Dynamic relocation of epigenetic chromatin markers reveals an active role of constitutive heterochromatin in the transition from proliferation to quiescence

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Accepted 13 September 2004
Journal of Cell Science 117, 6153-6162 Published by The Company of Biologists 2004
doi:10.1242/jcs.01537

Summary

Quiescent lymphocytes have small nuclei, filled with masses of facultative heterochromatin. Upon receiving mitogenic signals, these cells undergo nuclear enlargement, chromatin decondensation, the reactivation of cell proliferation, and changes in the intranuclear positioning of key genes. We examined the levels and intranuclear localization of major histone modifications and non-histone heterochromatin proteins in quiescent and reactivated mouse spleen lymphocytes. Dramatic and selective changes in localization of two heterochromatin-associated proteins, the histone variant macroH2A and HP1α, occurred during lymphocyte reactivation. Reciprocal changes in the locations of these two proteins were observed in activated lymphocytes and cultured mouse fibroblasts induced into quiescence. We also describe a new apocentric nuclear compartment with a unique set of histone modifications that occurs as a zone of chromatin surrounding centromeric heterochromatin in differentiated lymphocytes. It is within this zone that the most significant changes occur in the transition from proliferation to quiescence. Our results suggest that constitutive centromeric heterochromatin plays an active role in cell differentiation and reactivation.

Key words: Epigenetics, Heterochromatin, Histone modifications, MacroH2A, HP1

Introduction

During cellular differentiation, there is a progressive restriction in transcribable genes, until only the relatively small repertoire appropriate for the mature cell type remains. Concomitantly, much of the rest of the genome becomes epigenetically silenced, forming compact blocks of facultative heterochromatin (Francastel et al., 2000; Frenster, 1974). The succession of epigenetic modifications associated with facultative heterochromatin is normally unidirectional, but in cases in which differentiated cells resume proliferation, a partial or complete reversal must occur. Examples include tissue and organ regeneration (Echeverri and Tanaka, 2002; Stocum, 2002), cloning by injection of somatic nuclei into egg cytoplasm (Lu et al., 1999; Wangh et al., 1995), and many types of cancer (Schmutzler and Kohrle, 2000; Stein et al., 2000; Sugimura and Ushijima, 2000).

The identification of key histone modifications and non-histone proteins associated with specific genes and chromatin domains has greatly increased our understanding of the molecular markers that distinguish active euchromatin from silenced heterochromatin (Richards and Elgin, 2002). Nuclear proteins and histone modifications contributing to the establishment and maintenance of epigenetic silencing include the Polycomb group proteins and histone H3 methylation at lysine 27 (Czermin et al., 2002; Lavigne et al., 2004), heterochromatin protein 1 (HP1) and histone H3 methylation at lysine 9 (Lachner et al., 2001; Nielsen et al., 2002), SIR3 and hypoacetylation of histone H4 (Carmen et al., 2002), and MeCP2 and CpG methylation (Nan et al., 1998). These results support the concept that patterns of histone modifications and associated non-histone proteins uniquely determine the transcriptional competence of chromatin domains (Jenuwein and Allis, 2001; Turner, 2002).

Constitutive heterochromatin is a class of compact chromatin quite different from facultative heterochromatin, comprised of gene-poor DNA containing highly repetitive sequences. As its name indicates, constitutive heterochromatin is presumed to be independent of the state of cell differentiation and the level of transcriptional activity. The best studied examples of constitutive heterochromatin involve telomeric and centromeric DNA (Hennig, 1999; Richards and Elgin, 2002), and there is a wealth of evidence from many systems that genes translocated to regions of constitutive heterochromatin tend to be downregulated (Grunstein, 1998; Reuter and Spierer, 1992; Wallrath, 1998). There is also evidence that constitutive heterochromatin is inherently compact (Gilbert and Allan, 2001), whereas the level of compaction of facultative heterochromatin is variable and developmentally regulated. Whether similar compaction mechanism(s) are shared by both types of heterochromatin is...
unknown, although both feature similar core histone modifications (Cowell et al., 2002; Turner, 2002) and share some non-histone proteins that promote chromatin silencing (Chadwick and Willard, 2003; Gilbert et al., 2003).

Although telomeres and centromeres per se have well-defined and essential functions, the role(s) of the often large amounts of sub-telomeric and pericentromeric heterochromatin are not fully understood. We were therefore intrigued to find striking changes in two heterochromatin-associated proteins, macroH2A and HP1α, during the reactivation of quiescent mouse lymphocytes, a process involving a massive increase in nuclear volume, the breakup of blocks of facultative heterochromatin, re-entry into the cell cycle, and transcriptional upregulation. Similar changes in intranuclear localization could also be observed under more controlled conditions in cultured mouse fibroblasts. On the basis of our observations and the known functions of the mobile heterochromatin proteins, we propose that during the establishment of quiescence, HP1α moves from constitutive heterochromatin and assists in global chromatin repression and formation of facultative heterochromatin, whereas macroH2A accumulates in constitutive heterochromatin and may contribute to maintaining its repressed state.

Materials and Methods
Rabbit polyclonal anti-HP1α IgG (α-HP1α) (Filesi et al., 2002); Rabbit polyclonal IgG against histone H3 peptide 1-20 with trimethylated Lysine-9 (α-H3me3,K9) (Cowell et al., 2002); Rat monoclonal anti-HP1β (M31) IgG (α-HP1β) (Wreggett et al., 1994) and anti-HP1γ (M32) IgM (α-HP1γ) (Horsley et al., 1996) were as described. Rabbit polyclonal IgG against histone H3 peptide 6-13 with dimethylated Lysine-9 (α-H3me2,K9), and against histone H4 peptide 7-16 with acetylated Lysine-12 (α-H4acK12), were obtained from Upstate Biotechnology (Lake Placid, NY), monoclonal anti-BrDU antibody, conjugated with fluorescein was from Roche (Indianapolis, IN), and antibodies against Ikaros (Cobb et al., 2000) were kindly provided by S. Smale.

Lymphocyte isolation and reactivation
Primary mouse spleenocyte suspensions were prepared under sterile conditions by gently macerating fresh spleen between frosted microscopy glass slides. Cells were washed twice in RPMI medium (Sigma cat #R8785) by centrifuging at 200 g for 10 minutes and resuspended at a concentration of 2.5×10^6 cells/ml in RPMI supplemented with 10% heat-inactivated fetal bovine serum (Sigma, cat #16001, 2 mM L-glutamine, 0.05 mM 2-mercaptoethanol, 500 µg/ml gentamicin). For activation, 5 µg/ml of concanavalin (Con A, Sigma cat #C-7275) was added and cells were incubated at 37°C and 5% CO2 for the desired time and harvested by centrifugation at 200 g for 10 minutes.

To separate B- and T-cells from the initial spleenocyte population, polystyrene tissue culture plates were coated with anti-mouse Ig antibody diluted with PBS to 100 µg/ml (4 ml per 100 mm plate), incubated for 1 hour at room temperature or overnight at 4°C, and rinsed four times with PBS. Spleenocytes from up to 10 animals were resuspended in 10 ml RPMI-10/HEPES (RPMI 1640 without bicarbonate, containing 20 mM HEPES and 10% fetal calf serum), centrifuged for 10 minutes at 200 g (4°C), resuspended in 10 ml filter-sterilized LIS media [1 liter containing 25 ml Hank’s Balanced Salt Solution (308 mM), 50 ml 0.336 M HEPES, pH 7.2, 800 ml 0.308 M sorbitol, 125 ml 0.308 M glucose], and incubated for 10 minutes on ice. Cell suspensions in LIS were filtered through gauze, centrifuged for 10 minutes at 200 g, 4°C and resuspended in RPMI-10/HEPES at 4×10^7 cells/ml. Five ml of this suspension (2×10^9 cells) was placed in a coated plate and allowed to settle for 30 minutes at room temperature. Nonattached cells were transferred into a second coated plate, allowed to settle again, and the procedure was repeated one more time. Nonattached cells (T-lymphocytes) were washed by centrifuging at 200 g for 10 minutes and resuspended in PBS.

Expression plasmids and cell transfection
cDNA clones encoding histones H2A.1 (#875484R), H2A.Z (77385R), and H2A.X (#599273R) were obtained from ATCC (Manassas, VA). cDNA clones encoding macroH2A.2 and its N-terminal domain macroH2A.1-140 were obtained by reverse-transcription-mediated PCR from mouse erythroleukemia cells. To construct plasmids expressing macroH2A (pSG146), macroH2A1.2-140 (pSG147), H2A.X (pSG154), and H2A.1 (pSG155), all sequences were inserted into the Eco RI site of the pCDNA4/HisMax expression vector (Invitrogen) so that the histone ORF lies downstream and in frame with the Express™ epitope tag encoded in the vector. NIH/3T3 cells (ATCC CRL-658) were propagated in Dulbecco’s modified Eagle’s medium with 4.5 g/L glucose (Mediatech, USA), 1.5 g/L sodium bicarbonate, 1xantibiotic-antimycotic solution, and 10% (v/v) Fetal Calf Serum. Cells were grown in 5% CO2 at 37.5°C. Cells were transfected at approximately 80% confluency on 30 mm dishes using 12 µl of Lipofectamine-2000 reagent (Gibco-BRL) for 5 hours in serum-free growth media, as described in the vendor’s manual. After transfection, cells were allowed to continue growing in their normal media for 48 hours.

Nuclear isolation and western analysis
Isolation of nuclei from mouse lymphocytes and cultured NIH/3T3 cells, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE), western blotting and ECL detection were performed as described (Istomina et al., 2003). For detection of HP1 and histones, proteins were transferred to Immobilon-P PVDF membranes (Millipore) in 20% methanol. HP1β was detected according to Wreggett et al. (Wreggett et al., 1994) and HP1α according to Horsley et al. (Horsley et al., 1996). Modified histones were probed with Upstate Biotechnology antibodies (see above) as recommended by the manufacturer. For semiquantitative analysis of relative protein levels, the Coomassie-stained gels and autoradiographs were digitized and the intensity of protein bands was quantitated using the NIH image software package. Calibration curves were made by plotting the intensities of control protein bands against samples of known concentration and used to estimate the concentration of unknown samples. Protein to DNA ratios were estimated from parallel spectrophotometric measurements (A260=1 for 50 µg/ml of DNA).

BrdU labeling and microscopy sample preparation
Pulses of 10 µg/ml BrdU were added for 5 minutes to spleenocytes activated with concanavalin A, and further incubated for the desired time. Cells were then washed in PBS, incubated for 10 minutes with PBS containing 0.5% NP-40, then fixed in 4% paraformaldehyde, at 20°C, and deposited on slides using a Cytospin centrifuge. DNA was denatured by incubating slides in 4 N HCl for 30 minutes at room temperature. Slides were then rinsed in PBS and neutralized in 0.1 M borate buffer pH 8.5, followed with PBS. Samples were finally incubated with 5 µg/ml of anti-BrdU antibody diluted in 0.1% BSA/PBS for 1 hour at room temperature, and mounted in anti-fading media (Vectorshield).

Immunofluorescence microscopy and image analysis
Non-adherent cells were fixed and attached to glass slides by Cytospin centrifugation as above. Samples were then blocked in KCM [120 mM
KCl, 20 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.1% (v/v) Triton X-100) for 15 minutes containing 2% (w/v) BSA and 10% Nonfat dry milk for 15 minutes. Slides were treated with primary antibodies in KCM containing BSA and dry milk, incubated for 1 hour at room temperature, washed twice in KCM, overlaid with KCM containing BSA and dry milk for 5 minutes, and treated with fluorescent secondary antibodies, Alexa Fluor 488 and/or 594 (Molecular Probes, Eugene, OR).

NIH/3T3 cells were grown on cover glasses, fixed, and probed first with primary antibodies against macroH2A, HP1α, HP1β, H3K9Me2, H3K9Me3, and H4K12Ac, followed by secondary antibodies labeled with Alexa Fluor 488 and/or 594 (Molecular Probes) as described (Istomina et al., 2003). All samples were stained with 0.1 μg/ml Hoechst 33258 in PBS. Fluorescence microscopy was performed using a Nikon Eclipse microscope with a 60× or 100× plan apo lens. For deconvolution, 16-bit z-slices were captured at 0.4 μm steps with a cooled CCD camera and iteratively deconvolved (usually 20 cycles) using AutoDeblur software (AutoQuant Imaging, Watervliet, NY) as described (Irving et al., 2002). Deconvolved images in the figures represent a single ‘z’ slice through the center of the nucleus. Image intensity analysis was performed with Image-Pro Plus software (Media Cybernetics, Silver Spring, MD).

Results

Nuclear macroH2A in quiescent lymphocytes is concentrated in centromeric chromatin

A biochemical screen which discriminates between active euchromatin and inactive and condensed heterochromatin based on their differential self-association in vitro (Grigoryev et al., 1992; Kamakaka and Thomas, 1990; Ridsdale and Davie, 1987; Weintraub, 1984) identified the core histone variant macroH2A as strikingly enriched in the self-associating heterochromatin fraction, and thus likely to be preferentially located in highly compact chromatin in mouse lymphocytes (Woodcock et al., 2000) (T.N., S.A.G., J.R.P. and C.L.W., unpublished). In many cell types from female mammals, macroH2A is concentrated in inactive X chromosomes (Costanzi and Pehrson, 1998), and appears to play a role in maintaining but not establishing the X inactivation process (Csankovszki et al., 1999), thus contributing to the propagation of this type of facultative heterochromatin. The identification of macroH2A as possibly associated with highly compact heterochromatin suggested that its intranuclear localization would be of interest. We therefore examined the immunolocalization of macroH2A in quiescent lymphocytes from mouse spleen using a comprehensive range of fixation and other preparative conditions. In addition to general faint staining throughout the nucleus, the most striking feature of macroH2A distribution was a pronounced labeling of large chromatin foci (Fig. 1a). These co-localized precisely with bright DAPI or Hoechst staining (Fig. 1b,c,e), and were verified as pericentromeric heterochromatin formed around merged centromeres (chromocenters) by their colocalization with the centromeric marker protein CENP-B (Fig. 1d,e). The fraction of cells with pronounced pericentromeric anti-macroH2A was variable, up to a maximum of ~75% positive cells. Attempts to improve antigen accessibility by allowing nuclei to swell in low salt, or by pre-treatment with DNase did not significantly increase the yield of cells with positively staining centromeres, indicating that the variation in macroH2A immunofluorescence was a true reflection of native macroH2A content. Quiescent spleenocytes consist primarily of mature B-cells (~80%) and T-cells (~20%). When separated (see Materials and Methods), both T- and B-cell populations had a similar proportion of nuclei with positive centromeric localization of macroH2A. In contrast, thymocytes that consist largely of immature T-cells mostly showed a weak uniform distribution of macroH2A and the minor fraction with pericentromeric localization of the protein coincided with the smallest, most mature cells (not shown). In agreement with earlier reports (Costanzi and Pehrson, 1998), we observed a strong colocalization of macroH2A with Xi in hepatocytes from female mice (Fig. 1f-h), but never detected any localization of macroH2A over Xi in identically treated female lymphocytes. Conversely, no centromeric labeling with macroH2A was observed in hepatocytes. A third pattern of macroH2A localization, observed primarily in large immature thymocytes, was centrosomic. This was much more frequent in activated lymphocytes (see Fig. 5 below) and has also been reported in proliferating embryonic stem cells (Rasmussen et al., 2000).

Fig. 1. macroH2A in quiescent mouse lymphocytes and hepatocytes. Quiescent lymphocytes from mouse spleen (a-e) and liver cells (f-h) stained with antibodies against macroH2A (a,f) and Hoechst 22358 (b,g). Double-staining with antibodies against macroH2A and Hoechst (c,h), macroH2A and CENPB (d) and CENPB and Hoechst (e).
acetylated at lysine 12 (H4K12Ac) and lysine 8 (H4K8Ac), which are typically elevated in euchromatin, and histone H3 methylated at lysine 9 (H3K9Me), characteristic of heterochromatin (Jenuwein and Allis, 2001; Turner, 2002). H4K12Ac showed a very distinct intranuclear distribution in quiescent lymphocytes (Fig. 2a,c), being localized toward the interior of the nucleus, and completely excluded from the macroH2A-enriched centromeric foci. There was a clear region of exclusion of H4K12Ac in the form of a zone, approximately 0.5 µM in width around each chromocenter. We suggest the term ‘apocentric zone’ for this nuclear region. Antibodies against H4K8Ac showed a similar exclusion from centromeres and the apocentric zone (not shown). In contrast, antibodies generated against histone H3 dimethylated at lysine 9 (H3K9Me2), tended to bind most strongly to the apocentric zone (Fig. 2b,d) from which H4K12Ac and H4K8Ac were excluded. These observations suggest that there is an area of chromatin surrounding the chromocenters characterized by a unique pattern of core histone modifications and constituting a novel apocentric nuclear ‘compartment’. In contrast to the distribution of H3K9Me2 sites, an antibody prepared against the tri-methylated antigen (H3K9Me3) (Cowell et al., 2002) was largely confined to centromeric foci (Fig. 2e-g). This is in agreement with earlier data on non-centromeric localization of H3K9Me2 (Maison et al., 2002) and centromeric localization of H3K9Me3 (Cowell et al., 2002).

When these same antibodies were used with a non-differentiated mouse cell line (NIH/3T3 cells) induced to quiescence by serum deprivation, the results were very different, both H4K12Ac and H3H9Me2 being distributed throughout the nucleus (Fig. 2h-j). Thus, the segregation of H4K12Ac and H3H9Me2 that we observed in quiescent differentiated lymphocytes was absent from non-differentiated mouse cells. H3H9Me3 showed the same strong centromeric staining in proliferating NIH/3T3 cells as observed in lymphocytes (Fig. 2k-m).

Quiescent spleen lymphocytes may be reactivated in vitro, during which the cells leave the G0 state, and undergo several rounds of replication over a period of ~72 hours.
Epiogenetic chromatin markers

Concomitantly, the nuclei enlarge dramatically as much of the facultative chromatin decondenses (Setterfield et al., 1983). This transition allows direct comparisons to be made between the highly repressed state and active, cycling nuclei. T-lymphocytes from mouse spleen were reactivated with Concanavalin A (Con A), and the intranuclear localization of centromeric chromatin, macroH2A, H4K12Ac, H3K9Me2, H3K9Me followed. The relative amounts of macroH2A, H4K12Ac and H3K9Me2 in both quiescent and reactivating cells were also determined using semi-quantitative western blotting of total nuclear protein.

During reactivation, we observed significant changes in both the relative amounts and intranuclear localization of H4K12Ac and H3K9Me2. Western blots indicated a ~2.5-fold increase in H4K12Ac per nucleosome (Fig. 3a), and a ~2-fold decrease in H3K9Me2 per nucleosome during 70 hours of Con A-induced reactivation (Fig. 3b). H4K12Ac showed the same exclusion from centromeric heterochromatin as in quiescent cells, but now the apocentric zone of exclusion around the centromere was absent, and the immunofluorescence signal occupied much more of the nuclear volume (Fig. 3c). The distribution of H3 with methylated lysine 9 was little changed from the unreactivated pattern, with the highest abundance of the dimethylated form in the apocentric zone (Fig. 3d) and the trimethylated histone H3 occupying the centromeric foci (not shown). Thus, upon reactivation, acetylation of histone H4, a marker of transcriptionally active chromatin, spreads into the apocentric zone from which it was formerly excluded, and is no longer segregated from dimethylated histone H3K9Me2.

Changes in HP1 and Ikaros localization during T-cell reactivation

In proliferating mouse cells, HP1, especially the α and β isoforms, are associated with chromatin silencing and compaction and typically show strong staining of centromeric chromatin (Horsley et al., 1996; Wreggett et al., 1994). However, in quiescent mouse lymphocytes, HP1α gave weak staining distributed throughout the nucleus (Fig. 4a) and only HP1β showed strong enrichment in centromeric heterochromatin (Fig. 4e). HP1β retained its centromeric pattern throughout reactivation (Fig. 4f-h), whereas, in contrast, HP1α became localized in the vicinity of centromeric chromatin during reactivation (Fig. 4i,j).

Fig. 4. Localization of heterochromatin protein 1 (HP1) and Ikaros in resting and reactivated lymphocytes. Resting spleen lymphocytes (a,e) and lymphocytes treated with Con A for 36 hours (b-d,f-h) stained for HP1α (a,b), HP1β (c,f), Hoechst (c,g) and double-stained for HP1α and Hoechst (d), HP1β and Hoechst (h) and Ikaros and Hoechst (i,j).

Fig. 5. MacroH2A in reactivated lymphocytes. (a) Con A-activated lymphocytes from mouse spleen double-stained with antibodies against macroH2A and BrdU. (b) Nuclear proteins from resting (Con A –) and activated (Con A +) lymphocytes were stained with Coomassie (lanes 1, 2) and probed with antibodies against macroH2A (lanes 3, 4). (c) Fraction of proliferating, BrdU-positive lymphocyte nuclei (open columns) and fraction of nuclei with pericentromeric macroH2A distribution (solid columns), with standard errors. Lymphocytes were reactivated with Con A for 36 hours, pulsed with BrdU for 5 minutes and, at the times indicated, stained for macroH2A and BrdU. (d) Fraction of nuclei with pericentromeric macroH2A distribution. Lymphocytes were reactivated with Con A for 36 hours, then pulsed with BrdU for 1 hour [trichostatin A (TSA)] or 5 minutes (Na-but) and were incubated for 48 hours without histone deacetylase (HDAC) inhibitors (48), for the last 4 hours with 5 ng/ml TSA (TSA), or for last 24 hours with 2.5 mM Na-butyrate (Na-but). Cells were stained for macroH2A, and quantitated to reveal the distribution of macroH2A.
reactivation (Fig. 4b-d). However, unlike HP1\(\beta\) and macroH2A (see below), the distribution of HP1\(\alpha\) did not precisely coincide with the DAPI-bright centric foci, but extended beyond them (Fig. 4d) into portions of the apocentric zone defined by the high concentration of H3K9Me2.

During T-cell reactivation, there is a gradual build-up of the Ikaros transcriptional repressor, which, together with genes destined for repression, becomes localized close to pericentromeric heterochromatin (Brown et al., 1999). Indeed, when reactivated cells were stained for Ikaros, early in reactivation, we observed very little or no accumulation, whereas at later times, pericentromeric Ikaros became abundant (Fig. 4i,j). Further, in lymphocytes reactivated for 72 hours, the Ikaros signal extended beyond the boundary of the DAPI-defined pericentromeric chromatin into the apocentric zone (4j). Thus, in reactivated cells, the apocentric zone becomes enriched with two chromatin repressors, HP1\(\alpha\) and Ikaros, perhaps counteracting the increase in histone H4 acetylation in this subnuclear area.

Changes in macroH2A localization during lymphocyte reactivation

Reactivation of T-lymphocytes in vitro occurs in two phases: during the first 24-36 hours, cell and nuclear enlargement predominates, and is followed by a phase of rapid cell division. Most experiments were terminated by 72 hours after which cell death becomes significant. During the enlargement phase, little change in the distribution of macroH2A was observed, but this was followed by a steady and dramatic loss of centromeric macroH2A localization (Fig. 5a), until at 72 hours, this staining pattern was rare (Fig. 5c). Fig. 5b (lane 2) shows a substantial increase in the number of nuclear protein bands in ConA-treated cells that reflect the induction or upregulation of numerous nuclear proteins involved in lymphocyte reactivation and in cell proliferation. During the same period, there was only a small reduction of ~20% in total macroH2A per nucleus (Fig. 5b), suggesting that the loss of centromeric localization was not attributable to a simple dilution of macroH2A, and that synthesis of new macroH2A must accompany reactivation. Fully reactivated cells frequently showed a pronounced centrosomic localization of macroH2A (small arrows in Fig. 5a) as verified by co-localization with an antibody to gamma-tubulin (not shown). The centrosomic localization of macroH2A suggests that this cytoplasmic organelle could be a repository of protein displaced from the nucleus.

The apparent correlation between macroH2A redistribution and cell proliferation suggested a relationship between the loss of centromeric staining and replication. This possibility was investigated using BrdU and anti-BrdU antibodies to identify replicated and replicating nuclei (Fig. 5a). A 1-4-hour treatment with BrdU at the beginning of the proliferation phase, showed that by 36 hours, many cells had undergone at least one round of replication. We then focused on the changes in macroH2A localization in BrdU-positive cells between 36 hours and 72 hours of reactivation, and found a steady reduction in the proportion of BrdU-labeled cells with macroH2A in centromeres (Fig. 5c). This suggested that macroH2A was being progressively lost from the centromeres during, and perhaps as a consequence of the replication process.
We also wished to examine the effects of arresting the reactivation process. Simply withholding Con A leads to rapid apoptotic cell death, but treatment with the deacetylase inhibitors sodium butyrate or trichostatin A (TSA) has been shown to reversibly arrest proliferation in many cell types while retaining cell viability (e.g. Wharton et al., 2000). When reactivating lymphocytes at 48 hours were exposed to Na-butyrate or TSA, we observed a dramatic and rapid reaccumulation of macroH2A in centromeric foci (Fig. 5d). Thus, the loss of macroH2A in pericentromeric heterochromatin can be reversed in reactivated lymphocytes if proliferation is blocked by histone deacetylase (HDAC) inhibitors.

Intranuclear relocalization of macroH2A and HP1α in 3T3 cells

We then asked whether the specific pericentromeric chromatin-related relocalization of HP1α and macroH2A is unique to differentiated primary lymphocytes or reflects a general property of cell proliferation. Mouse 3T3 fibroblasts provide a particularly useful model system because they can be manipulated to undergo a transition from proliferation to quiescence (without differentiation) by contact inhibition, deprivation of growth factors or by treatment with HDAC inhibitors (Stiegler et al., 1998). Because, compared with lymphocytes, the level of endogeneous macroH2A is low in 3T3 cells, we transfected them with expression vectors encoding tagged protein fusions. To detect transitions in chromatin organization, we examined the localization of HP1α and macroH2A in 3T3 cells grown to ~50% confluency in the presence of 5 mM of Na-butyrate for 48 hours, by which time proliferation and replication cease, as documented by the absence of cells with metaphase chromosomes and the absence of BrdU incorporation (data not shown).

In proliferating 3T3 fibroblasts, full-length macroH2A was distributed nucleus-wide as small foci uncorrelated with the Hoechst-bright pericentromeric heterochromatin (Fig. 6, panels 1-2). In contrast, in quiescent cells, the localization of transfected macroH2A had changed dramatically, and became precisely coincident with pericentromeric heterochromatin (Fig. 6, panels 4 and 5) similar to that observed with quiescent lymphocytes. Murine macroH2A1.2 is a 372 amino acid long chimeric histone variant with an N-terminal histone H2A core domain more closely related to the H2A sequences of lower eukaryotes than vertebrates (Pehrson and Fuji, 1998), whereas the function of the 177 amino acid C-terminal ‘hismacro’ domain remains enigmatic (Ladurner, 2003). To determine which domain of macroH2A mediates its localization in heterochromatin, we construct containing only the histone H2A-like N-terminal domain of macro-H2A and lacking 229 of the C-terminal residues (mH2A1-140) was prepared. Upon transfection into 3T3 cells followed by treatment with Na-butyrate, the mutant H2A-like protein localized to pericentromeric heterochromatin (Fig. 6, panels 10, 11). It thus appears that the histone H2A N-terminal domain is sufficient for heterochromatic association. The N-terminal domain also contains sufficient information to target macroH2A to inactive X-chromosomes (Chadwick et al., 2001).

Because in these experiments macroH2A was ectopically expressed in 3T3 cells, we checked whether centromeric localization occurs with any overexpressed histone or is specific for macroH2A. 3T3 cells were co-transfected with macroH2A and either express-tagged histone H2A.1 or H2A.X. H2A.1 is an archetypal histone H2A (Albig et al., 1997) and H2A.X is involved in DNA repair (Sedelnikova et al., 2003). Cells were examined after double staining with a rabbit antibody against macroH2A (red) and mouse antibody against the express tag (green, Fig. 6 panels 12-15). The results clearly show that in these co-expressing cells only macroH2A became located to the centromeres upon Na-butyrate treatment, whereas the two other histones displayed a general nuclear staining independent of the state of quiescence.

In proliferating macroH2A-expressing 3T3 cells, the ectopically expressed macroH2A is distributed over the whole nucleus, whereas HP1α (Fig. 6, panel 3), and HP1β (not shown) both show a strong centromeric enrichment, in agreement with previous studies (Wreggett et al., 1994; Minc et al., 2001). However, when the proliferation of 3T3 cells was halted by butyrate treatment, HP1α became spread over the whole nucleus (Fig. 6, panel 6) at the same time as macroH2A accumulated in the centromeres (panel 4). As with lymphocytes, the heterochromatin markers, HP1β (panel 9) and H3K9Me3 (not shown) remained associated with centromeric heterochromatin regardless of the state of proliferation. Thus, in both mouse lymphocytes and 3T3 cells, HP1β and H3K9Me3 are constitutively enriched in centromeric heterochromatin whereas macroH2A and HP1α undergo striking changes in location. The similarity of the chromatin rearrangement suggests that in these two very different systems, the reciprocal exchange of macroH2A and HP1α may contribute to a common mechanism for epigenetic chromatin remodeling.

Discussion

The transitions of cells from the proliferative, cycling state to quiescence and vice versa are critical events in development, which, if disrupted, may lead to disease and death. Here we show that major changes in epigenetic chromatin markers in both the constitutive heterochromatin that surrounds centromeres and non-centromeric chromatin accompany the reactivation of quiescent T-lymphocytes from mouse spleen (Fig. 7). These changes involve the intranuclear localization of the core histone variant macroH2A, core histone modifications associated with gene activity (histone H4 acetylated at lysine 12 and 14) and repression (histone H3 dimethylated at lysine 9), and also in HP1α. Two other epigenetic markers common in transcriptional silencing, trimethylation of H3 lysine 9 and HP1β are confined to constitutive heterochromatin under all conditions examined. The reciprocal accumulation of macroH2A and HP1α observed during lymphocyte transitions were also found in 3T3 fibroblasts (Fig. 7), suggesting that these may reflect a general chromatin remodeling mechanism that can be activated in differentiated primary cells as well as in cell cultures.

In addition to changes in the components of pericentromeric heterochromatin, we observed an ‘apocentric’ zone, averaging approximately 0.5 µM in thickness around each centromere. This zone, defined in quiescent cells by the exclusion of H4K12Ac and enrichment in H3K9Me3, experiences the most prominent changes during the transition to proliferation (Fig.
H3K9Me2 is also associated with apocentromeric chromatin in differentiated mouse embryonic erythrocytes (Papova et al., 2003) but is more or less uniformly distributed in the nuclei of quiescent fibroblasts (Fig. 2), indicating that its pericentromeric localization is associated with terminal cell differentiation rather than quiescence. Remarkably, expression in quiescent fibroblasts of a single non-histone heterochromatin protein, MENT, is sufficient to cause a large-scale change in the location of H3K9Me2, which then appears in pericentromeric heterochromatin (Istominia et al., 2003). These authors suggested that the change might contribute to the irreversible inactivation of genes marked by H3K9Me2. Because MENT is not expressed in mouse lymphocytes, it remains to be seen which factor(s) lead to the large-scale chromatin remodeling reported here.

We view the apocentric zone not as a sharply demarcated nuclear compartment, but rather as a region of varying width that can be strongly influenced by the presence of pericentromeric heterochromatin. In Fig. 2b, for example, a gradient of H3K9Me2 is seen in the apocentric zone, with the highest concentration of this histone modification adjacent to the pericentromeric heterochromatin. Although this is the first cytological demonstration of an apocentric zone, evidence for a special nuclear environment surrounding pericentromeric heterochromatin has come from other studies. For example, genes destined to be repressed in maturing B- and T-lymphocytes become located in this region (Brown et al., 1999; Su et al., 2004). Concomitantly, the Ikaros transcriptional repressor is upregulated and co-localizes with the repressed genes (Brown et al., 1997). Similarly, repressed β-globin is located in the vicinity of centromeres (Schubeler et al., 2000), a location equivalent to the apocentric zone described here.

In many cell types, macroH2A enrichment is seen in the inactive X chromosome (Xi), and indeed, liver nuclei from female mice showed macroH2A foci over Xi and no centromeric label (Fig. 1f-h). In contrast, in lymphocytes there was no enrichment of macroH2A over the inactive X chromosome (Fig. 1a-e). The fact that in other tissues such as liver, there are many non-dividing cells that do not have large amount of pericentromeric macroH2A shows that the pericentromeric association is specific for certain cell types and is not simply a matter of proliferation state. Other experiments suggest that the two patterns of macroH2A distribution are not always mutually exclusive: a study of murine embryonic cells showed that macroH2A1.2 could associate with pericentromeric heterochromatin and inactive X chromosome in the same nucleus (Costanzi et al., 2000). During differentiation, macroH2A foci became co-localized with Xi by day 8, but by day 16, multiple foci that co-localized with DAPI-bright centromeric chromatin appeared in 50% of cells.

The finding that, in quiescent lymphocytes, macroH2A is concentrated in constitutive rather than facultative heterochromatin is surprising, because this protein might be expected to contribute to the spreading of non-centromeric facultative heterochromatin either by direct transcriptional inhibition at a promoter (Perche et al., 2000) or by interfering with chromatin remodeling by SWI/SNF (Angelov et al., 2003). However, macroH2A associates with the inactive X chromosome only after inactivation is established and thus is more likely to be involved in the maintenance of facultative heterochromatin rather than its initiation (Csankovszki et al., 1999; Mermoud et al., 1999). It is possible that the association of macroH2A with constitutive heterochromatin in quiescent cells reflects a similar involvement in the maintenance of repression, possibly compensating for the absence of repressive factors such as Ikaros and HP1α.

Because total macroH2A levels show little variation during lymphocyte activation, the changes in localization presumably involve a dynamic relocation process. The loss of macroH2A from pericentric heterochromatin during lymphocyte reactivation (Fig. 5) indicates a mechanism that is at least in part replication dependent. In contrast to the gradual loss of centromeric macroH2A seen during proliferation, imposing quiescence in activated lymphocytes or 3T3 cells by treatment with HDAC inhibitors resulted in the rapid accumulation of macroH2A over centromeres in the absence of replication. This result further supports a mechanism that directs intranuclear macroH2A deposition in a manner that is dependent on the proliferative state of the cell and/or histone modification state but not on replication per se. MacroH2A lacking the non-histone domain showed the same intranuclear localization pattern as the full-length protein, showing that its heterochromatin localization is determined by the histone portion. The mode of macroH2A deposition in chromatin provides an interesting parallel to other functionally significant histone variants such as H3.3 (Ahmad and Henikoff, 2002) and H2A.Z (Rangasamy et al., 2003) that are also independent of replication and may impose epigenetic marks on certain chromosomal domains leading to inhibition of heterochromatin spreading. H2A.Z is targeted to pericentromeric heterochromatin during early differentiation of undifferentiated mouse embryo cells into trophoblasts (Rangasamy et al., 2003). In these cells, macroH2A is not located at pericentromeric heterochromatin. However, in transformed cell lines, H2A.Z is not enriched in pericentromeric heterochromatin but located in the heterochromatin on the arms of chromosomes (Rangasamy et al., 2004). Therefore it appears that in the systems examined so far, the localizations of H2A.Z and macroH2A are mutually exclusive, perhaps allowing constitutive heterochromatin to fulfill different functions at different stages of development. The recent report of a special chromatin remodeling ATPase involved in the positioning of histone H2A.Z (Krogan et al., 2003) suggests that specific histone recruitment complexes may also exist for other H2A variants, including macroH2A.

HP1 is a universal epigenetic marker of constitutive heterochromatin found in all major types of eukaryotes and involved in chromatin-mediated repression and heterochromatin spreading, as well as in many other nuclear events (Li et al., 2002; Singh and Georgatos, 2002). Our work reveals a striking difference between HP1β, which remains confined to pericentromeric heterochromatin in both differentiated lymphocytes and 3T3 fibroblasts, and HP1α which is uniformly distributed in quiescent cells, but relocated to both apo- and peri-centromeric chromatin upon cell activation (Fig. 4d). Two other recent studies documented a unique property of HP1α in chromatin binding: it is the only HP1 subtype firmly associated with soluble chromatin (Gilbert et al., 2003) and it binds soluble heterochromatin in vitro via the hinge region rather than via the chromodomain that mediates HP1 binding to methylated histone H3 in centromeric heterochromatin (Meehan et al., 2003). This dual mode of
chromatin interaction makes HP1α a likely candidate to participate in the formation of facultative heterochromatin in differentiated mammalian cells.

These findings reveal a new layer of complexity in our understanding of the large-scale changes in nuclear organization that accompany the transitions between quiescence and proliferation. Surprisingly, major changes are seen not only in facultative chromatins where it might be expected, but also in the supposedly invariant constitutive heterochromatin surrounding the centromeres. The identification of an apocentric zone surrounding pericentric heterochromatin which accumulates repressive histone modifications, correlates well with previous data showing that genes destined for repression in activating lymphocytes are relocated to the neighborhood of the centromeres (Brown et al., 1997; Schubeler et al., 2000). The gradual disappearance of the boundary between the apocentric zone and the rest of the nucleus as lymphocyte activation proceeds, and its absence from undifferentiated cells suggest that it is directly involved in developmentally regulated chromatin repression associated with cell maturation and terminal differentiation.

We thank W. Earnshaw for antibodies against CENPB, S. Smale for antibodies against Ikaros, and B. Osborne for advice on spleen lymphocyte isolation and reactivation. This work was supported in part by NIH grants GM-43786 to C.L.W. and GM-59118 to S.A.G.

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