Methylation of Histone H3 by COMPASS Requires Ubiquitination of Histone H2B by Rad6*

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The DNA of eukaryotes is wrapped around nucleosomes and packaged into chromatin. Covalent modifications of the histone proteins that comprise the nucleosome alter chromatin structure and have major effects on gene expression. Methylation of lysine 4 of histone H3 by COMPASS is required for silencing of genes located near chromosome telomeres and within the rDNA (Krogan, N. J, Dover, J., Khorrami, S., Greenblatt, J. F., Schneider, J., Johnston, M., and Shilatifard, A. (2002) J. Biol. Chem. 277, 10753–10755; Briggs, S. D., Bryk, M., Strahl, B. D., Cheung, W. L., Davie, J. K., Dent, S. Y., Winston, F., and Allis, C. D. (2001) Genes. Dev. 15, 3286–3295). To learn about the mechanism of histone methylation, we surveyed the genome of the yeast Saccharomyces cerevisiae for genes necessary for this process. By analyzing ~4800 mutant strains, each deleted for a different non-essential gene, we discovered that the ubiquitin-conjugating enzyme Rad6 is required for methylation of lysine 4 of histone H3. Ubiquitination of histone H2B on lysine 123 is the signal for the methylation of histone H3, which leads to silencing of genes located near telomeres.

Heritable, quasistable modifications of histone proteins, such as acetylation, phosphorylation, and methylation, play essential roles in the regulation of gene expression in eukaryotic organisms and have been proposed to be required for developmental and cellular commitment in metazoa (1–3). Histone acetylations on histones H3 and H4 are the best characterized covalent modifications of histones and have been demonstrated to have wide ranging effects on the regulation of gene expression (1). Phosphorylation has been demonstrated to be an important modification of histone in transcriptional activity, condensation of chromosomes during mitosis and meiosis, and regulation of cell division.

Methylation of lysine 4 in the amino-terminal tail of histone H3, mediated by a multiprotein complex we call COMPASS (complex of proteins associated with Set1) (4–7, 9), is required for silencing of expression of genes located near chromosome telomeres and within the rDNA (5, 6). We and others have demonstrated that COMPASS includes Set1, a member of the chromatin-associated proteins (the Trithorax (Trx) group) that possess a sequence motif called the SET domain, and seven other polypeptides (4–7, 9). The SET domain takes its name from the Drosophila proteins Su(var)3-9, Enhancer of zest (E(z)), and Trx. Also, COMPASS contains another yeast protein related to the human Trx protein ASH2. SET domain-containing proteins have been implicated in histone methylation and regulation of transcription in several organisms (5, 8–12). Although the SET domain-containing proteins play fundamental roles in development and oncogenesis, their molecular function is poorly understood (13–16).

To better understand the mechanism of histone H3 lysine 4 methylation, we surveyed the genome of the yeast Saccharomyces cerevisiae for genes necessary in this process. Analysis of ~4800 mutant strains resulted in the discovery of the ubiquitin-conjugating enzyme Rad6 as a protein required for methylation of lysine 4 of histone H3. We have demonstrated that ubiquitination of histone H2B on lysine 123 is the signal for the methylation of histone H3 by COMPASS, which leads to silencing of genes located near telomeres.

MATERIALS AND METHODS

Preparation of Yeast Cell Extracts from 96-well Plates—Using a 96-well pinning device, the entire collection of 4800 yeast non-essential gene deletion mutants was inoculated from ~80 °C stocks onto agar plates containing YPD (1% yeast extract, 2% proteo-peptone, 2% dextrose) + 200 μg/ml Geneticin (Invitrogen), allowed to grow for 48 h, and used to inoculate 96-tube PCR plates filled with 100 μl of YPD. After 48 h of growth at 30 °C the plates were centrifuged at 2000 × g for 10 min. Medium was removed by wrist-snap inversion and draining into absorbent towels. Plates were then covered and frozen at ~80 °C for up to 1 week. Cells were thawed at room temperature, resuspended in 30 μl of lysis buffer (20 mM Tris, pH 7.5, 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 0.1% Nonidet P-40, 1 mg/ml Zymolyase 100T), and incubated at 37 °C for 15 min. 10 μl of 4× Laemmli loading buffer were added, and the samples were vortexed briefly before heating at 100 °C for 5 min.

Preparation of Total Yeast Cell Extracts and Analysis for Histone H3 Lys-4 Methylation—Yeast cells were grown to mid-log phase in YPD medium, pelleted, washed with distilled water, pelleted, and resuspended in lysis buffer (20 mM Tris, pH 7.5, 50 mM KCl, 1 mM EDTA, 0.1% Nonidet P-40, 1 mg/ml Zymolyase 100T), and incubated at 37 °C for 15 min. 10 μl of 4× Laemmli loading buffer were added, and the samples were vortexed briefly before heating at 100 °C for 5 min.

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by detection of the bound antibody with horseradish peroxidase-conjugated anti-rabbit IgG secondary antibodies (1:10,000 dilution).

RESULTS AND DISCUSSION

Development of a Biochemical Screen in 96-well Plates to Identify Genes Essential for Lys-4 Methylation of Histone H3—To learn more about the mechanism of histone methylation by COMPASS, we sought to identify all the (non-essential) proteins in S. cerevisiae necessary for methylation of Lys-4 of histone H3. As described under “Materials and Methods,” we developed a method to survey the genome of S. cerevisiae for genes required for this histone modification by testing extracts of each of the ~4800 viable mutants for the presence of Lys-4-methylated histone H3. Mutants missing a component of COMPASS are defective in this histone H3 modification. As shown in Fig. 1A, cells deleted for set1 are defective for the methylation of Lys-4 of histone H3. When this assay was performed in a 96-well plate, the mutant cell missing Cps50/Yar003 was found to be defective for the methylation of Lys-4 of histone H3. However, all of the other 95 mutants in this microtiter plate possess this modification (Fig. 1B).

Rad6 Is Essential for Lys-4 Methylation of Histone H3—Screening of all 52 microtiter plates containing the 4827 mutants we tested revealed that the rad6 deletion mutant is defective in histone H3 Lys-4 methylation (Fig. 2A). This was confirmed by testing histone H3 Lys-4 methylation in two independently generated rad6 mutants and the homozygous diploid generated from them (Fig. 2, B and C). Also, the H3 Lys-4 methylation-deficient phenotype of the rad6Δ cells was complemented by a plasmid containing RAD6 (Fig. 2D).

Lysine 123 of Rad6 Is Essential for Lys-4 Methylation of Histone H3—Rad6 is a ubiquitin-conjugating enzyme (E2) (17) involved in DNA repair (17, 18), DNA damage-induced mutagenesis (19, 20), meiosis (21), transposition of retrotransposons (22), and gene silencing (23). Rad6 catalyzes ubiquitination of Lys-123 of histone H2B (24). It seemed possible that this modification of histone H2B is responsible for the requirement of Rad6 for methylation of histone H3. Indeed, histone H3 is not Lys-4-methylated in a strain that is unable to attach ubiquitin to Lys-123 of histone H2B (due to a change of Lys-123 to Arg) (Fig. 3). We conclude that ubiquitination of H2B at Lys-123 is required for methylation of Lys-4 of histone H3. This is consistent with the observation that RAD6 is required for telomeric gene silencing (23) since methylation of histone H3 Lys-4 is required for telomeric and rDNA silencing (5, 6).

Involvement of Other Rad6-interacting Proteins in Lys-4 Methylation of Histone H3—Several proteins are involved with Rad6 in diverse cellular processes (17–22). However, our genome-wide survey of S. cerevisiae revealed that none of the mutants missing these (non-essential) Rad6-interacting proteins, including those that act with Rad6 in DNA damage repair (Rad18 and Rex4) and the N-end rule-dependent protein degradation pathway (Ubr1 and Ubr2), are involved in methylation of histone H3 Lys-4 (Fig. 3C). This indicates that Rad6 functions in the regulation of gene expression by controlling methylation of histone H3 independently of the above proteins.

To define the biochemical characteristics of Rad6 protein, we used Rad6-specific polyclonal antibody to determine whether Rad6 exists in a large macromolecular complex. Our analysis suggests that at least a portion of Rad6 protein is found in a large complex in yeast extracts (Fig. 3D). Perhaps this complex...
includes an E3 ligase required for the recognition of histone H2B as specific substrate.

Our understanding of the role of the Trx class of proteins in regulation of gene expression and development is rudimentary. The Set1 protein of yeast is similar to the Drosophila and human trithorax proteins (Trx and MLL, respectively). The Trx protein may function as a DNA-binding protein and appears to be a regulator of gene expression. Mutations affecting MLL, the human homologue of Trx, result in the development of hematological malignancies. Our molecular and biochemical characterization of the Set1-containing protein complex we call COMPASS is a first step toward understanding the function of SET domain-containing proteins in regulation of gene expression.

We and others have now provided evidence that COMPASS is a histone methyltransferase that catalyzes methylation of Lys-4 of histone H3 (4–7, 9).

Our analysis of −4800 mutant strains of the yeast S. cerevisiae for a defect in histone H3 Lys-4 methylation resulted in the discovery of the ubiquitin-conjugating enzyme Rad6 as a protein required for this process (Fig. 4). We have shown that lysine 123 of histone H2B, which is ubiquitinated by Rad6 (24), is essential for the methylation of histone H3 by COMPASS, which leads to silencing of genes located near telomeres. Our results presented here (Fig. 4) suggest that histone H2B in nucleosomes in “active” regions of chromatin is recognized by the Rad6 complex and ubiquitinated on Lys-123. The ubiquitination of histone H2B provides a signal for either direct or indirect recruitment and/or activation of COMPASS. COMPASS can then catalyze the methylation of lysine 4 of histone H3, which leads to silencing of that region of chromosomal DNA (5, 6). An important, unanswered question is whether ubiquitination and/or methylation of histones is limited to certain (silent) regions of chromosomes or whether these modifications occur throughout chromosomes. Ubiquitination has recently been demonstrated to be involved in the regulation of gene expression in eukaryotic cells (25). Although the work presented here cannot at this time rule out a role for protea-

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**Fig. 3.** Ubiquitination of lysine 123 of histone H2B is essential for Lys-4 methylation of histone H3. A, schematic representation of histone H2B and its lysine 123 that is ubiquitinated by Rad6 (24). B, cell extracts from haploid strains missing htb1-1 and htb2-1 and containing a CEN-URA3 plasmid with HTA1 and either wild-type HTB1 or htb1 with lysine 123 converted to arginine (K123R, generously provided by Mary Anne Osley, Ref. 24) were analyzed for the presence of Lys-4-methylated histone H3. C, the presence of methylation of Lys-4 of histone H3 in yeast strains missing genes encoding Rad6, Ubr1, Ubr2, Rad18, and Rex4 was determined by subjecting 2000 ng of whole cell extracts from cells deleted for these genes to 16% SDS-PAGE followed by immunoblotting as described above. D, the presence of Rad6-containing macromolecular complexes was determined by the application of wild-type yeast extract on a Superose-6 PC size exclusion column. 100-μl fractions were collected, and 25 μl were subjected to SDS-PAGE, then transferred to nylon membranes, and probed with a Rad6-specific polyclonal antibody. WT, wild type; Meth., methylated.

**Fig. 4.** Model of the role of Rad6 and COMPASS in regulating chromatin structure and gene silencing. Histone H2B within the active region of chromosomal DNA is recognized by the Rad6 protein complex and ubiquitinated on its lysine 123. This ubiquitination of histone H2B serves as a direct or indirect recognition signal and/or activation signal for COMPASS, which catalyzes methylation of lysine 4 of histone H3 and results in silencing of that region of chromosomal DNA. Ub, ubiquitination; CH₃, methylation.
somes in this process, our results suggest a mechanism by which ubiquitination of histone H2B can control transcription by regulation of methylation on histone H3 via COMPASS.

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