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Research article

G_1 phase-dependent nucleolar accumulation of human histone H1x

Stefan Stoldt*, Dirk Wenzel†, Ekkehard Schulze‡, Detlef Doenecke* and Nicole Happel*[1]

*Institute for Biochemistry and Molecular Cell Biology, University of Göttingen, Humboldtallee 23, 37073 Göttingen, Germany, †Department of Neurobiology, Max Planck Institute for Biophysical Chemistry, 37077 Göttingen, Germany, and ‡Institute for Biology III, Bioinformatics and Molecular Genetics, Albert-Ludwigs-University, Schaenzlestrasse 1, 79104 Freiburg, Germany

Background information: H1 histones are a protein family comprising several subtypes. Although specific functions of the individual subtypes could not be determined so far, differential roles are indicated by varied nuclear distributions as well as differential expression patterns of the H1 subtypes. Although the group of replication-dependent H1 subtypes is synthesized during S phase, the replacement H1 subtype, H1°, is also expressed in a replication-independent manner in non-proliferating cells. Recently we showed, by protein biochemical analysis, that the ubiquitously expressed subtype H1x is enriched in the micrococcal nuclease-resistant part of chromatin and that, although it shares common features with H1°, its expression is differentially regulated, since, in contrast to H1°, growth arrest or induction of differentiation did not induce an accumulation of H1x.

Results: In the present study, we show that H1x exhibits a cell-cycle-dependent change of its nuclear distribution. This H1 subtype showed a nucleolar accumulation during the G_1 phase, and it was evenly distributed in the nucleus during S phase and G_2. Immunocytochemical analysis of the intranucleolar distribution of H1x indicated that it is located mainly in the condensed nucleolar chromatin. In addition, we demonstrate that the amount of H1x protein remained nearly unchanged during S phase progression, which is in contrast to the replication-dependent subtypes.

Conclusion: These results suggest that the differential localization of H1x provides a mechanism for a control of H1x activity by means of shuttling between nuclear subcompartments instead of a controlled turnover of the protein.

Introduction

The basal structural unit of chromatin organization is the nucleosome. It consists of a core nucleosome, i.e. the core histone octamer with DNA wrapped around its surface, the linker DNA and the H1 histone, which is positioned in the DNA region between the nucleosome cores. H1 histones are considered to stabilize the compaction of chromatin into higher order structures (reviewed in Hansen, 2002; Hizume et al., 2005) and, in addition to their role as structural proteins, there is growing evidence for the participation of H1 histones in transcriptional regulation (reviewed in Brown, 2003; Harvey and Downs, 2004; Bustin et al., 2005). So far, 11 H1 homologous proteins have been described in human (reviewed in Ausio, 2006). These are the main class H1 subtypes, H1.1, H1.2, H1.3, H1.4 and H1.5, which are expressed in a replication-dependent manner and are present in all somatic cells (reviewed in Khochbin, 2001; Parseghian and Hamkalo, 2001; Wisniewski et al., 2007). The replacement subtype H1° is expressed in a replication-independent manner and thus it is restricted mainly to growth-arrested cells (reviewed in Zlatanova and Doenecke, 1994). The group of tissue- and developmental-stage-specific subtypes consists of H1t, H1T2, HILS1 and H1Foo (Seyedin et al., 1981; Tanaka et al., 2001, 2005; Yan et al., 2003; Martianov et al., 2005). The subtype H1x is a ubiquitously expressed H1 subtype (Yamamoto and Horikoshi, 1996). Although it shares some features with H1°, its regulation of expression differs from that of this replacement histone (Happel et al., 2005).

The varied composition of the individual H1 subtypes in different tissues, cell types and developmental stages suggests specific functions of the

[1]To whom correspondence should be addressed (email nhappel@gwdg.de).

Key words: cell cycle, histone H1, histone H1x, nuclear localization, nucleolus.

Abbreviations used: ActD, actinomycin D; DAPI, 4′,6-diamidino-2-phenylindole; DFC, dense fibrillar component; FC, fibrillar centre; GC, granular component; UBF, upstream binding factor.

individual H1 subtypes. This conclusion is also supported by the varied intranuclear distribution of the H1 subtypes (reviewed in Parseghian and Hamkalo, 2001). Microscopy analysis revealed that the H1 subtype H1.2 is uniformly distributed, reflecting the DNA distribution within the cell nucleus. In contrast to this, the distributions of H1.3 and H1.4 are non-uniform, but exhibit punctate staining patterns (Parseghian et al., 1994). A preferential localization at the nuclear periphery was shown for H1.5 (Parseghian et al., 1993), and H1° was observed in condensed chromatin areas and in perinucleolar regions (Breneman et al., 1993; Gorka et al., 1993). Analysis of the H1 subtype distribution by immunoprecipitation of cross-linked chromatin revealed that H1.3 and H1.4 appear to be preferentially associated with inactive regions, whereas H1.2 and H1.5 appear more prevalent in active regions of chromatin (Parseghian et al., 2000; 2001).

Recently we reported on peculiar properties of the subtype H1x. We showed that it exhibits lower acid solubility compared with that of bulk H1 histones, and that H1x was enriched in the micrococcal nuclease-resistant part of chromatin. H1x and H1° share the common features in that their genes are solitarily located and that their mRNAs are polyadenylated. Since this is in contrast to the replication-dependently expressed main class subtypes, we analysed if their genes are regulated similarly. We showed that the genes of H1x and H1° are regulated differentially, since growth arrest or induction of differentiation did not lead to an accumulation of H1x in contrast to H1° (Happel et al., 2005).

In the present study, we characterized the intranuclear localization of human H1x by immunofluorescence labelling of cultured human cells and found that it exhibits a G_1 phase-dependent nucleolar accumulation, which is the first observation of a cell-cycle-dependent shuttling of a histone between nuclear subcompartments. Analysis of the H1x expression at different stages of the cell cycle interphase revealed that the H1x protein amount is neither affected by a cell cycle arrest nor by S phase progression. Thus, regulation of H1x expression differs not only from that of the replication-independently expressed subtype H1°, but also from those of the replication-dependent H1 subtypes. This cell-cycle-dependent nucleolar accumulation may provide a mechanism for the control of H1x activity by intranuclear shuttling instead of regulation at the protein level.

Results

H1x exhibits partial nucleolar accumulation

In order to understand the function of the histone subtype H1x, its intranuclear localization was determined. Indirect immunofluorescence detection of H1x in non-synchronized HeLa cells revealed quite different localization patterns. H1x was either distributed all over the nucleus or it was localized in large clusters (Figure 1A). The same distribution could be found in human SV-80 fibroblasts (Figure 1A). In comparison with this, labelling with a general anti-H1-antibody, which showed no labelling of H1x by Western blot analysis of a histone extract from HeLa cells (Happel et al., 2005), was uniformly distributed, whereas the nucleoli appeared to be stained to a much lesser extent than the other parts of the nuclei (Figure 1B). Laser scanning microscopy of HeLa cells revealed that H1x, which was not located in the large clusters, showed a punctate distribution, whereas the main H1 histones were distributed more homogeneously (Figure 1C).

To confirm this antibody-labelling, we generated FLAG-tagged fusion constructs of H1x and, for comparison, H1.2. H1x–FLAG was also partially localized in clusters, whereas H1.2 showed a distribution similar to the pattern of the general H1 antibody (Figure 2).

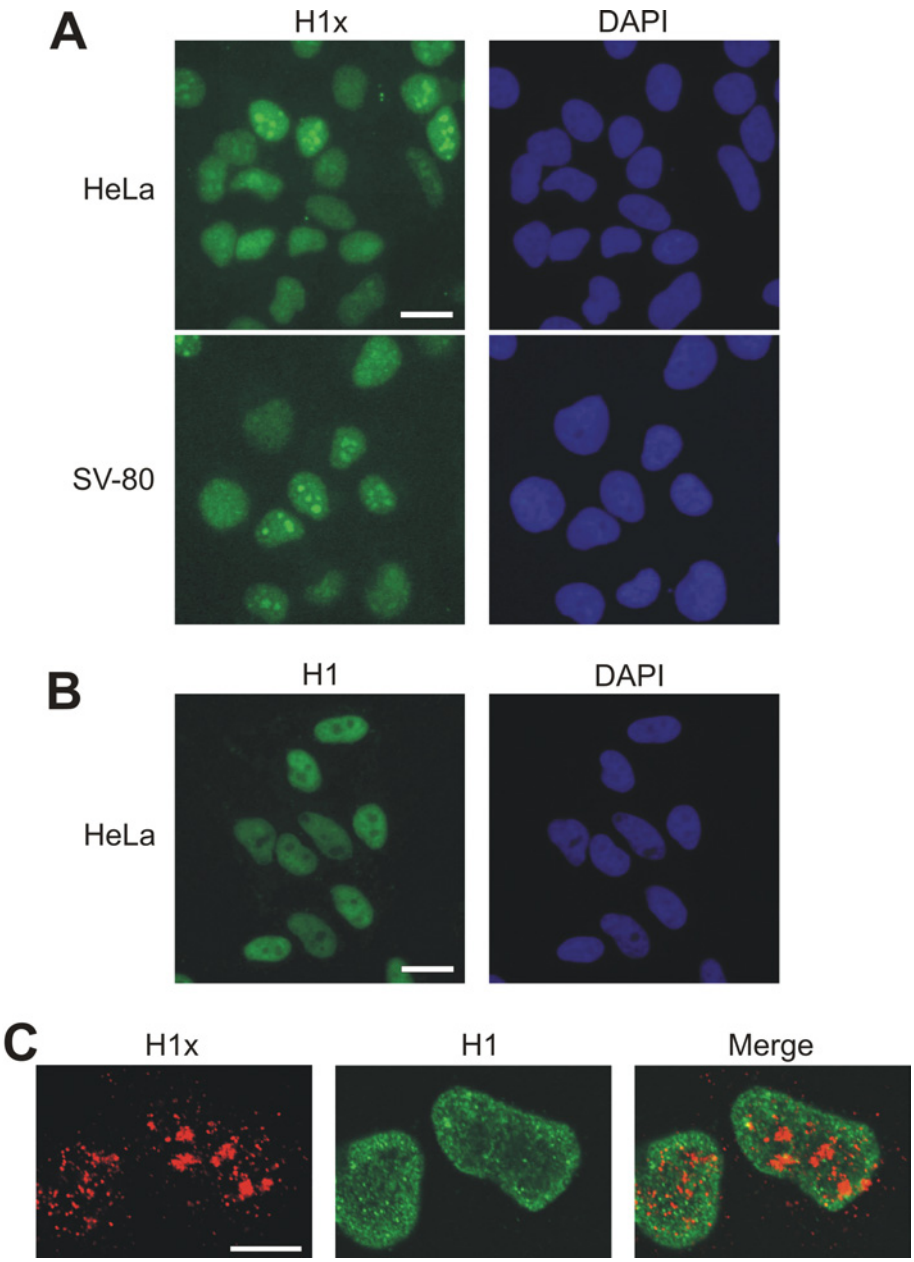
Comparison of the H1x distribution with DAPI (4′,6-diamidino-2-phenylindole) staining suggested that the observed clusters are located at the nucleoli. To resolve this distribution further, we performed double-labelling with the nucleolar marker proteins fibrillarin and nucleolin. Both nucleolar proteins are located primarily in the dense fibrillar regions of the nucleolus, and nucleolin has also been assigned to the granular component (Biggiogera et al., 1990; Matera et al., 1994; Ginisty et al., 1999; Snaar et al., 2000). As shown in Figure 3 the large H1x clusters are indeed located within nucleoli. However, besides some overlap H1x did not co-localize with fibrillarin or nucleolin.

Nucleolar localization of H1x is not linked to ribosomal gene transcription

To analyse if the nucleolar localization of H1x is functionally connected to ribosomal gene transcription,

Figure 1 | Intranuclear localization of H1x in human cell lines

(**A**) Indirect immunofluorescence detection of H1x in non-synchronized HeLa cells and SV-80 fibroblasts. H1x exhibits partial nucleolar accumulation. Scale bar, 20 μm. (**B**) Indirect immunofluorescence detection of main class H1 histones in HeLa cells revealed a uniform distribution of these subtypes outside the nucleoli. Scale bar, 20 μm. (**C**) Localization of H1x and main class H1 histones in HeLa cells analysed by laser scanning microscopy after indirect immunofluorescence labelling. Scale bar, 10 μm.

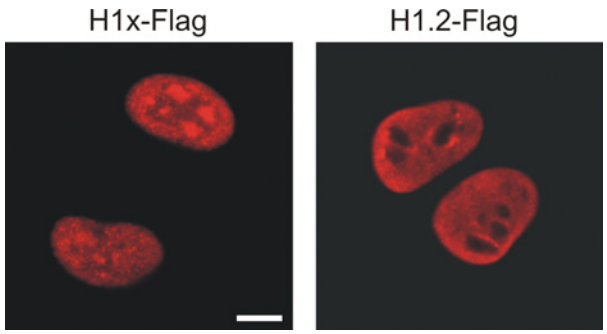


we performed a double-labelling of H1x and the RNA polymerase I transcription factor UBF (upstream binding factor), which revealed that both proteins did not co-localize exactly, but were arranged at adjacent sites within nucleoli (Figure 4A). Thus, it can be concluded that the nucleolar H1x is not located at the sites of ribosomal gene transcription.

Furthermore, the effect of specific inhibition of RNA polymerase I by a low dose of Actinomycin D on the nucleolar localization of H1x was examined.

Figure 2 | Intranuclear distribution of H1x–FLAG and H1.2–FLAG

Localization of FLAG-tagged fusion constructs of H1x and H1.2 in HeLa cells analysed by laser scanning microscopy. Scale bar, 10 μm.



Although it is known that the nucleoli segregate under this condition (reviewed in Hernandez-Verdun, 2006), nucleolar localization of H1x was still visible. H1x seems to be located in part in the central body, which is associated with the UBF-containing caps (Figure 4B).

Cell-cycle-dependent localization of H1x

Since the observed nucleolar localization of H1x was only visible in part of the non-synchronized HeLa cells, it was probable that this was due to its dependence on the cell cycle phase. To address this question, we examined the H1x localization in HeLa cells during the different stages of the cell cycle interphase. For this analysis, HeLa cells were treated with sodium butyrate, which leads to a cell cycle arrest and to an accumulation of cells in G_1 phase (Darnell, 1984), as indicated by a complete loss of S phase cells (Figure 5A). Arrest of HeLa cells at the G_1/S transition with aphidicolin and subsequent release causes a synchronized progression of cells into S phase. At 2 h after release from arrest, the cells accumulated in early S phase and at 6 h after release they accumulated in mid-S phase (Figure 5A). Indirect immunofluorescence labelling revealed that H1x was located mainly in nucleoli in sodium butyrate-treated cells,

Figure 3 | H1x is located partially in nucleoli

Nucleolar localization of H1x shown by laser scanning microscopy after double-labelling with antibodies against H1x and the nucleolar proteins fibrillarin and nucleolin respectively. Besides some overlap, H1x did not co-localize with fibrillarin or nucleolin. Scale bar, 10 μm.

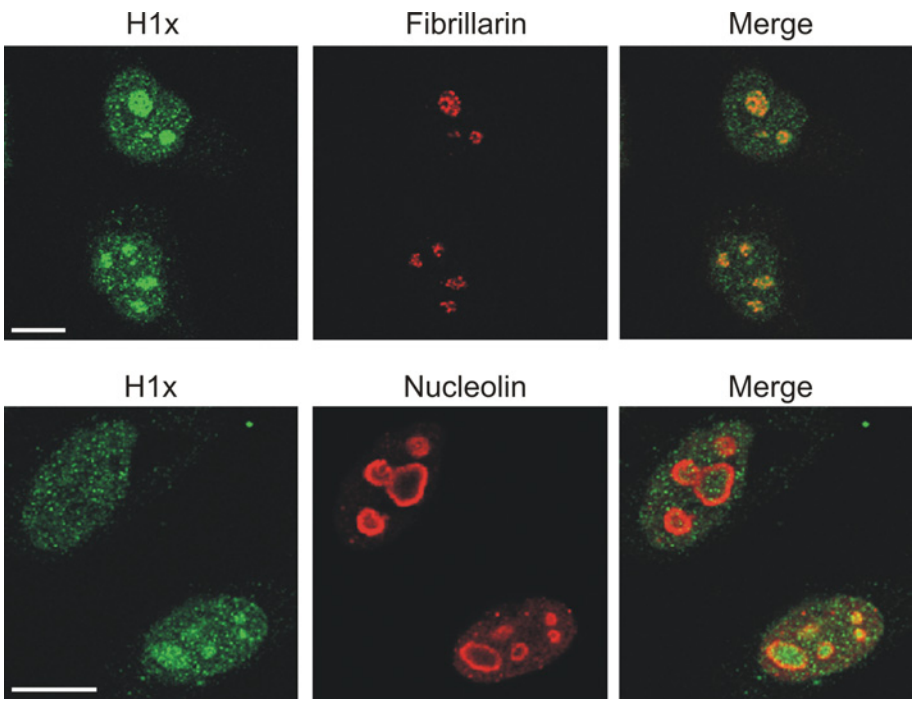
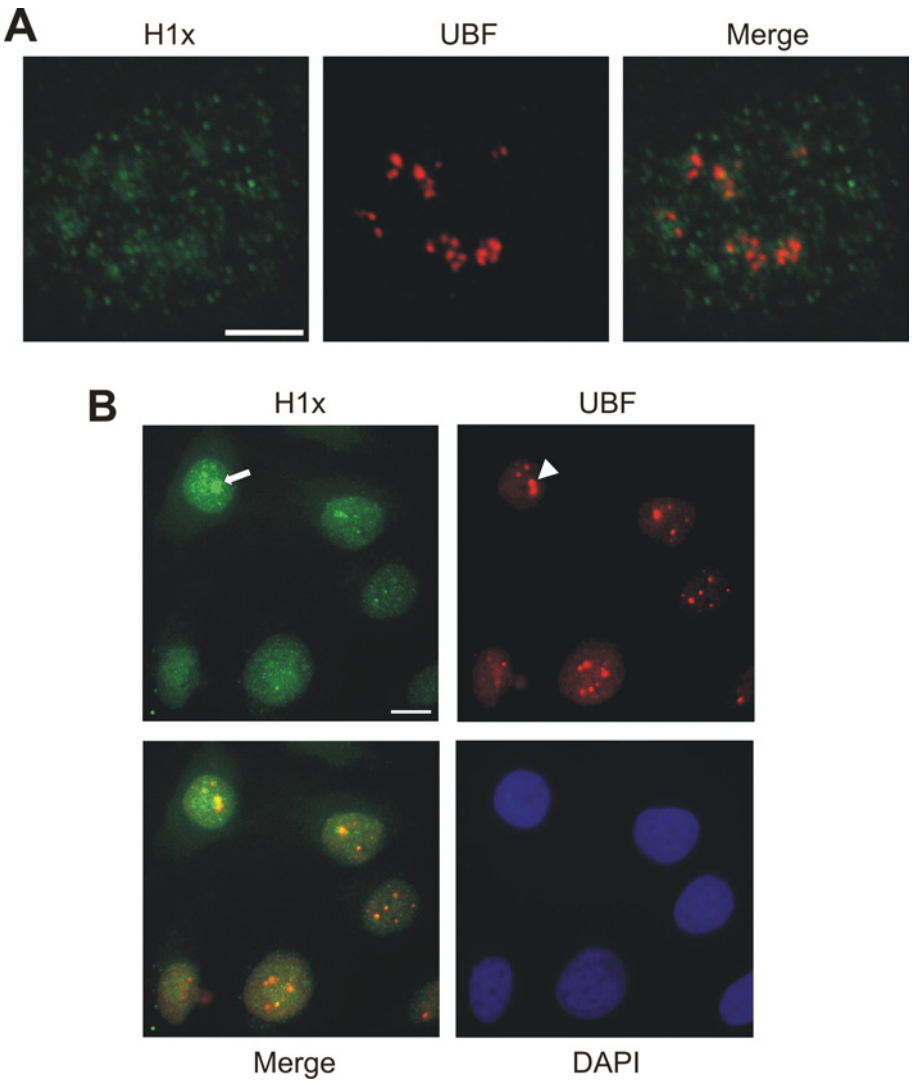


Figure 4 | Nucleolar localization of H1x is not linked to ribosomal gene transcription

(**A**) Indirect immunofluorescence detection of H1x and the nucleolar RNA polymerase I transcription factor UBF in HeLa cells. Scale bar, 5 μm. (**B**) Double-labelling of H1x and the RNA polymerase I transcription factor UBF after inhibition of RNA polymerase I transcription by treatment of HeLa cells with ActD. Nucleolar localization of H1x persists after RNA polymerase I inhibition. H1x seems to be located in the central body of the segregated nucleoli (arrow). The arrowhead marks UBF that is localized within a cap, which is associated with the central body of the nucleolus as described by Hernandez–Verdun (2006).

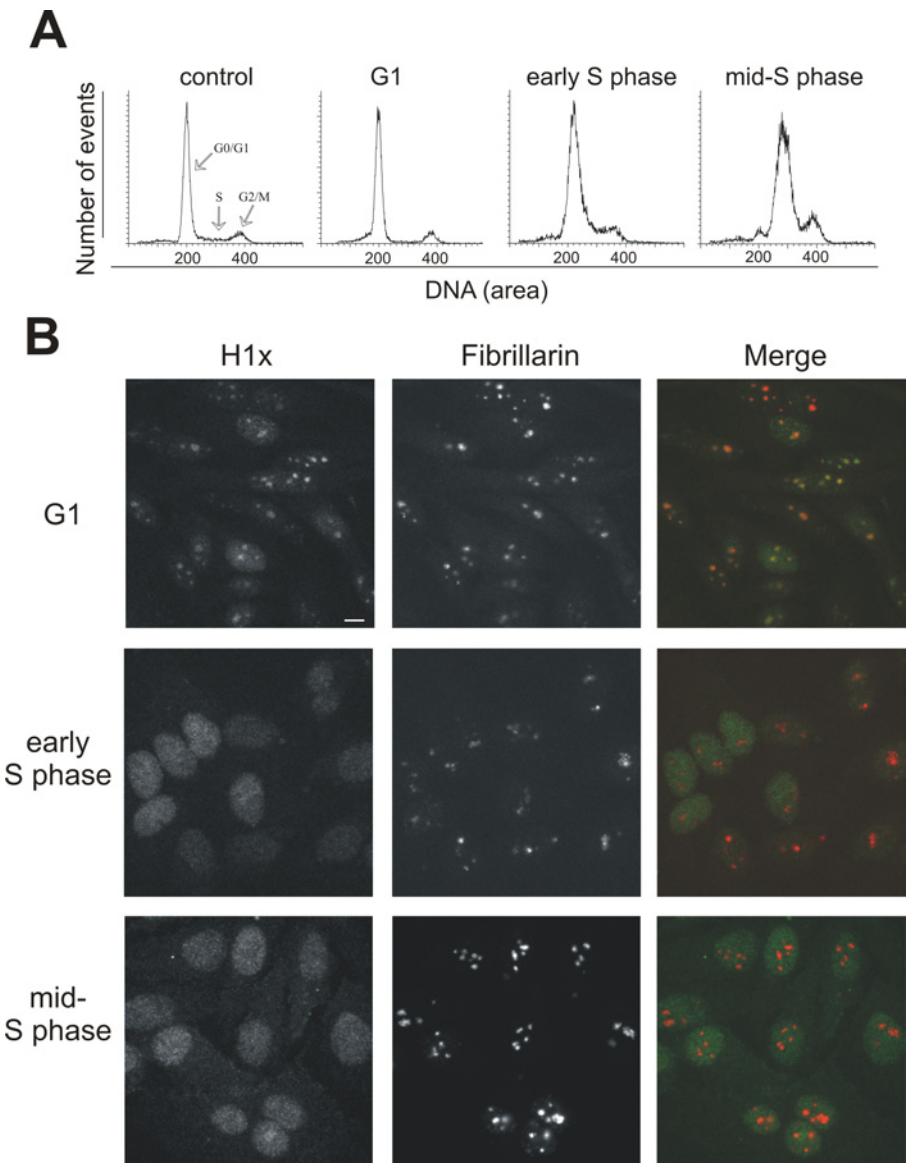


and thereby G_1 arrested cells, whereas cells in the different stages of S phase showed no nucleolar accumulation of H1x (Figure 5B). This result was confirmed by protein biochemical analysis of H1x in purified nucleoli. An increased amount of H1x could be found in nucleoli derived from G_1 cells in comparison with nucleoli from non-synchronized and S-phase cells respectively (Figure 6).

To investigate the intranuclear distribution of H1x during the G_2 phase of the cell cycle, i.e. before the nucleoli disassemble in the mitotic prophase (reviewed in Dimario, 2004), double labelling of H1x and histone H3 phosphorylated at Ser^{10} was performed. Phosphorylation of histone H3 is low in interphase cells and increases strongly during mitosis. It was shown that the phosphorylation at Ser^{10} starts

Figure 5 | Cell-cycle-dependent localization of H1x

(**A**) DNA content of synchronized HeLa cells measured by flow cytometry. HeLa cells were either treated with sodium butyrate for arrest in G_1, or with aphidicolin followed by release from the block for synchronization in S phase. Early S phase cells were harvested at 2 h and mid-S phase cells at 6 h after release from the aphidicolin block respectively. Non-synchronously grown cells served as control. (**B**) Indirect immunofluorescence detection of H1x and the nucleolar protein fibrillarin in G_1 arrested HeLa cells after treatment with sodium butyrate or in early and mid-S phase by blocking with aphidicolin and subsequent release for 2 and 6 h respectively. Scale bar, 10 μm.

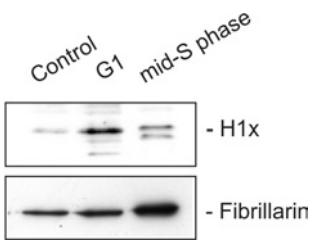


in late G_2 in a non-random manner in pericentromeric heterochromatin (Hendzel et al., 1997). As shown in Figure 7 H1x is evenly distributed in G_2 cells.

To narrow down the nucleolar localization of H1x in G_1, we analysed the localization of H1x in parallel to the occurrence of the transcription factor E2F-1. The expression of the *E2F-1* gene is induced strongly when cells progress from G_1 to S phase. During mid-G_1 phase, E2F-1 is released from the binding to its inhibitory protein Rb (retinoblastoma). The active E2F-1 initiates transcription of genes required for

Figure 6 | G_1-dependent nucleolar accumulation of H1x analysed by protein biochemistry

Immunoblot analysis of H1x in purified nucleoli from non-synchronized (control), G_1-arrested and mid-S phase cells. H1x is enriched in nucleoli from butyrate-treated G_1-arrested cells. The amount of the nucleolar marker protein fibrillarin was used as the loading control.



G_1/S transition, and positively regulates its own transcription (reviewed in Dyson, 1998; Matsumura et al., 2003). Since cells with high E2F-1 expression levels showed no nucleolar localization of H1x, it can be concluded that the nucleolar accumulation of H1x occurs in early to mid-G_1 phase (Figure 8). This result was also obtained with the H1x–FLAG construct (see Supplementary Figure 1 at http://www.biolcell.org/boc/099/boc0990541add.htm).

Analysis of nucleolar H1x localization by immunocytochemistry

To further specify the nucleolar localization of H1x we performed an immunocytochemical approach on cryosections of HeLa cells, which were transfected with the H1x–FLAG construct and arrested in G_1 by treatment with sodium butyrate. The cryosections were labelled either with the affinity-purified H1x antibody (Figure 9) or with an anti-FLAG antibody (data not shown). Both antibodies reacted with electron dense regions within the nucleoli, and partially, labelling was detected in the GC (granular

Figure 7 | Intranuclear distribution of H1x in G_2

Double-labelling of H1x and histone H3 phosphorylated at Ser^{10} [pH3(S10)] in non-synchronized HeLa cells. Phosphorylation of Ser^{10} of histone H3 starts in late G_2. H1x is evenly distributed in the nucleus in the pH3(S10) positive cells in the G_2 phase of the cell cycle. Scale bar, 10 μm.

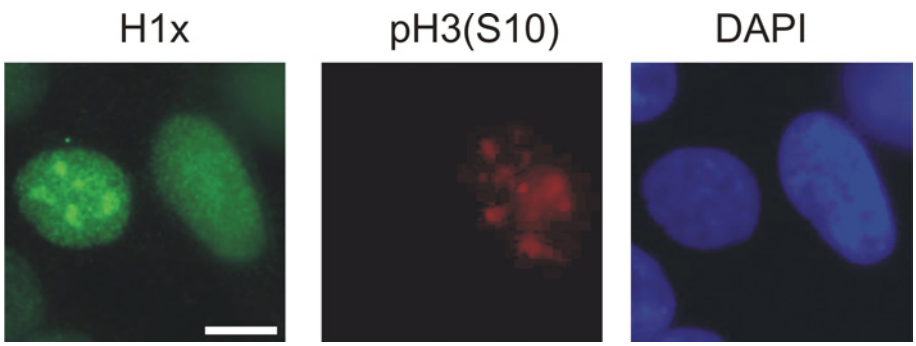


Figure 8 | Nucleolar accumulation of H1x is restricted to early to mid-G_1

Double-labelling of H1x and E2F-1 in non-synchronized HeLa cells. Cells which highly express E2F-1 exhibit no nucleolar accumulation of H1x. Scale bar, 20 μm.

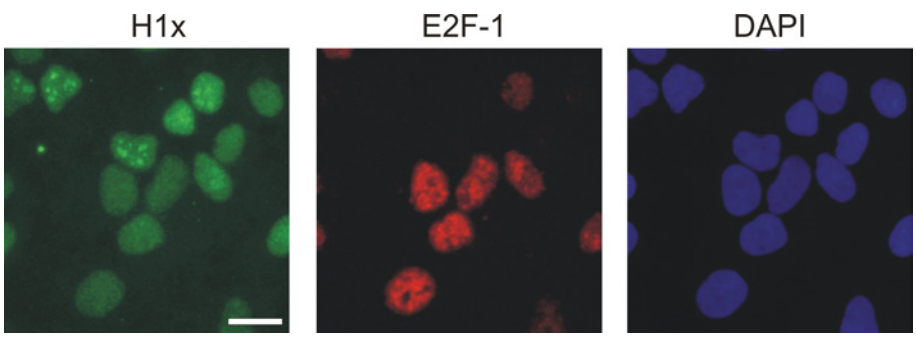
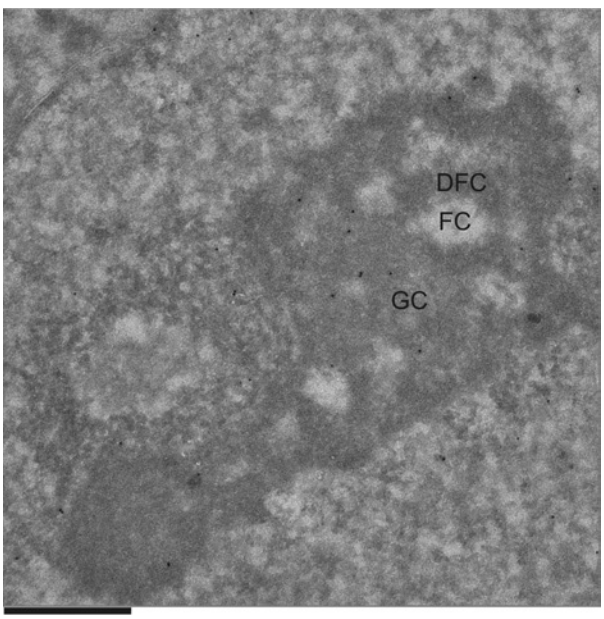


Figure 9 | Intranucleolar distribution of H1x

Ultrathin cryosections of HeLa cells, transfected with the H1x–FLAG construct and arrested in G_1 by treatment with sodium butyrate, were labelled with affinity-purified anti-H1x antibody. H1x is located mainly at electron dense structures and partially in the GC. FC, fibrillar centre; DFC, dense fibrillar component. Scale bar, 500 nm.



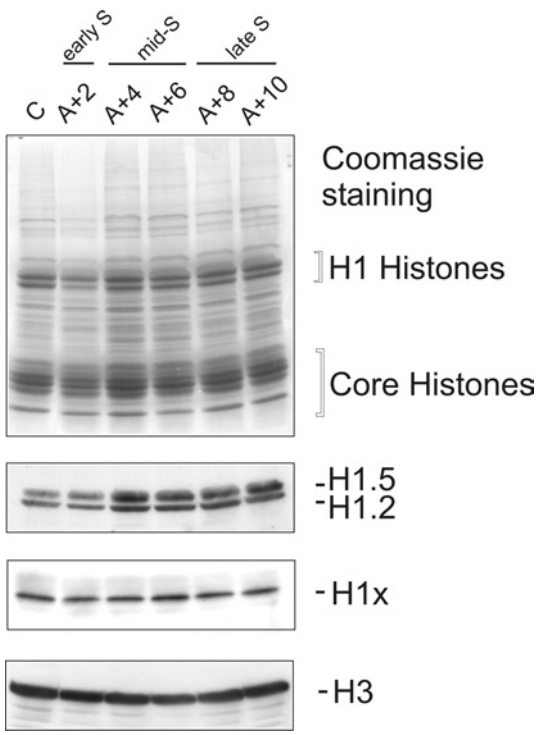
component). Essentially, no H1x was detected in fibrillar centres (Figure 9).

Expression of H1x during S phase in comparison with replication-dependent H1 subtypes

The next question was how the expression of the *H1x* gene is regulated throughout S phase of the cell cycle. In a previous study, we raised the question if the *H1x* gene (which, like the gene of the replacement subtype H1°, is located solitarily and gives rise to a polyadenylated mRNA) is regulated in a similar manner to the replacement histone H1°. We showed that this is not the case since the protein level of H1x did not change after cell cycle arrest with sodium butyrate or after triggering differentiation (Happel et al., 2005). To analyse the amount of H1x protein during S phase, histones were extracted from synchronized HeLa cells, and the amount of core histones was taken as a loading control, by monitoring the

Figure 10 | Analysis of the H1x protein amount during S phase progression

Determination of relative protein levels of the replication-dependent expression of histone subtypes H1.2 and H1.5, and of H1x by Western blot analysis. Whole histones were extracted from synchronized or non-synchronized (control, C) HeLa cells. Numbers indicate time periods in hours after release from an aphidicolin arrest (e.g. A+2 means 2 h after release). As control for equal loading, equal amounts of core histones were loaded as shown by Western blot analysis of histone H3. Immunoblot analysis was carried out for H1x and with an antiserum reacting with H1.2 and H1.5 (Ab4112; see Supplementary Figure 2 at http://www.biolcell.org/boc/099/boc0990541add.htm). In contrast to the replication-dependent expression of the histone subtypes, the amount of H1x remained unchanged during S phase.



expression level of the core histone H3. Immunoblot analysis of H1.2 and H1.5 showed changes in the H1/core histone ratio during S phase. The H1/core histone ratio was low in early S phase, whereas the amount of the somatic H1 histones increased sharply in mid-S phase (D'Anna and Tobey, 1984; D'Anna et al., 1985). In contrast to these two subtypes, detection of the H1x protein revealed nearly no change during S phase (Figure 10).

Discussion

The replication-dependent histone subtypes H1.1 to H1.5 are expressed during the S phase of the cell cycle in order to assemble chromatin with the newly replicated DNA, and the so called replacement subtype H1° accumulates when the cells stop proliferating and start to differentiate. It is controversially discussed whether H1° replaces replication-dependent subtypes in chromatin at active sites or if it is involved in transcriptional repression (reviewed in Zlatanova and Doenecke, 1994). In the present study, we analysed the histone subtype H1x and demonstrate that its expression is regulated in a manner similar to neither the replacement subtype H1° nor to the replication-dependent subtypes, since the protein amount of H1x did not change after cell cycle arrest in G_1 or during S phase progression. On the other hand, a G_1-dependent nucleolar accumulation of this H1 histone subtype was shown.

Nucleoli consist of three distinct substructures. These are the FCs (fibrillar centres), which are surrounded by the DFC (dense fibrillar component), which again are embedded within the GC (reviewed in Scheer and Hock, 1999). Among these three main nucleolar components, condensed chromatin is visible on the ultrastructural level (reviewed in Derenzini et al., 2006). Fibrillarin, which is a component of snRNP (small nuclear ribonucleoprotein) particles and thus is directly involved in post-transcriptional processes, including pre-mRNA processing and ribosome assembly (Tollervey et al., 1993), has been shown to be located within the DFC (Ochs et al., 1985). Since the portion of H1x that was shown to be located in nucleoli in G_1 phase was hardly co-localized with fibrillarin, it can be concluded that the nucleolar H1x is located mainly in structures other than the DFC. The absence of H1x in areas containing the RNA polymerase I transcription factor UBF, which is also located in the DFC and at the periphery of the FCs (Roussel et al., 1993), demonstrates that H1x is not located directly at the sites of ribosomal gene transcription. Furthermore we could show that the nucleolar localization is independent of RNA polymerase I activity, since it was not abolished through inhibition of this enzyme. This result fits very well the observation that transcriptionally active ribosomal genes are free of regularly spaced nucleosomes (Dammann et al., 1993), and it is consistent with the finding that UBF out-competes H1 histones for binding on the linker DNA in reconstituted nucleosomes (Kermekchiev et al., 1997). Analysis of the intranucleolar distribution of H1x on the ultrastructural level revealed localization in electron dense structures and partial localization to the GC. Since H1x did not co-localize with UBF and hardly with fibrillarin, we can conclude that H1x is not located in the DFC in the main. Thus, the electron dense structures in the cryosections labelled with the anti-H1x antibody are presumably condensed nucleolar chromatin. Thus, it can be assumed that H1x is located at the inactive ribosomal genes, which have been shown to be organized in regularly spaced H1-containing nucleosomes (reviewed in Derenzini et al., 2006). The question remains why H1x leaves these nucleolar sites in S phase. A cell cycle-dependent shuttling between nucleolus and nucleoplasm has been described for several other proteins. For example, the Ki-67 antigen, which is thought to be required for maintaining cell proliferation, is localized in the nucleoplasm in early G_1 and is located in the nucleolus during S phase and G_2 (Kill, 1996). Although it is thought that the nucleolar localization of proteins is caused by their specific nucleolar activities and not due to specific targeting (Scheer and Weisenberger, 1994), there is also growing evidence for a function of the nucleolus as a subnuclear storage site. The nucleolus seems to serve as a general sequestration centre for the temporary inactivation of regulatory proteins, for example p53 regulating proteins or the yeast phosphatase Cdc14p (reviewed in Olson et al., 2000).

The results of the present study suggest that the observed cell-cycle-dependent shuttling of H1x is a mechanism to control H1x activity. Further experiments have to be done to find out if H1x fulfils a specific function in nucleoli in G_1 or whether it acts primarily during S phase and has to be sequestered into nucleoli in G_1 instead of regulating the turnover of its protein level.

Materials and methods

Cell culture, cell cycle arrest and synchronization

The cell line HeLa (cervix carcinoma) was obtained from the DSMZ (Braunschweig, Germany), the cell line SV-80 [SV-40 (simian virus-40) transformed fibroblast] from CLS (Heidelberg, Germany). The cells were maintained in the recommended medium supplemented with 10% (v/v) foetal calf serum at 37°C in a humidified 5% CO_2 atmosphere. HeLa cells were treated with 5 mM sodium butyrate (Sigma) for 24 h to arrest the cell cycle

and to accumulate cells in G_1 (Darnell, 1984). For synchronization, cells were incubated with aphidicolin (10 μM for 24 h; Qbiogene-Alexis, Grünberg, Germany). Cells were released from this block by washing the monolayer with PBS and growth medium. After different time periods of growing in the regular medium, the synchronized cells were harvested. Inhibition of transcription by RNA polymerase I was done by adding 0.04 μg/ml ActD for 2 h to the growth medium.

Flow cytometry

To estimate the cell cycle distribution, control and treated HeLa cells (1×10^6 cells) were fixed in 7% (v/v) ethanol at −20°C for at least 1 h. Cells were then centrifuged (500 *g* for 5 min at 4°C) and the DNA was stained by suspending the cells in 500 μl PBS containing 100 μg/ml RNase A and 50 μg/ml propidium iodide. After incubation for 30 min at room temperature (22 °C) cells were measured with a FACSCalibur Flow Cytometer (BD Bioscience, San Jose, USA). Cell cycle distribution was analysed using the software Modfit LT (Verity Software House, Topsham, USA).

Histone extraction

The purification of whole histones from HeLa cells was done as described earlier (Happel et al., 2005) using 0.2 M sulfuric acid.

Purification of nucleoli

Nuclei from HeLa cells were extracted as described by Bunce et al. (1988) and nucleoli from these nuclei were purified according to the method described by Zalta et al. (1971) using a Branson Digital Sonifier (45% amplitude).

Immunoblotting

Proteins were separated by SDS/PAGE (15% gels) and electrophoretically transferred on to nitrocellulose membranes. Uniform blotting was checked by staining the nitrocellulose filter with Ponceau S (Sigma). Blots were probed with polyclonal anti-H1x-specific antiserum in a 1:1000 dilution (Happel et al., 2005), anti-(histone H3) antibody (#9715, CST, New England Biolabs, Frankfurt, Germany), anti-fibrillarin antibody (clone 38F3, #MC-992, Kamiya Biomedical Company, Seattle, USA) or a polyclonal antiserum specific for H1.2 and H1.5 (Ab4112; see Supplementary Figure 2 at http://www.biolcell.org/boc/099/boc0990541add.htm). The Ab4112 antiserum was generated by using $HClO_4$-extracted H1 histones from HL60 cells as the antigen. Immunization of the rabbit and generation of the serum was performed by Bioscience (Göttingen, Germany). The immunoreactive proteins were visualized using the chemiluminescence ECL® Plus detection reagents (Amersham Bioscience, Freiburg, Germany) after incubation with a horseradish peroxidase-conjugated goat anti-rabbit antibody (#A0545; Sigma) or horseradish peroxidase-conjugated goat anti-mouse antibody (#A9917; Sigma). Stripping of blotting membranes for reprobing was done by incubation of the membranes in stripping buffer [62.5 mM Tris/HCl, pH 6.7, 100 mM 2-mercaptoethanol and 2% (w/v) SDS] for 30 min at 50°C with occasional agitation.

H1x– and H1.2–FLAG expression in HeLa cells

For the generation of a FLAG-tagged fusion construct of H1x, the coding region for H1x was amplified from the H1x cDNA cloned in the vector pOTB7, which was obtained from the NIH (National Institutes of Health)-MGC (Mammalian Gene Collection) Project (Genbank accession no. BC000426, I.M.A.G.E. Consortium No. 2819756; distributor: RZPD German Resource Center for Genome Research, Berlin, Germany) by PCR using the following PCR primers (forward primer ONH62, including the restriction site HindIII: 5′-CAAGCTTACCATGTCCGTGGAGCTCGAG-3′ and reverse primer ONH63, including the restriction site XbaI: 5′-CTCTAGACTTGCGGCCCTTGGGCAC-3′). The PCR product was cloned in the vector pGMTeasy (Promega, Mannheim, Germany) and sequenced. Then the coding region was excised from this vector using the restriction enzymes HindIII and XbaI and cloned into the expression vector pFLAG-CMV-5.1 (Sigma), which had been linearized using the same restriction enzymes. The generation of the H1.2–FLAG construct was performed in the same way, using the coding region of H1.2 as template (Albig et al., 1998) and the following PCR primers: (forward primer ONH70, including the restriction site HindIII: 5′-CAAGCTTAACATGTCCGAGACTGCTCCTG-3′ and reverse primer ONH71, including the restriction site XbaI: 5′-CTCTAGATTTCTTCTTGGGCGCCGCCTTC-3′). The resulting plasmids were used for the transfection of HeLa cells using the Effectene transfection reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions. After 24 h of incubation at 37°C in a humidified 5% CO_2 atmosphere, the cells were used for indirect immunofluorescence detection or immunocytochemistry as described.

Immunofluorescence

For indirect immunofluorescence detection, cells were fixed with 3% (v/v) paraformaldehyde in PBS for 15 min, permeabilized with 0.5% Triton X-100 in PBS for 10 min, and blocked with 3% (w/v) BSA in PBS. The following antibodies were used for indirect immunofluorescence labelling: Affinity purified anti-H1x antibody at a final concentration of 1–2 μg/ml. Affinity purification was performed using a SulfoLink column (Pierce/Perbio Science, Bonn, Germany) on to which 1 mg of the corresponding peptide used as the antigen had been coupled. To confirm the specificity of the anti-H1x antibody, a pre-adsorption experiment was performed. The diluted antibody was pre-incubated with recombinantly expressed H1x (Happel et al., 2005) coupled on to NHS (*N*-hydroxysuccinimido)-activated Sepharose (Amersham Bioscience) for 1 h at 37°C. Application of the pre-adsorbed antibody resulted in the absence of specific labelling. Mouse monoclonal anti-(histone H1) antibody (# 05-457, Upstate, Charlottesville, U.S.A.) at a concentration of 1 μg/ml; mouse monoclonal anti-FLAG (#F3165, Sigma) at a final concentration of 1 μg/ml; mouse monoclonal anti-fibrillarin (clone 38F3, Kamiya Biomedical Company, Seattle, U.S.A.) in a 1:250 dilution; mouse monoclonal anti-nucleolin (clone 4E2) in a 1:4000 dilution (Abcam, Cambridge, U.K.); human anti-UBF (kindly provided by Prof. I. Grummt and Dr R. Voit, German Cancer Research Center, Heidelberg, Germany) diluted 1:1200; anti-(phospho-Ser^{10}-histone H3) [pH3(S10)] in a 1:50 dilution (clone 6G3, CST, New England Biolabs); anti-E2F-1 (#DLN-11362, Dianova, Hamburg, Germany) in a 1:200 dilution. The following secondary antibodies were used in a 1:1000 dilution: Alexa® $Fluor^{488}$ anti-mouse IgG (#A11017), Alexa® $Fluor^{555}$ anti-mouse IgG (#21425), Alexa® $Fluor^{488}$ anti-rabbit IgG (#11070) and Alexa® $Fluor^{555}$ anti-rabbit IgG (#21430) from Molecular

Probes (distributed by MoBiTec, Göttingen, Germany). Anti-(human IgG–Cy3) was used at a 1:400 dilution. The nuclei were visualized with DAPI using Vectashield Mounting Medium with DAPI (Vector Laboratories, Burlingame, U.S.A.). For fluorescence microscopy an Axioskop was used (Zeiss, Göttingen, Germany). Confocal light scanning microscopy was performed using a Zeiss LSM 310 series equipped with a Zeiss Plan Apochromat 63×/1.40 and Carl Zeiss LSM version 3.95 software, a Zeiss Axioplan 2 microscope equipped with a laser scanning module LSM 510, Nomarski differential interference contrast, and conventional epifluorescence optics or a Leica TCS SP5.

Electron microscopy

Ultrathin cryosections were prepared as described previously (Tokuyasu, 1973, 1980; Kreykenbohm et al., 2002). HeLa cells transfected with the H1x–FLAG construct, were fixed with 2% PFA [1 vol. growth medium and 1 vol. 4% (w/v) paraformaldehyde] for 30 min at room temperature. Cells were post-fixed with 4% and 0.1% glutaraldehyde in PBS for 2 h on ice. After being washed twice with PBS containing 0.02% glycine, the cells were embedded in 10% (w/v) gelatine, cooled on ice, and cut into small blocks. The blocks were infused with 2.3 M sucrose in PBS at 4°C overnight, mounted on metal pins and frozen in liquid nitrogen. Sections (70 nm) were cut at approx. −110°C using a diamond knife (Diatome) in an ultracryomicrotome (Leica Microsystems, Wetzlar, Germany), collecting with a 1:1 mixture of 2% methyl-cellulose and 2.3 M sucrose (Liou et al., 1996). For immunolabelling, sections were incubated with affinity-purified antibody against H1x (0.5 μg/ml) or with a polyclonal anti-FLAG antibody (#F7425, Sigma) for 20 min, followed by incubation with Protein A–gold (10 nm) for 20 min. As a negative control, labelling with just Protein A–gold was performed. Sections were contrasted with uranyl acetate/methyl cellulose for 10 min on ice, embedded in the same solution and examined with a Philips CM120 electron microscope. Electron Micrograph Images were taken with a CCD camera (TVIPS, Gauting, Germany).

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