

## Genetically Encoded Fluorescent Reporters of Histone Methylation in Living Cells

Chi-Wang Lin, Cindy Y. Jao, and Alice Y. Ting\*

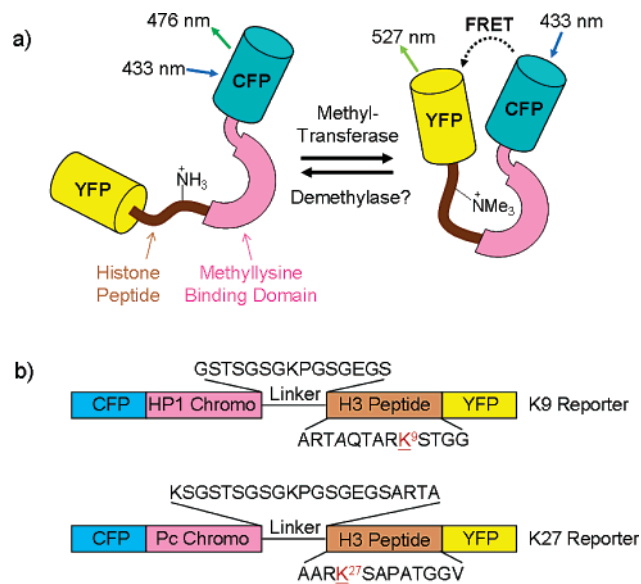
Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Received October 3, 2003; E-mail: ating@mit.edu

Post-translational protein modifications are used by cells to dynamically regulate protein structure and function. This regulation is particularly pronounced on histone proteins, the scaffolding proteins around which DNA is wrapped in chromatin. Phosphorylation, acetylation, and methylation of the N-terminal tails of histone proteins strongly influence transcription of neighboring genes by altering steric access to DNA and by introducing or removing protein docking sites.<sup>1</sup> Despite the importance of these modifications, few methods exist for detecting them, and none are effective in intact, living cells. Here, we describe a new class of fluorescent reporters that can be used in single, living cells to track histone methylation with high spatial and temporal resolution.

The general reporter design is shown in Figure 1a. A peptide substrate corresponding to the methylation site of interest within histone protein H2A, H2B, H3, or H4 is joined via a flexible linker to a methyllysine binding domain known as a chromodomain. Recently discovered, chromodomains are small (~55 amino acid), monomeric domains found in a variety of nuclear signaling proteins that bind with 1–200  $\mu\text{M}$   $K_d$  to lysine-methylated peptides but not to their unmethylated counterparts.<sup>2</sup> This fusion construct is sandwiched between a pair of fluorescence resonance energy transfer (FRET)-capable green fluorescent protein (GFP) mutants, cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP). On enzymatic methylation of the histone-derived peptide, the chromodomain forms an intramolecular complex with the methyllysine side chain, altering the spatial relationship between the flanking CFP and YFP units, and changing the FRET level. Reversal of this FRET change would be evidence for a conjectured but so far undetected histone demethylase activity. This general design, in which a conformationally sensitive natural or engineered protein is sandwiched between two GFP mutants capable of FRET, has been successfully used to detect a range of intracellular molecules and biochemical events, including  $\text{Ca}^{2+}$ , cGMP, Ras activity, protease activity, and phosphorylation.<sup>3</sup> Our reporters are the first for detecting protein methylation.

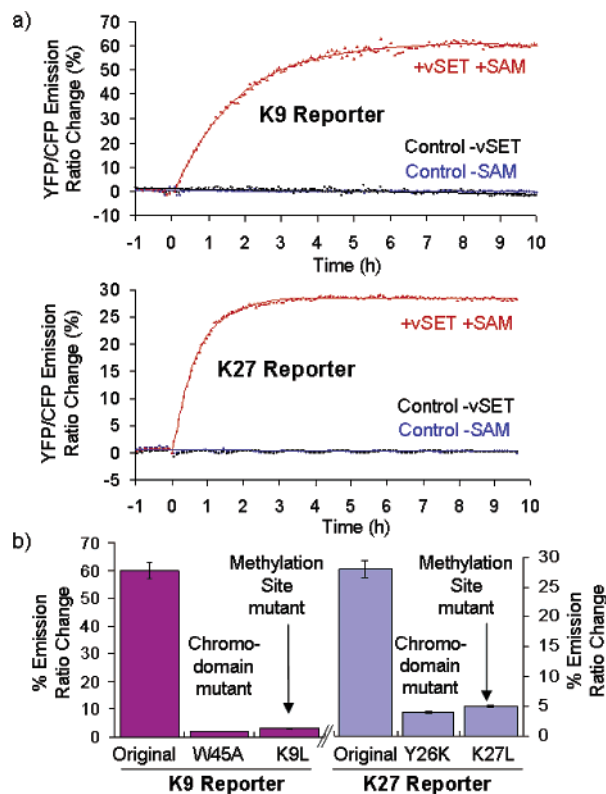
We constructed methylation reporters for two lysine positions in histone H3 (K9 and K27) known to be involved in transcriptional repression,<sup>4</sup> X-inactivation,<sup>5</sup> and cellular differentiation.<sup>6</sup> The genes for the two reporters (domain structures shown in Figure 1b) were assembled by standard cloning methods (GenBank accession nos. AY422822 and AY422823; see Supporting Information for details). The K9 reporter uses the HP1 chromodomain (residues 21–76) to recognize lysine 9 methylation in the context of residues 1–13 of histone H3,<sup>7,8</sup> while the K27 reporter uses the Polycomb (Pc) chromodomain (residues 21–78) to recognize lysine 27 methylation in the context of residues 24–35 of histone H3.<sup>9,10</sup> When H3 peptides spanning both lysines are incorporated, each chromodomain shows some crossover binding (Ting and Lin, unpublished observations); therefore, these shorter substrate sequences must be used. Crystallographic data<sup>7–10</sup> and peptide modification experiments<sup>11</sup> suggest that these shorter sequences are still specific



**Figure 1.** (a) General histone methylation reporter design. (b) Domain structures of two methylation reporters. The K9 reporter, top, uses the HP1 chromodomain for recognition of methylated K9 from the histone H3 N-terminus. An alternative methylation site at K4 in the original substrate has been mutated to alanine (italicized). The K27 reporter, bottom, uses the Polycomb (Pc) chromodomain for recognition of methylated K27 from histone H3.

substrates for histone methyltransferase enzymes and retain binding affinity for chromodomains. We estimated from cocrystal structures<sup>7–10</sup> that 15- and 21-residue linkers for the K9 and K27 reporters, respectively, would suffice to allow intramolecular complexation between the two linked domains.

Both reporters were overexpressed in *E. coli* and purified by nickel affinity chromatography. Reporter responsivity was assessed in vitro through measurement of the YFP/CFP emission ratio ( $I_{527\text{ nm}}/I_{476\text{ nm}}$ ) in response to methylation by vSET, a histone H3 methyltransferase from *Paramecium bursaria* chlorella virus.<sup>11</sup> Figure 2a shows that both K9 and K27 reporters give emission ratio increases (60% and 28%, respectively) that depend on the presence of both enzyme and methyl donor, *S*-adenosyl methionine (SAM). To confirm a correlation between reporter FRET level and methylation state, and to examine the specificity of enzymatic methylation, we digested reporter protein from each reaction shown in Figure 2a with the protease Glu-C and analyzed the peptide fragments by MALDI-TOF (data in Supporting Information). The K9 reporter from the +vSET/+SAM reaction was cleanly trimethylated at lysine 9 after 10 h (no mono- or dimethylation detected), while the –SAM negative control contained only unmodified reporter protein. The K27 reporter was di- and trimethylated in the +vSET/+SAM reaction, while the corresponding negative controls contained only unmodified protein.

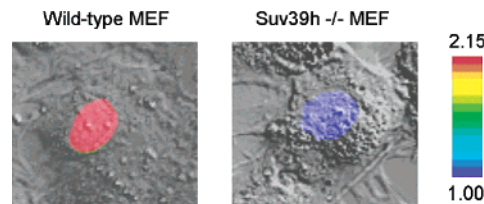


**Figure 2.** (a) Time-course graphs showing the emission ratio responses of the K9 and K27 reporters to enzymatic methylation by vSET (excitation at 433 nm). (b) Maximal emission ratio changes observed in vSET methylation reactions with various reporter mutants.

Site-directed mutagenesis was also used to examine the specificities and FRET response mechanisms of the two reporters (Figure 2b). Mutation of lysine 9 of the K9 reporter to a methylation-incompetent leucine (K9L) abolished the FRET response to vSET methylation. Similarly, mutation of lysine 27 of the K27 reporter (K27L) eliminated that reporter's response. The chromodomains of each reporter were also modified to eliminate affinity for methylated lysine (W45A<sup>8</sup> for the K9 reporter, and Y26K<sup>10</sup> for the K27 reporter). Both chromodomain mutants were unresponsive to enzymatic methylation, consistent with a FRET increase mechanism based on complexation between methyllysine side chain and chromodomain.

To test the K9 reporter in living cells, we appended a nuclear localization sequence (PKKKRK) to the C-terminal end of the reporter gene. The plasmid containing the reporter gene was then introduced by lipofection into both wild-type mouse embryonic fibroblasts (MEFs) and sister cells lacking the major K9-H3-specific methyltransferases Suv39h1 and Suv39h2 (Suv39h<sup>-/-</sup>).<sup>12</sup> Previous studies have shown that these cells exhibit drastically reduced levels of K9-H3 methylation in comparison to wild-type MEFs.<sup>12</sup> The reporter was efficiently expressed in both cell lines after 12 h. Measurement of YFP/CFP emission ratios showed that FRET levels were lower on average in Suv39h<sup>-/-</sup> cells than in wild-type MEFs (Figure 3;  $n = 278$  cells,  $\sigma = 0.163$ ,  $p < 0.001$ ), demonstrating that our K9 construct can effectively report variations in methylation levels across different cell lines.

In conclusion, we have developed new fluorescent reporters of histone methylation that are effective in vitro and in single living



**Figure 3.** Basal YFP/CFP emission ratios of the K9 reporter expressed in wild-type mouse embryonic fibroblasts (MEFs) and sister cells lacking the K9-H3 methyltransferases Suv39h1 and Suv39h2. The bar on the right shows the correlation between YFP/CFP emission ratio and cell pseudocolor.

cells. By permitting investigators to record information nondestructively over time, our methodology should complement traditional techniques for detecting protein methylation, such as immunofluorescence or immunoprecipitation.<sup>13</sup> Although not demonstrated here, the spatial resolution could be greatly improved if the reporter were targeted through genetic fusion to specific intranuclear sites, such as transcription factor complexes or chromatin itself. Particularly in light of recent reports that histone methylation can be quite dynamic,<sup>4,14</sup> these reporters should find application in studies of gene silencing, X-inactivation, and cell differentiation, as well as in screening for the conjectured histone demethylase activity and for small-molecule inhibitors of methyltransferase activity.

**Acknowledgment.** We thank Sean Liu, Michael A. Gelman, Christine Yu, C. David Allis, and Yi Zhang for their advice and assistance. C.-W.L. and C.Y.J. were supported by the Merck/MIT fellowship and MIT's UROP program, respectively. The Polycomb, HP1, and vSET genes were gifts from Yi Zhang, Sepideh Khorasanizadeh, and Ming-Ming Zhou, respectively. The wild-type MEFs and Suv39h<sup>-/-</sup> sister cells were gifts from Thomas Jenuwein. We thank the NIH (K22-HG002671-01), ONR (N00014-03-1-0456), Dreyfus Foundation (NF-02-003), and MIT for funding.

**Supporting Information Available:** MALDI-TOF spectra of methylated K9 and K27 reporter proteins. Experimental protocols for cloning, reporter expression, methylation assays, and cell imaging. This material is available free of charge via the Internet at <http://pubs.acs.org>.

## References

- (1) Felsenfeld, G.; Groudine, M. *Nature* **2003**, *421*, 448–453.
- (2) Jones, D. O.; Cowell, I. G.; Singh, P. B. *BioEssays* **2000**, *22*, 124–137.
- (3) Zhang, J.; Campbell, R. E.; Ting, A. Y.; Tsien, R. Y. *Nat. Rev. Mol. Cell Biol.* **2002**, *3*, 906–918.
- (4) Kim, J.; Jia, L.; Tilley, W. D.; Coetzee, G. A. *Nucleic Acids Res.* **2003**, *31*, 6741–6747.
- (5) Plath, K.; Fang, J.; Mlynarczyk-Evans, S. K.; Cao, R.; Worringer, K. A.; Wang, H.; de la Cruz, C. C.; Otte, A. P.; Panning, B.; Zhang, Y. *Science* **2003**, *300*, 131–135.
- (6) Ait-Si-Ali, S.; Guasconi, V.; Fritsch, L.; Yahi, H.; Sekhri, R.; Naguibneva, I.; Robin, P.; Cabon, F.; Poleskaya, A.; Harel-Bellan, A. *EMBO J.* **2004**, *23*, 605–615.
- (7) Nielsen, P. R.; Nietlispach, D.; Mott, H. R.; Callaghan, J.; Bannister, A.; Kouzarides, T.; Murzin, A. G.; Murzina, N. V.; Laue, E. D. *Nature* **2002**, *416*, 103–107.
- (8) Jacobs, S. A.; Khorasanizadeh, S. *Science* **2002**, *295*, 2080–2083.
- (9) Min, J.; Zhang, Y.; Xu, R. M. *Genes Dev.* **2003**, *17*, 1823–1828.
- (10) Fischle, W.; Wang, Y.; Jacobs, S. A.; Kim, Y.; Allis, C. D.; Khorasanizadeh, S. *Genes Dev.* **2003**, *17*, 1870–1881.
- (11) Manzur, K. L.; Farooq, A.; Zeng, L.; Plotnikova, O.; Koch, A. W.; Sachidanand; Zhou, M. M. *Nat. Struct. Biol.* **2003**, *10*, 187–196.
- (12) Peters, A. H.; O'Carroll, D.; Scherthan, H.; Mechtler, K.; Sauer, S.; Schofer, C.; Weipoltshammer, K.; Pagani, M.; Lachner, M.; Kohlmaier, A.; Opravil, S.; Doyle, M.; Sibilia, M.; Jenuwein, T. *Cell* **2001**, *107*, 323–337.
- (13) Lachner, M.; Jenuwein, T. *Curr. Opin. Cell Biol.* **2002**, *14*, 286–298.
- (14) Sacconi, S.; Natoli, G. *Genes Dev.* **2002**, *16*, 2219–2224.

JA038854H