Opinion

Histone modifications: from genome-wide maps to functional insights

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Abstract

A large number of histone modifications has been implicated in the regulation of gene expression. Together, these modifications have the potential to form a complex combinatorial regulatory code. Genome-wide mapping approaches provide new opportunities to decipher this code, but they may suffer from systematic biases. Integration of datasets and improved technologies will provide the way forward.

The DNA in the nucleus of a eukaryotic cell is packed into chromatin, the fundamental building block of which, the nucleosome, consists of an octamer of the four histones H2A, H2B, H3 and H4, around which the DNA is wrapped. The histones within chromatin are subject to extensive post-translational modification, including acetylation, methylation, phosphorylation, ubiquitination, and ribosylation. Many enzymes have been identified that are responsible for the addition or removal of modifications at one or a few specific histone amino-acid residues, and many histone modifications are believed to play important roles in the regulation of transcription. Although some histone modifications may cause alterations in the structure or overall charge of the nucleosome [1,2], it is likely that most act by controlling the docking of specific regulatory factors. For example, the chromodomain of heterochromatin protein 1 (HP1) binds to the tail of histone H3 only when lysine 9 of H3 (H3K9) is methylated, and this may contribute to repression of transcription [3]. Similarly, the bromodomains of various transcriptional activators and nucleosome-remodeling factors recognize specific acetylated lysine residues within histone H3 or H4 [4]. While some modifications attract specific regulatory factors, others appear to block protein binding. This is illustrated by the inhibitory effect of acetylation on the binding of the silencing protein Sir3 to histone H4 [5]. Undoubtedly, many more factors will be discovered that recognize particular modification states of histones.

Given the large number of histone modifications that appear to be involved in the control of gene expression, the integration of their regulatory roles is an important issue. How do these modifications work together? For example, H3K4 methylation and H3K9 acetylation have both been implicated in gene activation; do these two modifications typically act together on a common set of genes, or are they part of separate signaling pathways each of which controls different sets of genes? Can repressive signals (such as H3K9 methylation) and activating signals (such as H3K4 methylation) occur on the same nucleosome and, if so, how are their effects integrated? Do the different modifications control transcription mostly by simple additive or redundant mechanisms that are the same for different genes, or do they constitute a complex combinatorial code, whereby the effect of one modification differs between genes and depends on the local context of other modifications (Figure 1)? Because of the myriad combinations that may exist, these questions cannot be solved by single-gene studies. Rather, it is necessary to compare a large number of genes to identify the global patterns, interrelationships, and functional roles of histone modifications.

In the past few years, chromatin immunoprecipitation (ChIP) combined with microarray detection (ChIP-on-chip) has become the principal technique for mapping genome-wide patterns of histone modifications [6,7]. Most laboratories use

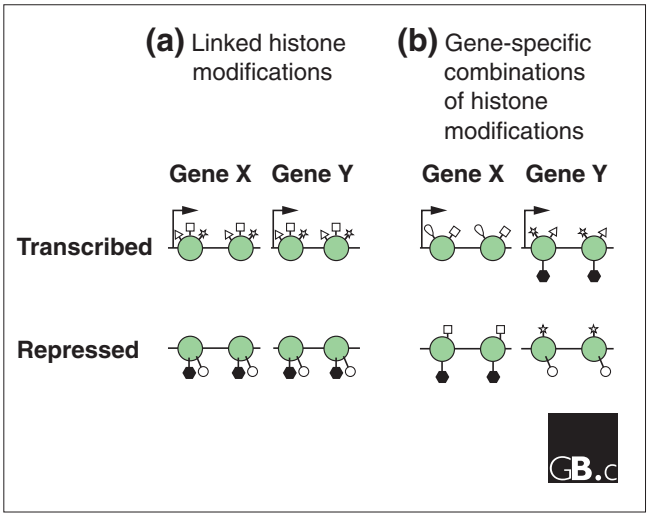

Figure 1
Two models for the role of the different histone modifications. **(a)** Histone modifications are linked, such that all active genes are marked by an identical combination of 'active' histone modifications, and all inactive genes by another common set of 'repressive' histone modifications. **(b)** Histone modifications are not linked but form a complex combinatorial code, such that various combinations of modifications mark (groups of) active genes, and various other combinations mark inactive genes. Note that some organisms (such as budding yeast) may lack 'repressive' histone modifications.

a similar ChIP protocol, which involves cross-linking of protein-DNA complexes *in vivo* by addition of formaldehyde to living cells, followed by lysis of the cells and mechanical shearing of the chromatin to yield fragments of about 0.5-2 kilobases in size. This is followed by immunoprecipitation of cross-linked protein-DNA complexes using antibodies against a specific epitope, such as a certain histone modification. DNA is then recovered from the immunoprecipitated complexes and labeled and hybridized to DNA microarrays that bear thousands of sequences representing a large fraction of the genome. The presence or absence of the histone modification at each genomic location can then be inferred from the microarray signals, assuming that the cross-linking efficiency and the epitope recognition by the antibody are uniform across the entire genome. Several laboratories have begun a systematic genome-wide dissection of the relationships between histone modifications and transcriptional regulation, using combinations of ChIP-on-chip, mRNA expression profiling, and other methods. Here, we review the recent progress made (and some problems encountered) in this new and exciting field.

Genome-wide distribution of nucleosomes

Before discussing whole-genome maps of histone modifications, it is useful to consider the genomic distribution of nucleosomes (that is, the histone octamers) themselves. The positioning of nucleosomes is believed to be crucial for gene regulation. Two recent papers [8,9] describe the whole-genome mapping of histone-genome interactions in the budding yeast *Saccharomyces cerevisiae*. ChIP-on-chip experiments were performed with epitope-tagged histone H2B or H4, and endogenous H3 (using an antibody that is presumably insensitive to the H3 modification state). The results indicate that there are locus-specific differences in nucleosome density. Most notably, the upstream regions of highly active genes display a reduced nucleosome density compared to the upstream regions of inactive genes. Depletion of histone H2B in promoter regions was also detected by a genome-wide mapping technique that combines ChIP with mass sequencing of short sequence tags [10]. A similar depletion of nucleosomes, although of lower magnitude, was observed in coding regions of highly active genes, when compared to inactive genes [8,9]. Although it cannot be completely ruled out that local differences in cross-linking efficiency or epitope accessibility account for the observed differences, the results are in agreement with detailed studies of selected regions, such as the Pho5 promoter [11,12]. Thus, these data suggest that, as a general rule, gene activation in yeast is accompanied by reduced nucleosome density.

Histone modification maps: dealing with possible biases

In order to understand the interplay between different histone modifications during transcriptional regulation, it is necessary to construct systematic genome-wide location maps of all histone modifications and the proteins that control and interact with them. Two laboratories have taken important steps towards this goal by global mapping of a large number of histone modifications. Kurdistani *et al.* [13] performed ChIP-on-chip in *S. cerevisiae* using 11 antibodies, each of which specifically recognizes a different acetylated histone lysine residue. They used whole-genome arrays of coding regions (ORFs) and intergenic regions (IGRs), thereby creating complete genomic maps of these histone acetylations. Schübeler *et al.* [14] performed similar ChIP-on-chip experiments in a cultured cell line from *Drosophila melanogaster*, using antibodies against six distinct histone modifications. But because the complete *Drosophila* genome is not yet available on microarrays, these mapping experiments were done with cDNA arrays, thus providing a view of transcribed regions only. One modification (H3K4 dimethylation) was also mapped using a genomic tiling array covering the left arm of chromosome 2. Strikingly, both papers report that the genomic distributions of virtually all tested histone modifications are highly similar to one another. Schübeler *et al.* [14] found that H3 and H4 acetylation, H3K4 di- and trimethylation, and H3K79 di-methylation, are all strongly correlated with each other, having pairwise Pearson correlation coefficients of at least +0.7. Along the same lines, Kurdistani *et al.* [13] report that all pairs of 11 tested acetylation sites show significant positive correlations, with the majority of

correlation values (both in ORFs and in IGRs) being well above +0.5.

Before we reflect on the potential biological implications, the possibility should be considered that some kind of biological or experimental bias is responsible for the strong correlations. One possible source of bias could be local differences in nucleosome density. It is conceivable that in genomic regions with high nucleosome densities ChIP analysis of any histone modification might yield stronger signals than in regions with low nucleosome density [15] (Figure 2). Unfortunately, the papers by Schübeler *et al.* [14] and Kurdistani *et al.* [13] did not address this putative bias by including control experiments with antibodies against histone epitopes that lack potential modification sites. To illustrate the putative consequences of this bias, we compared the yeast histone acetylation data from Kurdistani *et al.* [13] with yeast nucleosome-density maps [8,9] (Table 1). Strikingly, this analysis reveals that especially within IGRs there is a considerable correlation between H2B, H3 or H4 density and ChIP of several of the lysine acetylation variants. These results argue that ChIP of histone modifications may be biased by local differences in nucleosome density. Given the strict 2:2:2:2 stochiometry of histones in the nucleosome, it is likely that the bias is similar for modifications of all four histones.

Kurdistani *et al.* [13] reported that the acetylation levels in IGRs of all tested lysines in histones H2A, H2B and H4 are negatively (although weakly) correlated with expression levels of the corresponding genes (Table 1). But when the results are corrected for the nucleosome-density bias, it may well be that these modifications are positively correlated with gene expression. Likewise, the reported weakly positive correlations between gene expression and acetylation of H3K9, H3K18 and H3K27 in IGRs may in fact be more positive when corrected for nucleosome density. To further investigate this bias, we compared the correlations of each lysine acetylation with nucleosome density and with gene expression levels (Figure 3). The striking linear relationship between points on the plot shown in Figure 3 indicates that these correlations are not independent, underscoring the notion that the links between histone modification and gene expression cannot be interpreted safely without a correction for nucleosome density. Obviously, the bias caused by nucleosome-density differences may have implications for further analyses and interpretations of all (previously published) histone-modification maps.

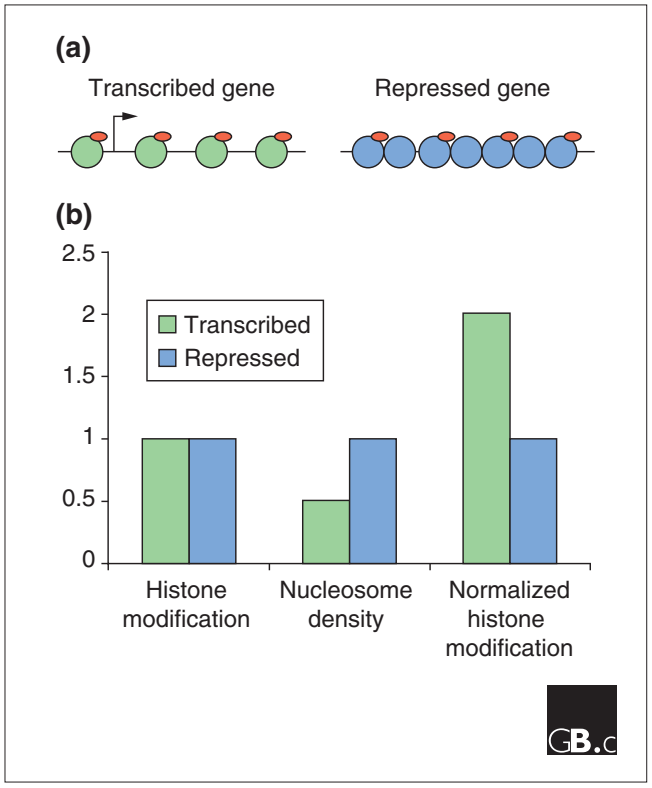

Figure 2
Effect of nucleosome distribution on chromatin immunoprecipitation. Uneven genomic distribution of nucleosomes **(a)** may cause a bias in the results of chromatin immunoprecipitation experiments **(b)**. The bias may be solved by including ChIP controls for nucleosome density (see text) and subsequent normalization of the histone-modification ChIP data.

Table 1

Correlations between intergenic histone acetylation patterns, levels of gene expression and histone density

ChIP epitope	Gene expression	H4 density	H3 density	H2B density
H4K16	-0.18**	+0.42**	+0.43**	+0.35**
H4K8	-0.15**	+0.35**	+0.41**	+0.35**
H4K12	-0.08*	+0.30**	+0.38**	+0.30**
H2AK7	-0.10*	+0.29**	+0.30**	+0.27**
H2BK11	-0.13**	+0.29**	+0.32**	+0.26**
H2BK16	-0.13**	+0.28**	+0.32**	+0.26**
H3K14	-0.10*	+0.26**	+0.34**	+0.27**
H3K23	-0.04	+0.15**	+0.22**	+0.16*
H3K9	+0.07	+0.06	+0.08*	+0.07
H3K27	+0.08*	-0.03	+0.00	+0.02
H3K18	+0.21**	-0.08*	-0.10*	-0.05

The correlations shown are between intergenic histone acetylation patterns [13] and levels of gene expression (data taken from [13]), and the experimentally mapped densities of tagged histone H4 [8], and untagged H3 and tagged H2B [9]. Pearson correlation coefficients are shown, which can range from -1 (perfect inverse correlation) through 0 (no correlation) to +1 (perfect positive correlation). Correlations were calculated for 1,580 intergenic regions that were represented in all three datasets; significance, *$p < 10^{-3}$, **$p < 10^{-8}$.

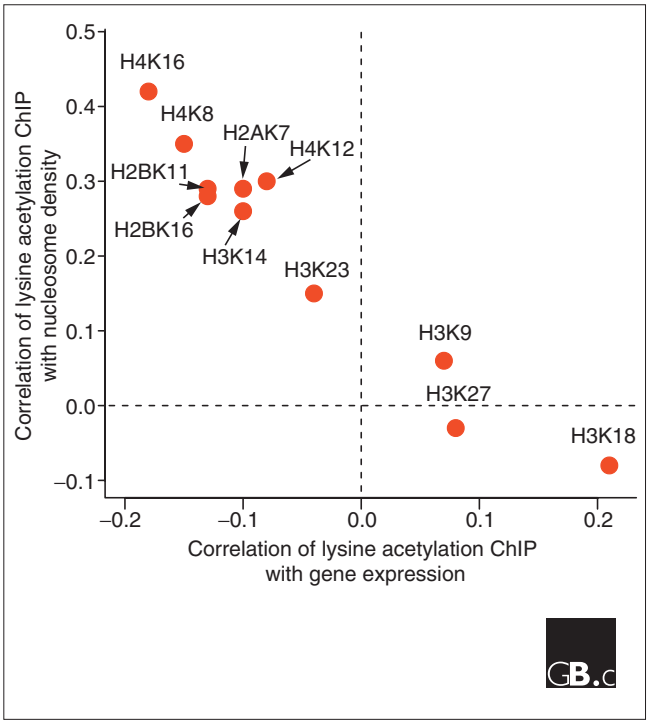

Figure 3
Comparison of the correlations of ChIP of each lysine acetylation with nucleosome density and with gene expression levels. Data are from Table 1.

It is not known whether depletion of nucleosomes in transcriptionally active regions is a general phenomenon in *Drosophila*. Schübeler *et al.* [14] performed ChIP-on-chip with an antibody against phosphorylated H3S10 as a control, and reassuringly found that this epitope does not correlate with any of the other modifications, although one might argue that this is not an entirely satisfying control because H3S10 phosphorylation is primarily a mitosis-specific mark [16]. Importantly, Schübeler *et al.* [14] report strong positive correlations between all mapped acetylation and methylation patterns and gene expression levels. Correction for nucleosome depletion in active regions would further increase these correlations, and therefore would only strengthen the conclusion that H3 and H4 acetylation, H3K4 di- and tri-methylation, and H3K79 di-methylation are all enriched in regions of high transcriptional activity. Although other experimental biases cannot be ruled out, these results argue that, at least in coding regions of the fly genome, these histone modifications are closely linked.

Interestingly, a recent mapping study of histone modifications in human cells suggests that linkage between active histone modifications might be a common theme in eukaryotic genomes. By combining ChIP with hybridization on tiling oligonucleotide arrays, Bernstein *et al.* [17] showed that more than 90% of H3K4 methylated sites on human chromosomes 21 and 22 are also H3K9K14-acetylated. Virtually no variation was found in H3 density, suggesting that in human cells differences in nucleosome occupancy are minor, and thus are unlikely to cause severe biases in the mapping of histone modifications.

Genome-wide functional analysis of histone modifications

Unraveling the role of histone modifications can be greatly facilitated by genome-wide analysis of histone-modifying enzymes. Such studies of histone deacetylases (HDACs) and histone acetyltransferases (HATs) in yeast argue that acetylation of most histone lysine residues is in general positively, not negatively, associated with gene expression. ChIP-on-chip mapping of histone acetylation patterns after deletion of either Rpd3 or Hda1 (both are HDACs) revealed increased acetylation of several histone lysine residues [18]. Comparison of these 'deacetylation maps' with expression profiles obtained after deletion of either HDAC revealed that increased acetylation is generally accompanied by increased transcription [18]. This was found for six different acetylation positions, including H4K12, H4K16, and H2BK16. Furthermore, genome-wide mapping of the binding sites of the HATs Gcn5 and Esa1 in yeast show that these enzymes are primarily recruited to active genes [19]. These data further support the view that for many lysines on the nucleosome acetylation is generally positively linked to transcription. While there seems to be a general correlation between histone acetylation and gene expression, some histone deacetylases seem to positively regulate gene expression, possibly by maintaining the amount of histone acetylation that is optimal for efficient transcription [20].

Another study employed expression profiling of yeast strains in which lysines on histone H4 were systematically mutated to arginine (which mimics unacetylated lysine) [21]. Mutations of lysines 5, 8, and 12 had identical effects on the expression of a broad set of genes. These effects were cumulative: changes in expression were proportional to the number of mutations. In contrast, mutation of H4K16 affected a distinct small group of genes. These results point to a nonspecific, cumulative regulatory role for H4K5, H4K8, and H4K12 acetylation.

Linked histone modifications?

Theoretically, the large number of possible histone modifications creates the potential for an extremely complex regulatory code. Nevertheless, careful comparison of the recent whole-genome studies of histone modifications, as discussed above, suggests a rather simple picture: most of the mapped modifications, including acetylation of all tested nucleosomal lysines and methylation of H3K4 and H3K79, are positively correlated with levels of gene expression. This raises the possibility that the different 'active' histone marks are linked. So far, no histone modifications have been identified

that mark transcriptionally inactive regions in budding yeast. In other eukaryotes, however, a number of such repressive marks have been reported, such as methylation of H3K9, H3K27, and H4K20. The degree of global linkage of these repressive histone marks remains to be established.

How may the linkage of various active histone modifications be established? In part, it may be a consequence of the broad substrate-specificity of some histone-modifying enzymes. For example, the HDAC Rpd3 and the HAT Gcn5 both control the acetylation state of several lysines on H3 and H4 [18,22]. Furthermore, several histone-modifying enzymes might act at the same nucleosome because they interact with each other (for example, the TAC1 chromatin-remodeling complex has both H3K4 methyltransferase activity and HAT activity [23]), or because they are recruited by the same signal (for example, the H3K4, H3K36, and H3K79 methyltransferases in yeast all require components of the Paf1 complex for their activity [24]). In addition, certain histone modifications may recruit complexes containing other modifying enzymes and thus trigger cascades of histone modifications (for example, methylation on H4R3 by PRMT1 facilitates acetylation by p300 on H4K8 and H4K12 [25]) It is conceivable that such cascades could be initiated by different initial modifications, yet ultimately result into the same set of modifications.

Why would multiple histone modifications be employed in parallel to control transcription? Partially redundant functions of histone marks in chromatin might confer robustness on epigenetic regulation. Thus, transcriptional responses might be largely independent of small changes in any single modification and the cell might have several options to induce transcriptional changes (changes in transcription can be elicited by inhibition or activation of one of several different histone-modification pathways) [26]. Despite the evidence that many active histone modifications are globally linked, it is likely that there are also differences between combinations of histone modifications that mark specific genes (Figure 1). It should be emphasized that the current ChIP-on-chip maps do not contain information about the timing of (sequential) events, as the maps were generally obtained from asynchronous cell populations. Moreover, ChIP-on-chip does not have single-nucleosome resolution, which may complicate the interpretation: it is formally possible that certain nucleosomes in a particular IGR or coding region carry functionally important modifications that differ from those in the surrounding nucleosomes. Indeed, detailed ChIP analysis of individual genes suggests that trimethyl-H3K4 occurs predominantly in the 5′ part of transcribed regions whereas dimethyl-H3K4 has a much broader distribution [17,27-29]. Furthermore, the different phenotypes and expression profiles that result from inactivation of the enzymes that control the active marks argue that the respective histone modifications can have distinct effects on gene expression.

The high correlations between histone modifications as reported by Schübeler *et al*. [14] and Kurdistani *et al*. [13] may have been overestimated as a result of differences in nucleosome densities or other unknown biases. Interestingly, for a series of additional analyses of the yeast histone acetylation maps, Kurdistani *et al*. [13] employed a data-normalization procedure called variance normalization. The first step of this normalization involves the subtraction (for each probed locus) of the average of all 11 measured acetylation levels from each individual acetylation level. If the presumed nucleosome-density bias is indeed similar for all histone epitopes, then this normalization procedure would at least partially correct for the bias, although the residual values after such a subtraction may have a high noise content. Intriguingly, after variance normalization and the subsequent use of a clustering algorithm, Kurdistani *et al*. [13] identified clusters of functionally related genes that were enriched in certain histone-acetylation patterns. Further experiments are needed to reveal the functional relevance of the relationships between histone-modification patterns and gene functions.

Histone-modification maps in the future

In addition to revealing interesting biological information, recent whole-genome mapping studies have also revealed some technical hurdles that still need to be overcome if we are to construct highly reliable epigenetic maps. First, improved experimental methods are required. Inclusion of control ChIPs, of modification-free histone epitopes or tagged histones, may help to solve the potential bias problems associated with ChIP-on-chip. It will also be crucial to verify whole-genome ChIP results by an independent method because the exact choice of procedures used for ChIP (such as the type of cross-linker, the extent of cross-linking, fractionation of chromatin, and experimental conditions that may cause cellular stress responses) can affect the outcome of a ChIP-on-chip experiment [19,30-32]. At present, no alternative genome-wide method is available for ChIP analysis of histone modifications. The DamID technology, which employs tethered Dam methyltransferase [33], provides an independent means for verifying the global binding data of histone-modifying enzymes and other chromatin-associated proteins, but it is not suitable for mapping histone modifications.

A second challenge lies in the integration of histone-modification maps with other whole-genome chromatin datasets, such as gene-expression profiles, global maps of DNA methylation, DNase-hypersensitive sites, and the binding of histone-modifying enzymes and other chromatin-interacting proteins. For example, a recent mapping study in *Arabidopsis* revealed tight links between methylation of H3K9, hypomethylation of H3K4, and methylation of DNA in a heterochromatic region of the genome [34]. Whole-genome datasets are extremely complex, however, and each mapping

approach yields different information. Moreover, most of the current data should be regarded as probabilistic because of the rather high noise levels. Advanced statistical techniques, such as Bayesian network models [35], may be essential for the successful integration of different types of data and the eventual construction of useful biological models. Despite these challenges, the genome-wide exploration of chromatin structure and function is well on its way.

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