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Joseph Landry  
_Stony Brook University_

Ann Sutton  
_Stony Brook University_

Tina Hesman  
_Washington University School of Medicine in St. Louis_

Jinrong Min  
_Cold Spring Harbor Laboratory_

Rui-Ming Xu  
_Cold Spring Harbor Laboratory_

See next page for additional authors

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Set2-Catalyzed Methylation of Histone H3 Represses Basal Expression of \textit{GAL4} in \textit{Saccharomyces cerevisiae}

Joseph Landry,1 Ann Sutton,2 Tina Hesman,3 Jinrong Min,4 Rui-Ming Xu,4 Mark Johnston,3 and Rolf Sternglanz2*

* Corresponding author. Mailing address: Department of Biochemistry and Cell Biology, Stony Brook University, Stony Brook, NY 11794-5215. Phone: (631) 632-8565. Fax: (631) 632-8575. E-mail: rolf@life.bio.sunysb.edu.

\textit{Saccharomyces cerevisiae} has six proteins with a SET domain, two of which have been characterized. Set1 catalyzes methylation of lysine 4 of H3 and has been correlated with transcriptional activity (3, 12, 27, 30). Set2 methylates lysine 36 of H3 and represses transcription when tethered to a reporter gene (33). Several recent papers have shown that Set2 binds to RNA polymerase II and specifically to the elongating form of the C-terminal tail of the largest subunit (17, 18, 40). The functional significance of this binding and of H3 lysine 36 methylation is not known. Here we describe experiments showing that methylation by Set2 is required for repression of basal transcription of a specific gene, \textit{GAL4}.

\section*{MATERIALS AND METHODS}

\subsection*{Plasmid construction.}

Unless specified, all PCRs were performed with \textit{Pfu} Turbo DNA polymerase (Stratagene) according to the manufacturer's instructions. The DNA sequence of each cloned PCR product was determined to ensure the fidelity of the polymerase.

Using primers 5'-GGCGGCGACTTCCATGTCGGAAGACAAACAGT-3' and 5'-TTAGCCGCTGAGACATATGAGTACGCGC-3', the recombinant glutathione S-transferase (GST)-Set2 expression vector was constructed by amplying the first 900 bases of the \textit{SET2} open reading frame (ORF) from yeast genomic DNA. Using the \textit{BamH}I and \textit{SalI} sites, the amplon was then subcloned into pGEX-KG, resulting in an in-frame N-terminal fusion with GST. Primers used for cloning are P1L15 (5'-ATGCGGCAAACACTGCTGCTGAGGAGTCTTGAACGAT-3' and 5'-TTAGCCGAACTGCTGCTGAGGAGTCTTGAACGAT-3'), and additional PCR primers P1L13 (5'-GGCAAACTGCTGCTGAGGAGTCTTGAACGAT-3' and 5'-TTAGCCGAACTGCTGCTGAGGAGTCTTGAACGAT-3').

Recombinant Drosophila histone expression vectors pJWL124 (H3 K9R), pJWL110 (H3 K7R), and pJWL112 (H3 K36R) were constructed by site-directed mutagenesis to generate pJWL129 (GST-Set2 C82Y) and pJWL108 (GST-Set2 C201A), respectively.

\section*{RESULTS}

The effects of methylation are believed to be a result of either direct changes in protein structure or, in some cases, creation of a binding site for proteins with a chromodomain (2, 15, 24). Similarly, in \textit{Drosophila} and \textit{mammals} (2, 15), ordered nucleosomes are generally transcriptionally active. In both cases the fundamental unit of chromatin is the nucleosome, composed of 146 bp of DNA wrapped around a histone octamer consisting of two copies of each histone protein, H2A, H2B, H3 and H4 (39). Ordered nucleosomes are generally transcriptionally inactive and a less condensed form of a binding site for proteins with a chromodomain (2, 15, 24).

Methylation of specific lysines of histone proteins can target chromatin for activation or repression of transcription (11, 43). For example, methylation of lysine 9 of histone H3 recruits HP1 via its chromodomain and establishes heterochromatin in flies and mammals (2, 15, 24). Orderly nucleosomes are generally more repressive but can be modified in a number of ways to alter rates of transcription (36). Posttranslational modifications of histone N-terminal tails are important mechanisms by which transcription of open chromatin is modulated (9, 32).

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The highly conserved SET domain is found in many proteins implicated in modulating chromatin structure, including members of the \textit{Polycomb} and \textit{Trithorax} groups of proteins, and proteins involved in position effect variegation and gene silencing in budding and fission yeasts (8). The SET domain is associated with lysine HMT (23, 25, 34, 37, 41), and it interacts with the N-terminal tails of histones, signaling proteins, components of nucleosome remodeling factors, and proteins involved in DNA damage repair (4, 10, 29).
TCCTCACACCTCGTTGCGTGG-3') and JL36 (5'-CCATCTACCGGTTGGGAAGGTCATAGAGA-3'), respectively. PCR product was then introduced with pBM3352 into YM6119 (H3 K27R mutation. Using the EcoRI and SacI sites, the PCR product was recombined into the H3 K36R mutation. Using the EcoRI and SacI sites, the PCR product was used to gap repair an H9251. The PCR product was then introduced with pBM3352 into YM6119 (H3 K27,36R) was constructed using pJWL110 as a template and primer set JL407, and ORFs were identified in a 200-μl volume containing 50 mM Tris-Cl (pH 8.5), 10 mM β-mercaptoethanol, 250 mM sucrose, 1 μl of S-adenosyl-l-[3H]methylene (Amersham catalog no. TRK685) (820 Ci/mmol), 1.5 μg of recombinant Drosophila H3/H4, and 5 μg of GST-Set2, GST-Set2(C82Y), GST-Set2(C201A), and GST-CII were transformed into BL21(DE3) Codon+ RIL (Strategene catalog no. 230245). Expression was performed in 250-ml cultures of Luria broth with 50 μg of ampicillin/ml and 34 μg of chloramphenicol/ml. The cultures were grown at room temperature to an optical density at 600 nm of approximately 0.2 and then chilled on ice. Ethanol and IPTG (isopropyl-β-D-thiogalactopyranoside) were added to achieve final concentrations of 3% (vol/vol) and 0.45 mM, respectively. The cultures were induced at 18°C overnight with gentle mixing. The cells were pelleted at 5,000 × g for 10 min at 4°C, resuspended in 20 ml of ice-cold 1× phosphate-buffered saline, and then lysed by sonication. Insoluble material was removed by centrifugation at 12,000 × g for 20 min at 4°C. Fusion proteins were purified on a column using 1 ml of glutathione Sepharose (Amersham catalog no. 17-5132-01) according to the manufacturer's instructions. Eluted proteins were dialyzed in 4 liters of 1× phosphate-buffered saline at 4°C. Glycerol and dithiothreitol were added to the purified protein to achieve final concentrations of 10% (vol/ vol) and 1 mM, respectively. Protein concentrations were estimated with a Biorad gel for analysis by SDS-PAGE. The renaturedCHO cells were allowed to air dry and added to 4 ml of Ecosint. Tritium was quantified using a liquid scintillation counter.

Extracts for the CAT assay of GAL4::UAS-Gal-4 reporter strains were made as described previously (6). A total of 20 μg of protein from the extracts was assayed using a FAST CAT (deoxy) assay kit (Molecular Probes catalog no. F-6616) according to the manufacturer's instructions. Activity is expressed as the amount of acetylated BODIPY FL 1-deoxycholoramphenicol fluorescence.

Chromatin immunoprecipitation. Samples were processed for chromatin immunoprecipitation as described previously (13). In brief, YM4192 and BCY1 were grown in 200 ml of yeast extract-peptone-dextrose to an A600 of ~0.8 and then fixed with formaldehyde. Whole-cell extracts were made from fixed cells and sonicated to shear the chromatin to an average size of 400 bp. Using 1 μl of anti-H3 histyl lysine 36 antibody (a generous gift from B. Strahl) and protein A-Sepharose, a total of 1/10 of the shared chromatin was then immunoprecipitated in a 200-μl volume. Following immunoprecipitation, the cross-links were reversed and the DNA fractions were extracted for PCR.

PCR amplification of target loci was accomplished using Herculease polymerase (Strategene) and the following primer sets: for ACT1, 5'-CCAATGTCGGCAGAGATT-3' and 5'-CATGATACCTTGTTGCTGCTGCT-3'; for HMR-E, 5'-GCGGCTAGGACTTCGTC-3' and 5'-GTTCGCCATGCGTC-3'; and for the GAL4 promoter, 5'-AGTTTCAAACGATCCTCGTGTGCT-3' and 5'-CTGATAGAAGAGACTGC-3'. Products were resolved on a 1.5% agarose gel and visualized with ethidium bromide.
RESULTS

Set2 is a repressor of GAL4 transcription. The GAL4 promoter, which does not seem to contain a TATA box, is regulated by three cis-acting elements: a UAS necessary for maximal GAL4 expression, an upstream essential sequence that is essential for GAL4 expression, and Mig1 binding sites that mediate glucose repression of GAL4 expression (5). Mutants from which the GAL4 UAS has been deleted have a low basal level of GAL4 expression that is insufficient to support the utilization of galactose (5). These mutants revert to a Gal− phenotype at a frequency of approximately 10−6 per plated cell. The mutations responsible for suppression of the Gal− phenotype caused by the UAS deletion are all recessive, implying that they affect genes encoding negative regulators of GAL4 transcription.

The gene corresponding to one of the mutant complementation groups was isolated and found to be SET2. The strongest mutant allele was designated set2-1. Assays were conducted to evaluate the effects of set2-1 on GAL4 expression levels by using strains with a gal4-cat reporter gene and measuring CAT activity. Consistent with our expectation that Set2 is a negative regulator of GAL4 expression, the set2-1 allele significantly increased expression of a UAS-less GAL4 gene (0.2 U of CAT activity [31] compared to 0.02 U for a comparable SET2 strain). This mutation does not increase activity from an intact GAL4 promoter (1.4 U compared to 1.6 U for a wild-type SET2 strain), suggesting that Set2 affects basal, but not activated, GAL4 expression. Set2 does not appear to be a global repressor of gene expression, because the set2-1 mutation did not affect expression of three other genes we tested (GDN4, CTS1, and HIS3) and Tyl (data not shown).

The SET and SAC domains of Set2 are necessary for the repression of GAL4. The Set2 protein contains a highly conserved SET domain, flanked by two SAC (SET domain-associated cysteine-rich) domains (Fig. 1). SAC domains are found in many, but not all, SET-containing methyltransferases (25, 37) and have been shown to be essential for in vitro activity (24). The set2-1 allele results in a C82Y conversion in the SACI domain (Fig. 1). Because the set2-1 mutation resides in the SAC domain, we wanted to identify other residues in the SAC and SET domains that are necessary for repression of GAL4 expression. To do this, we mutagenized the SAC and SET domains and selected nonfunctional set2 mutants by the method used to isolate set2-1. The region of SET2 encoding the SAC and SET domains (aa 1 to 296) was amplified using mutagenic PCR, and the product was inserted into a SET2-containing plasmid (pBM3670) by recombination (gap repair; see Materials and Methods for details). Plasmids containing nonfunctional set2 genes were selected as Gal− transformants and rescued in E. coli DH5α, and their set2 DNA sequences were determined.

Most of the recovered mutations affected highly conserved amino acids in the SAC and SET domains (Fig. 1). Mutated residues in the SACI domain were predominantly cysteines (including cysteines 82, 88, 97, and 109). SACII domain mutations also predominantly affected cysteines (including cysteines 248, 250, and 255). Amino acid conversions in the SET domain, while more variable, were most often found in highly conserved amino acids (including residues G151, F196, H199, S200, G219, and E231) but also in less conserved residues (H170, C197, and Q223).

Biological characterization of the SAC and SET domains of Set2. We found that purified recombinant GST-Set2 has robust methyltransferase activity on chicken erythrocyte histones. Analysis of the methylated histones by triton acid urea electrophoresis revealed that the predominant substrates are H3 and, to a much lesser extent, H4 (data not shown).

To confirm that the HMT activity detected was due to Set2, we introduced two single-amino-acid changes in highly conserved cysteines located in the SAC and SET domains. We chose to mutate C82 (because it is mutated in set2-1) as well as C201 (because previous studies determined that the corresponding mutation abolished the enzymatic activity of the Suv39 h1 and G9a HMTs [26, 34]). Each of these mutants was assayed for its ability to methylate recombinant H3/H4 tetramers. Both mutations resulted in a complete loss of HMT activity, showing that the in vitro activity we detected was due to Set2 (Fig. 2). The fact that the set2-1 mutation abolished in vitro activity suggests that Set2 enzymatic activity is essential for repression of GAL4.

The catalytic domain of Set2 predominantly methylates lysine 36 on H3. We used GST-Set2 to methylate recombinant H3 and determined that lysine 36 was methylated (data not shown), confirming a previous report (33). To complement this analysis, we undertook a mutational analysis of the H3 N-terminal tail. Single lysines 9, 27, and 36 and lysines 27 plus 36 on recombinant H3 were converted to arginine. The resulting mutated H3 proteins were expressed and mixed with wild-type recombinant H4 and then used as the substrates in methyltransferase reactions with GST-Set2. In agreement with the results from the sequence data, GST-Set2 does not transfer methyl groups to histones with lysine 36 on H3 converted to arginine (Fig. 3). As a control, the well-characterized Clr4 methyltransferase (24) was able to methylate H3 except when lysine 9 is converted to arginine (Fig. 3).

Histone H3 lysine 36 is essential for GAL4 repression by SET2. To test the effect of the histone H3 lysine 36 mutation on GAL4 transcription, a ΔUAS gal4:cat reporter was introduced into strains which contained four different SET2 alleles: SET2, set2 C82Y, set2 C201A, and Δset2. Each strain also contained either wild-type HHT2 or an hht2 K36R mutation. Extracts from multiple transformants of each reporter strain were tested for CAT activity.

While the Δset2 mutation caused a significant increase in CAT activity in a HHT2 strain, the methyltransferase-defective set2 alleles differed somewhat in their abilities to repress cat expression. The set2 C82Y allele had the same levels of cat expression as the Δset2 allele, providing additional evidence that the methyltransferase activity of Set2 is necessary for its repressive effects on ΔUAS gal4:cat. The set2 C201A allele was found to have ~50% repressive ability (Fig. 4), while this mutant protein was completely defective in vitro (Fig. 2). Possibly this allele is less defective in vivo than it is in vitro (see Discussion).

The hht2 K36R mutation caused a significant increase in CAT activity in the ΔUAS gal4:cat reporter strain. This increase in activity is independent of the SET2 allele, demonstrating that the ability to methylate lysine 36 is epistatic to SET2 and placing the hht2 K36R allele downstream of SET2 in...
the pathway of repression of ΔUAS gal4::cat. It is interesting that the Δset2 hht2 K36R strain has lower CAT activity than the Δset2 HHT2 strain (Fig. 4). This could be due to a global effect on gene expression because of the presence of the mutated H3 throughout the genome.

We next used chromatin immunoprecipitation to show that histones in the ΔUAS gal4::cat chromatin are a direct target of Set2 methylation. Using an antibody specific to H3 methyl lysine 36, we were able to selectively precipitate the ΔUAS gal4::cat promoter and coding region from extracts of a SET2 strain but not from those of a Δset2 strain (Fig. 5). The loss of methylation at lysine 36 in the Δset2 strain demonstrates a direct involvement of Set2 in the modification of the ΔUAS gal4::cat region. These data support previous results demonstrating that Set2 methylates H3 at the promoter and coding region of GAL4 (40). In our experiments HMR-E was not precipitated in a SET2 background and served as a negative control. It is interesting that we also precipitated sequences from the ACT1 ORF in a SET2 strain, suggesting that ACT1 is also a target of Set2 methylation.

**DISCUSSION**

We first identified SET2 in a selection for genes involved in the basal repression of GAL4. Three set2 mutants were isolated, the strongest of which was designated set2-1, which changes a highly conserved cysteine residue (C82) in the catalytic domain of Set2 to tyrosine. This suggests that Set2 represses basal transcription of GAL4 through its methyltransferase activity.

We identified other residues in the catalytic domain that are necessary for GAL4 repression (Fig. 1). Conserved cysteine residues found in the SACI and SACII domains and highly conserved residues located in the SET domain are important for Set2 function. The structures of several SET domain proteins have been determined recently (7, 14, 21, 22, 35, 38, 42),
and two of the solved structures, *S. pombe* Clr4 and *Neurospora crassa* Dim-5, contain cysteine-rich SAC domains. The structures show that cysteines in the N-terminal SAC domain have a structural role in coordinating the binding of a triangular zinc cluster, while the cysteine corresponding to C201 in Set2 has been suggested to contact the C-terminal cysteine-rich SAC domain to form a cofactor-substrate binding site (22, 42).

Given this structural information, we believe that the C82Y mutant alters the zinc cluster structure and that the enzyme loses activity because of structural changes. On the other hand, the C201A mutation should cause minimal structural changes but the purified protein may be unable to form an intact cofactor or substrate binding site in vitro. It is possible that such a binding site can be restored to some degree in vivo in the presence of other proteins. These structure-based interpretations are consistent with our results shown in Fig. 4. In addition, three of the mutations recovered in the selection, C97, H199, and Q112 (similar to Clr4 R320), were found to ablate HMT activity in previously characterized HMT enzymes, leading us to believe that the defects in set2 affect catalysis rather than protein-protein interactions (24, 25, 41).

We found that the catalytic domain of Set2 has HMT activity in vitro (Fig. 2) and, in agreement with a recent report (33), that the prominent site of methylation is lysine 36 on histone H3. We showed that GST-Set2 cannot methylate a histone H3 substrate when lysine 36 is converted to an unmethylatable arginine, confirming its preference for lysine 36 (Fig. 3).

We believe that the HMT activity of Set2 on H3 lysine 36 is responsible for its basal repression of *GAL4* for four reasons.

First, the set2-1 mutant (C82Y) isolated in our original screening, as well as the C201A mutant, is catalytically inactive in vitro (Fig. 2). Second, these catalytically inactive mutants have a significantly reduced ability to repress the *ΔUAS gal4::cat* reporter gene (see Results and Fig. 4). Third, the ability of Set2 to repress *GAL4* expression is dependent on the availability of lysine 36 on H3 for methylation, because the *hht2 K36R* change causes a loss of repression of the *ΔUAS gal4::cat* reporter gene.

### FIG. 2. Mutations in the SET and SAC domains result in loss of GST-Set2 methyltransferase activity. (Upper panel) The locations of the two amino acid changes made to highly conserved cysteine residues in the GST-Set2 expression plasmid are indicated. (Lower panel) GST-Set2, GST-Set2 (C82Y), GST-Set2 (C201A), and a GST control protein were purified and assayed for methyltransferase activity. The bar graphs represent averages of three independent assays.

### FIG. 3. GST-Set2 preferentially methylates lysine 36 of H3. (A) GST-Set2 was used to methylate recombinant wild-type (W.T.) and mutant histone H3/H4 tetramers. Levels of GST-Set2 activity on the various histone H3 mutants are indicated. Activity was lost when lysine 36 was mutated to the unmethylatable arginine. (B) Clr4 was used as a control to monitor the quality of the histone tetramers. Methylation reactions were performed as described for Fig. 2 except that recombinant GST-Clr4 was used instead of GST-Set2. Consistent with published results, GST-Clr4 was unable to methylate the H3 K9R mutant. For each panel, the bar graph represents the averages of two experiments.

### FIG. 4. Methyltransferase activity of Set2 and the presence of lysine 36 on histone H3 are required for the repression of *ΔUAS gal4::cat*. CAT assays were performed on extracts from *ΔUAS gal4::cat* reporter strains containing *SET2, set2 C82Y, set2 C201A, and Δset2 alleles in an *HHT2* or *hht2 K36R* background. The data show derepression of *ΔUAS gal4::cat* with enzymatically defective Set2 mutants. In addition, significant derepression of the reporter was seen with the introduction of an unmethylatable arginine at residue 36 on H3. The bar graph represents the averages of four independent experiments. W.T., wild type.
porter gene that is the same whether the SET2 gene is present or has been deleted (Fig. 4). Finally, the chromatin immunoprecipitation experiments show that SET2 is directly responsible for methylation of lysine 36 at the GAL4 gene (Fig. 5). The combination of genetic and biochemical evidence strongly suggests that repression of GAL4 by Set2 is mediated by methylation of lysine 36 on histone H3.

The repressive effects of Set2 methylation on transcription in vivo are in agreement with a previous report (33). In that report, LexA-Set2 was found to repress transcription 20-fold when tethered to a CYC1-lexAoperator-lacZ reporter. The differences in the level of repression by Set2 at GAL4 and CYC1-lexAoperator-lacZ may be due to differences in the recruitment of Set2 to these promoters. In agreement with our results, a C201A mutation in LexA-Set2 resulted in a 50% loss in repression (33). This partial effect could be due to the different activity levels of the mutant protein in vitro (where it was completely defective; Fig. 2) versus that seen in vivo. Or perhaps Set2 has a repression function independent of its methylation activity.

It is not easy to reconcile our results regarding the repression of basal transcription of GAL4 with the numerous recent reports that Set2 binds to the elongating form of RNA polymerase II (17, 18, 40). Perhaps Set2 acts as a backup system for transcriptional repression. Under conditions of repression, transcriptional repressors bind to the promoter region of regulated genes, preventing the assembly and subsequent clearance of an RNA polymerase II transcription complex. But occasionally, "leaky" transcription can occur under repressive conditions. Perhaps Set2 methyletes the promoter and the coding part of the gene when this leaky transcription occurs, thus marking the chromatin and preventing subsequent transcription. It is also possible that the methylation of lysine 36 by Set2 has different functions at promoters than at coding regions of genes.

The actual repression mechanism resulting from lysine 36 methylation is still not known. One model is that the methylation of lysine 36 causes an alteration of nucleosome structure that is repressive in nature. To test this hypothesis, we conducted MNase protection assays on hht2 K36R Δset2 and *HHT2* SET2 strains at the *GAL4* promoter. We found no difference in digestion patterns, suggesting that nucleosome positioning had not been altered in the absence of methylation (data not shown). It is still possible that K36 methylation changes chromatin structure in a way that cannot be detected with MNase assays. A second model is that methylation of lysine 36 might recruit a chromodomain-containing protein that acts as a repressor of transcription. This would be similar to the mechanism used for the establishment of heterochromatin by HP1 binding to methyl lysine 9 on H3 (2, 15, 24).

In summary, we have shown that Set2 methylation is involved in the repression of basal transcription of *GAL4*. The fate of the methylated histones under conditions of transcriptional activation is unknown. It is possible that methylation of K36 at *GAL4* is permanent and that its repressive effects are overcome through the recruitment of transcriptional activators. It is also possible that a demethylating enzyme exists. Finally, there may be a mechanism whereby methylated histones are replaced by unmethylated ones upon transcriptional activation (1).

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