Molecular Cell, Vol. 9, 1201–1213, June, 2002, Copyright ©2002 by Cell Press

PR-Set7 Is a Nucleosome-Specific Methyltransferase that Modifies Lysine 20 of Histone H4 and Is Associated with Silent Chromatin

Kenichi Nishioka,[1] Judd C. Rice,[2] Kavitha Sarma,[1] Hediye Erdjument-Bromage,[3] Janis Werner,[6] Yanming Wang,[2] Sergei Chuikov,[1] Pablo Valenzuela,[4] Paul Tempst,[3] Ruth Steward,[5] John T. Lis,[6] C. David Allis,[2] and Danny Reinberg[1,7]

[1]Howard Hughes Medical Institute
Division of Nucleic Acids Enzymology
Department of Biochemistry
University of Medicine and Dentistry of New Jersey
Robert Wood Johnson Medical School
Piscataway, New Jersey 08854

[2]Department of Biochemistry and Molecular Genetics
University of Virginia Health System
Box 800733 Jordan Hall, Room 6222
Charlottesville, Virginia 22908

[3]Molecular Biology Program
Memorial Sloan Kettering Cancer Center
1275 York Avenue
New York, New York 10021

[4]BIOS Chile IGSA and Millennium Institute
for Fundamental and Applied Biology
1943 Marathon Avenue
Santiago, Chile

[5]Waksman Institute
Department of Molecular Biology and Biochemistry
Rutgers University
Piscataway, New Jersey 08854

[6]Department of Molecular Biology and Genetics
Cornell University
Ithaca, New York 14853

Summary

We have purified a human histone H4 lysine 20 methyltransferase and cloned the encoding gene, *PR/SET07*. A mutation in *Drosophila pr-set7* is lethal: second instar larval death coincides with the loss of H4 lysine 20 methylation, indicating a fundamental role for PR-Set7 in development. Transcriptionally competent regions lack H4 lysine 20 methylation, but the modification coincided with condensed chromosomal regions on polytene chromosomes, including chromocenter and euchromatic arms. The *Drosophila* male X chromosome, which is hyperacetylated at H4 lysine 16, has significantly decreased levels of lysine 20 methylation compared to that of females. In vitro, methylation of lysine 20 and acetylation of lysine 16 on the H4 tail are competitive. Taken together, these results support the hypothesis that methylation of H4 lysine 20 maintains silent chromatin, in part, by precluding neighboring acetylation on the H4 tail.

Introduction

In the nuclei of eukaryotic cells, DNA is stored in the form of chromatin; this structure is repressive to most, if not all, processes that require access of proteins to DNA. The basic building block of chromatin is the nucleosome, which is composed of two copies of each of four core histone proteins (H2A, H2B, H3, and H4) wrapped by 146 base pairs of DNA (Luger et al., 1997). The N-terminal tails of the core histone proteins protrude from the nucleosome. These histone tails appear to be unstructured and are believed to participate in the formation of higher order chromatin structure by mediating internucleosomal interactions (Luger et al., 1997), as well as contact with DNA that is positioned between the nucleosomes (linker DNA) (Angelov et al., 2001).

Longstanding cytological studies have generally defined two types of chromatin: euchromatin, which appears as an extended structure and is transcriptionally active, and heterochromatin, a compacted, transcriptionally silent structure (Grunstein et al., 1995). The mechanisms by which euchromatin is converted to heterochromatin and vice versa are poorly defined. However, an extensive literature documents that histone tails undergo a variety of posttranslational modifications, including acetylation, phosphorylation, methylation, and ubiquitination (Strahl and Allis, 2000; Zhang and Reinberg, 2001). Recent work has provided compelling evidence that these alterations affect chromatin structure and its functional properties. For example, acetylation of core histone tails correlates with the opening of the chromatin structure to allow transcription (Roth et al., 2001).

Other types of covalent modifications of chromatin components, including methylation of histones H3 and H4, also appear to play critical roles. First described in 1964, histones have long been known to be substrates for methylation (Murray, 1964). Early studies that used metabolic labeling followed by amino acid sequencing of bulk histones showed that several lysine residues, including lysines 4, 9, 27, and 36 of H3 and lysine 20 of H4, are preferred sites of methylation (van Holde, 1988; Strahl et al., 1999; Rice and Allis, 2001; Zhang and Reinberg, 2001).

An important breakthrough in the identification of enzymes that carry out histone-lysine methylation came from studies of suppressors of position effect variegation (PEV) in *Drosophila* (Reuter and Spierer, 1992). Suppressors of PEV, such as the Su(var)3-9, the polycomb-group protein Enhancer of zeste, and the trithorax-group protein Trithorax, all contain an evolutionarily conserved sequence motif termed the SET domain (Jenuwein et al., 1998). The SET domain of the human homolog of *Drosophila* Su(var)3-9 (Suv39h1) was later found to share sequence similarity with several previously identified SET domain-containing methyltransferases from plants (Klein and Houtz, 1995; Zheng et al., 1998). This observation led Jenuwein and colleagues to discover that Suv39h1 and its *S. pombe* homolog Clr4 each contain an intrinsic histone methyltransferase activity (Rea et al., 2000) that specifically methylates histone H3 at lysine 9 (H3-K9). Mutagenesis studies with Suv39h1 revealed that the SET domain and two adjacent cysteine-rich regions (the pre-SET and post-SET domains) are

[7]Correspondence: reinbedf@umdnj.edu

required for enzymatic activity. Subsequent studies resulted in the isolation of another enzyme, G9a, which displays substrate specificity similar to that of Suv39h1 but also methylates H3-K27 (Tachibana et al., 2001). Recently, enzymes that methylate H3-K4 have also been identified in yeast (Briggs et al., 2001; Roguev et al., 2001; Nagy et al., 2002) and higher eukaryotes (Wang et al., 2001a; Nishioka et al., 2002). However, it has been difficult to identify enzymes that methylate the tail of histone H4. Recent studies demonstrated that the arginine-specific methyltransferase, PRMT1, methylates H4-R3, but the enzyme that methylates H4-K20 has not been identified.

In the present study, we describe the identification, characterization, and functional analyses of a novel, mammalian histone methyltransferase (HMT) specific for lysine 20 of histone H4. We found that this enzyme, PR-Set7, resides as a single polypeptide and is highly specific for nucleosomal histones. We also show that methylation of H4-K20 is associated with silent, transcriptionally inactive regions within euchromatin. Methylation of histone H4-K20 may maintain this higher order chromatin structure by inhibiting the acetylation of histone H4-K16. Taken together, these studies help to shed light on mechanisms that regulate chromatin structure through a series of concerted enzymatic reactions that ultimately "mark" functionally distinct chromatin domains.

Results

Isolation of an H4-K20-Specific HMT

To identify and analyze HMTs present in human cells that specifically methylate histone H4, nuclear extracts from HeLa cells were fractionated on several chromatographic resins. Fractions from the columns were assayed for HMT activity using either core histone polypeptide or mono- and oligonucleosomes, in the presence and absence of histone H1, as substrates. Proteins were separated as illustrated in Figure 1A. The separation of proteins in the DEAE-cellulose flowthrough (unbound) fraction on a negatively charged column (phosphocellulose) resulted in the resolution of two HMT activities, each with a different substrate and histone specificity. The histone H3-specific activity was eluted from the column at a lower salt concentration and was able to methylate core histone polypeptides as well as oligonucleosomes (Figures 1A and 1B). This activity was specific for the K9 residue of H3 and was identified as Suv39h1 (K.N., K.S., and D.R., unpublished data). The other major HMT activity was eluted from the phosphocellulose column at a higher salt concentration and exclusively methylated nucleosomal histone H4 (Figures 1A and 1B). Further separation of the H4-specific HMT on a gel filtration column demonstrated that the activity had an apparent native mass of approximately 70 kDa (Figure 1C). The final step of the purification scheme, fractionation on a Heparin agarose column, showed that the H4 HMT activity correlated with the appearance of a single polypeptide of approximately 40 kDa (Figure 1D). It was later found by gel-filtration analysis that the enzymatically active 40 kDa protein resides as a homodimeric complex (data not shown).

The purified native enzyme was subjected to further analysis to more clearly define its substrate specificity. We conducted assays with known substrates for several previously characterized protein methyltransferases and found that the newly purified enzyme was highly specific for nucleosomal histone H4 (data not shown). A reaction mixture that contained nucleosomal histone H4, ^{3}H-labeled S-adenosyl methionine (SAM), and the purified enzyme was then subjected to Edman degradation, and this analysis demonstrated that the target site for methylation is lysine 20 (Figure 1E). Moreover, when we carried out an HMT assay using nucleosomes reconstituted with an H4 species that contained an alanine in place of a lysine at position 20 (K20A), the newly purified HMT was unable to methylate the substrate, demonstrating further that this enzyme is specific for H4-K20 (Figure 1F, lane 7).

Mass spectrometric analysis of peptides derived from the protein that coeluted with the nucleosomal H4-specific HMT activity allowed us to generate probes with which to isolate a full-length cDNA clone. cDNA sequence analysis revealed that the activity was encoded by a gene that is absent in lower eukaryotes but is present in worms, flies, and vertebrates (Supplemental Figure S1A [http://www.molecule.org/cgi/content/full/9/6/1201/DC1]). The cDNA sequence matched perfectly with a sequence recently deposited in GenBank referred to as PR/SET domain containing protein 07 (accession number NP065115). For simplicity, we termed the enzyme PR-Set7.

Sequence analysis of PR-Set7 showed that it contains an evolutionarily conserved SET domain, a motif that is shared by all other known lysine-directed HMTs (Supplemental Figure S1B [http://www.molecule.org/cgi/content/full/9/6/1201/DC1]). In contrast to other HMTs, except for Set9 (Nishioka et al., 2002; Wang et al., 2001a), PR-Set7 is devoid of the Pre- and Post-SET domains, which were thought to be required for enzymatic activity (Rea et al., 2000).

Recombinant PR-Set7 Protein Targets Histone H4-K20

For biochemical characterization, we produced a His-tagged, full-length, wild-type recombinant PR-Set7 (rPR-Set7) protein in bacteria, as well as a mutant version that contained a single arginine-to-glycine substitution (rPR-Set7-R265G) (Figure 2A) and purified these as described in Experimental Procedures. Arginine 265 corresponds to a key conserved residue within the SET domain that was shown to be required for HMT activity (Rea et al., 2000). The wild-type and mutant recombinant proteins were then tested for HMT activity using both octamers and nucleosomes as substrates (Figure 2C), and, as expected, only the wild-type rPR-Set7 was active. Much like the native protein, rPR-Set7 preferentially methylated nucleosomal substrates rather than core histone polypeptides (Figure 2C). The specific site of the modification by rPR-Set7 was further verified using nucleosomes that had been reconstituted with a mutant form of histone H4 where lysine 20 was replaced by alanine (K20A); as predicted, the activity of rPR-Set7 was significantly reduced (Figure 2C, lane 9). Therefore, we concluded that PR-Set7 is a histone H4-K20-specific

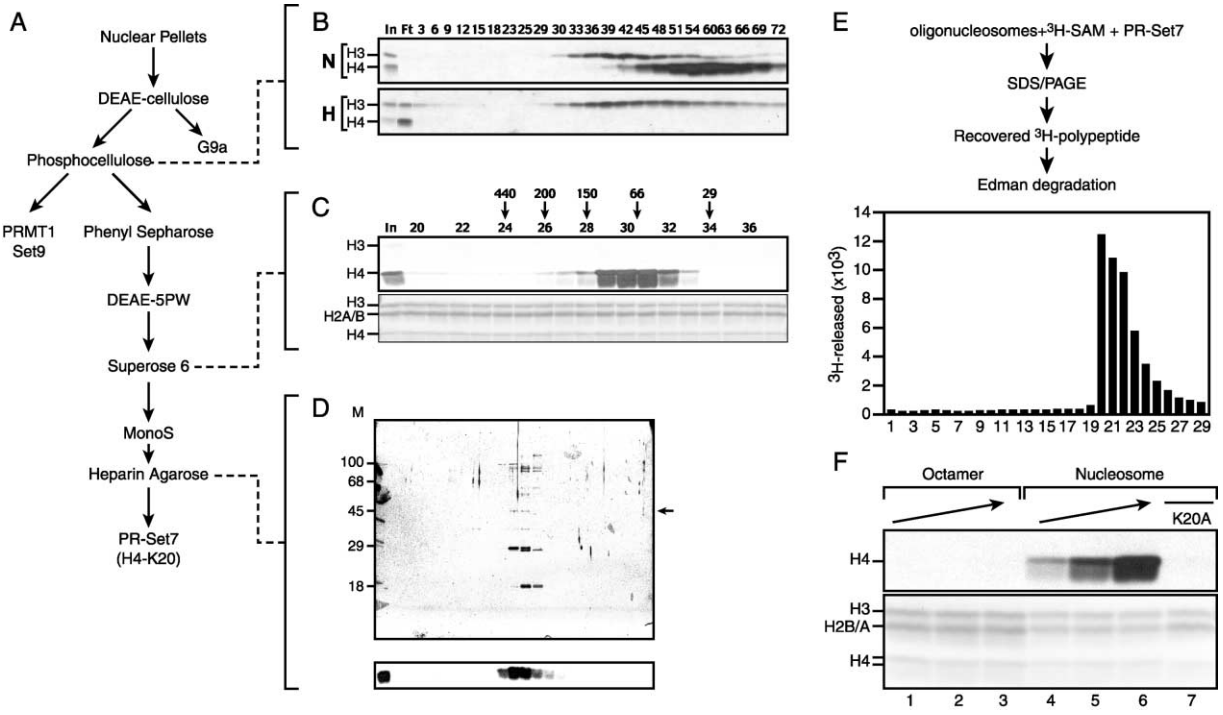

Figure 1. Isolation and Identification of a Human H4-K20 HMT Specific for Nucleosomal Substrates

(A) Schematic representation of the HMT activities present in HeLa-derived extracts and their fractionation during purification of PR-Set7. The DEAE-cellulose column binds a 100 kDa core histone H3-K9-specific HMT (G9a-like activity), while the phosphocellulose column binds two HMTs that were reactive on oligonucleosomes: a 650 kDa H3-K9 activity (Suv39h1-like activity) and an H4-K20 activity. The phosphocellulose flowthrough contains both PRMT1 (H4-R3) and Set9 (H3-K4) activities (Nishioka et al., 2002).

(B) HMT assays for phosphocellulose column fractions using the indicated substrate (H, core histones; N, oligonucleosomes), where signals at H3 correspond to a Suv39h1-like activity and those for H4 correspond to H4-K20 activity.

(C) An aliquot of the DEAE-5PW flowthrough fraction was applied onto a 2.4 ml Superdex S200 column (SMART System). The top panel shows the result of HMT assay performed with recombinant oligonucleosomes. Arrows indicate each peak of size-marker (kDa). The bottom panel shows Coomassie staining of the membrane used for the HMT assay.

(D) Analysis of Heparin agarose fractions. The top panel shows silver staining of the column fractions separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Molecular weights markers (kDa) are indicated on the left. The bottom panel shows corresponding HMT activity for each fraction. The arrow indicates a 40 kDa PR-Set7 polypeptide.

(E) Radiolabeled histone H4 polypeptide sequencing was carried out as indicated in the flow diagram. The graph shows the results of sequencing, where the *x* axis indicates the position of amino acid residues relative to the N terminus.

(F) Titration of native PR-Set7 enzyme using either a recombinant octamer or oligonucleosomes as substrates. Reactions were performed as described in Experimental Procedures using increasing amounts of purified PR-Set7 in the reaction mixture. The K20A indicates mutant oligonucleosomes in which H4-K20 was substituted with an alanine.

HMT and that the SET domain is responsible for the catalytic activity of the enzyme, as observed with other lysine-specific HMTs (Rea et al., 2000; Tachibana et al., 2001; Wang et al., 2001a; Nishioka et al., 2002).

Interestingly and unexpectedly, the strict nucleosomal substrate specificity of PR-Set7 was lost upon the generation of a recombinant PR-Set7 protein lacking the first 14 amino acids at the N-terminal end of the protein (ΔN14-rPR-Set7) (Figure 2A, bottom, and Figures 2D and 2E). In contrast to rPR-Set7, ΔN14-rPR-Set7 was able to methylate nucleosomes as well as octamer and the H4 polypeptide (Figure 2E). It is currently unclear if the deletion of the first 14 amino acids of PR-Set7, the cloning of ΔN14-rPR-Set7 into a different vector, the differences in purification schemes, or all of these directly contributed to the observed alterations in substrate specificity. Regardless, this mutant form of rPR-Set7 methylated histone H4 polypeptides exclusively at lysine 20 as previously observed with the native protein (Figures 1E and 1F). We confirmed by a gel-filtration analysis that this relaxed substrate recognition was not due to aggregation of the mutant enzyme (data not shown). These results demonstrate striking alterations in substrate recognition (i.e., free histone versus nucleosome) of known HMTs resulting from deletions in the protein far removed in primary sequence from the catalytic SET domain.

Is Methyl H4-K20 a Higher Eukaryote-Specific Modification?

As mentioned above, obvious PR-Set7 homologs are absent from yeast. This observation prompted us to look for the presence of methylated H4-K20 in a variety of organisms, including the yeast *Saccharomyces cerevisiae*. Toward this end, polyclonal antibody that specifically recognized methyl H4-K20 (α-methyl H4-K20) was generated as described in Experimental Procedures. This antibody was highly specific for methylated H4-K20

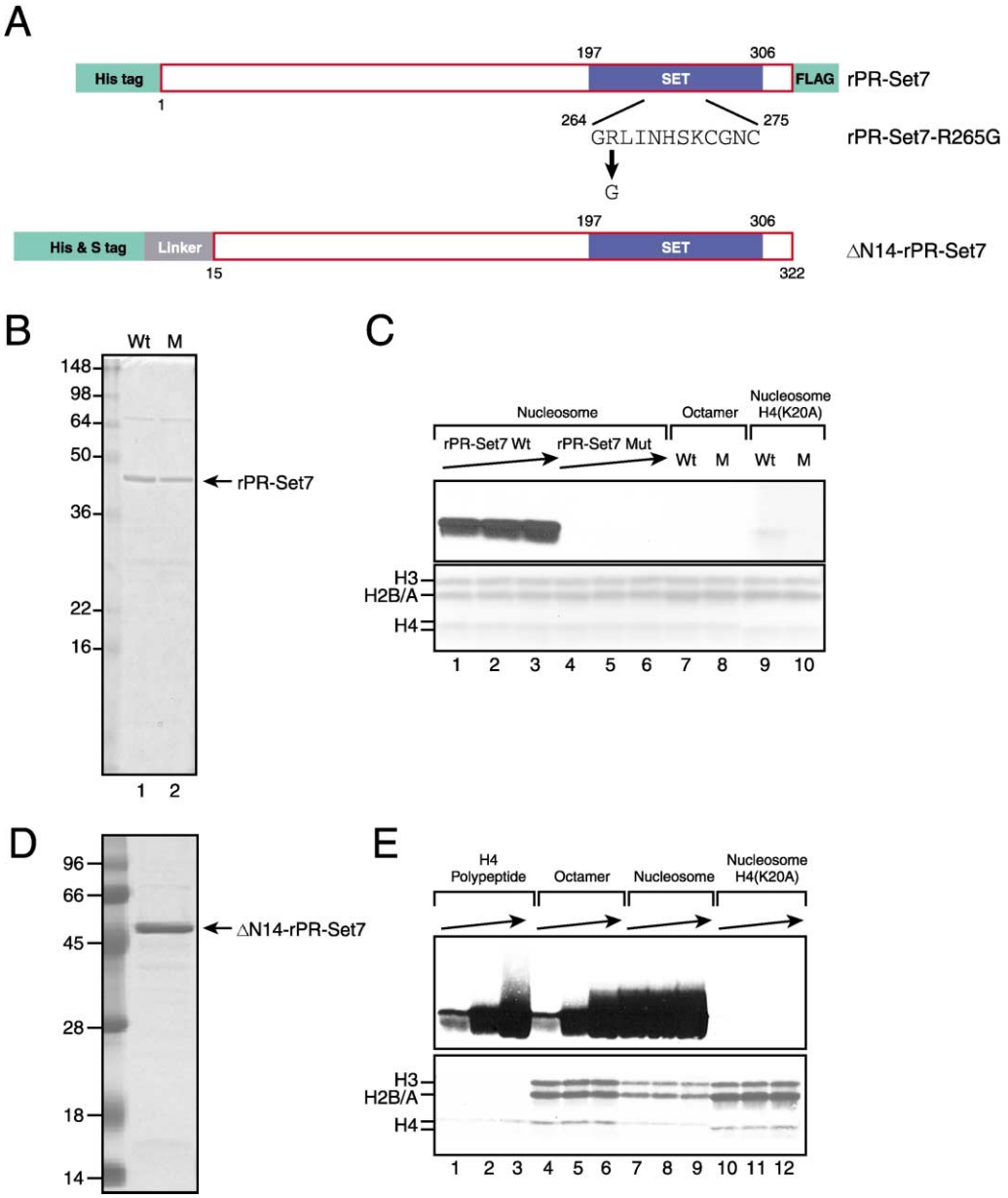

Figure 2. Specificity of Recombinant PR-Set7

(A) Structures of recombinant PR-Set7 (rPR-Set7) proteins. The open red box shows the original structure of PR-Set7 protein, where the SET domain is shown in blue. The mutant PR-Set7 protein has an R265G substitution at the indicated position in the core SET domain. In the ΔN14-rPR-Set7 derivative, the gray box shows 18 amino acids of linker sequence that replaced a 14 amino acids deletion of the N-terminal part of PR-Set7 protein. The green box indicates tag-sequences, which were either an N-terminal 6XHis (6XHis+S) sequence derived from vector or C-terminal FLAG. Each bacterially expressed protein was analyzed on a gel-filtration column, and it was confirmed that the protein was not aggregated.

(B) SDS-PAGE analysis of rPR-Set7 proteins visualized by Coomassie staining. Lane 1 is wild-type rPR-Set7 and lane 2 is the R265G mutant. Molecular weight markers are indicated on the left of the gel (kDa).

(C) Specificity of rPR-Set7. Wild-type and mutant rPR-Set7 were titrated in HMT assays using recombinant oligo-nucleosomes or octamer as substrates. The top panel shows HMT activity with H4 in the context of nucleosomes. Wt, wild-type; Mut or M, mutant rPR-Set7. The amounts of rPR-Set7 used in lanes 7–10 are the same as those used in lanes 3 and 6. The bottom panel shows Coomassie staining of the membrane used for the HMT assay.

(D) SDS-PAGE analysis of the ΔN14-rPR-Set7 protein visualized by Coomassie staining. Molecular weight markers are indicated at the left of the gel (kDa).

(E) Specificity of ΔN14-rPR-Set7. ΔN14-rPR-Set7 was titrated in HMT assays using recombinant H4 polypeptides (lanes 1–3), octamer (lanes 4–6), or oligonucleosomes (lanes 7–12) as substrates. The K20A indicates mutant oligonucleosomes in which H4-K20 was substituted to an alanine (lanes 10–12). The top panel shows HMT activity on H4 polypeptide. The bottom panel shows Coomassie staining of the membrane used for the HMT assay.

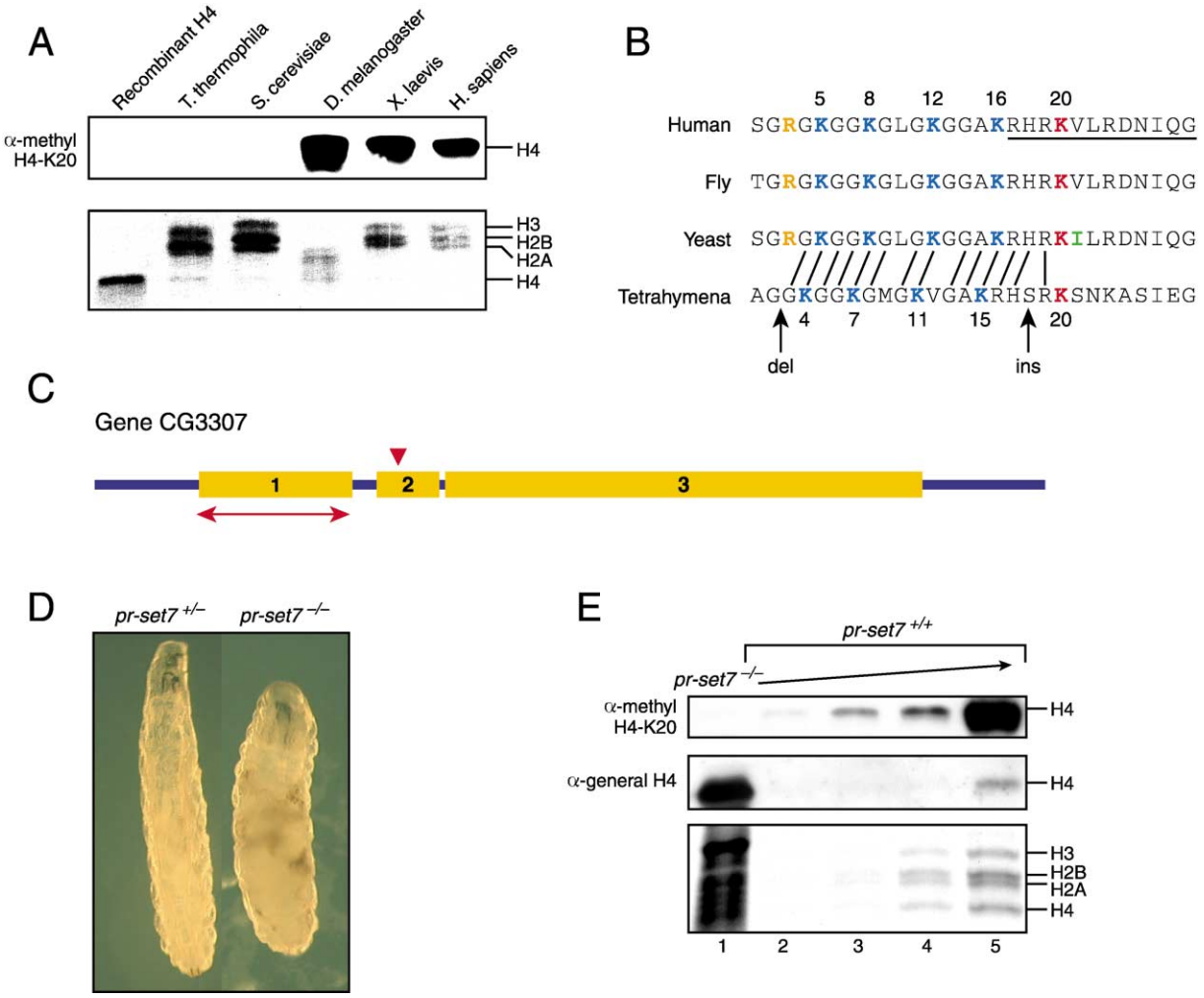

Figure 3. Species Analysis of H4-K20 Methylation and Analysis of *Drosophila pr-set7* Mutants

(A) Histones were extracted from the indicated organisms as described in Experimental Procedures and were fractionated by SDS-PAGE. Top panel shows Western blot analysis using antibody against methyl H4-K20. Bottom panel shows Ponceau staining of histones; the histone polypeptides are indicated at the right of the gel.

(B) Sequence alignment of histone H4 tails. Histone H4 tails (amino acid residues 1 to 25) from various species are aligned; where acetylatable lysines are blue, the methylatable arginines are orange, and each lysine 20 is red. In the lower panel, a comparison between *S. cerevisiae* and *Tetrahymena* is shown. Yeast has an isoleucine 21 instead of valine, while *Tetrahymena* has arginine-deletion (del) and serine-insertion (ins) at the indicated positions.

(C) Gene structure of the *D. melanogaster pr-set7 (CG3307)* gene. This gene is located at position 88B3 of chromosome 3R. Exons are shown in orange. Insertion of the P element is in the 1st exon of the gene (bi-headed arrow). The arrowhead indicates a hypothetical translation initiation site.

(D) Picture of the second instar larvae. *pr-set7*$^{+/-}$, heterozygote; *pr-set7*$^{-/-}$, homozygote *l(3)neo41*.

(E) Western blot analysis of mutant and wild-type larval extracts using antibody against methyl H4-K20. Histones were extracted from wild-type and mutant larvae as described in Experimental Procedures and were fractionated by SDS-PAGE. The top panel shows a Western blot analysis using an antibody to methyl H4-K20. The middle panel shows a result with an α-general H4 antibody. The bottom panel shows Coomassie staining of histones. *pr-set7*$^{+/+}$, wild-type; *pr-set7*$^{-/-}$, homozygote *l(3)neo41*.

and failed to recognize an unmodified H4 polypeptide as well as other well known methylated lysine residues including histone H3 lysine 4 or lysine 9 (Supplemental Figure S2 [http://www.molecule.org/cgi/content/full/9/6/1201/DC1] and data not shown). Western blot analyses with the antibody were then performed with acid-extracted histones derived from several organisms (Figure 3A). Interestingly, the H4-K20 modification is detectable only on histone H4 derived from higher eukaryotes such as fly, frog, and human. In agreement with the observation described above, we failed to detect the modification in yeast histone H4. Because yeast histone H4 has replaced an isoleucine for a valine at position 21 (Figure 3B, highlighted in green), it was possible that the antibody was unable to recognize methylated yeast histone H4 for this reason. We therefore used rPR-Set7 to methylate yeast nucleosomal H4 in vitro and subjected the modified H4 to Western blotting using the α-methyl H4-K20 antibody (data not shown). Indeed, the antibody was able to recognize the in vitro-methyl-

ated K20 in yeast H4. Therefore, our findings strongly suggest that H4 from *S. cerevisiae* is not methylated at position K20 in vivo.

We also failed to detect the presence of methylated H4-K20 in the ciliated protozoan *Tetrahymena* (Figure 3A). The sequence of the *Tetrahymena* H4 tail is significantly different from that of other eukaryotes (Figure 3B, bottom) including the loss of an arginine at position 3. However, by insertion of a novel serine at position 18, *Tetrahymena* has established a lysine residue at position 20. Our inability to detect H4-K20 methylation in *Tetrahymena* may be due to inability of the antibody to recognize this sequence variation. In addition, neither rPR-Set7 nor ΔN14-rPR-Set7 was able to methylate *Tetrahymena* histone H4, indicating that either *Tetrahymena* is not methylated at H4-K20 or that PR-Set7 possesses a strict amino acid specificity for enzymatic activity that is not present in the *Tetrahymena* sequence. Therefore, it remains an open question whether methylation at H4-K20 occurs in *Tetrahymena*.

Methylation of H4-K20 by PR-Set7 Is Essential for Development in *Drosophila*

Because a PR-Set7 homolog is present in *Drosophila* as a gene product of *CG3307* (Supplemental Figure S1A [http://www.molecule.org/cgi/content/full/9/6/1201/DC1]), and because we were able to demonstrate methylation of H4-K20 in this species (Figure 3A), we chose *Drosophila* as a model system to analyze the biological significance of this modification. The catalytic SET domain of *Drosophila pr-set7* is about 40% identical in amino acid composition to that of human PR-Set7 (Supplemental Figure S1A). In the *Drosophila* gene disruption project, a single, homozygous lethal P element insertion into the 5′-untranslated region of the first exon of *CG3307* (*l(3)neo41*) was isolated (Figure 3C). This P element is deleted by *Df(3R)red31* and is lethal over the deficiency (Spradling et al., 1999). We crossed this mutant with a green fluorescent protein (GFP)-tagged *TM3* balancer chromosome in order to identify homozygous mutants (*pr-set7*$^{-/-}$). Although heterozygous mutants (*pr-set7*$^{+/-}$) generated no obvious phenotype, homozygote mutants died as late second instar larvae (Figure 3D).

In order to determine if the homozygote mutant larvae lacked methylation on H4-K20, we collected wild-type, homozygote mutant and heterozygote, GFP-positive sibling second instar larvae (Figure 3D). Figure 3E shows Western blot of homozygote mutant and wild-type extract using the indicated antibodies. The results demonstrate that the methyl H4-K20 modification clearly exists in the wild-type (*pr-set7*$^{+/+}$) larval extract (Figure 3E, lanes 2 and 3) as well as in their heterozygous siblings (*pr-set7*$^{+/-}$) (data not shown). In contrast, the homozygous (*pr-set7*$^{-/-}$) larval extract yielded no detectable methyl H4-K20 (Figure 3E, lane 1). These observations demonstrate that *Drosophila pr-set7* encodes the major H4-K20 HMT in *Drosophila* and that methylation at this residue is essential for development and viability.

Methyl H4-K20 Is Localized to Chromatin-Dense and Transcriptionally Silent Regions

To gain insights into the function of H4-K20 methylation, we used the highly specific methyl H4-K20-specific antibodies to analyze the distribution of methylated H4-K20 on *Drosophila* polytene chromosomes and mouse embryonic fibroblasts (MEFs). Methylation of histone H4-K20 on polytene chromosomes coincided with condensed chromosomal regions, including chromocentric heterochromatin and numerous bands on the euchromatic arms (Figures 4A–4C). Competition experiments were performed to verify that the observed staining pattern was specific for methyl H4-K20. In these experiments, the staining was completely removed with peptides that contained the H4 tail methylated at lysine 20, but not with unmodified peptides or by peptides that contained the H3 tail methylated at lysine 4 or lysine 9 (data not shown). Moreover, costaining of polytene chromosomes with antibodies raised in different organisms, a rabbit polyclonal and a mouse monoclonal antibody to methyl H4-K20, shows complete overlap (Supplemental Figure S3 [http://www.molecule.org/cgi/content/full/9/6/1201/DC1]).

Having the monoclonal antibody described above allowed us to compare directly the distribution of methylated H4-K20 and that of other modifications that occur on the H3 and H4 tails (Zhang and Reinberg, 2001), using existing rabbit polyclonal antibodies. Comparison of the distribution of methyl H4-K20 and methyl H3-K9 on polytene chromosomes established that the H4-K20 methylation pattern is distinct from the predominantly chromocentric pattern of methylated H3-K9 (Figures 4D–4F), a modification that has been associated with constitutive heterochromatin in various species (Jacobs et al., 2001; Nakayama et al., 2001; Litt et al., 2001; Maison et al., 2002).

We next analyzed the distribution of the methyl H4-K20 with respect to transcriptionally active or competent genes. To accomplish this, we compared the staining pattern on *Drosophila* polytene chromosomes obtained with the polyclonal antibody to methyl H4-K20 to that observed with monoclonal antibody to the transcription-engaged form of RNA polymerase II. This analysis demonstrated nonoverlapping patterns for each of the antibodies in the entire chromosomes, except at regions that were not fully spread (Figures 5A–5C), and thus allowed us to conclude that methylated H4-K20 was very low or absent from transcriptionally competent regions. Similar results were obtained when the staining pattern of the antibody to methyl H4-K20 was compared to that of the transcriptionally active form of RNA polymerase II at heat shock loci under heat shock (transcriptionally permissive) conditions (data not shown).

To further analyze the association of the methyl H4-K20 mark with transcriptionally silent chromatin, we compared the methyl H4-K20 staining pattern to that obtained with antibodies specific to methyl H3-K4, a mark that has been correlated with transcriptionally competent genes in higher eukaryotes (Litt et al., 2001; Noma et al., 2001). Consistent with the results presented above with RNAPII staining, nonoverlapping patterns for methyl H3-K4 and methyl H4-K20 were observed in the entire polytene chromosomes, except at regions that were not fully spread (Figures 5D–5F). Thus, we conclude that the methylated H4-K20 modification marks transcriptionally silent chromatin.

Because H3-K9 methylation is associated with constitutive heterochromatin in various species (Jacobs et al., 2001; Nakayama et al., 2001; Litt et al., 2001; Maison et

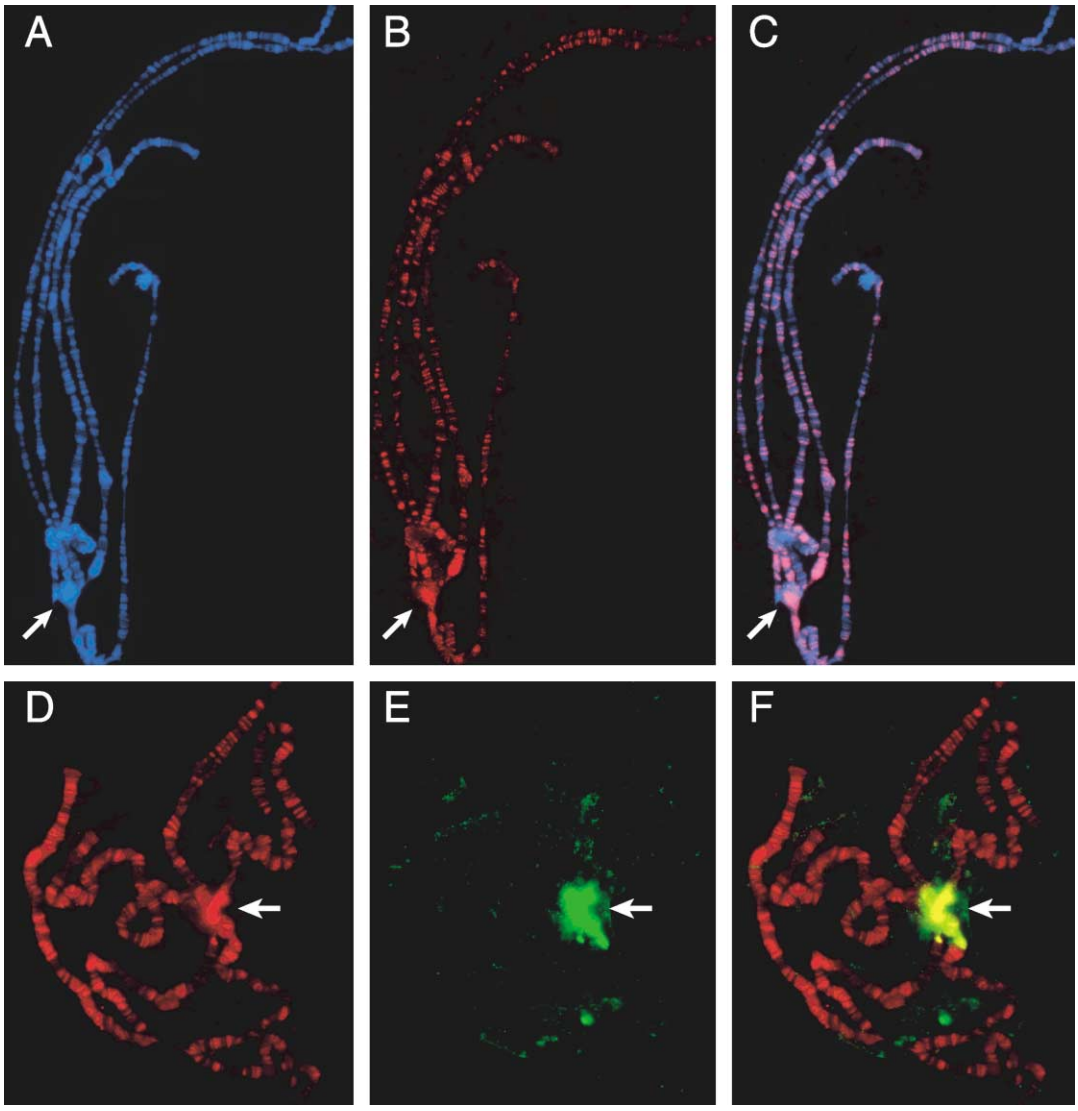

Figure 4. Immunofluorescence Microscopic Analysis of *Drosophila* Polytene Chromosomes

Polytene chromosomes were prepared as described previously (Lis et al., 2000). (A) Hoechst staining to show DNA levels or chromatin density. (B) Chromosomal staining with the polyclonal α-methyl H4-K20 antibody. (C) Merged image of (A) and (B). (D) Chromosomal staining with the monoclonal α-methyl H4-K20 antibody. (E) Chromosomal staining with polyclonal α-methyl H3-K9. (F) Merged image of (D) and (E). In all panels, the arrows indicate the chromocenter.

al., 2002), we analyzed the staining pattern of methyl H4-K20 in MEFs lacking the known H3-K9 HMTs *Suv39h1* and *Suv39h2* (Peters et al., 2001). The localization of methyl H4-K20 in wild-type MEFs was to euchromatin arms and was absent in the constitutive heterochromatin (data not shown). Double knockout MEFs demonstrated unchanged localization of methyl H4-K20 (data not shown), corroborating the findings in *Drosophila* that methyl H4-K20 and methyl H3-K9 are separate and independent modifications. These data suggest that the methyl H3-K9 and methyl H4-K20 modifications may direct the formation of distinct types of silent chromatin.

Negative Interplay between H4-K16 Acetylation and H4-K20 Methylation

Previous studies have established that interplay occurs between acetylation and methylation of residues within the H3 and H4 histone tails (Rea et al., 2000; Wang et al., 2001a, 2001b; Nishioka et al., 2002). The H4 tail can be acetylated at lysines 5, 8, 12, and 16; acetylation of lysines 5 and 12 correlates with histone deposition of newly synthesized histones in *Drosophila* and human cells (Sobel et al., 1995). Acetylation of lysine 16 is observed in the transcriptionally hyperactive male X chromosome in *Drosophila* (Turner et al., 1992) and was enriched in transcriptionally active chromatin in human cells (Johnson et al., 1998). In light of our above results suggesting that methyl H4-K20 is associated with transcriptionally silent chromatin, we tested whether methyl H4-K20 affects acetylation of H4-K16. Given that hyperacetylated H4-K16 is a hallmark of the hyperactive *Drosophila* male X chromosome (Turner et al., 1992), we analyzed for the presence of methylated H4-K20 and acetylated H4-K16 on this chromosome. As previously

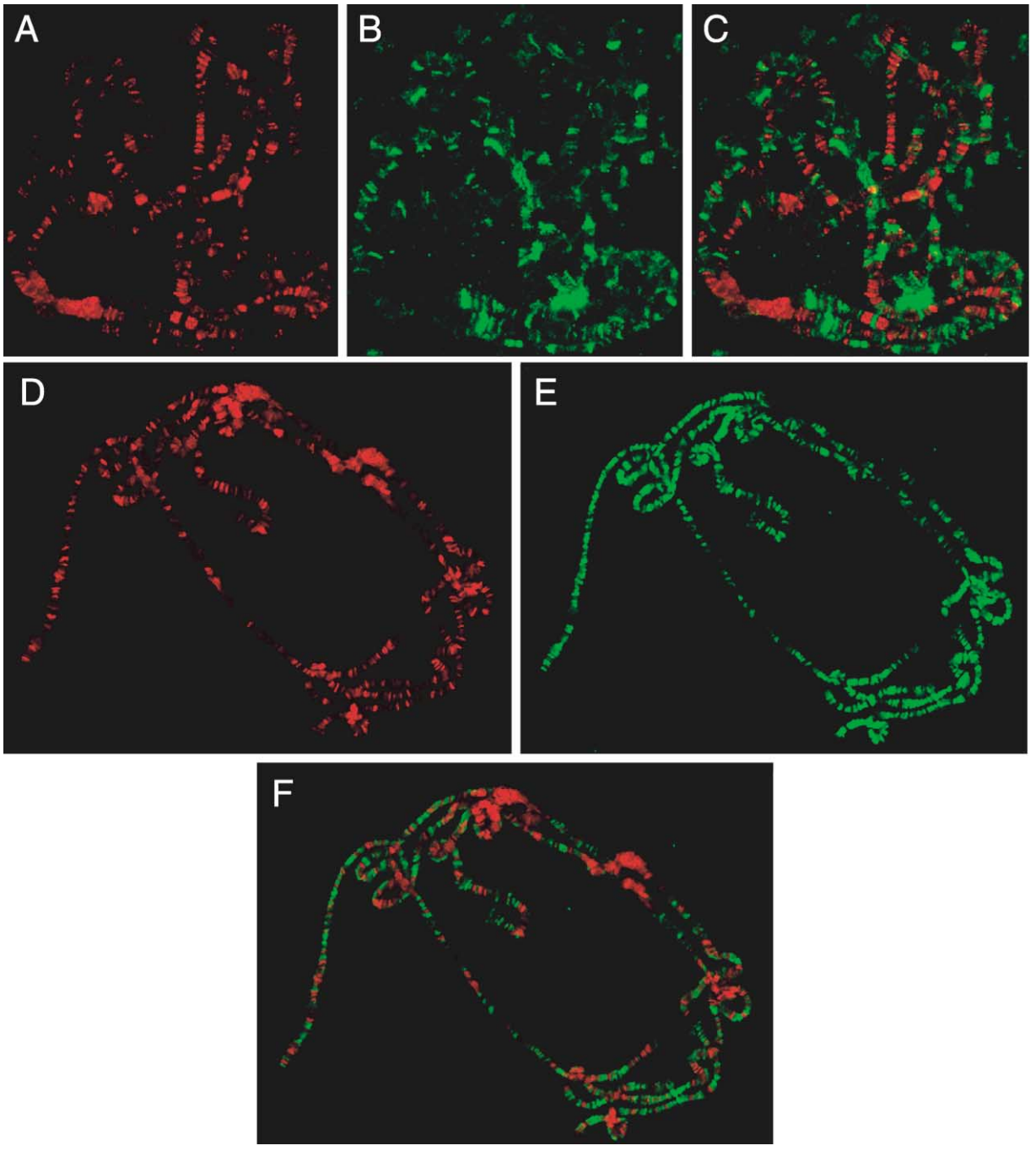

Figure 5. H4-K20 Methylation Is Undetectable in Transcriptionally Competent Regions on *Drosophila* Polytene Chromosomes

Drosophila polytene chromosomes were prepared as previously described (Lis et al., 2000) and stained with the polyclonal or monoclonal methyl H4-K20 antibody ([A] or [D], respectively) and with monoclonal antibody to the serine 2-phosphorylated form of RNA polymerase II (B), or the polyclonal methyl H3-K4 antibody (E). (C) Merged image of (A) and (B). (F) Merged image of (D) and (E).

reported (Turner et al., 1992), significant staining with antibody specific to acetylated H4-K16 was detected on the *Drosophila* male X chromosome and not on that of female (Figures 6A–6C and data not shown). In contrast, low levels of methylated H4-K20 were detected on the male X chromosome, which was comparable to that of female (Figures 6B and 6C and data not shown). Thus, there is an inverse correlation in the number and intensity of bands containing methyl H4-K20 and acetyl H4-K16. These data suggest a negative interplay between methylation of H4-K20 and acetylation of H4-K16.

To determine whether there is a biochemical link between these modifications, we tested the ability of the histone acetyltransferase (HAT), p300, to acetylate H4-K16 in the presence or absence of methyl H4-K20. An unmodified and a methyl H4-K20-containing peptide

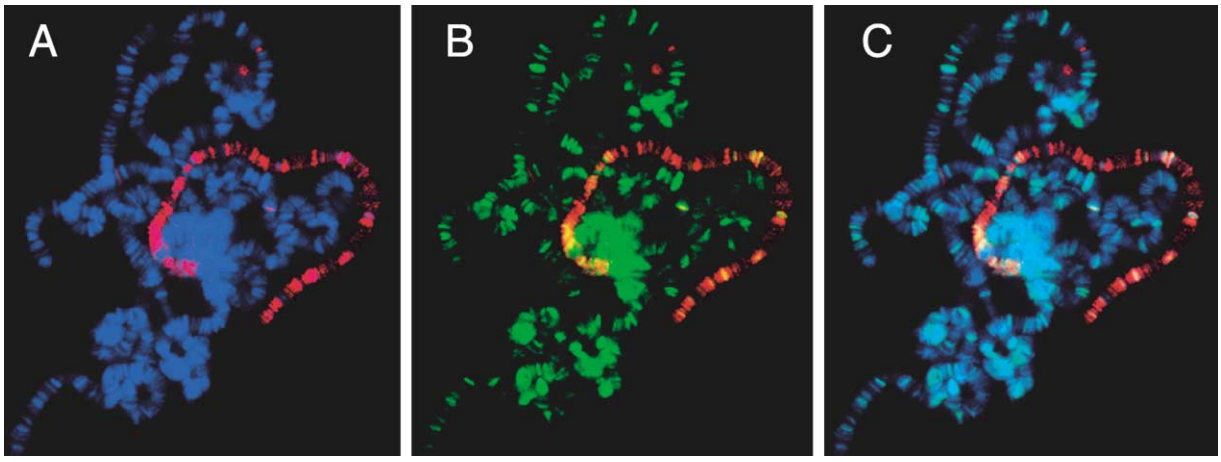

Figure 6. Interplay between Methyl H4-K20 and Acetyl H4-K16 on *Drosophila* Polytene Chromosomes

Drosophila male polytene chromosomes were costained with the antibodies specifically recognizing methyl H4-20 (green) and acetyl H4-K16 (red), where DNA was counterstained with DAPI (blue). (A) DAPI and acetyl H4-K16. (B) Methyl H4-K20 and acetyl H4-K16. (C) Merged image of DAPI, methyl H4-K20, and acetyl H4-K16.

from residues 9 to 25 were created and used as substrates in the assay. We found that methylation of H4-K20 inhibited acetylation by p300 by 50%–60% compared to the unmodified peptide (Figure 7A). Since this peptide set contains lysines 12 and 16, both of which are known targets of p300 HAT activity (Schiltz et al., 1999), the question of which lysine residue was affected by methyl H4-K20 had to be resolved. Dot blot analysis using site-specific acetyl-H4 antibodies demonstrated that the p300-mediated acetylation of lysine 16, but not acetylation of lysine 12, was inhibited by methylation of lysine 20 (Figure 7B). Using the same experimental approach, we discovered that the acetylation of H4-K16 inhibited the ΔN14-rPR-Set7-mediated methylation of H4-K20 (Figure 7C). Taken together, these data indicate that acetylation of H4-K16 and methylation of H4-K20 are mutually restrictive both in vivo and in vitro, indicating that methyl H4-K20 maintains silent chromatin, in part, by precluding neighboring acetylation on the histone H4 tail.

Discussion

Although histone proteins have long been recognized to be methylated at specific residues in vivo (van Holde, 1988), the enzymes that catalyze the modification reaction and the functions of these modifications have only recently begun to be revealed (Zhang and Reinberg, 2001). In this study, we isolated an HMT, PR-Set7, which specifically methylates lysine 20 of histone H4 exclusively within a nucleosomal context. Prior to this study, the function(s) of lysine methylated histone H4 was obscure, but was largely believed to be associated with transcriptionally active rather than repressed genes (Hendzel and Davie, 1989, 1991). However, here we established that methylated H4-K20 is associated with silent chromatin. We also demonstrated, in support of the "histone code hypothesis" (Strahl and Allis, 2000), that methylation at H4-K20 inhibits acetylation of H4-K16 and vice versa. Consistent with the notion that an enzyme that alters the establishment of silent chromatin should have a tremendous impact on gene expression, our studies establish that the absence of methyl H4-K20 in vivo impairs the development and viability of a multicellular organism. Based upon the available evidence, we favor the view that a lack of, or diminishment of, H4-K20 methylation may alter patterns of gene expression, by perturbing a generally repressive, higher order chromatin structure that critically depends upon H4-K20 methylation.

PR-Set7 Catalyzes Methylation of Nucleosomal H4-K20

The enzymatic activity of PR-Set7 is contained within a single polypeptide of ~40 kDa that appears to exist as a homodimer, as the native protein elutes from a gel filtration column with an apparent mass of ~70 kDa. The results demonstrate that PR-Set7 is an H4-K20-specific HMT, as the enzyme did not methylate any other residue on histone H4 or on any other histone (Figures 1E and 2C). In agreement with previous studies demonstrating that the SET domain can be a signature for lysine-HMTs (Rea et al., 2000; Tachibana et al., 2001; Wang et al., 2001a; Nishioka et al., 2002), PR-Set7 contains a SET domain, and a single substitution of a conserved arginine to glycine within the SET domain abolished its enzymatic activity (Figures 2A–2C). Interestingly, PR-Set7 is devoid of the Pre- and Post-SET domains (Supplemental Figure S1B [http://www.molecule.org/cgi/content/full/9/6/1201/DC1]), demonstrating that these domains, although important for the functions of other HMTs, are not absolutely required for HMT activity. PR-Set7 was found to be highly specific for nucleosomes, as no activity could be demonstrated when histones were used as a substrate (Figures 1F and 2C). This lack of activity on nonnucleosomal histones is not likely to be due to the absence of the Pre- and Post-SET domains, because we recently isolated an HMT with specificity for H3-K4 that exclusively methylates free histones and lacks both of these domains (Nishioka et al., 2002; also see Wang et al., 2001a).

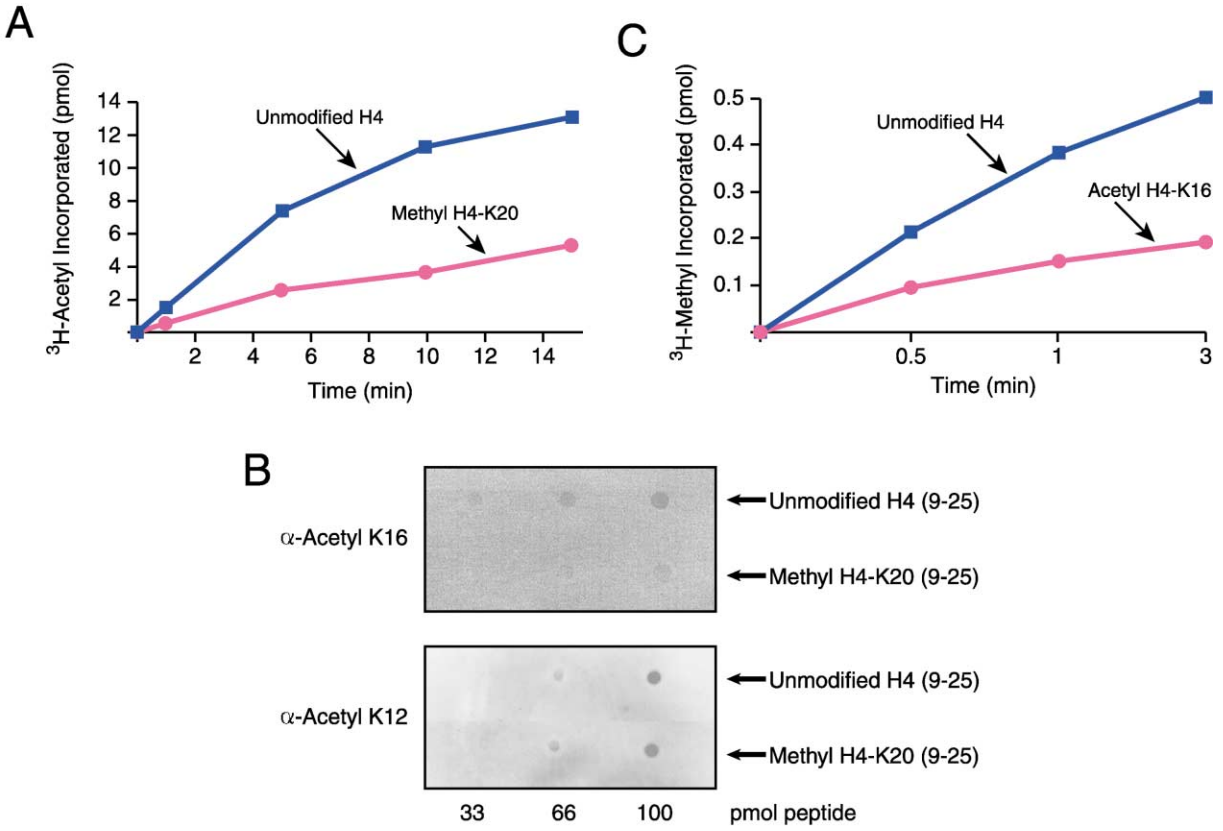

Figure 7. Interplay between Methylation and Acetylation on Histone H4 Tail

(A) Effect of methylated H4-K20 on the histone acetyltransferase (HAT) activity of p300. Recombinant p300 was purified from baculovirus-infected SF9 cells and was incubated for the indicated number of minutes with either unmodified or di-methyl K20-containing synthetic histone H4 tail peptides (H4 tail amino acid residues 9 to 25) as substrates. Incorporated ^{3}H-acetyl moieties were detected and plotted on the *y* axis.

(B) Dot blot analysis of the HAT activity of p300. HAT assays using both p300 and the synthetic peptides described above were performed, and the reaction mixtures were dotted onto nitrocellulose membrane. Western blot analysis with titration of amounts of peptides was performed using antibodies to acetyl H4-K16 and acetyl H4-K12.

(C) Effect of acetyl H4-K16 on the HMT activity of PR-Set7. ΔN14-rPR-Set7 was incubated for the indicated number of minutes with either unmodified or monoacetylated K16-containing synthetic histone H4 tail peptides (H4 tail amino acid residues 9 to 25) as substrates. Incorporated ^{3}H-methyl moieties were detected and plotted on the *y* axis.

PR-Set7 and Methyl H4-K20 Are Found Only in Higher Eukaryotes

Enzymes that affect the formation of silent chromatin are expected to be present in all organisms capable of compacting their DNA. However, our studies indicate that both the homolog of PR-Set7 and methyl H4-K20 are absent from the yeast *S. cerevisiae*. An explanation for this apparently disparate finding may reside in the way that nucleosomes are packaged in yeast. Recent crystallographic studies have established that intern-ucleosomal interactions are different in higher eukaryotes (frog) and yeast (Luger et al., 1997; White et al., 2001). In nucleosomes of higher eukaryotes, a patch of basic residues, amino acids 16–25, within the histone H4 tail has been reported to interact with a patch of acidic residues present on the surface of the H2A-H2B dimer in the neighboring nucleosome (Luger et al., 1997). Therefore, enzymes that can modify residues within these regions, including acetylation of H4-K16 and methylation of H4-K20, may likely alter or control higher order chromatin structure (Luger and Richmond, 1998). Importantly, structural studies of yeast nucleosome revealed that the histone H4 tail does not interact with the patch of acidic residues in the neighboring nucleosome (White et al., 2001). These observations, combined with the fact that yeast exhibits more relaxed and less condensed chromatin, provide a plausible explanation to our finding that H4-K20 methylation was not detectable in yeast.

Methyl H4-K20 as a Basic Element of Silent Chromatin?

Our data show that methylated H4-K20 and acetylated H4-K16 are mutually restrictive modifications (Figures 6 and 7) and that methyl H4-K20 is localized in transcriptionally silent chromatin (Figure 5). According to previous observations by chromatin immunoprecipitation analysis, acetylated H4-K16 was enriched in transcriptionally active chromatin (Johnson et al., 1998). In a related finding, the ATPase activity of *Drosophila* ISWI, a protein present in complexes that was suggested to maintain higher order chromatin structure (Deuring et al., 2000), is suppressed by the presence of acetylated H4-K16 (Clapier et al., 2002; Corona et al., 2002). Taken

together with the results obtained from structural studies of nucleosomes (Luger et al., 1997; White et al., 2001), these observations strongly suggest that methylated H4-K20 not only renders chromatin "tightly closed" by inhibiting the acetylation of histone H4-K16 but may provide an appropriate substrate for ISWI to generate regularly spaced higher order chromatin structure. Moreover, and related to these observations, previous studies have established that the localization of ISWI on polytene chromosomes is predominantly associated with regions that are not highly transcribed and thus would be expected to overlap the pattern of methylated H4-K20 (Deuring et al., 2000). Although we have not detected specific binding of human ISWI to methyl H4-K20-containing peptides or alterations of the ISWI ATPase activity by methylated H4-K20 (K.N. and D.R., unpublished data), it is tempting to speculate that ISWI is recruited to chromatin containing methylated H4-K20.

Can PR-Set7 Regulate Mechanisms for Propagating Cellular Memory?

It is known that histone H4 is temporally hyperacetylated during (or shortly after) DNA replication and is deacetylated after nucleosome deposition, although some parts of the chromosomes remain hyperacetylated during mitosis (Jeppesen, 1997; Jasencakova et al., 2000). A hypothesis has been put forth that acetylation of histone H4 could provide a mechanism for propagating the appropriate chromatin structure, presumably transcription-competent chromatin, to newly synthesized DNA (Jeppesen, 1997). However, a counterregulatory machinery that maintains the integrity of transcriptionally silent chromatin has not been discovered. Particularly, none of the histone deacetylase activity targeting "nucleosomal H4-K16" has been identified. The data presented here indicate that methylated H4-K20 and acetylated H4-K16 are mutually restrictive modifications (Figures 6 and 7), and we suggest that these modifications are coordinately regulated during the cell cycle. In other words, we speculate that during mitosis, PR-Set7 marks H4-K20 residues with methyl groups according to the map of nonacetylated H4-K16 on mitotic chromosomes, and then the methyl H4-K20 can counteract acetylation of H4-K16 during the next cell cycle. Thus, methylated H4-K20 could represent an epigenetic mark of silent chromatin that is propagated during cell division.

Experimental Procedures

Purification of PR-Set7

HeLa-derived nuclear extracts were prepared as previously described (LeRoy et al., 1998). All procedures were performed at 4°C. Approximately 20 g of proteins derived from the nuclear pellet fraction was loaded onto a 2000 ml DEAE-52 (Whatman) column equilibrated with buffer D (50 mM Tris-HCl [pH 7.9], 0.2 mM EDTA, 10% glycerol, 1 mM DTT, and 0.2 mM PMSF) containing 0.1 M ammonium sulfate. The column was eluted with a 5 column volume linear 0.1 to 0.65 M ammonium sulfate gradient in buffer D. PR-Set7 activity was in the flowthrough, and this fraction then was loaded onto a 1000 ml phosphocellulose (Sigma) column equilibrated with buffer D containing 0.1 M ammonium sulfate. The column was eluted with a 10 column volume linear 0.1 to 0.6 M ammonium sulfate gradient in buffer D. A Suv39h1-like activity eluted between 0.1 to 0.2 M ammonium sulfate with the bulk of the protein, while PR-Set7 activity eluted between 0.15 to 0.25 M ammonium sulfate. The HMT active fractions were pooled and adjusted to 1 M ammonium sulfate.

The pooled sample was loaded onto an 8 ml phenyl-superose (Amersham Pharmacia Biotech) column. The column was eluted with a 10 column volume linear ammonium sulfate gradient (1 to 0 M) in buffer D. The HMT activity eluted between 0.5 to 0.75 M ammonium sulfate. The active fractions were pooled, dialyzed against buffer D containing 0.05 M ammonium sulfate, and loaded onto a 3.3 ml DEAE-5PW (TosoHaas) column. PR-Set7 activity was again in the flowthrough. The sample was concentrated using a small amount of phosphocellulose resin and loaded onto either a 480 ml Superose 6 (Amersham Pharmacia Biotech) column or a 2.4 ml Superdex S200 column (SMART system, Amersham Pharmacia Biotech) equilibrated in buffer D containing 0.5 M ammonium sulfate. PR-Set7 activity eluted in a set of fractions that corresponded to an approximately 70 kDa molecular size. The eluate was dialyzed against buffer H (20 mM HEPES-KOH [pH 7.9], 0.2 mM EDTA, 10% glycerol, 1 mM DTT, and 0.2 mM PMSF) containing 0.1 M ammonium sulfate and was loaded onto a 1 ml MonoS (Amersham Pharmacia Biotech) column. The column was eluted with a 15 column volume linear 0.1 to 0.5 M ammonium sulfate gradient in buffer D. The PR-Set7 activity eluted between 0.3 to 0.4 M ammonium sulfate. The active fractions were pooled, dialyzed against buffer D containing 0.1 M ammonium sulfate, and loaded onto a 1 ml HiTrap Heparin (Amersham Pharmacia Biotech) column. The column was eluted with a 10 column volume linear 0.1 to 1 M ammonium sulfate gradient in buffer D. The PR-Set7 activity eluted between 0.4 to 0.5 M ammonium sulfate. The active fractions were pooled and analyzed by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The polypeptide that coeluted with the PR-Set7 activity was excised from the gel and analyzed by mass spectrometry.

Protein Identification

Gel-bound polypeptides were subjected to in-gel tryptic digestion and mass spectrometric analysis as previously described (Erdjument-Bromage et al., 1998). Identification of the spectra corresponding to known peptide sequences in the NCBI nonredundant database was facilitated using the PeptideSearch (Mann et al., 1993) algorithm. The data obtained from PR-Set7 polypeptides revealed that the peptide masses matched a protein called PR/SET domain containing protein 07 (accession number NP065115) in the NCBI database.

Cloning and Plasmid Construction for rPR-Set7

The open reading frame of PR-Set7 (GenBank accession number AY064546) was amplified from a Marathon cDNA library derived from HeLa cells (Clontech) using the polymerase chain reaction (PCR) and 5′-NdeI site-containing primer and a 3′-FLAG-tag and HindIII site-containing primer. The PCR-amplified gene was then c cloned into the pCR2.1 vector (Invitrogen). The FLAG-tagged PR-Set7 cDNA was inserted into the NdeI-HindIII sites of the bacterial expression vector pET-28b (Novagen). The vector encoding ΔN14-rPR-Set7 was generated by insertion of an EcoRI-HindIII fragment derived from clone CS0DK003YG13 (Research Genetics) into the corresponding sites of pET-30c, resulting in an extra 18 amino acids of linker sequence instead of a 14 amino acids deletion in the N-terminal part of PR-Set7. The recombinant proteins were expressed in *E. coli* strain BL21(DE3)pLysS (Invitrogen), which was incubated at 37°C for 3 hr, and purified on Ni-NTA agarose according to the manufacturer's instructions (Qiagen). Because ΔN14-rPR-Set7 was expressed in inclusion bodies in bacteria, the purified protein was renatured by step dialysis. Each purified protein preparation was dialyzed against buffer D containing 0.1 M ammonium sulfate, and it was confirmed by Superdex S200 gel-filtration that the various PR-Set7 proteins did not aggregate. Site-directed mutagenesis of the PR-Set7 expression plasmid was carried out using the Quick Change Site-Directed Mutagenesis Kit according to the manufacturer's instructions (Stratagene).

Histone Methyltransferase Assay and Preparation of Its Substrates

The samples or rPR-Set7 protein were incubated at 30°C for 1 hr in a reaction buffer that contained 50 mM Tris-HCl (pH 8.5), 5 mM

$MgCl_2$, 4 mM DTT, and 1 μM ^{3}H-labeled SAM (Amersham Pharmacia Biotech). Two micrograms of either histone octamer, oligonucleosomes, or mononucleosomes was used as substrates. The total volume of the reaction mixture was adjusted to 25 μl. The reaction was stopped by addition of SDS sample buffer, and the reaction mixtures were fractionated by 15% SDS-PAGE. Separated histones were then transferred to a PVDF membrane and visualized by Coomassie staining. The membrane was sprayed with EN^{3}HANCE (NEN) and exposed to Kodak XAR film overnight.

Native octamers, oligonucleosomes, and mononucleosomes were purified from HeLa cells as previously described (Orphanides et al., 1998). Recombinant *Xenopus* octamers and oligonucleosomes were prepared as described (Luger et al., 1999), where 5S ribosomal DNA repeats were used for assembly of oligonucleosomes. Purified calf thymus histone H1 was purchased from Roche Molecular Biochemicals.

Radiolabeled Histone Sequencing

Recombinant *Xenopus* histone H4 assembled into nucleosomes was labeled with ^{3}H-SAM using purified native enzyme and fractionated by a 15% SDS polyacrylamide gel. The polypeptide was then transferred to a PVDF membrane and subjected to Edman degradation amino acid analysis coupled with scintillation counting.

Histone H4 Di-Methyl Lysine 20-Specific Antibodies

A conjugated peptide derived from the N terminus of human histone H4 residues 17 to 28, which contains a single di-methyl lysine at position 20 (RHRKVLRDNIQGC), was conjugated to keyhole limpet hemocyanin, and the resulting molecule was used for immunization of rabbits. Polyclonal antisera were processed by affinity purification using a modified peptide-conjugated sepharose column, and the bound antibody fraction was then loaded onto an unmodified peptide-conjugated column. The unbound fraction was collected and subjected to Western blotting or immunofluorescence microscopy.

Monoclonal antibody against the same peptide was developed by Bios-Chile using the general procedure of Kohler and Milstein (1975). The peptide was coupled to ovalbumin and used to immunize 2-month-old female Balb/c mice. Hybridoma supernatants were screened by ELISA using multi-well plates activated with the methylated peptide. Positive cells were recloned by the limiting dilution technique. For ascites, fluid hybridomas were grown in the peritoneal cavity of Balb/c mice previously injected with 0.5 ml of Pristane (Sigma). Characterizations of these antibodies are provided in Supplemental Figures S2 and S3 (http://www.molecule.org/cgi/content/full/9/6/1201/DC1).

Interplay between Methylation and Acetylation

The HMT and HAT assays were performed under the same conditions as described previously (Nishioka et al., 2002), where ^{3}H-labeled SAM was used for the HMT assays and ^{3}H-labeled acetyl-CoA was used for the HAT assays. For analysis of site specificity of HAT activity, the reaction mixtures were dotted onto nitrocellulose membrane with titration of amounts of peptides and Western blotted using antibodies to acetyl H4-K16 and acetyl H4-K12 (Serotec). A histone H4 N-terminal peptide was synthesized that contained amino acid residues 9 to 25, followed by a GG-linker and a biotinylated lysine. Modified peptides were synthesized using either dimethylated lysine or monoacetylated lysine in indicated position.

Immunofluorescence Microscopy

For *Drosophila* polytene chromosomes preparation, salivary glands of third instar larvae were dissected, and polytene chromosomes were isolated and mounted on slides as previously described (Lis et al., 2000). Polytene chromosomes were stained with a 1:5 dilution of mouse monoclonal, a 1:20 dilution of the rabbit polyclonal α-methyl H4-K20 antibodies, a 1:50 dilution of the α-methyl H3-K9 antibody (Upstate), a 1:50 dilution of α-methyl H3-K4 antibody (Upstate), or a 1:10 dilution of α-Pol II antibody (Covance H5). The fluorescence-conjugated secondary antibodies were used as described in Park et al. (2001).

Drosophila Mutant Analysis

Fly stocks were obtained from Bloomington Stock Center. Second instar larvae were raised on standard *Drosophila* egg-lay plates at room temperature and collected about 55 hr after egg-lay. The larvae (heterozygote or mutant) were homogenized in 100 μl of extraction buffer (20 mM Tris-HCl [pH 8.0], 10 mM EDTA, 150 mM NaCl, 0.2% NP-40, and 0.2% Triton X-100). Ten microliters of the extract from either mutant or heterozygous larvae and increasing amounts of extract from the wild-type larvae were fractionated by a 15% SDS-polyacrylamide gel and Western blotted using a 1:1000 dilution of the α-methyl H4-K20 antibody (Upstate Biotech) or a 1:1000 dilution of an α-general histone H4 antibody (Upstate Biotech).

Organismal "Zoo" Blot

For zoo blot analysis, 200 ng of *Xenopus* recombinant histone H4 and 2 μg each of the core histones from human HeLa cells, *Xenopus* XTC cells, *Drosophila* S2 cells, *S. cerevisiae*, and *T. thermophila* were acid extracted and fractionated by a 15% SDS-polyacrylamide gel. The histones were then transferred to a PVDF membrane, Ponceau stained, and blotted with a 1:1000 dilution of α-methyl H4-K20 antibody.

Acknowledgments

We thank members of the Allis, Lis, and Reinberg laboratories for helpful discussions and to Sherrie Hu for technical assistance. We thank Brian Schwartz for analyzing the distribution of RNA Polymerase II (regardless of CTD-phosphorylation) with respect to methyl H4-K20 on *Drosophila* polytene chromosomes (see cover). This work was supported by grants from NIH (GM-37120) and the Howard Hughes Medical Institute to D.R., NIH (GM-53512) to C.D.A., NIH (GM-25232) to J.T.L., NCI Cancer Center Grant P30 CA08748 to P.T., and an NIH training grant (GM-64279) to J.C.R. We also wish to thank Upstate Biotechnology, especially Mary Anne Jelineck and Rene Rice, for their support and development of the polyclonal methyl H4-K20 antibody. We thank Thomas Jenuwein for the use of the wild-type and *Suv39h1/h2* double knockout MEFs.

Received: May 10, 2002

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