Histone H4-Lysine 20 Monomethylation Is Increased in Promoter and Coding Regions of Active Genes and Correlates with Hyperacetylation*

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Methylation and acetylation of position-specific lysine residues in the N-terminal tail of histones H3 and H4 play an important role in regulating chromatin structure and function. In the case of H3-Lys4, H3-Lys9, H3-Lys27, and H4-Lys20, the degree of methylation was variable from the mono- to the di- or trimethylated state, each of which was presumed to be involved in the organization of chromatin and the activation or repression of genes. Here we investigated the interplay between histone H4-Lys20 mono- and trimethylation and H4 acetylation at induced (β-major/β-minor globin), repressed (c-myc), and silent (embryonic β-globin) genes during *in vitro* differentiation of mouse erythroleukemia cells. By using chromatin immunoprecipitation, we found that the β-major and β-minor promoter and the β-globin coding regions as well as the promoter and the transcribed exon 2 regions of the highly expressed c-myc gene were hyperacetylated and monomethylated at H4-Lys20. Although activation of the β-globin gene resulted in an increase in hyperacetylated, monomethylated H4, down-regulation of the c-myc gene did not cause a decrease in hyperacetylated, monomethylated H4-Lys20, thus showing a stable pattern of histone modifications. Immunofluorescence microscopy studies revealed that monomethylated H4-Lys20 mainly overlaps with RNA pol II-stained euchromatic regions, thus indicating an association with transcriptionally engaged chromatin. Our chromatin immunoprecipitation results demonstrated that in contrast to trimethylated H4-Lys20, which was found to inversely correlate with H4 hyperacetylation, H4-Lys20 monomethylation is compatible with histone H4 hyperacetylation and correlates with the transcriptionally active or competent chromatin state.

It has been proposed that distinct post-translational histone modifications act sequentially or in combination to form a “histone code” within chromatin (1). Acetylation and methylation of specific histone lysine residues can serve as a mark of either euchromatin or silent heterochromatin. Although methylation of H3-Lys4, H3-Lys9, and H3-Lys27 has been linked to transcriptional activation and protection of euchromatin, H3-Lys9, H3-Lys27, and H4-Lys20 methylation is generally thought to be associated with gene repression and heterochromatin formation (2–4). In this regard it must be noted that histone lysine residues can be mono-, di-, or trimethylated (5), thus extending the coding potential of a methylatable lysine position. Previous studies, however, focused only on detection of H3 (for review see Refs. 3 and 4) or H4 (6–8) lysine methylation regardless of the methylation status. Recently, it was shown that a distinction between di- and trimethylation of various lysines of histone H3 is important for processes of transcriptional regulation or gene silencing (9–11). Moreover, studies that focused on the *in vivo* distribution of mono-, di-, and trimethylated H3-Lys9 and H3-Lys27 demonstrated that mono- and dimethylated H3-Lys9 and H3-Lys27 are specifically localized to silent domains within euchromatin, whereas trimethylated H3-Lys9 and monomethylated H3-Lys27 were enriched at pericentric heterochromatin (12, 13). In contrast to findings suggesting a role for H4-Lys20 methylation in regulating gene expression, a recently published study demonstrates that H4-Lys20 methylation, in particular trimethylation, plays no apparent role in gene regulation or heterochromatin function but is involved in DNA damage response in fission yeast (14).

The first studies on methylation of H4-Lys20 with antibodies to dimethylated H4-Lys20 reported that dimethylated H4-Lys20 acts in antagonizing H4-Lys16 acetylation (7, 8) and does not correlate with gene activity (6). Trimethylated H4-Lys20 was found to be a marker of constitutive heterochromatin in murine interphase and metaphase cells (15) enriched at pericentric heterochromatin (16). Furthermore, it was shown that trimethylated H3-Lys9 is required for induction of H4-Lys20 trimethylation and that trimethylation of histone H3-Lys9 and H4-Lys20 functions as a repressive mark in gene-silencing mechanisms (5). ChIP experiments demonstrated that the human histone deacetylase SirT1-induced deacetylation of H4-Lys16 is accompanied by H1 enrichment and the spreading of trimethylated H3-Lys9 and monomethylated H4-Lys20 at the promoter region of a repressed reporter system (17). Histone H3-Lys27 trimethylation and H4-Lys20 monomethylation were shown to be associated with Xist expression in ES cells and seem to mark the initiation of X inactivation. However, both H3-Lys27 trimethylation and H4-Lys20 monomethylation are maintained in the absence of transcriptional repression (18). Further investigations indicating a role of methylated H4-Lys20 in gene activation were performed by Beisel *et al.* (19), who found that the *Drosophila* epigenetic activator ASH-1, a histone methyltransferase, activates transcription by dimethylation of H3-Lys4, H3-Lys9, and H4-Lys20 at the promoter of target genes. Significant differences in subnuclear localization of the mono- and trimethyl versions of histone H4-Lys20 were recently observed during mouse embryogenesis (20). H4 monomethyl Lys20 was shown to be elevated in proliferating cells; in contrast, histone H4 trimethyl Lys20 became enriched in differentiating cells during the mouse developmental process. Most recently, we investigated the changes in Lys20 methylation

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2 The abbreviations used are: ChIP, chromatin immunoprecipitation; RnApol II, RNA Polymerase II; RT-PCR, Reverse transcription-polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PMSF, phenylmethylsulfonyl fluoride; MEL, mouse erythroleukemia; DAPI, 4,6-diamidino-2-phenylindole; MeSO, dimethyl sulfoxide; HILIC, hydrophilic interaction liquid chromatography; ESI-MS, electrospray ion-mass spectrometry; Ab, antibody; FITC, fluorescein isothiocyanate; fwd, forward; rev, reverse.
states of various acetylated H4 histones as well as the interplay between the various H4-Lys\textsuperscript{20} methylation states and acetylation during in vitro differentiation of mouse erythroleukemia cells (16). We found that trimethylated H4-Lys\textsuperscript{20} histones increase during the differentiation process of mouse erythroleukemia (MEL) cells and that these hypermethylated H4 histones completely preclude histone H4 tri- and tetracetylation.

With these observations in mind, we decided to investigate the methylation states of H4-Lys\textsuperscript{20} in correlation with acetylation at induced (β major/minor globin) or repressed (c-myc) genes or at silent genes like embryonic β-globin in the MEL cell system. The β-globin locus, containing the erythroid-specific and developmentally regulated β-globin genes, is a particularly informative system for investigating the structure/function of histone modification patterns. The present study indicates that monomethylated H4-Lys\textsuperscript{20} is not a principal feature of repressed gene regions. On the contrary, after induction of expression of the adult β-globin gene, we found increased monomethylated H4-Lys\textsuperscript{20} paralleled by hyperacetylation of H4 at the β-major and β-minor promoter and β-globin transcribed region. Increased levels of monomethyl H4-Lys\textsuperscript{20} and hyperacetylated H4 at the promoter (exon 1) and the transcribed exon 2 region were also found at the highly expressed c-myc gene. Most interestingly, monomethylation and hyperacetylation of the H4 histone also persist at low expression levels of c-myc, indicating that neither hypoacetylation nor decreased monomethylation of H4 is a prerequisite for c-myc gene down-regulation.

To confirm the findings obtained with ChIP, we investigated the H4 acetylation/methylation pattern using hydrophilic interaction liquid chromatography. These experiments revealed that monomethylation of H4-Lys\textsuperscript{20} excludes neither acetylation of H4-Lys\textsuperscript{16} nor hyperacetylation of the respective H4 histone molecule. These experiments therefore support our ChIP results showing a negative correlation between the patterns of trimethylated H4-Lys\textsuperscript{20} and H4 hyperacetylation but a positive correlation between the patterns of monomethylated H4-Lys\textsuperscript{20} and H4 hyperacetylation at distinct gene regions of the c-myc and β-globin genes. Immunofluorescence microscopy studies showed that trimethyl H4-Lys\textsuperscript{20} is enriched mainly within DAPI-dense regions, which almost completely overlap with HP1β-stained heterochromatin largely excluded, however, from active chromatin (RNP10 II) regions. In contrast, monomethyl H4-Lys\textsuperscript{20} mainly overlaps with RNP10 II-stained euchromatic regions and is largely excluded from HP1β-stained heterochromatin, indicating an association with transcriptionally engaged chromatin. To summarize our results, we conclude that hyperacetylated and monomethylated H4-Lys\textsuperscript{20} may be important in maintaining the transcriptionally active or competent chromatin state.

**MATERIALS AND METHODS**

**Cell Culture**—MEL cells (line F4N) were grown in Dulbecco’s minimum Eagle’s medium containing 2× nonessential amino acids, 1× penicillin/streptomycin, and 10% fetal calf serum. Cells were cultured at non-saturating density of 5×10\textsuperscript{4}/ml at 37 °C and 5% CO\textsubscript{2}. Differentiation was induced by the addition of 2% Me2SO for 96 h or 1.75 mM sodium butyrate for 72 h. DNA was purified by two extractions with phenol/chloroform and precipitated with ethanol. Purified DNA was resuspended in 50 μl of water and quantified using picogreen fluorescence (Molecular Probes).

**PCR**—The following primers were used for PCR: HS6 (product size 245 bp) fwd 5’-TCTTTAGTGAGGCAACATTC-3’; rev 5’-AGACGAGGAAAGGAGGAGT-3’;

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**Reverse Transcription (RT)-PCR**—Expression analysis by RT-PCR was performed with the Titanium One-step RT-PCR kit (Clontech) by using the following primer pairs: GAPDH fwd 5’-AGCGGAAGCCACTCCACATCTTCCA-3’ and rev 5’-ATCCAGACGAGGACACATGTGGGAGA-3’; myc fwd 5’-GGCTGAGATTTCTTGGCGGTGGA-3’ and rev 5’-TCTTTGCGCGCAGCCTGGTAGGA-3’; and β-globin fwd 5’-GGATTCTTACACCCTTGGGACCCCACG-3’ and rev 5’-GGATTCTTACACCCTTGGGACCCCACG-3’. The RT-PCR analysis of the two adult β-globin genes (β-minor and β-major) was performed with a primer pair that amplifies β-minor and β-major globin cDNAs. To distinguish the two β-globin products, restriction enzyme digestion with PvuI was performed, which generates two β-minor globin products (234 and 107 bp) and one undigested β-major globin product (341 bp). PCR samples were separated on a 2% agarose gel, standardized by using control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) signal, and quantified.
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CTGATGAGGATCAATGTTAG-3′, rev 5′-TCATCATCTCTG-AGCAGGCTGTA-3′; HS2 (product size 295 bp) fwd 5′-TCTTTAATCTGCTTAAAGAAGGC-3′, rev 5′-GGACTCTATACGCTGAA-3′; HS1 (product size 260 bp) fwd 5′-CTATCCCTTGAGACATAAACAGAAG-3′, rev 5′-AGAAACTCCCTCACTGAA-3′; E\textsubscript{y}-globin promoter (product size 245 bp) fwd 5′-AGAGGATTTTTTGGAGAGGA-3′, rev 5′-CACAGGATGTCCACGAAGATCTAGA-3′; B-H\textsubscript{1}-globin promoter (product size 246 bp) fwd 5′-AGTCTCCAGGTGAAGAATGAAAAG-3′, rev 5′-ATGAAACCTTGCAAATTTCTGC-3′; B-major globin promoter (product size 179 bp) fwd 5′-GTCATCCAGGATGAGAAATG-3′, rev 5′-AGCACTGATCCTACCTCCT-3′; B-\textsubscript{\beta}-globin transcribed region (product size 179 bp) fwd 5′-CCACGCGGTCTTTAGTGATAG-3′, rev 5′-ATGCAAGAAGTAATGAGGATAAGA-3′, rev 5′-ATGCAATCATTCTGAGTGCAAAGTA-3′, rev 5′-GCAATGCTCACAACAGACTTCA-3′; B-\textsubscript{\beta}-globin transcribed region 2 (product size 246 bp) fwd 5′-CCACGCGGTCTTTAGTGATAG-3′, rev 5′-AGATAGCCAGGGAAGAAATG-3′, myc exon 1 and 2 (product size 264 bp) fwd 5′-GGAGAGGAGGAAAGAGATTG-3′, rev 5′-CTGGAATATTACACTGCGTACA-3′; and myc exon 2 transcribed region (product size 239 bp) fwd 5′-TTTCATCTACGAAGACAGCTAGC-3′, rev 5′-CTCCTCTCAAGTACTGGATCT-3′.

W\textsubscript{e}B began with the isolation of total histones and the fractionation of histone proteins by reversed-phase HPLC. The histone isoforms obtained by HILIC were identified by ESI-MS as described (23).

RESULTS

Distribution of Monomethyl H4-Lys\textsuperscript{20} and Trimethyl H4-Lys\textsuperscript{20} during Interphase and at Metaphase in MEL Cells—To localize modified histones, we used immunofluorescence with monospecific antibodies. The distribution of mono- and trimethylated H4-Lys\textsuperscript{20} during MEL cell interphase was investigated by dual immunofluorescence staining (Fig. 1). We found that trimethylated H4-Lys\textsuperscript{20} is enriched mainly in DAPI-dense regions with almost complete overlapping with HP1\textsuperscript{+} stained heterochromatin (Fig. 1A), which is largely excluded from active chromatin (RNApol II) (Fig. 1B) regions. In contrast, monomethylated H4-Lys\textsuperscript{20} mainly overlaps with RNApol II-stained euchromatic regions (Fig. 1D) and not with DAPI-dense regions (Fig. 1C), thus indicating an association with transcriptionally engaged chromatin. A comparable distribution pattern of both monomethylated (data not shown) and trimethylated H4-Lys\textsuperscript{20} was also seen in differentiated MEL cells (16).

Mono- and Trimethyl H4-Lys\textsuperscript{20} Histones Are Enriched after Induction of MEL Cell Differentiation—We next asked whether the levels of modified histones change during the MEL cell differentiation process using Me\textsubscript{SO}. Histones from induced and uninduced MEL cells were prepared and run on SDS-PAGE. After blotting to nitrocellulose, specific antibodies to mono-, di-, and trimethylated H4-Lys\textsuperscript{20} acetylated...
specific sequence enrichment in the mouse β-globin gene region. In order to determine the enrichment or depletion of immunoprecipitation for a specific antibody, the total amount of DNA in the bound fraction was measured with the pico green DNA quantification method, and an equal amount of input DNA (before immunoprecipitation) was run in parallel as a reference standard. Fig. 4 shows, as a representative panel of data, the specific PCR products generated after different cycle numbers for the β-globin gene exon 2 region using anti-tetracetylated H4 antibody for immunoprecipitation. To calculate the relative abundance of each sequence, we used the difference in the number of PCR cycles for the bound and the input fraction needed to reach a fixed threshold value. The enrichment or depletion of a sequence in the bound fraction was calculated from the difference between the threshold cycle number for the bound and for the input fraction (28). By using this data analysis method, we were able to calculate a 13-fold increase in hyperacetylated H4 at the β-globin exon 2 region in the bound fraction as compared with the input fraction of induced MEL cells. Data from the analysis of MEL cells before and after induction of differentiation are given in Fig. 5, which shows the distribution of hyperacetylated H4 and the acetyl H4-Lys16 (Fig. 5, A and B, respectively) as well as the mono- and trimethylated H4-Lys20 (Fig. 5, C and D, respectively) across the mouse β-globin locus. In uninduced MEL cells, hyperacetylated histone H4 was enriched to about 5-fold in the locus control region at hyperacetylated H4 and enriched at about 1.5-fold and that of trimethylated H4-Lys20 about 2-fold (Fig. 2). The level of acetylated H4-Lys16 and the level of hyperacetylated histone H4 were clearly diminished in Me2SO-treated MEL cells as compared with untreated controls (Fig. 2). These results prompted us to analyze the relationship between the distribution of various epigenetic marks and gene activation or repression during MEL cell differentiation.

Because it is well known that during MEL cell differentiation the adult β-globin gene is largely expressed (24) and the c-myc gene is rapidly down-regulated (25–27), we used these gene loci to investigate histone H4 acetylation and methylation status at distinct sites. As determined by RT-PCR (Fig. 3), the increase in β-major globin transcript is about 20-fold and that in β minor about 3-fold after 96 h of induction (Fig. 3, A and B). The myc transcripts decreased by about 10–15-fold after 96 h of induction, which is consistent with earlier reports (25–27) on the down-regulation of this gene, whereas the GAPDH transcript levels remained unchanged before and after induction (Fig. 3, C and D).

Examination of Acetyl H4-Lys16 and Hyperacetyl H4 Pattern in Differentiated and Untreated MEL Cells at the β-Globin Gene Region Using ChIP—Using various antibodies specific to mono- or trimethylated H4 lysine 20 and acetylated H4 lysine 16 as well as hyperacetylated H4 in chromatin immunoprecipitation studies, we determined the extent of

H4-Lys20, and hyperacetylated H4 were used for immunological Western blot analysis. Although the level of dimethylated H4–Lys20 did not change after treatment with Me2SO, the monomethylated H4–Lys20 level increased about 1.5-fold and that of trimethylated H4–Lys20 about 2-fold (Fig. 2). The level of acetylated H4–Lys16 and the level of hyperacetylated histone H4 were clearly diminished in Me2SO-treated MEL cells as compared with untreated controls (Fig. 2). These results prompted us to analyze the relationship between the distribution of various epigenetic marks and gene activation or repression during MEL cell differentiation.

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Pattern of Hyperacetylation and Lys\textsuperscript{20} Monomethylation of H4

FIGURE 3. Expression of the β-major globin and β-minor globin and c-myc gene in MEL cells before and after induction. A, RT-PCR analysis of two adult β-globin genes performed with primer pairs that coamplify the respective β-minor and β-major globin cDNAs. After 25 PCR cycles, products were digested and thereafter loaded on a 2% agarose gel. Quantification of digested PCR products (see “Materials and Methods”) of cDNA from uninduced (−DMSO) and induced Me\textsubscript{2}SO (+DMSO) MEL cells was performed. B, representative gel is shown. C, RT-PCR analysis of the c-myc and the GAPDH gene. Quantification of PCR products of the respective cDNAs from untreated (−DMSO) and induced (+DMSO) MEL cells was performed. D, representative gel is shown. M, DNA molecular weight marker; +D, with Me\textsubscript{2}SO (DMSO) treatment; −D, without Me\textsubscript{2}SO treatment.

FIGURE 4. A representative panel of data generated by semiquantitative PCR using a primer pair for the β-globin gene exon 2 region of induced MEL cells. An antibody to tetraacetylated H4 was used for immunoprecipitation. PCR was stopped after the given numbers of cycles and the samples were loaded on a 2% agarose gel. The gel was scanned using a Typhoon imager (Amersham Biosciences) and quantified with the Bio-Rad Quantity One software. Intensity is given in arbitrary units. A representative gel is shown below the curve of the bound and the input fractions. Enrichment of a sequence in the bound fraction was calculated from the difference between the threshold cycle number for the bound fraction (C\texttextsuperscript{b}) and the threshold cycle number for the input DNA (C\texttextsuperscript{d}). As control, we used an isotype-matched antibody for immunoprecipitation. M, DNA molecular weight marker.

molecules, using a specific antibody able to recognize acetylated H4-Lys\textsuperscript{16}. The acetylation pattern of H4-Lys\textsuperscript{16} across the β-globin region did not resemble the hyperacetylation pattern of H4 with the exception of the HS3 region, which showed a 3-fold increase after induction. This increase is comparable with the 4-fold increase in hyperacetylated H4 histone (Fig. 5B). In this context it must be mentioned that the anti-acetylated H4-Lys\textsuperscript{16} antibody used, although mainly and specifically detecting acetylated lysine 16 of histone H4, is not able to distinguish whether or not other H4 sites are also acetylated. Likewise, we cannot rule out the possibility that the anti-tetraacetylated H4 antibody used, which reacts mainly with hyperacetylated H4 histones (di-, tri-, and tetraacetylated isoforms), also detects monoacetylated H4. Despite this possible unspecificity, the anti-tetraacetylated H4 antibody is used for detecting transcriptionally active chromatin regions (for example see Ref. 30).

Pattern of Monomethyl and Trimethyl H4-Lys\textsuperscript{20} in the β-Globin Gene Region—To investigate further whether mono- and/or trimethylation of histone H4-Lys\textsuperscript{20} could be related to activation or inactivation of the β-globin gene, we determined the methylation pattern of H4 histone lysine 20 (Fig. 5, C and D). Although in untreated control MEL cells the monomethylated H4-Lys\textsuperscript{20} was uniformly low across the entire region with a slight increase only in the β-minor promoter region, the monomethylation peaks significantly increased by about 3–4-fold at the β-major promoter and in the transcribed exon 2 region of the globin gene after induction of differentiation (Fig. 5C). Distribution of trimethylated H4-Lys\textsuperscript{20}, however, was quite different (Fig. 5D). Whereas the inactive Ey and H1B promoters showed an enrichment of trimethylated H4-Lys\textsuperscript{20}, all other regions displayed normal or diminished trimethylation. In this context, the various H4-Lys\textsuperscript{20} mono- and trimethylation patterns of HS6 and the other hypersensitive sites examined (HS1–3) are worth noting. In contrast to the modest H4-Lys\textsuperscript{20} monomethylation of HS1–3 in uninduced and induced MEL cells, we observed an increase in H4-Lys\textsuperscript{20} monomethylation of HS6 after MEL cell induction (Fig. 5C). On the other hand, a decrease in H4-Lys\textsuperscript{20} trimethylation of HS6 similar to that found in HS1–3 after induction was not detected (Fig. 5D). Summarizing our results, we concluded that a pronounced negative correlation exists between patterns of H4-Lys\textsuperscript{20} trimethylation and H4 hyperacetylation (compare Fig. 5, A and D), whereas a positive correlation exists between the patterns of H4-Lys\textsuperscript{20} monomethylation and H4 hyperacetylation in distinct regions of the β-globin gene (compare
Fig. 5. A and C, β-major promoter and transcribed β-globin gene region.

Acetylation and Methylation Mapping of Other Mouse Genes—We conducted similar experiments with the c-myc exon 1 and exon 2 region. During the induction of MEL cells with Me2SO, the c-myc mRNA level decreased early (2 h) and late (96 h, see Fig. 3B) with a transient increase at 24 h after inducer addition (25–27). We used the c-myc gene to determine the consequences of down-regulation of a gene during the Me2SO-induced differentiation process on the acetylation and methylation status of histone H4. Both the myc exon 1 region, where the P1 and P2 myc promoters are situated (27), and the transcribed myc exon 2 region showed a considerable amount of hyperacetylated H4 before induction of differentiation (Fig. 6A). A decrease in histone H4 hyperacetylation was not found at the promoter nor in the
exon 2 region after 96 h of Me2SO treatment (Fig. 6A). In the promoter region only 3-fold enrichment of monomethylated H4-Lys20 was detected, whereas the exon 2 region showed substantial H4-Lys20 monomethylation (Fig. 6C). Similar to the result achieved with hyperacetylation, a decrease in monomethylated H4-Lys20 was not found in exon 1 nor in exon 2 region of the c-myc gene after induction of differentiation (Fig. 6C). At neither of the myc exons analyzed was an enrichment of trimethylated H4-Lys20 observed before or after induction (Fig. 6D).

Histone H4-Lys20 Monomethylation Is Compatible with Histone H4 Hyperacetylation—Investigations by Nishioka et al. (8) concerning the human methyltransferase PR-Set7 have shown that methylation of H4-Lys20 and H4-Lys16 acetylation are inversely correlated. Because acetylation of H4-Lys16 in human cells is regarded as a mark of transcriptionally active chromatin (31), the authors suggested that methylated H4-Lys20 maintains silent chromatin. The study by Nishioka et al. (8) did not discriminate between the individual methylation states. As PR-Set7 is known to be a monomethyltransferase, the incompatibility observed between H4-Lys20 methylation and H4-Lys16 acetylation obviously should be caused by H4-Lys20 monomethylation. Most interestingly, the ChIP experiments in the present study revealed a positive correlation between patterns of monomethylated H4-Lys20 and hyperacetylated H4 or acetylated H4-Lys16 in the case of myc exons 1 and 2 (Fig. 6, A–C) and between monomethylated H4-Lys20 and hyperacetylated H4 in the β-major promoter and the exon 2 region of the β-globin gene (Fig. 5, A and C). To confirm our ChIP results, we used immunofluorescence to examine the distribution of mono- or trimethylated H4-Lys20 and hyperacetylated H4 during MEL cell interphase by dual immunofluorescence staining (Fig. 7). We found that trimethylated H4 Lys20 excludes epitope recognition of the anti-tetraacetylated H4 antibody (Fig. 7A), whereas monomethylated H4 Lys20 mainly overlaps with hyperacetylated H4 regions (Fig. 7B). To further support our findings with an additional method, we induced MEL cells with the deacetylase inhibitor butyrate for 72 h, and we investigated the H4 acetylation/
methylated pattern using a hydrophilic interaction liquid chromatographic procedure enabling the simultaneous separation of non-, mono-, di-, and trimethylated lysine 20 of non-, mono-, di-, tri-, and tetraacetylated H4 histones (Fig. 8). As shown, H4-Lys20 mono- or dimethylation does not preclude acetylation of the neighboring H4-Lys16 (Fig. 8, arrows), and tetraacetylated H4 exists in an H4-Lys20 mono- (Fig. 8, arrowhead) and dimethylated form. A negative interplay, however, exists between H4-Lys20 trimethylation and H4 hyperacetylation, which was previously demonstrated by Sarg et al. (16) and is also supported by the present report (Figs. 5–7).

DISCUSSION

The goal of this study was to investigate the relationship between acetylation/methylation marks in histone H4 and gene activation or repression in the course of in vitro differentiation of MEL cells. We performed chromatin immunoprecipitation assays to analyze genes largely expressed like β-major and β-minor globin genes, as well as quiescent genes like the embryonic Ey and βH1 genes, and the rapidly down-regulated but transcriptionally competent genes like the c-myc gene.

As to the function of histone H4-Lys20 methylation, there is no clarity at present. Although in the past methylated H4-Lys20 histone was largely believed to associate with transcriptionally active rather than repressed genes (32, 33), recently published reports indicate that methylation histone H4-Lys20 is associated with silent chromatin (7, 8). However, many of these reports disregard the fact that a specific lysine of histones can be methylated in vivo to form monomethyl-, dimethyl-, or trimethyl-lysine (34). Therefore, it is not possible to surmise the transcriptional state of a gene based on the fact that a given lysine in its histone is methylated or not. Instead, the precise methylation state of the lysine has to be used as an indicator of an active or repressed gene (9).

By using specific antibodies, this study demonstrates that mono- and trimethylated H4-Lys20 histones are localized to distinct domains of chromatin (Fig. 1). Trimethylated H4-Lys20 is mainly enriched in DAPI-dense regions (Fig. 1, A and B, and Fig. 7A), reflecting the characteristic accumulation at pericentric heterochromatin (data not shown). Major functions of trimethylated H4-Lys20 heterochromatin include the formation of a specialized chromatin structure around centromeres, which is vital to the structural integrity of the centromere, and the protection of genome stability by silencing transposable elements (35, 36). However, outside the DAPI-dense regions, we found additional weak trimethylation of H4-Lys20 (Fig. 1, A and B, and Fig. 7A). The 2–3-fold increase in trimethylated H4-Lys20 at the embryonic quiescent Ey or βH1 promoter region (Fig. 5D) might reflect this type of H4-Lys20 trimethylation. It must be mentioned that the repressed myc promoter and transcribed gene region did not show enrichment of trimethylated H4-Lys20 in our ChIP experiments (Fig. 6), indicating that trimethylated H4-Lys20 might play a role in repression of some genes but is not a generalized mark of gene repression.

In contrast to H4-Lys20 trimethylation, monomethylated H4-Lys20 histones are excluded from DAPI-dense regions and mainly overlap with RNApol II-stained euchromatic regions (Fig. 1) and regions where histone H4 is hyperacetylated (Fig. 7A), indicating an association with transcriptionally engaged chromatin. Our ChIP experiments support this assumption, because we observed increased monomethylation of H4-Lys20 at the promoters and transcribed gene regions of active genes (β-major globin gene, c-myc gene). There is a pronounced positive correlation between patterns of hyperacetylated and monomethylated histone H4-Lys20, most obviously in the β-major promoter and the exon 2 region of the β-globin gene (Fig. 5, A and C) as well as in the c-myc exon1 (promoter region) and c-myc exon 2 (transcribed gene) (Fig. 6). Most unexpectedly, we also found high levels of hyperacetylated and monomethylated histone H4-Lys20 in the c-myc exon 1 and exon 2 region after induction of MEL cells (96 h of Me2SO treatment), when c-myc expression is substantially diminished. The down-regulation of the c-myc gene in MEL cells induced to differentiate by Me2SO treatment is known to be the result of both a transcriptional inhibition consisting of a block of transcript elongation and a post-transcriptional regulation consisting of an increased rate of mRNA degradation (37). Because the expression of c-myc is known to decline in a polyphasic manner during the MEL cell differentiation process (25, 26), we suppose that the c-myc gene is still engaged in the transcription initiation process, persisting in a transcriptionally competent condition and thus explaining the consistently high levels of hyperacetylated and monomethylated H4 histones. The finding that the RNA pol II complex is located at the c-myc P2 promoter (exon 1), even when c-myc is not expressed (27), might explain our results. Furthermore, a correlation between acetylation and active transcription is not imperative. For instance, the inactive β-major and β-minor globin
gene promoter (22, 24) and the inactive transcribed globin gene region (Fig. 5A) of induced MEL cells or the transcriptionally inactive β-mi-
nor globin promoter in yolk sac (22) are all hyperacetylated. Therefore, we assume that hypoacetylation of H4 is not a prerequisite of c-
or globin promoter in yolk sac (22) are all hyperacetylated. Therefore, we assume that hypoacetylation of H4 is not a prerequisite of c-
We further investigated whether hyperacetylation in down-regulated c-
terned MEL cells with the deacetylase inhibitor butyrate (Fig. 8). Taken together, in contrast to H4-Lys20 trimethylation at the promoter of target genes in Drosophila. This trivalent methylation pattern established by ASH-1 seems to prevent the interaction of repressors with
We showed recently (16) that histone H4 hyperacetylation (tri- and
tetraacetylation) precludes Lys20 trimethylation of H4. The present study supports this finding, because ChIP experiments (Figs. 5 and 6) and
immunofluorescence studies (Fig. 7A) revealed a negative correlation between patterns of trimethylated H4-Lys20 and H4 hyperacetyla-
tion. A positive correlation, however, was found between monometh-
ylation, which is associated with chromatin compaction and repression of gene activity (16), H4-Lys20 monomethylation correlates with
the transcriptionally active or competent chromatin state.

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