Monomethylation of Lysine 20 on Histone H4 Facilitates Chromatin Maturation[∇]

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Histone modifications play an important role in shaping chromatin structure. Here, we describe the use of an in vitro chromatin assembly system from *Drosophila* embryo extracts to investigate the dynamic changes of histone modifications subsequent to histone deposition. In accordance with what has been observed in vivo, we find a deacetylation of the initially diacetylated isoform of histone H4, which is dependent on chromatin assembly. Immediately after deposition of the histones onto DNA, H4 is monomethylated at K20, which is required for an efficient deacetylation of the H4 molecule. H4K20 methylation-dependent dl(3)MBT association with chromatin and the identification of a dl(3)MBT-dRPD3 complex suggest that a deacetylase is specifically recruited to the monomethylated substrate through interaction with dl(3)MBT. Our data demonstrate that histone modifications are added and removed during chromatin assembly in a highly regulated manner.

All DNA in a eukaryotic cell is assembled into chromatin to fit it into the restricted nuclear space and to organize the genome (29, 56). As the DNA content of a cell doubles during S-phase of the cell cycle, the cell has to provide sufficient histone molecules to package the newly replicated DNA into chromatin. This is achieved mainly by a coupling of histone and DNA synthesis (37). The progression of the DNA replication machinery disrupts the nucleosome in front of the replication fork, which is then reassembled onto the newly synthesized DNA strands in a random manner (15, 20). The remaining gaps are subsequently filled up with newly translated histone molecules, leading to a mosaic pattern of new and old histone octamers (2, 21). This process is assisted by the action of nuclear chaperones, which bind to the histones before deposition (14, 34). The newly deposited histones are more loosely associated with the DNA than the bulk histones and mature slowly into a more stable chromatin structure. Assembly is achieved via an ordered deposition of H3 and H4, followed by the binding of H2A/H2B dimers and finally the interaction of the linker histone H1 with the chromatin fiber (19–22, 49).

Posttranslational modifications of histone molecules are generally considered to play an important role during the establishment and maintenance of chromatin structures. The combination of histone modifications has been proposed to

constitute a “histone code” (23, 55), which is involved in the maintenance of epigenetic information. However, it is unclear how histone modifications are replicated during cellular division and DNA repair when newly translated histones are deposited onto the DNA.

Modification marks can be stably maintained through multiple mitotic divisions (3, 23, 30, 55). However, for most histone modifications, the molecular mechanisms that regulate this maintenance have so far remained elusive. A key aspect of this maintenance or reestablishment of modification patterns is the precise copying of histone modification patterns from the parental histone to the newly synthesized ones. As the newly synthesized histones carry a specific histone modification pattern that is distinct from the histones found within chromatin (32), this copying not only involves a deposition of particular marks but also the removal of others. Newly synthesized histone H4 is acetylated at position K5 and K12 (5, 32, 50). Recently, a class II histone deacetylase (HDAC) activity (Thd2) has been isolated that specifically deacetylates the replication-dependent H4 modifications in *Tetrahymena thermophila* (48). Thd2 localizes specifically to the micronucleus of the ciliate and is involved in chromatin maturation, which further demonstrates the importance of the deacetylation for chromatin fiber formation. Histone H3 is acetylated mainly at K9 and K14 in HeLa cells and at K14 and 23 in *Drosophila* cells, suggesting a lower degree of conservation of the H3 acetylation sites (7, 32, 50). This acetylation pattern is rapidly removed when histones are deposited. In the case of H4, deacetylation happens within minutes, making the analysis of the diacetylated H4 isoform during chromatin assembly difficult (5, 40).

In order to get a better understanding of the processes that occur during histone deposition, we performed a detailed,

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time-resolved analysis of histone modifications during chromatin assembly. The use of an in vitro assembly system allows a precise analysis of the turnover of histone H4 modification during histone deposition, which is difficult to analyze in vivo due to its low half-life. We find that deacetylation is nonprocessive in that either the acetyl group at K12 or the one at K5 is removed from the H4 molecule independently of each other. After deposition, H4 is rapidly monomethylated at K20, which facilitates the subsequent deacetylation through the recruitment of a complex containing dl(3)MBT and the deacetylase dRpd3.

MATERIALS AND METHODS

Antibodies and immunoblotting. Polyclonal anti-dl(3)MBT antibody was raised in rabbits coinjected with three unrelated specific peptides (P1, PSGED KTRSTQKNKQNTSASC; P2, YFERPLYDRPGRPSAC; and P3, CLPEQS QTNGYKTDHDDQELS; Peptide Specialty Laboratories). For generation of monoclonal anti-dl(3)MBT antibodies, rats were immunized with either peptide P1 or P3 (antibodies MBT P1 6E6 and MBT P3 8F10, both isotype immunoglobulin G1) (see above). Anti-glutathione *S*-transferase (anti-GST) antibody was produced in rats. Anti-dRpd3 and anti-dHDAC3 antibodies were generous gifts from J. Müller and B. Turner, respectively. Monoclonal mouse anti-FLAG M2 antibody is commercially available (Sigma). For immunoblotting, proteins were transferred onto a polyvinylidene difluoride membrane (Roth), probed with the indicated antibodies, detected with enhanced chemiluminescence-labeled secondary antibodies, and detected using the Immobilon Western kit (Millipore).

Chromatin assembly extract (*Drosophila* assembly extract [DREX]). Zero to 90 min after egg laying, *Drosophila* embryos were rinsed in water and allowed to settle into embryo wash buffer (0.7% NaCl, 0.05% Triton X-100) on ice to arrest further development. After four successive collections, the pooled harvest was dechorionated. The wash buffer was decanted and replaced with wash buffer at room temperature, and the volume was adjusted to 200 ml. After the addition of 60 ml of 13% hypochlorite, the embryos were stirred vigorously for 3 min, poured back into the collection sieve, and rinsed extensively with tap water. Then they were allowed to settle in 200 ml of wash buffer for about 3 min, after which the supernatant (containing the chorions) was aspirated off. Two more settlings were performed, one in 0.7% NaCl and one in extract buffer (10 mM HEPES [pH 7.6], 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM EGTA, 10% glycerol, 10 mM 3-glycerophosphate, 1 mM dithiothreitol [DTT], and 0.2 mM phenylmethylsulfonyl fluoride [PMSF], added freshly) at 4°C. The embryos in extract buffer were settled in a 60-ml glass homogenizer on ice for about 15 min, and the volume of the packed embryos was estimated. The supernatant was aspirated, and the embryos were homogenized by one complete stroke (3,000 rpm) and 10 strokes at 1,500 rpm with a pestle connected to a drill press. Homogenate was supplemented with an additional 3.5 mM MgCl₂ from a 1 M MgCl₂ stock solution and quickly mixed (final MgCl₂ concentration, 5 mM). Nuclei were pelleted by centrifugation for 10 min at 10,000 rpm in an SS34 rotor (Sorvall). The supernatant was clarified by centrifugation for 2 h at 45,000 rpm (190,000 × g) in an SW 56 rotor (Beckman) and collected with a syringe, avoiding the floating layer of lipids. Aliquots (300 to 500 µl) were frozen in liquid nitrogen. Protein concentrations were determined with the Bradford assay, using bovine serum albumin (BSA) as the standard (4).

Chromatin assembly on immobilized DNA. One microgram of rDNA was immobilized onto 0.3-mg paramagnetic streptavidin beads (Dyna) in EX100 buffer (10 mM HEPES [pH 7.6], 100 mM NaCl, 1.5 mM MgCl₂, 0.5 mM EGTA, 10% [vol/vol] glycerol, 0.2 mM PMSF, 1 mM DTT) and, after being washed extensively, blocked for 30 min with BSA (1.75 µg/µl) in EX100. The DNA on the beads was concentrated on a magnetic concentrator (Dyna) and resuspended in a total volume of 240 µl containing 80 µl DREX and ATP regenerating system (3 mM ATP, 30 mM creatine phosphate, 10 µg creatine kinase/ml, 3 mM MgCl₂, and 1 mM DTT). Whenever indicated, TSA was added in a total concentration of 50 nM sodium butyrate in a final concentration of 2 mM, and *S*-adenosyl-homocysteine (SAH) was added as stated. The reaction mixture was rotated at 26°C for the denoted time period in order to reconstitute chromatin. After extensively washing the chromatin with EX500 (10 mM HEPES [pH 7.6], 500 mM NaCl, 1.5 mM MgCl₂, 0.5 mM EGTA, 10% [vol/vol] glycerol, 0.2 mM PMSF, 1 mM DTT), it was subjected to mass spectrometry, micrococcal digestion, or Western blotting.

Expression and purification of recombinant proteins. dl(3)MBT cDNA was obtained from the Berkeley *Drosophila* Genome Project (clone no. LD05287). This sequence was used for generation of a vector expressing FLAG-tagged

dl(3)MBT [pPac-FLAGdl(3)MBT] (9) by PCR (details available upon request). For generation of a stable S2 line, cells (8 × 10⁶) cultured at 26°C in Schneider's *Drosophila* medium (10% fetal bovine serum; Sigma) were cotransfected with 20 µg pPacFLAG-dl(3)mbt and 1 µg pPuro (a kind gift from E. Izaurralde) using calcium phosphate transfection according to standard procedures. Two days posttransfection, cells were selected for 4 to 6 weeks using 5 µg/ml puromycin (Sigma), clones were picked and expanded, and expression was verified by Western blotting. For baculovirus-mediated expression, FLAG-tagged dl(3)MBT and a FLAG-tagged construct containing the three MBT domains were cloned into pVL1392. Virus production and protein purification have been previously described (10). A construct containing the three MBT domains of dl(3)MBT was cloned into pGex4T1 by PCR (details available upon request). Expression and purification of GST fusion proteins were performed following standard procedures.

Histone purification and nucleosome assembly by salt dialysis. Recombinant histones or histones purified from *Drosophila* embryos (native histones) were expressed, purified, and reconstituted into octamers as described previously (12). Chromatin was reconstituted by salt dialysis overnight at 4°C using NaCl concentrations of 2 M to 0.1 M.

HDAC assay. HDAC assays were performed as described before (10). In some reactions, 500 µM TSA was added.

Immunoprecipitations. Four hundred micrograms of S2 nuclear extract was incubated with 50 µl monoclonal antibodies in a final volume of 350 µl phosphate-buffered saline for 2 h at 4°C with rotation. Five microliters of Protein G beads (GE Healthcare) was added, and incubation was continued for 1 h. Following extensive washing with phosphate-buffered saline, beads were used immediately for HDAC assays (see above). FLAG immunoprecipitations (FLAG-IP) were carried out with S2 nuclear extracts (400 µg total protein) and 5 µl anti-FLAG agarose (Sigma) in a total volume of 400 µl FLAG-IP buffer (25 mM HEPES [pH 7.6], 12.5 mM MgCl₂, 0.1 mM EDTA [pH 8.0], 10% glycerol, 0.1% NP-40, 150 mM NaCl). Incubation was performed for 3 h at 4°C with rotation. After being extensively washed with FLAG-IP, buffer beads were either used immediately in HDAC assays or analyzed by Western blotting. In some experiments prior to immunoprecipitation, nuclear extracts were incubated for 30 min with ethidium bromide (50 µg/ml).

Mass spectrometry. Histones were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Coomassie blue-stained bands were excised. Digestions were carried out overnight with 200 ng sequencing grade trypsin (Promega); in this case, histones were chemically modified beforehand by being treated with propionic anhydride to convert free amino groups to propionic amides of lysine residues. In order to purify the peptides from the contaminating salts or acrylamide, the peptide solution was passed over a pipette tip containing small amounts of C₁₈ reversed phase material (ZipTip; Millipore). After three 10-µl wash steps with 0.1% trifluoroacetic acid, the bound peptides were eluted with 1 µl of prepared matrix solution (saturated α-cyanohydroxycinnamic acid [Sigma] dissolved in 50% acetonitrile [vol/vol], 0.3% trifluoroacetic acid [vol/vol]) directly onto the target plate. Samples were air dried to allow cocrystallization of the peptides and the matrix. The target plate was loaded in a Voyager DE STR spectrometer, and spectra were analyzed using the in-house-developed software Manuelito. For tandem mass spectrometry (MS-MS), collision-induced decay spectra were recorded on a Q-Star XL instrument with manually adjusted collision energies. Fragment spectra were interpreted manually.

Methyltransferase activity. Assays were carried out in 25 µl of methyltransferase buffer (50 mM Tris-HCl [pH 8.0], 0.5 mM DTT) containing the indicated substrate and 500 nCi *S*-adenosyl-(methyl-³H)-L-methionine (25 µCi/ml) (Amersham) as the methyl donor. Reactions were stopped by spotting 20 µl on P81 filter paper. Filter papers were then washed three times for 10 min in 50 mM carbonate buffer (pH 9.2) and dried, and ³H incorporation was measured by scintillation counting.

Micrococcal nuclease digestion. Chromatin from 2 µg DNA was resuspended in EX100 containing 5 mM CaCl₂ and 100 Boehringer units of MNase (Sigma). After 30, 90, and 300 s at room temperature, the digestion was stopped by adding 4% SDS and 100 mM EDTA. The suspension was subjected to RNase A and proteinase K treatment, and precipitated DNA was separated onto a 1.3% agarose gel. A 123-bp ladder (Invitrogen) was used as a size marker.

Nuclear extract preparation. Nuclear extracts from S2 cells were prepared as described previously (35).

Peptide pull-down assay. H4 peptides comprising amino acids 16 to 25 with K20 that were either unmodified or mono-, di-, or tri-methylated were coupled to SulfoLink coupling gel (Pierce) via a C-terminal cysteine according to the manufacturer's protocol. Immobilized peptides were preblocked with 1 µg/µl BSA in binding buffer (25 mM Tris [pH 8.0], 150 mM NaCl, 2 mM EDTA, 0.5%

FIG. 1. DREX-mediated chromatin assembly. (a) Scheme of chromatin reconstitution protocol. The DNA used for chromatin assembly is a linearized biotinylated fragment containing 12 repeats of a 5S nucleosome positioning sequence. Two micrograms of DNA bound to paramagnetic beads was reconstituted into chromatin for 1 to 6 h at 26°C using 80 μ l DREX. (b) Corresponding histones stained with Coomassie blue. When boiling streptavidin-coated beads, a strongly stained streptavidin band appeared at the same molecular weight as H4. It is therefore labeled H4/streptavidin. (c) Micrococcal nuclease digestion pattern of the reconstituted chromatin. M, lanes containing the 123-bp ladder as a molecular weight marker; MNase, micrococcal nuclease.

NP-40) for 1 h at 4°C. Recombinant GST-3MBT or FLAG-tagged 3MBT were incubated with preblocked immobilized peptides for 2 h at 4°C in binding buffer with agitation. After extensive washing with binding buffer, peptide-bound protein was analyzed by Western blotting.

RESULTS

In order to determine the kinetics of histone modifications during chromatin assembly, we used a well-characterized S-150 chromatin assembly extract (4) prepared from early *Drosophila* embryos. This extract can be used to assemble large fragments of DNA into an ordered nucleosomal array that closely resembles the chromatin structure observed in vivo (6). For our experiments, we used an array of nucleosome positioning sequences derived from the sea urchin 5S rRNA gene (16). The linearized DNA was biotinylated and subsequently immobilized using magnetic streptavidin beads (4, 12) (Fig. 1a). Although the histone deposition is very fast and occurs within the first hour (4) (Fig. 1b), properly spaced chromatin can be detected only after 4 to 5 h of assembly, as judged by micrococcal nuclease digestion (Fig. 1c). Histones carry specific modifications before deposition onto chromatin in vivo (5, 14, 32), which are rapidly removed after they have been loaded onto DNA. Therefore, we tested whether we could observe similar modification kinetics in the in vitro assembly system.

In accordance with the modifications that were observed on storage histones in *Drosophila* and *Xenopus* embryos early in development (7, 11, 46), histone H4 is found acetylated at the N terminus in a mainly diacetylated form in the extract that we used for assembly (Fig. 2a). We mapped the position of the two acetyl groups to positions K5 and K12 using MS/MS (Fig. 2c). In order to investigate the dynamic changes of histone modifications, chromatin was isolated at various times during assembly and the modification patterns were analyzed by mass spectrometry and Western blotting. As seen in vivo and in the *Xenopus* assembly system (46), the diacetyl form of H4 is

deacetylated during chromatin assembly. Interestingly, the deacetylation is relatively slow, with a half-time of 150 min for the diacetylated form as measured by mass spectrometry and Western blotting (Fig. 2b and data not shown). The deacetylation is not a rapid processive event, as we see an intermediate accumulation of the monoacetylated form (Fig. 2b).

In addition to that, we do not observe a preferred order of deacetylation, as acetyl groups are removed from K5 and K12 with similar kinetics independently of each other (data not shown). The deacetylation is inhibited by the addition of HDAC inhibitors, such as sodium butyrate or TSA, suggesting that class I or class II HDAC activity is targeted to the newly assembled chromatin (Fig. 2d; compare to Fig. 2b for a 0-h time point). During longer assembly times, we do observe some residual deacetylation in the presence of TSA and butyrate, albeit with a much lower efficiency (Fig. 2d). No significant deacetylation occurs when the extract is incubated for the same time in the absence of DNA, showing that the process of assembly is essential for the removal of the acetyl groups (Fig. 2b).

We next wondered whether additional modifications would influence the deacetylation of H4K5 and H4K12. The only methylation that we observed on any histone within the 6 h of chromatin assembly is a monomethylation of H4 at lysine 20. This methylation is strictly dependent on the assembly of H4 into chromatin, as it is not detected in H4 isolated from the assembly extract prior to the addition of DNA (Fig. 3b, lane 1). Monomethylation of H4K20 in H4 increases during assembly as judged by mass spectrometry and Western blotting (Fig. 3). We do not observe a further methylation of H4 during chromatin assembly, leading to di- or trimethylated forms.

The key monomethylase specific for H4 is PR-SET7 (43). As the enzyme is clearly present in DREX, as judged by Western blotting (data not shown), we hypothesized that this enzyme is responsible for the modification in the extract. In order to

FIG. 2. Dynamics of modifications on H4 peptide 4-17 during DREX assembly. (a) Top, sequence of H4 peptide 4-17; bottom, time course analysis of H4 peptide 4-17 by matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF) analysis. Chromatin was reconstituted as described above. The reconstitution was stopped after 1, 2, 3, 4, 5, and 6 h, chromatin attached to paramagnetic beads was washed, and histones were separated by 18% SDS-PAGE. H4 was cut out and subjected to mass spectrometric analysis. (b) Quantification of MALDI-TOF data that are shown in panel a. As a control, DREX was incubated at 26°C without DNA for 0 h or 6 h and labeled as –DNA 0 h/6 h. unmod, unmodified; ac, acetylation. (c) MS-MS spectra generated from the diacetylated peak from 1 h DREX assembly. (d) Quantification of MALDI-TOF analysis. HDAC inhibitors were added to the chromatin assembly reaction, and H4 was analyzed as described above. TSA, trichostatin A; Na-butyrate, sodium butyrate.

better study the *Drosophila* PR-SET7 enzyme, we expressed the full-length protein in bacteria and studied its enzymatic properties (Fig. 4). The recombinant PR-SET7 enzyme is a very efficient nucleosomal methyltransferase but only poorly methylates recombinant H4 when it is not within a nucleosomal context (Fig. 4a and b). As it methylates preacetylated H4 in our system, we tested whether PR-SET7 may methylate chromatin that is acetylated on K5 and 12 preferentially. In order to do this, we fully acetylated H4 on positions 5 and 12

using a recombinantly expressed yHAT1 enzyme (38), assembled it into chromatin, and investigated its ability to serve as a substrate for PR-SET7. Consistent with the substrate requirements of the human enzyme, we do not find an effect on the methylation efficiency after acetylation at positions 5 and 12 in vitro (Fig. 4c and d).

The dependency of PR-SET7 on the presence of nucleosomal DNA and the fact that the enzyme does not distinguish between differentially acetylated substrates let us conclude that

FIG. 3. H4 becomes monomethylated at K20 after assembly. (a) Chromatin was assembled as shown before using DREX. Corresponding histones were separated by 18% SDS-PAGE and then digested with AspN prior to mass spectrometric analysis. Also shown is a comparison of posttranslational modifications of the H4 peptide 20-23 1 h (top) and 4 h (bottom) after the assembly reaction. (b) Western blot analysis of kinetics of H4K20me₁. H3 is serving as a loading control. M, molecular weight marker; α H3, anti-H3; α H4K20me₁, anti-H4K20me₁.

PR-SET7 senses the proper nucleosome assembly rather than the acetylation state of the histone.

In order to study the effect of monomethylation on the process of histone modification maturation, we used SAH to inhibit methyltransferase activity during chromatin assembly. SAH is the reaction product of all methyltransferases that use SAM as cofactors and acts as a competitive inhibitor. In order to test which concentration to use in the assembly reaction, we first analyzed whether PR-SET7 could indeed be inhibited by SAH. In vitro methyltransferase assays showed that a concentration of 500 μ g/ml very efficiently inhibits PR-SET7 (Fig. 5a). When we used SAH in an assembly, we could indeed reduce the level of monomethylation in a concentration-dependent manner (Fig. 5b and d, top). However, we did not observe a major difference in chromatin spacing or in the level of chromatin assembly as judged by nuclease digestion (Fig. 5c).

When we determined the degree of H4 deacetylation of untreated chromatin with the SAH-treated chromatin, we found a substantially increased level of H4 acetylation when monomethylation is inhibited by SAH, arguing for a decrease in acetylation efficiency in the presence of SAH (Fig. 5d, bottom). This was not due to an inhibition of HDAC's presence in

the assembly extract, as the overall levels of HDAC activity in the extract did not decrease (Fig. 5e). Therefore, we wondered whether a specific deacetylase may be targeted to the monomethylated chromatin in order to ensure efficient deacetylation. We first tested the hypothesis that the most abundant deacetylase in the extract, dRpd3, may preferentially deacetylate a premethylated peptide. However, we did not observe a significant increase in the HDAC activity of recombinant dRpd3 when a K20-methylated H4 peptide was used as substrate (Fig. 5f). Another possible explanation for our observation could be that the deacetylase responsible for the removal of the acetyl groups from K5 and K12 after chromatin assembly is brought to methylated H4 by an additional targeting factor. However, HDAC complexes identified to date lack domains capable of binding methylated H4K20. One of the domains that binds monomethylated H4K20 in vitro is the MBT domain (28, 31, 54). *Drosophila* sp. have three genes that code for MBT-containing proteins SCM, dSMBT, and dl(3)MBT. Mutations in SCM and dSmbt result in homeotic transformations, whereas mutations in dl(3)MBT show effects on the cell cycle, such as a block in mitosis (58). The cell cycle phenotype displayed by dl(3)MBT mutants is consistent with a role in chromatin as-

FIG. 4. PR-SET7 needs nucleosomes as a substrate. (a) Recombinant dPR-SET7 was cloned into a pMyb vector, expressed in bacteria, purified over chitin affinity chromatography according to the manufacturer's instructions (New England Biolabs), and assayed for HMTase activity on different substrates. The histogram shows the radioactivity incorporated. rec. nuc., recombinant nucleosomes; rec. octamer, recombinant octamer. (b) Autoradiography of the experiment described for panel a. (c) Methylation of recombinant nucleosomes and nucleosomes acetylated at K5 and K12 by dPR-SET7. (d) Autoradiography of the experiments described for panel c.

sembly. Moreover, similar phenotypes have been observed in neuroblasts that are deficient for PR-SET7 (43).

We wondered whether dl(3)MBT might be recruited to chromatin during assembly. To check this, we used an antibody that specifically recognizes the dl(3)MBT protein and observed an efficient recruitment of this protein to in vitro-assembled chromatin similar to the monomethylation of H4K20 (Fig. 6a). We then verified that the MBT domains of dl(3)MBT can indeed bind to methylated histone H4 tails in vitro. We incubated a GST fusion containing the three dl(3)MBT MBT domains with immobilized histone H4 peptides. As has been shown previously for the human orthologue, the dl(3)MBT MBT domains bound to histone H4 tails in this assay and displayed a strong preference for mono- and dimethylated H4K20 (54) (Fig. 6b). The MBT domains of dl(3)MBT bound more strongly to tails with a methylated K20 residue than to the unmodified tail (Fig. 6b, compare lanes 2 to 5). In contrast to human hl(3)MBTL1 (31), dl(3)MBT bound with increased

affinity to all three methylated H4K20 peptides with an enhanced affinity for H4K20me₁ and H4K20me₂. To exclude the possibility that a posttranslational modification may influence dl(3)MBT's ability to distinguish between the differentially methylated peptides, we also expressed FLAG-tagged MBT domains using a baculoviral expression system. Binding studies using the protein expressed in a eukaryotic system did not differ significantly from the bacterially expressed protein (Fig. 6b, compare top and bottom). These results suggest that dl(3)MBT has the potential to specifically associate with H4K20-methylated H4 tails during chromatin assembly. We then investigated if dl(3)MBT associates with HDAC activity. We generated an S2 line stably expressing FLAG-tagged dl(3)MBT. Anti-FLAG immunoprecipitates were generated from this line and control S2 cells and subjected to HDAC assays in vitro (Fig. 6c, top). Whereas immunoprecipitation from control extracts yielded only background HDAC activity, immunoprecipitates from FLAG-tagged dl(3)MBT-containing

FIG. 5. Deacetylation of H4K5/K12 is dependent on H4K20 monomethylation. (a) A histone methyltransferase assay was performed in the presence and absence of SAH with and without PR-SET7. (b) Quantification of Western blot analysis. Chromatin was reconstituted for 3 h using DREX treated with and without SAH. Chromatin was washed, separated on an 18% protein gel, blotted to a nitrocellulose membrane, and detected with H4K20me₁ antibody. H3 was used as a loading control. (c) Micrococcal digestion pattern of the reconstituted chromatin incubated with or without SAH. M, lanes containing the 123-bp ladder as a molecular size marker. (d) Chromatin was assembled for 4 h. Histones were separated by 18% SDS-PAGE and then digested with trypsin prior to mass spectrometric analysis. Top two panels, comparison of unmodified H4K20 and monomethylated K20 peak of chromatin treated with or without SAH (shown is the peptide 20-23) (Fig. 3); bottom two panels, comparison of acetylation ratios of peptide 4-17 of chromatin that has been treated with and without SAH. (e) HDAC assay using 5 μ l of chromatin assembly extract in the presence of either 500 μ M TSA or 500 μ g/ml SAH. (f) Deacetylation of an in vitro-acetylated peptide containing amino acids 9 to 29 of H4 carrying a methylation or no modification at K20.

FIG. 6. Binding of dl(3)MBT to chromatin is inhibited by SAH, and dl(3)MBT is found in a complex with dRpd3. (a) After chromatin was reconstituted using DREX, bound dl(3)MBT was detected by Western blotting. (b) Immobilized H4 peptides (amino acids 16 to 25) were incubated with recombinant baculovirally expressed FLAG-3MBT (top) or recombinant GST-3MBT expressed in bacteria (bottom). Binding of GST- and FLAG-tagged proteins was analyzed by immunoblotting using anti-GST and anti-FLAG antibodies. unmod, unmodified H4 16-25 peptide; me1, me2, and me3, H4 peptide mono-, di-, and tri-methylated at K20; control, beads without peptide. (c, top) HDAC assays were performed with anti-FLAG immunoprecipitates (IP αFlag) from nuclear extracts of S2 cells (mock) and an S2 line stably expressing FLAG-tagged dl(3)MBT [Flag-dl(3)MBT]. Immunoprecipitations were carried out in the absence or presence of ethidium bromide (EtBr), and HDAC reactions were performed with or without TSA as indicated (middle and bottom). Anti-FLAG immunoprecipitates were analyzed by Western blotting for the presence of Flag-dl(3)MBT, dRpd3, and dHDAC3 as indicated. Ten percent of nuclear extracts were loaded as input. (d) HDAC assays were performed with immunoprecipitates obtained with a mixture of two monoclonal anti-dl(3)MBT antibodies (at a ratio of 1:1), anti-GST antibody, or no antibody (beads only) (top), and S2 nuclear extract was precipitated with dl(3)MBT antibody (bottom). Immunoprecipitations were subjected to Western blotting using anti-dRpd3 and anti-dl(3)MBT antibody as shown. (e) Chromatin was reconstituted for a specific time and treated with or without SAH (500 µg/ml). The reconstitution was stopped after 15 and 45 min. Chromatin attached to beads was washed (100 mM NaCl) and separated by 18% SDS-PAGE. dl(3)MBT and dRpd3 were detected by a polyclonal antibody.

extracts had robust HDAC activity. To rule out that association of HDAC activity was a result of spurious coprecipitation of HDAC-containing chromatin fragments, immunoprecipitations were carried out in the presence of ethidium bromide to dissociate protein-DNA interactions. Ethidium bromide had no significant effect on dl(3)MBT-associated HDAC activity. Immunoprecipitated HDAC activity was sensitive to inhibition by TSA, suggesting that it was due to a class I HDAC. We therefore probed immunoprecipitates for the presence of the class I HDACs dRPD3 and dHDAC3 by Western blotting (Fig. 6c, middle and bottom). dRPD3 specifically coprecipitated with FLAG-tagged dl(3)MBT, and this interaction was not affected by the presence of ethidium bromide, confirming that it is due to a protein-protein interaction. In contrast, dHDAC3 could not be detected in the immunoprecipitates (Fig. 6c, bottom).

In order to verify that endogenous dl(3)MBT associates with HDAC activity (Fig. 6d, top), we immunoprecipitated S2 extracts with dl(3)MBT antibodies. Indeed, HDAC activity was precipitated by the dl(3)MBT but not by the control antibody. In addition, immunoprecipitation of dl(3)MBT coprecipitated endogenous dRPD3 (Fig. 6d, bottom).

Taken together, these results demonstrate that dl(3)MBT forms a complex with the HDAC dRPD3. In order to finally test whether this interaction and hence the recruitment might be responsible for the efficient deacetylation of newly assembled chromatin, we investigated the recruitment of dl(3)MBT and dRPD3 to chromatin during assembly. By using specific antibodies against dl(3)MBT and dRPD3, we could see an increase in dl(3)MBT and dRPD3 binding to chromatin with increasing assembly time (Fig. 6e, lanes 2 and 4). However, when the monomethylation of H4K20 was inhibited, this recruitment was greatly reduced (Fig. 6e, compare lanes 2 and 4 with lanes 1 and 3), suggesting a model in which the monomethylation of H4K20 indeed facilitates the recruitment of the deacetylase by virtue of its interaction with dl(3)MBT, thereby assisting the maturation of histone modifications during chromatin assembly.

DISCUSSION

In this work, we use an in vitro chromatin assembly system to recapitulate and dissect the changes in histone modifications during chromatin assembly. We find that H4 histone molecules carrying a diacetyl mark at K5 and K12 assemble onto DNA added to a *Drosophila* embryo extract. Histone H4 is monomethylated at K20 immediately after histone deposition. A candidate enzyme for carrying out this methylation is PR-SET7, which we show to (mono)methylate H4K20 within nucleosomal substrates. Concomitantly with H4K20 monomethylation, dl(3)MBT associates with chromatin. This is then followed by the removal of the two acetyl groups of H4. The three MBT domains of dl(3)MBT have the capacities to bind methylated H4K20 in vitro, and the full-length protein forms a complex with the HDAC dRPD3 in vivo. Our results strongly support a model of stepwise maturation of histone marks, which may help to guarantee the fidelity of chromatin assembly.

Lysine methylation within histone tails has been suggested to be a key player during epigenetic inheritance. However, in

contrast to the methylation of H3, methylation of H4 has not been so clearly associated with transcriptional regulation (30, 41, 60). The mapping of H4 species methylated at K20 gave somewhat confusing results. Whereas H4K20me₃ is a rare modification that has been clearly associated with transcriptionally silent chromatin (45), most H4 molecules carry a dimethyl mark in the model systems analyzed so far (7, 39, 40, 59). H4K20me₁, on the contrary, has been reported to be enriched within promoter and coding regions of active genes (52) as well as inactive regions of the genome (47). Furthermore, the monomethyl mark has been suggested to play a role in chromatin assembly rather than transcriptional regulation, which is based mainly on observations made on PR-SET7, the methyltransferase that is responsible for H4K20 monomethylation in mammalian cells (57) and in *Drosophila* cells (27). The enzyme localizes to the replication fork (53) and is required for S-phase progression (24). Furthermore, H4K20me₁ levels have been shown to increase during late S- and G₂-phase (13, 40, 42). Recent findings also show that H4K20 (mono)methylation not only plays a role during chromatin assembly during S-phase but is also involved in the regulation of DNA repair. In the fission yeast *Schizosaccharomyces pombe*, the methyltransferase that methylates H4K20, SET9, is required for an efficient targeting of a known checkpoint protein crb2 to sites of DNA damage, thereby mediating cell cycle arrest (44). A lack of H4K20me₁ also leads to the accumulation of DNA breaks and cell cycle arrest before the entry into mitosis in mammalian and *Drosophila* tissue culture cells (18, 43). The arrest seems to be initiated by a G₂/M checkpoint, as the lack of PR-SET7 induces the activation of the checkpoint kinase ATM (18). The role of H4K20me₁ in repair processes is further confirmed by the fact that the simultaneous mutation of the *Drosophila* checkpoint gene *ami1* strongly increases the survival rate of PR-SET7 mutant larvae (43).

Our results strongly support the idea that the monomethylation of K20 within H4 plays a crucial role during chromatin assembly by marking the fully assembled nucleosome after histone deposition. Recombinant dPR-SET7 methylates H4 only after it has been assembled into a nucleosome, which is consistent with the observations that have been made on mammalian PR-SET7 (13, 36). The fact that the enzymatic activity as well as the expression of PR-SET7 is highly regulated during different phases of the cell cycle (18, 53) suggests that the methylation of H4 after nucleosome assembly might have an important regulatory function that has to be tightly controlled.

The effects of histone methylations are thought to be mediated by Tudor domain-containing factors (33). One of these factors that has been shown to bind a peptide carrying H4K20me₁ is l(3)MBT, which has been suggested to act as a chromatin lock to stabilize a higher-order chromatin structure (25, 54). Other factors that bind to H4K20me are p53BP1 and crb2, which mediate key processes in DNA repair (8). Our findings that dl(3)MBT interacts with the HDAC and is recruited to newly assembled chromatin via a mechanism dependent on H4K20me₁ could explain why H4K20me₁ has been mapped to transcriptionally active as well as inactive regions. Both cellular processes, DNA synthesis and transcription, involve a disruption of chromatin structure and are often accompanied by a de novo histone assembly (1, 14, 17). We show that the monomethylation of H4K20 is a very early step after dep-

osition that stimulates the recruitment of a complex of dl(3)MBT and dRpd3 immediately after nucleosome formation. The association of the complex greatly facilitates the deacetylation of the newly deposited histones. The effect we observe is clearly a kinetic effect, as we eventually observe deacetylation even in the absence of any monomethylation, which is very likely due to the abundance of HDAC enzymes in the extract. In this light, it is interesting that the kinetics of K12 deacetylation after deposition have been shown to differ when comparing early and late-replicating chromatin (51). It will be interesting to investigate whether this is due to the different degrees of monomethylation of H4.

Chromatin has a very high rate of disruption and reassembly at transcriptionally active genes, which may explain the fact that the monomethyl mark can be found at transcriptionally active promoters (52). However, the HDAC recruitment after nucleosome assembly may at the same time lead to a removal of acetyl groups from histones at these promoters, leading to transcriptional repression (25) if the primary signal of gene activation (such as the binding of a transcription factor) is gone. This may explain the contradictory results of H4K20me₁ mapping data.

Given that we are using linear DNA fragments and a de novo assembly system, it may well be that our in vitro system mimics repair-coupled chromatin assembly and that the monomethylation is part of a mechanism that governs histone modifications during chromatin repair. Based on our in vitro observations and the reported in vivo data, we suggest that the monomethylation mark may indeed function as an important step to ensure the proper establishment of histone modification marks during and after assembly. In this light, it is also interesting that H4K20 monomethylated histones have been found in a non-chromatin-bound histone pool in *Drosophila* S2 cells (26), which may contain the H4 molecules that are ejected during RNA transcription and repair.

Based on our experiments, we would argue that, at least in the *Drosophila* system, the kinetics of H4K5ac and H4K12ac deacetylation are controlled by PR-SET7-mediated H4K20 methylation. It will be an interesting task for the future to analyze how the PR-SET7 activity is regulated in vivo in response to cellular stress and what downstream factors play a role in sensing improper chromatin assembly.

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