The existence of local concentrations of specific proteins has been proposed to compartmentalize chromatin regions, creating an index system for the nucleus (Gasser and Laemmli, 1987; Stone and Pillus, 1998). The local concentration of specific proteins, such as Hp1 α and - β , could also be critical for defining domains that should be repressed. In our study, no major changes in the distribution of Hp1 α and - β were observed during DNA replication of heterochromatin regions (Fig. 1; and not shown). This was surprising at first, since the presence of Hp1 α and - β may reflect a high level of DNA condensation (Cavalli and Paro, 1998) that may not necessarily be compatible with the replication process. We thus conclude that, if the replication fork is destabilizing the nucleosome (Sogo and Laskey, 1995), this is a local phenomenon that does not affect the stability of the larger scale organization in a detectable manner. The maintenance of the local concentration of Hp1 α and - β as part of nucleoprotein complexes, either directly interacting or simply in close proximity with DNA, may ensure the rapid reassociation of the replicated regions into heterochromatin. Furthermore, the ability of HP1 proteins to oligomerize has led to the hypothesis that they can cross-link chromatin domains by the formation of concatenated multiprotein complexes (Cowell and Austin, 1997). Thus, Hp1 α and - β appear to be components that favor the maintenance of a heterochromatin region. Interestingly, in mouse cells, association of some genes with an HP1 β domain has been shown to correlate with their transcriptional repression (Brown et al., 1997). One would thus predict that in vertebrates, overexpression of HP1 β could result in a spreading or expansion of these domains, possibly producing phenotypes resembling PEV (Festenstein et al., 1996; Milot et al., 1996). Additional modification adding to stability in heterochromatin, such as methylation of DNA in vertebrates (Bird, 1992), should also be incorporated into the overall picture. Future experiments will provide insights into these issues.

The Replication-associated Acetylated Isoforms of Histone H4, Together with CAF-1, Provide a Window of Opportunity in Heterochromatin

Analysis of the distribution of CAF-1 and H4Ac 5, compared with DNA synthesis at heterochromatin regions, demonstrated a dynamic behavior that was not strictly parallel to DNA synthesis (Figs. 4, 5, and 6). No major differences were observed between the location of CAF-1 and patches of DNA corresponding to individual replication foci observed within 20 min after their labeling in HeLa cells. For this chase time, we established that two DNA synthesis events are already clearly separated. The maintenance of CAF-1 association with these patches of DNA after their synthesis is further emphasized by the detection of CAF-1 foci beyond the DNA replication time. This CAF-1 enrichment at the location of newly synthesized DNA soon after its synthesis, and its persistence, exceeds the time involved in synthesizing the DNA length

corresponding to a nucleosome and depositing one set of H3 and H4 at the replication fork (less than five minutes, according to Smith et al., 1984). This extended time period is comparable to the postreplicative phase required for proper chromatin maturation (Levy and Jakob, 1978; Worcel et al., 1978; Smith et al., 1984). An attractive hypothesis is that it could define the time needed to process a unit of a size between 50 and 200 Kb, reminiscent of the chromatin loops. This would be compatible with the recent report on CAF-1-coupled inheritance of chromatin acting postreplicatively in vitro (Shibahara and Stillman, 1999). Similarly, we determined that newly synthesized DNA sequences were associated with H4Ac 5 for ~20 min in late replication foci. The existence of a pool of acetylated histone H4 concentrated in the region of heterochromatin at the time of replication constitutes a strong argument for believing that these histones are used to form the new nucleosomes at these foci. It is striking though, that, at a large scale level, this replication-associated acetylated isoform could not be specifically detected at early replication foci although CAF-1 was still found (Figs. 2 and 3). Since the biochemical approach has indicated a general usage of H4Ac 5 and 12 (Annunziato, 1995) throughout the genome, however, multiple acetylation/deacetylation events occurring concurrently in these regions may mask changes associated specifically with replication. In heterochromatin, remarkably, the acetylation level of H4 associated with histone deposition at the replication fork appears as a dominant event. The parallel between CAF-1 and H4Ac 5 and 12 (Figs. 3, 5, and 6) in heterochromatin raises the possibility that CAF-1 association with acetylated histones helps to coordinate loading of acetylated histone H4 with a deacetylation event. This latter event could be promoted through the interaction of the smallest subunit of CAF-1 with a histone deacetylase previously detected in human cell extracts (Taunton et al., 1996). The heterochromatin specific effect of CAC deletions in yeast strains leading to the redistribution of the telomeric protein Rap1p, together with a loss of silencing (Enomoto et al., 1997; Monson et al., 1997; Enomoto and Berman, 1998), could be explained by defects in such a coordination when replicating heterochromatin regions. Future experiments should address whether perturbation of CAF-1 in higher eucaryotes similarly affects heterochromatin function. This may also relate to the possible role of CAF-1 associated with DNA repair (Gaillard et al., 1996; Kaufman et al., 1997; Martini et al., 1998). By facilitating the deacetylation step during the chromatin dynamics associated with DNA repair, CAF-1 may help to avoid the persistence of inappropriate chromatin organization.

Our data are consistent with the existence of a general mechanism for nucleosome assembly employed throughout the genome. In this pathway, histone deposition would make use of acetylated histones chaperoned by CAF-1 that would facilitate deacetylation. Depending on the local environment, particularly the presence of HATs/HDACs and their regulators, it may be possible to generate distinct acetylated chromatin configurations at a steady state. Inhibition of histone deacetylase by TSA showed that the dynamics of acetylation of histone H4 in heterochromatin is restricted to K5 and K12, and to the time of the DNA replication (Fig. 7). Prolonged exposure to the drug led to the

disruption of heterochromatin domains (HP1 staining, not shown, Taddei, A., and G. Almouzni unpublished results). These observations are reminiscent of those obtained in *Saccharomyces pombe* in which a TSA treatment disrupted centromeric organization and delocalized the swi6 protein (Ekwall et al., 1997). Further investigations are necessary to determine the exact basis of this phenomenon. It is intriguing that H4 acetylation seems to not be required for general nucleosome formation, per se, either in vitro or in vivo (Ma et al., 1998b; Zhang et al., 1998). It has been proposed that the so-called futile cycles of enzymatic metabolism may be needed for rapid response to changes in the environment and for evolution (Gerhart and Kirschner, 1997). It is interesting to consider the possibility that the acetylation/deacetylation cycle chaperoned by CAF-1 serves the same purpose. In that respect, it is noteworthy that mutations in histone tails affecting all the acetyltable sites in histone H4 gave rise in yeast to a checkpoint phenotype (Megee et al., 1995; Hartzog and Winston, 1997). The importance of the alternative states generated by the futile cycle could be envisaged at two different levels. At a cellular level, this could help to monitor S phase progression, DNA damage processing, and perhaps determine how S phase events are coordinated. At the level of a population, a certain degree of adaptability can be advantageous during evolution, as was discussed for the epigenetic inheritance of centromeres (Murphy and Karpen, 1998). This is exemplified in yeast with the switching mechanism at mating type loci that depends on replication (Laurenson and Rine, 1992).

We studied here how a preestablished structure could be propagated, the next challenge will be to understand how these structures can be formed de novo, which will be critical during the development of an organism.

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