Correlation Between Histone Lysine Methylation and Developmental Changes at the Chicken β-Globin Locus

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Methylation of histones at specific residues plays an important role in transcriptional regulation. Chromatin immunoprecipitation of dimethylated lysine 9 on histone H3 across 53 kilobases of the chicken β-globin locus during erythropoiesis shows an almost complete anticorrelation between regions of elevated lysine 9 methylation and acetylation. Lysine 9 is methylated most over constitutive condensed chromatin and developmentally inactive globin genes. In contrast, lysine 4 methylation of histone H3 correlates with H3 acetylation. These results lead us to propose a mechanism by which the insulator in the β-globin locus can protect the globin genes from being silenced by adjacent condensed chromatin.

The pattern of histone acetylation over the chicken β-globin locus changes during erythroid cell development, consistent with the role played by acetylation in regulation of gene expression (*1*). Early in erythroid development, neither the globin genes nor the upstream folate receptor genes (Fig. 1) display high levels of acetylation of either histones H3 or H4, but large changes occur as development proceeds and the genes are activated or inactivated. At certain places, however, there is little variation in acetylation; over a 16-kb region of condensed chromatin, no significant acetylation is observed, whereas at a pair of sites on either side of this region, there are sharp peaks of acetylation that are maintained at every stage and in every cell type. One of these sites marks the insulator element 5′HS4 (*2*, *3*), which lies just 3′ of the condensed chromatin region at the 5′ border of the globin gene cluster (Fig. 1).

Other histone modifications, including phosphorylation, ubiquitylation, and methylation, appear to be involved directly or indirectly in regulation of gene expression (*4*–*6*). Attention has focused recently on methylation of histone H3 at lysines 4 and 9 (K4 and K9) (*7*, *8*), correlated, respectively, with activation and inactivation of expression.

Because we already had detailed information about the acetylation of the chicken β-globin locus (*1*), we decided to analyze the patterns of methylation on K9 and K4 as a function of erythroid developmental stage. We made use of antibodies specific to these sites (see legend to Fig. 2) in chromatin immunoprecipitation (IP) studies. As in earlier work (*1*), we determined the extent of specific sequence enrichment with the use of polymerase chain reaction (PCR) primers distributed at fairly close intervals within the entire 53-kb region (Fig. 1). Figure 1 summarizes the state of chromatin structure and individual gene expression in cells corresponding to several stages of erythroid development and in 10-day embryonic chick brain. The folate receptor gene (FR) is expressed only in 6C2 cells arrested at the CFU-E (colony forming unit–erythroid) stage of development (*9*); the globin genes are expressed only at a later stage (10-day embryonic cells) (*10*), and the odorant receptor (OR) is expressed in a subset of embryonic brain cells (*11*).

The pattern of K4 methylation in each of the cell types across the entire region is shown in Fig. 2, A to C. There are marked peaks of methylation in 6C2 and 10-day embryonic erythrocytes at deoxyribonuclease I–hypersensitive sites HSA and 5′HS4. In 10-day erythrocytes, where the globin genes are quite active, there are also large concentrations of K4 methylation over the HS2 globin locus control element and near the β^A-globin gene. This resembles the pattern for H3 monoacetylation at K9 or diacetylation at K9 and K14 (Fig. 2, A to C), where peaks of acetylation are seen over HSA and 5′HS4 in all cell types. In 10-day erythrocytes, there is a trough of acetylation (Fig. 2C, sites at 31.977, 34.715, and 36.882 kb) over the β-globin locus separating two peaks (Fig. 2C, 27.729 and 39.807 kb) that matches the K4 methylation pattern. However, a small peak of K4 methylation over the 3′HS upstream of the OR gene is not mirrored in acetylation.

The distribution of methylation at K9 of histone H3 is quite different (Fig. 2, E to G). Methylation levels are uniformly high across the ~16-kb condensed chromatin region that

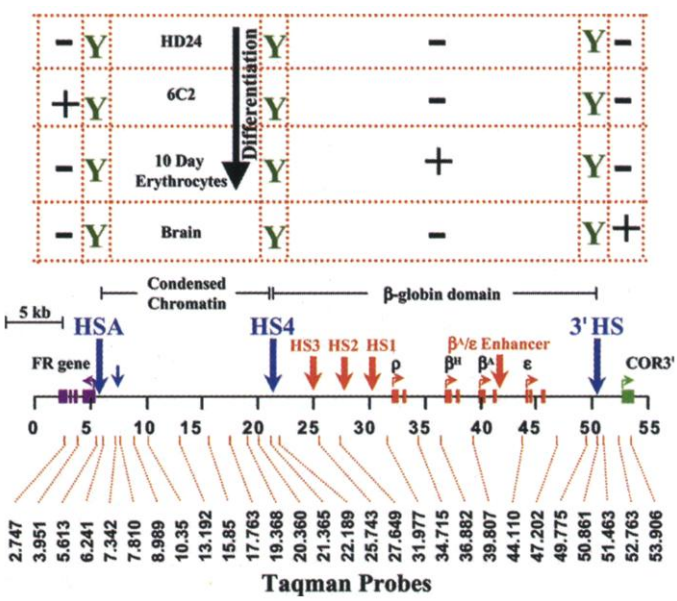

Fig. 1. Map of the chicken β-globin locus. At the 5′ end is a folate receptor gene (FR). HSA is a hypersensitive site associated with FR. A second hypersensitive site, HS4, marks the insulator element of the β-globin locus. The 16 kb between HSA and HS4 is occupied by a region of condensed chromatin. Beyond HS4 are found the hypersensitive sites HS3 to HS1, part of the globin locus control region. A strong enhancer, $\beta^{A/\varepsilon}$, lies between the β^A- and β^ε-globin genes. The 3′ end of the locus has another hypersensitive site (3′HS); beyond that is an odorant receptor gene (COR3′). Below the map are shown the name and location of primer pairs and Taqman probes used for analysis (*1*). The probe numbers correspond to the map positions. Above the map are shown data for four chicken cell types (9). HD24 is arrested at the BFU-E (burst forming unit–erythroid) stage, 6C2 is arrested at the CFU-E (colony forming unit–erythroid) stage, and 10-day erythrocytes are taken from embryonic circulation. Brain is isolated from 10-day chicken embryos. Expression of the individual genes in each cell type is indicated by a + or −. The presence of a hypersensitive site is indicated by Y.

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lies between the FR and globin genes. Variable levels of methylation are observed over the globin locus. There is a peak in all cell types studied at 36.882 kb that is adjacent to CR1 sequence repeat, presumably packaged as condensed chromatin. Unlike the K4 methylation, enrichments in methylation at K9 rarely exceed twofold. Numbers significantly less than 1 indicate depletion (see legend to Fig. 2), notably at either end of the 16-kb condensed chromatin region (HSA and HS4; see map in Fig. 1). These low levels of enrichment probably reflect the high overall levels of K9 methylation in this genome: It is reported that about 20% of chicken H3 K9 residues are methylated (*12*), so that the maximum theoretical enrichment is fivefold (*1*). It is perhaps surprising that there is such widespread distribution of a signal thought to be associated largely with constitutive heterochromatin. Such a role raises questions about mechanisms for removal of methyl groups if reactivation is required.

Results in Fig. 3 compare normalized data for H3 diacetylation, and K4 and K9 methylation. There is a pronounced negative correlation (Fig. 3A) between patterns of H3 acetylation and K9 methylation, most obviously within the ~16-kb condensed chromatin region that lies between the FR and globin genes. Throughout the region, there is negligible H3 acetylation, but a high level of K9 methylation. The two peaks of acetylation surrounding this region (5.613 and 21.365 on the map) are not detectably methylated at K9. In contrast, there is almost complete correlation between the patterns of H3 acetylation and H3 K4 methylation (Fig. 3B). Peaks and troughs of acetylation and K4 methylation occur most often within the same regions of the locus. A direct comparison of methylation at K4 and K9 (Fig. 3C) makes it clear that there is a strong anticorrelation between the two modifications.

We have carried out similar experiments using 10-day embryonic chick brain (Fig. 2, D and H). The major peak of K4 methylation over 5′HS4 remains, as does the peak of K9 monoacetylation at this site. The inactive FR gene and the adjacent 16-kb condensed chromatin region show elevated methylation at K9. However, the HSA site lying between them is devoid of K9 methylation; so is the insulator element at 5′HS4.

Our results confirm that transcriptional activation is associated with methylation at H3 K4, which parallels acetylation of H3 [as well as H4 (*1*)]. Inactive chromatin is marked by K9 methylation; the distribution of this modification across the locus is inverse to that of the other two. These data suggest a possible role for the chicken β-globin insulator at 5′HS4. As shown here and earlier, there is a strong constitutive peak of histone acetylation near the insulator site. It has been pointed out elsewhere that regions of condensed chromatin containing K9-methylated H3 could serve to propagate the methylated and condensed state (*13*–*15*). We suggest that one role of the insulator is to provide a center of H3 K9 acetylation, which could serve as a chain terminator for the propagation process initiated in the highly K9-methylated 16-kb condensed chromatin region (Fig. 4). This is related to, but distinct from, the mechanism for establishing boundaries in *Saccharomyces cerevisiae*, where a site that binds certain histone acetylases is sufficient to prevent the extension of silencing from HMR-E (*16*). The propagation process in yeast is not yet understood. In the case of the chicken β-globin locus, maintaining high levels of acetylation at K9 prevents methylation at that site, and thus could protect the β-globin locus, located down-

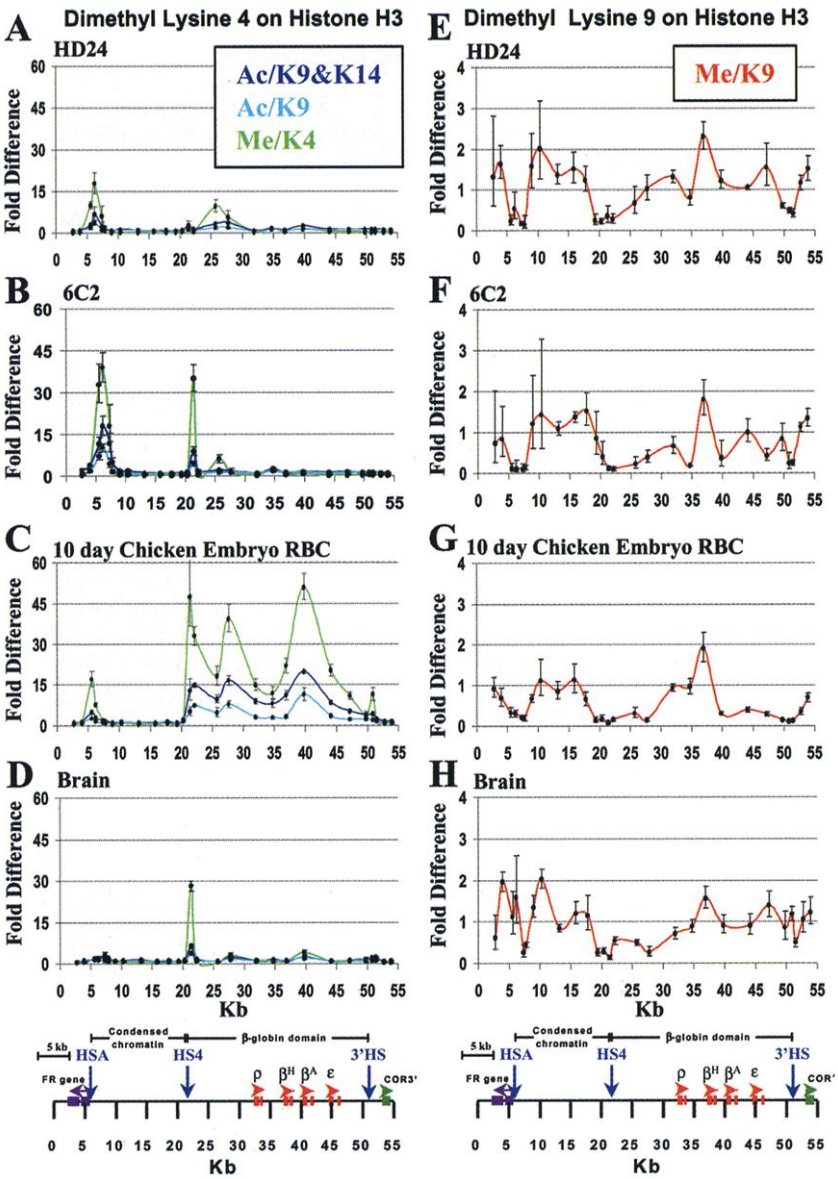

Fig. 2. Histone H3 acetylation and methylation across the β-globin locus as a function of erythroid developmental stage. Results of chromatin IP with antibodies to methylated and acetylated lysine sites on histone H3. For each IP, chromatin consisting of mono- and dinucleosomes fractionated on a 5 to 30% sucrose gradient was prepared from nuclei from each cell type digested with micrococcal nuclease (*1*). Immunoprecipitated samples were analyzed by RealTime PCR methods as described (*1*). Each data point indicates the average of three independent PCR analyses of an IP with the standard deviation shown by the error bars. Similar results were obtained in repeat IPs for Me/K9 and Ac/K9. (**A** to **C**) H3 methylated on lysine 4 (Me/K4 in green) at three developmental stages compared with H3 either monoacetylated on lysine 9 (Ac/K9 in light blue) or diacetylated on lysines 9 and 14 (Ac/K9 and K14 in blue). (**D**) Same for 10-day chicken embryonic brain. (**E** to **G**) H3 methylated on lysine 9 (Me/K9 in red) in erythroid cells. (**H**) Same for brain. Enrichments significantly less than 1 indicate underrepresentation in the IP fraction relative to the abundance of methylated sites in the genome. Specific antibodies for diacetylated lysine 9 and 14 of histone H3, acetylated lysine 9 of histone H3, and dimethylated lysine 9 of histone H3 were from Upstate Biotechnology. Anti–dimethyl lysine 4 of histone H3 is described (*20*).

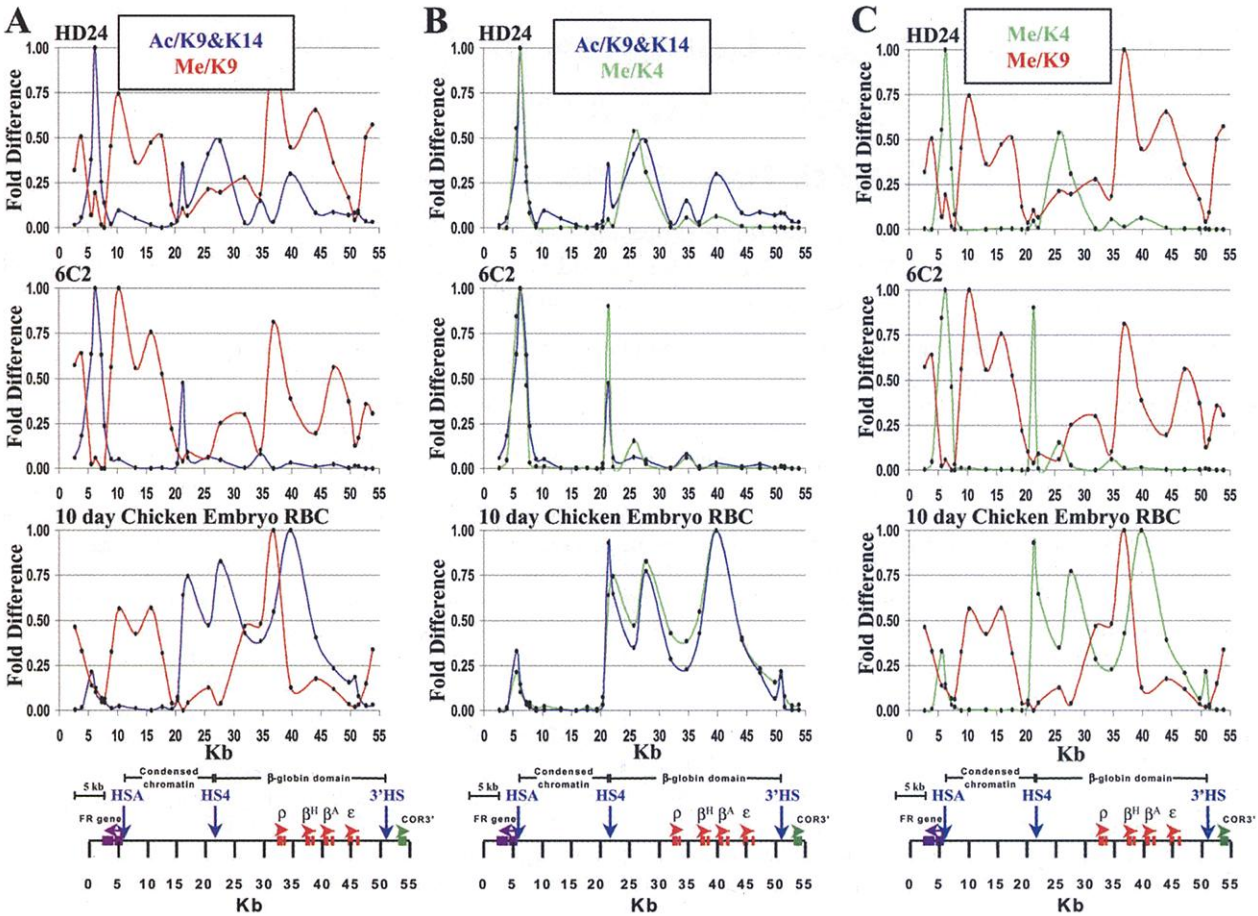

Fig. 3. (**A**) H3 diacetylated at K9 and K14 (Ac/K9&K14) compared with H3 methylated at K9 (Me/K9). (**B**) H3 diacetylated at K9 and K14 compared with H3 methylated at K4 (Me/K4). (**C**) H3 methylated at K4 or at K9. Ac/K9&K14 are shown in blue, Me/K4 in green, and Me/K9 in red. Data for each curve have been normalized with the lowest relative-difference data point set equal to 0 and the highest to 1.0.

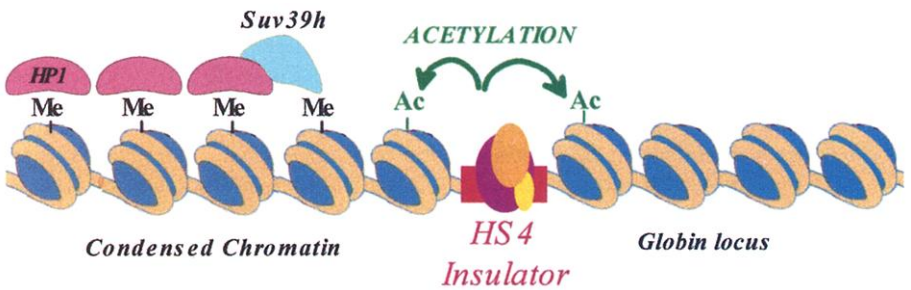

Fig. 4. A proposed model for how the β-globin insulator could block the extension of a condensed chromatin region into the β-globin gene cluster. Histones adjacent to the insulator are known to be sites of strong acetylation [(*1*) and Fig. 2]. Data presented in Figs. 2 and 3 show that histone H3 in the extended condensed chromatin region is enriched for methylation on K9. It has been suggested that highly methylated regions such as this one would bind HP1 (*14*, *15*), which in turn can bind the histone methylase Suv39h (*21*), resulting in propagation of methylation beyond the region. High levels of acetylation on H3 K9 will inhibit further methylation and could prevent this propagation. The proposed role of the insulator is to provide a source of acetylase activity. We suggest that this may be the basis of the ability of 5′HS4 to protect against position effects. This is distinct from its ability to block enhancer action, which is mediated by binding of the protein CTCF and is separable from the position-effect activity (*19*).

stream of the condensed 16-kb region, from silencing. This might also partially explain the ability of 5′HS4 to protect against position effects in transformed cell lines and animals (*17*–*19*).

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