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Distinctive Signatures of Histone Methylation in Transcribed Coding and Noncoding Human β-Globin Sequences▽†

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The establishment of epigenetic marks, such as methylation on histone tails, is mechanistically linked to RNA polymerase II within active genes. To explore the interplay between these modifications in transcribed noncoding as well as coding sequences, we analyzed epigenetic modification and chromatin structure at high resolution across 300 kb of human chromosome 11, including the β-globin locus which is extensively transcribed in intergenic regions. Monomethylated H3K4, K9, and K36 were broadly distributed, while hypermethylated forms appeared to different extents across the region in a manner reflecting transcriptional activity. The trimethylation of H3K4 and H3K9 correlated within the most highly transcribed sequences. The H3K36me3 mark was more broadly detected in transcribed coding and noncoding sequences, suggesting that K36me3 is a stable mark on sequences transcribed at any level. Most epigenetic and chromatin structural features did not undergo transitions at the presumed borders of the globin domain where the insulator factor CTCF interacts, raising questions about the function of the borders.

The amino-terminal tails of histones are subject to various posttranscriptional modifications in eukaryotic cells, including acetylation, methylation, phosphorylation, and ubiquitinylation (5). These modifications have the potential to alter chromatin structure and affect chromatin function. They may also act in a concerted fashion to provide a code by which downstream events related to transcription activation and repression are initiated or maintained and epigenetic information is transmitted to subsequent cell generations (17, 62). The acetylation of lysine residues in histone tails is usually associated with active genes, while the methylation of lysine residues can be associated with active or repressed genes (30, 55). Furthermore, lysines residues can be mono-, di-, or trimethylated. The functional interplay of the different levels of histone methylation has been the focus of considerable recent interest (33, 39).

Methylation can occur on four lysine residues in the H3 tail: K4, K9, K27, and K36. The methylation of K4 and K36 marks active chromatin. In the yeast *Saccharomyces cerevisiae*, Set1 and Set2, which methylate H3K4 and K36, respectively, are associated with different phosphorylated forms of the carboxy-terminal heptad repeat domain (CTD) of RNA polymerase II (pol II). Set1 associates with the initiation and early elongation-competent pol II phosphorylated at Ser5 (31, 44). Accordingly, K4me2 and me3 peak early in active genes; however, me2 is also found at inactive but potentially active genes (6, 42, 44, 51, 53, 54). K36me2 and me3 marks peak toward the 3′ end of transcribed coding sequences, consistent with the association of Set2 with the processively elongating Ser2 phosphorylated form of pol II (2, 32, 36, 66). Mammalian homologues of these methylases have been characterized, and the human H3 K36 methylase HYPB has been reported to interact with hyperphosphorylated pol II (14, 60). In contrast to K4 and K36 methyl marks in active chromatin, the methylation of K9 marks heterochromatin and repressed genes in euchromatic regions (33). Surprisingly, recent studies report the detection of H3K9me3 in the coding regions of active genes (7, 63).

The transcription of nongenic sequences in developmentally regulated mammalian loci is a well-known phenomenon (reviewed in reference 13). In some cases, the transcripts have been shown to depend on locus control region (LCR) activity and to play a critical role in the activation of target genes (23, 40). Implicit in these findings is the idea that LCR activity is limited to the relevant locus, possibly by insulators (65, 68). One such element, DNase I hypersensitive site 4 (HS4), flanks the 5′ side of the chicken β-globin locus and is presumed to both circumscribe LCR activity within the locus and protect the locus from the encroachment of upstream heterochromatin (12). The CTCF site in 5′ HS4 is required for enhancer blocking and participates in the intranuclear tethering of 5′ HS4 (3, 67).

Chicken 5′ HS4 and 3′ HS1 (also a site of CTCF interaction) define the boundaries of acetylated H3 and H4, dimethylated H3K4, and the general sensitivity to DNase I characteristic of this locus in erythroid cells (22, 37, 38, 50). The mouse and human globin loci are also flanked by constitutive HS sites with CTCF motifs, although their insulator activity varies considerably (9, 16). The mouse and human constitutive HS sites participate in the formation of the globin chromatin hub which brings the corresponding sequences into physical proximity when the globin locus is active (46, 61). In the mouse locus, the

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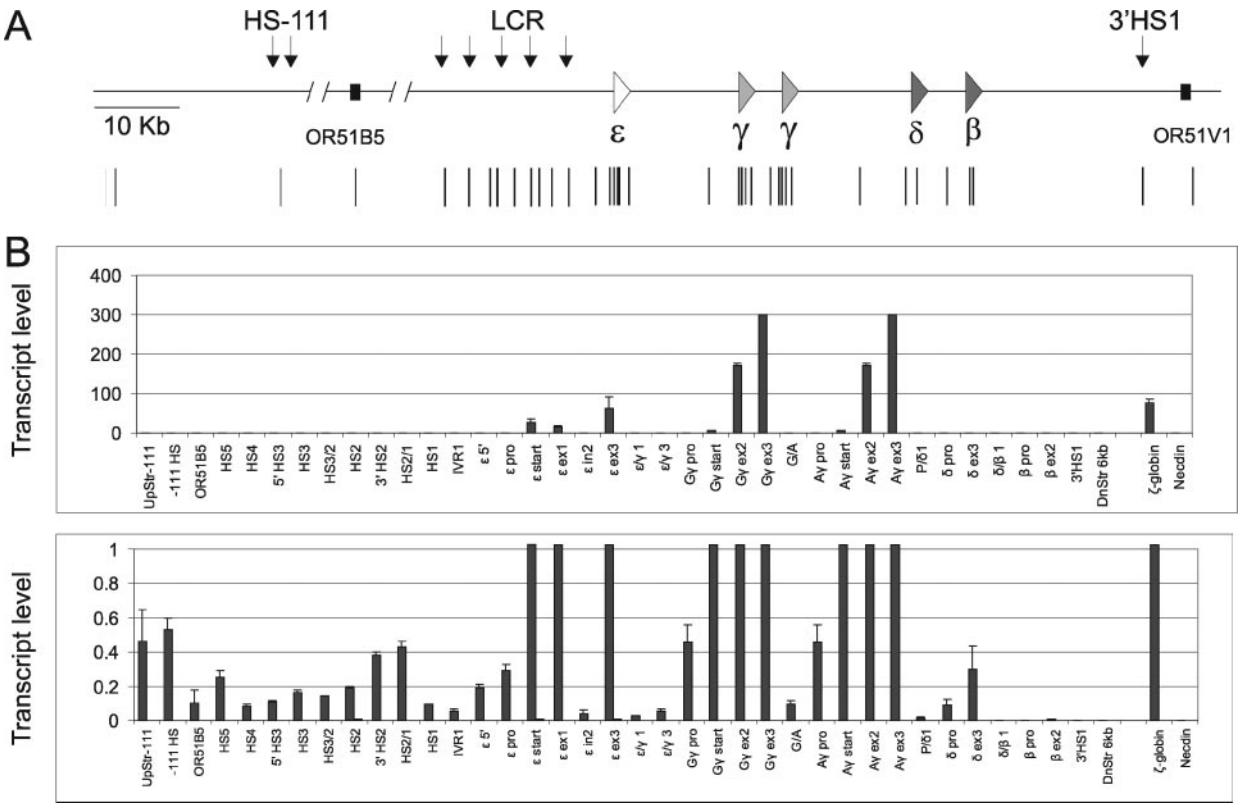

FIG. 1. Transcript levels within and flanking the human β-globin locus. (A) Arrowheads represent individual globin genes in the human β-globin and indicate the direction of transcription. Vertical black arrows represent the LCR and locus flanking DNase I hypersensitive sites. Odorant receptor genes in flanking sequences are represented by black squares. Gaps in the sequence diagram are denoted by double-hashed lines representing gaps of 21 kb (left) or 40 kb (right). Vertical bars below the diagram represent the locations of the TaqMan probes. (B) Total RNA from K562 cells was isolated, and transcript levels at specific regions were assayed by RT-qPCR and compared to a genomic DNA control of known concentration by the comparative C_T method ($n = 2$). Probes within γ-globin coding sequences do not distinguish between the Gγ and Aγ genes but probes up- and downstream and between these genes amplify unique sequences. Black bars, with reverse transcriptase; gray bars, without reverse transcriptase. Controls include necdin (brain specific) and ζ-globin (unlinked α-like embryonic globin). (C) The data of panel B are presented at an expanded scale. Error bars indicate standard errors of the means ($n = 3$).

boundaries of acetylated and methylated histones and DNase I sensitivity do not correlate precisely with each other or with the putative insulator/boundary sites (8, 18). The relationship between the hub sites and intranuclear tethering is unknown.

Intergenic transcription in the human β-globin locus has been well documented (1, 20, 21, 47). In the current study, we investigated the relationship between methylation states of histone H3K4, K36, and K9 and genic and intergenic transcription across the β-globin locus and extended flanking regions in human erythroid K562 cells. We also defined the extents of histone acetylation and sensitivity to DNase I. Monomethylated K4, K9, and K36 were detected broadly in the intergenic regions and inactive genes. The trimethylation of H3K4, K9, and K36 marked the coding regions of the highly transcribed genes where, conversely, the monomethyl mark was lost, consistent with a transcription-coupled mechanism of enzymatic conversion. In addition to its presence at transcribed genes, H3K36 trimethylation was observed across and beyond the globin locus in a pattern consistent with the idea that K36me3 is a stable mark on chromatin that is transcribed at any level. Transitions in histone methylation, in general, did not correlate with boundary sites in the human globin locus, nor did general DNase I sensitivity, indicating a complex multilevel organization of chromatin.

MATERIALS AND METHODS

Cell culture. K562 cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum. All experiments were performed at a confluence of 4×10^5 to 6×10^5 cells per milliliter in growing cells.

Reverse transcription quantitative PCR (RT-qPCR). RNA was prepared from 1×10^6 to 2×10^6 K562 cells using the Purescript kit (Gentra). Two micrograms of RNA was treated with RNase-free DNase I, and then 1 μg of RNA was reverse transcribed with random hexamers using the SuperScript II first-strand synthesis kit as suggested by the manufacturer (Invitrogen). cDNA was diluted to 400 μl, and 5 μl of cDNA was amplified in a 25-μl reaction volume by real-time PCR using TaqMan chemistry in the Prism 7900 (Applied Biosystems). The relative enrichment of specific cDNA sequences was compared with a genomic DNA standard using the comparative cycle threshold (C_T) method (37). Three preparations of RNA were analyzed. TaqMan primers and probes were designed using PrimerExpress (Applied Biosystems) and are listed in Table S1 in the supplemental material.

ChIP and antibodies. Chromatin immunoprecipitation (ChIP) was carried out as previously described (24) with minor modifications. Briefly, 2.5×10^7 K562 cells were incubated in growth medium containing 1% formaldehyde for 10 min at 25°C and the cross-linking reaction was quenched by adding glycine to 0.125 M. Isolated nuclei were digested with 200 U of MNase at 37° for 15 min, followed by sonication to produce chromatin of primarily mononucleosome size (150 bp).

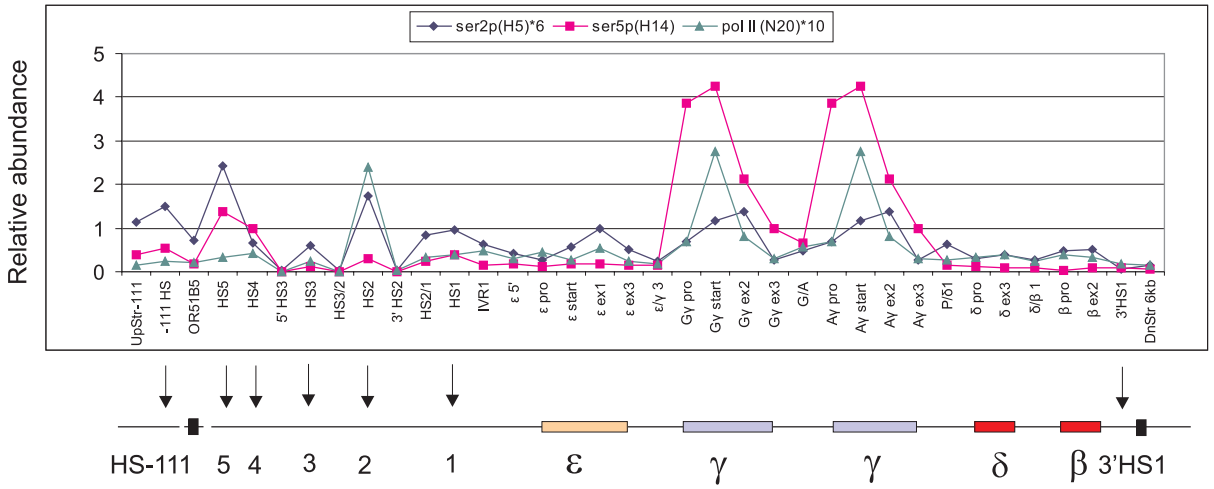

FIG. 2. RNA polymerase II association at transcribed genic and nongenic β-globin sequences. ChIP was performed using antibodies specific to the pol II N-terminal region (N-20; Santa Cruz), the initiation/elongation Ser5P CTD form of pol II (H14; Covance), and the elongation/termination Ser2P CTD form of pol II (H5; Covance). Values obtained by real-time qPCR were normalized to input by using the comparative C_T method and may be rescaled, i.e., divided or multiplied by a factor, as indicated, to accommodate individual antibody efficiencies and allow for direct comparison of the patterns of pol II localization. The results are the averages of three RNA preparations.

For RNA pol II antibody ChIP, whole-cell extracts were employed as described previously (43); however, more-stringent sonication conditions were substituted. Fragmented chromatin was reacted with antibodies after being precleared with protein A- or A/G-agarose. All sequences were represented equally in the digested chromatin as judged by comparison to genomic DNA fragmented with a restriction enzyme. A fraction of the material was reserved as input, and aliquots of the remainder (0.8 to 1 ml) were incubated at 4°C overnight with specific antibodies or with normal immunoglobulin G (IgG) or no antibody as controls. Protein-DNA complexes were bound to protein A or A/G agarose beads, washed, and eluted as described previously, followed by the reversal of formaldehyde cross-links at 65°C in 0.25 M NaCl for a minimum of 4 h. Between two and four preparations of chromatin were examined with each antibody. The antibodies used in these studies were obtained from Abcam (Cambridge, United Kingdom), Covance, or Upstate (Lake Placid, NY) and are listed in Table S2 in the supplemental material. Abcam literature indicates that antibody ab8988 against K9me3 has low cross-reactivity with K27me3, with which it shares an epitope. We tested a specific anti-K27me3 antibody (ab6002) and found little reactivity over background in the globin locus but a robust signal at the inactive necdin gene as expected for this repressive mark.

Real-time qPCR. Purified DNA was amplified by real-time qPCR using TaqMan chemistry in the Prism 7900 (PE Applied Biosystems). Data were collected at the threshold where amplification was linear. The relative intensity for each primer pair was determined by comparing the amount of target sequence in 1.25% of immunoprecipitated DNA to the amount of target sequence in 0.1% of input DNA using the comparative C_T method.

Preparation of nuclei and DNase I digestion. Nuclei were prepared from K562 cells (5×10^6) as described previously (19). Aliquots of approximately 1×10^6 nuclei were digested with 20 to 200 U DNase I (Invitrogen). The purified DNA was resuspended in 200 μl of 1× Tris-EDTA. Samples were diluted 1/20, and 5 μl was used for real-time qPCR analysis of DNase I sensitivity as previously described (41). The analysis was performed for three preparations of chromatin.

RESULTS AND DISCUSSION

Extragenic transcription and RNA pol II far upstream of the β-globin domain. The human globin genes are sequentially expressed in erythroid cells during development under the regulatory control of the β-globin LCR (57). K562 cells model the embryonic/fetal stage at which the ε- and γ-globin genes, but not the adult δ- and β-globin genes, are expressed. To provide a context for histone methylation studies, we used RT-qPCR to obtain a sensitive measure of accumulated transcripts for the globin genes and extragenic sequences over almost 300 kb on chromosome 11 in K562 cells. TaqMan probes across the globin locus and upstream and downstream of the globin chromatin hub sites (HS-111 and 3′ HS1) were employed (Fig. 1A and see Table S1 in the supplemental material).

The γ-globin genes were robustly expressed, and RNA accumulated to a level almost 10-fold higher than that for ε-globin (Fig. 1B). At a magnified scale (Fig. 1C), intergenic transcripts were revealed to be about 1,000-fold less abundant than γ-globin transcripts but up to 10-fold more abundant than unspliced, nascent transcripts within intron 2 of ε-globin (Fig. 1C). These results reflect steady-state transcript accumulation: we do not know if all are equally stable. A trace level of δ-globin but no β-globin transcription was detected, and intergenic transcripts were not observed in the 3′ end of the locus, consistent with earlier data that intergenic transcription in a human globin transgene in mouse erythroid cells is related to the stage of differentiation (20). Interestingly, robust extragenic transcription occurred upstream of the LCR, both in the silent odorant receptor gene (ORG) domain beyond HS5 and upstream of the HS-111 chromatin hub site.

Globin locus intergenic transcripts are sensitive to α-amanitin and DRB treatment and are thus associated with RNA polymerase II transcription (1, 26). However, pol II detection is limited primarily to the promoters of active genes and the LCR HS sites to which pol II is recruited and where the density of stabilized pol II is presumably greatest (25, 29, 52). We used three antibodies of widely differing affinities against different phosphorylated forms of RNA pol II in ChIP experiments to visualize pol II association. After cells were cross-linked with formaldehyde, nuclei were digested with MNase, followed by the sonication of chromatin to produce fragments of primarily mononucleosome size (see Fig. S1 in the supplemental material), and real-time qPCR was carried out with TaqMan probes (Fig. 1A).

To compare the overall patterns of pol II detection, the

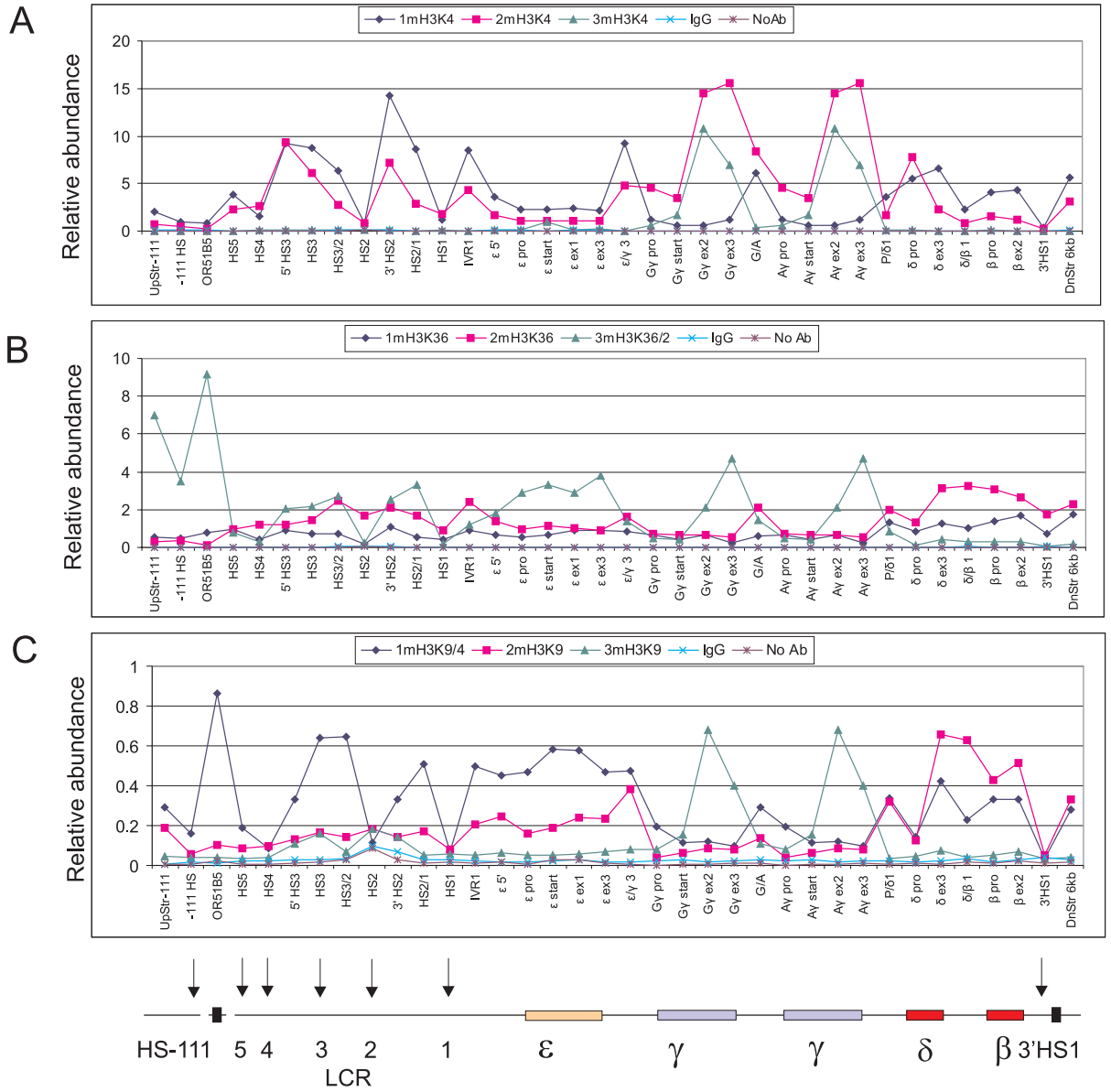

FIG. 3. Histone methylation patterns in and flanking the human β-globin locus. ChIP was performed using antibodies specific to the different methylated forms of histone H3K4, K9, and K36 (see Table S2 in the supplemental material for antibody sources). (A) Histone H3K4 mono-, di-, and trimethylation. Values were obtained by real-time qPCR and, in some instances, were rescaled (see the legend for Fig. 2) to allow for direct comparison of histone methylation patterns obtained with different antibodies. The results are averages of three different chromatin preparations. No Ab, no antibody. For additional control reactions where the scale has not been changed, including error bars, see Fig. S2 in the supplemental material. (B) Histone H3K36 mono-, di-, and trimethylation. (C) Histone H3K9 mono-, di-, and trimethylation. The diagram at the bottom is not drawn to scale.

results for antibodies of various affinities were rescaled as indicated in Fig. 2. All three antibodies detected pol II in the highly transcribed γ-globin genes. The peak of detection for antibodies against total pol II and against the ser5P-initiating/early elongating form of pol II was at the γ-globin start site, whereas the ser2P processively elongating form of pol II peaked at a more 3′ position as expected (11). Much lower levels of pol II were detected in the weakly transcribed ε-globin gene and across sequences between the genes and the LCR. Pol II was detected at LCR HS sites 1 through 5 at various levels by the different antibodies, although the ser5-initiating form of pol II was not detected at HS2, even though a pol II initiation site for reporter gene transcription was mapped there (49). The detection of pol II with these antibodies in the silent 3′ of the globin locus was below the level of the IgG (or IgM) control (not shown). In contrast, pol II was robustly detected upstream of the LCR, both in the silent ORG domain beyond HS5 and upstream of the HS-111 chromatin hub site, providing evidence that the far upstream transcripts we observed are pol II related.

Overlapping and distinct histone methylation in genes and nongenic transcribed sequences. Having established a wide-

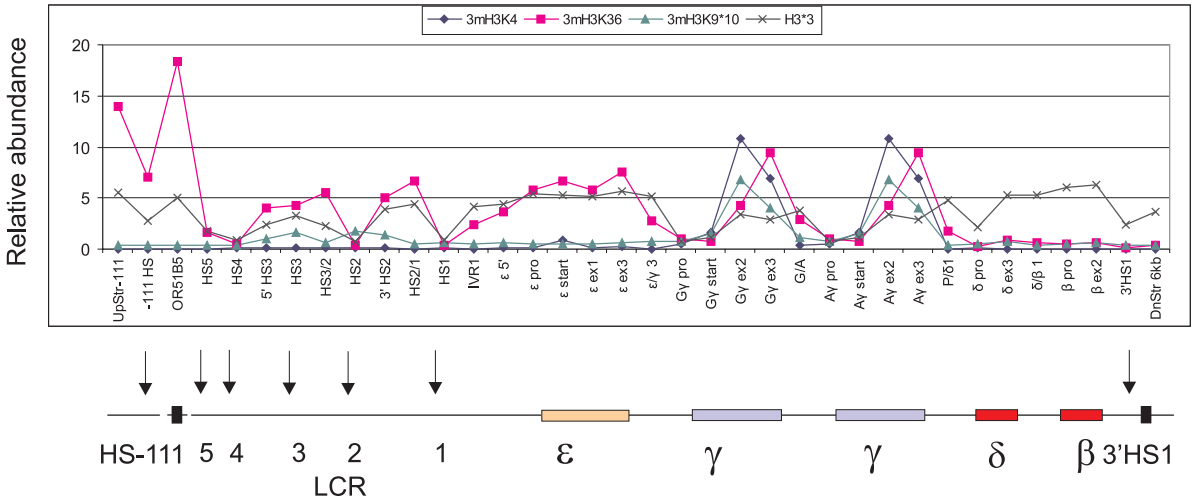

FIG. 4. H3K4, K9, and K36 trimethylation patterns in the human β-globin locus. The me3 patterns for H3K4, K36, and K9 from Fig. 3A, B, and C are plotted together and in some instances were rescaled (see the legend to Fig. 2) to allow for direct comparison of the methylation patterns obtained with different antibodies. ChIP was performed using antibodies to H3. All values are normalized to input using the comparative C_T method ($n = 3$), and the final values obtained were multiplied by 3 in order to compare the pattern of H3 distribution to those of histone trimethylation. For additional control reactions where the scale has not been changed, including error bars, see Fig. S2 in the supplemental material.

spread pattern of various levels of transcription and pol II localization within and flanking the human globin locus, we determined the pattern of mono-, di-, and trimethylation of H3K4, K36, and K9 by using ChIP with antibodies to these specific modified histones (see Table S2 in the supplemental material). The small size of the DNA fragments used for ChIP (see Fig. S1 in the supplemental material) produced a high-resolution picture of histone methylation through and surrounding the globin locus.

H3K4me1 and me2 were prominent in the LCR although not at the HS sites themselves (Fig. 3A) (see below). K4me1 levels were higher in the repressed δ- and β-globin genes than in the active ε- and γ-globin genes and showed peaks in the regions between the genes. Thus, K4me1 was very widely distributed. K4me3 was detected in only the coding region of the highly transcribed γ-globin genes, where it peaked along with K4me2, similar to the control actin gene (see Fig. S2A in the supplemental material). The elevated level of K4me2 and me3 within the highly transcribed γ-globin genes appears to be established at the expense of K4me1, consistent with frequent passage of pol II associated with a human Set1 complex. H3K4 methyl marks were very low in the transcribed sequences upstream of the globin domain and absent from the inactive brain-specific necdin gene (see Fig. S2A in the supplemental material).

Wide distribution of H3K36me3 across sequences transcribed at different levels. K36me1 was detected at a low level at all positions tested (Fig. 3B). However, even at the inactive β-globin gene, levels of K36 monomethylation were higher than those at the repressed necdin gene (see Fig. S2B in the supplemental material). Thus, K36me1 marks chromatin very broadly across the locus similarly to K4me1. K36me2 and H3K36me3 correlated with transcription activity in opposite ways. H3K36me2 was highest over the silent adult genes and had peaks in intergenic regions in the locus, while H3K36me3 marked the transcribed ε- and γ-globin genes where H3K36me2 was low. Notably, and in contrast to K4 methyl marks, K36me3 extensively marked the LCR-transcribed intergenic sequences and the distant 5′ sequences where transcription and pol II were detected, suggesting it stably marks sequences transcribed at any level.

Methylation of H3K9 in the globin locus and flanking regions. K9me1 was detected broadly in the globin locus and in the upstream transcribed region but was very low in the γ-globin genes and was high in the weakly transcribed ε-globin gene and the silent δ- and β-globin genes. The high level of K9me1 in both the ε-globin gene and the repressed globin genes compared to that in the necdin gene (see Fig. S2C in the supplemental material) and its detection in intergenic regions suggest that this modification may also mark chromatin generally in this locus. H3K9me3 was prominently detected in the highly transcribed γ-globin genes where it appeared at the expense of both me1 and me2 marks (Fig. 3C). Interestingly, me2, which is linked to repression in euchromatin (33), is low across the locus except at the silent adult genes. If the low level of H3K9me2 in the γ-globin genes is necessary to maintain active γ-globin transcription, then a mechanism to ensure complete conversion of mono- to trimethylation must exist.

Mono- and dimethylation patterns and transcription. Taken together, certain patterns emerge for the different levels of methylation at K4, K36, and K9. Monomethylated H3K4, K9, and K36 were widely distributed, and their elevated levels compared to that of the repressed necdin gene suggest that monomethylation marks "competent" chromatin that either is transcribed or will be transcribed at a different developmental stage. The loss of K4me1 and K36me1 at the actively transcribed genes is consistent with their conversion to higher methylated states by pol II-associated human homologues of the Set1 and Set2 methylases, and our data suggest that H3K9 trimethylation is also linked to transcription (63). Consistent

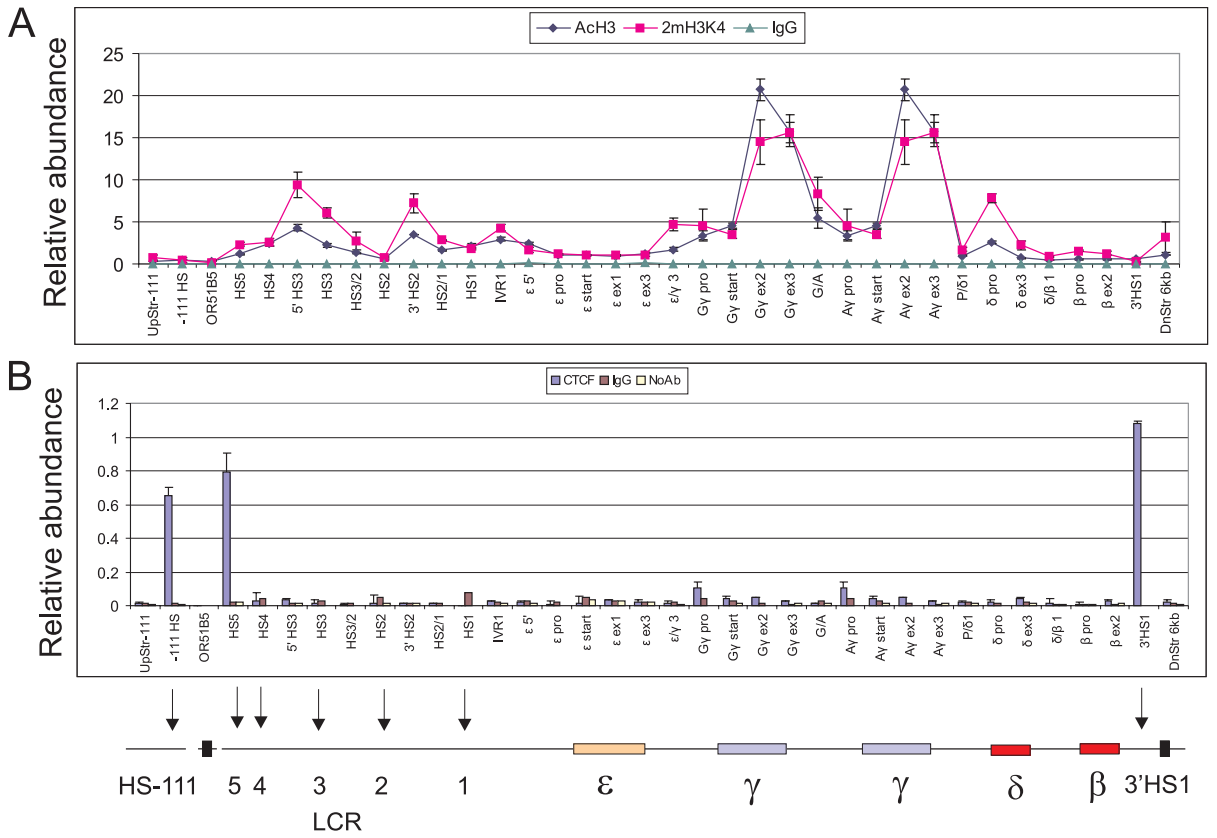

FIG. 5. Limits of subdomains of H3 hyperacetylation and K4 dimethylation and CTCF interactions in the human globin locus. (A) ChIP was performed using antibodies to diacetylated H3 (K9 and K14), and the results were compared to the pattern for H3K4me2 from Fig. 3A. Error bars represent standard errors of the means (SEM) (n = 3). No Ab, no antibody. (B) ChIP was performed using antibodies to CTCF. No Ab, no antibody. Error bars represent SEM (n = 3).

with this idea, it has been shown that the monomethylation of H3K4 marks the lysozyme locus in undifferentiated chicken monoblasts and is increasingly converted to H3K4me3 during cell differentiation (35). However, since we observe this mark across the silent adult genes where transcripts, at least at the level of detection of RT-qPCR, are not detected, the linkage between establishing the monomethyl mark and transcription is not complete and monomethylation may not be transcription coupled. How monomethylation is established will be important to determine.

K4, K36, and K9 each had a unique pattern of dimethylation. H3K4me2 marked the γ-globin genes, along with H3K4me3, which was similar to findings with yeast and chicken cells (51, 54). However, H3K36me2 decreased across γ-globin coding sequences as the me3 form increased, in contrast to the pattern on chicken genes (2). H3K9me2 was consistently low across the γ genes, in contrast to the results from another work (63), and it was the me1 form which was seen to diminish through the coding sequences as me3 increased. It should be noted that H3K36me2 and K9me2 marks were detectible at low levels in γ-globin coding sequences and it was only when intergenic as well as gene sequences were compared that it became clear that the dimethyl marks are actually at a low point within the genes. The dimethyl forms of K36 and K9 were highest over the silent adult genes in the K562 globin locus. While the role of K9me2 at silent genes in euchromatin was not unexpected, the K36me2 mark in the silent genes was surprising. However, data obtained for yeast indicate that methylation at K36 by Set2 can play a repressive role in chromatin (28, 34, 59).

Varying correlation of trimethyl marks with transcription. Figure 4 compares the patterns for the trimethylated forms of H3K4, K9, and K36, which all had peaks in the highly transcribed γ-globin genes. H3K4me3 and H3K9me3 are coincident in the γ-globin coding region as reported for several other mammalian genes (63). K36me3 overlapped with K4me3 and K9me3 in these genes but peaked further toward the 3′ end of the coding sequences, consistent with the results of earlier work (2). However, the general distributions of the different me3 marks across other coding and transcribed noncoding sequences differed significantly. K36me3 but not K4me3 or K9me3 marked the more weakly transcribed ε-globin gene, the LCR, and the intergenic regions to an extent similar to that of the γ-globin genes and was detected in the far upstream transcribed regions. This suggests that in mammals H3K36me3 is a stable mark of pol II passage. Interestingly, in yeast, dimethylated H3K36 is a genome-wide, transcription-coupled mark that does not correlate with transcription rate (48).

Epigenetic modification and chromatin structure. In Fig. 3 and 4, most of the methylation marks are absent from the HS sites in the LCR and the γ-globin promoters. Nucleosomes are generally assumed to be altered or depleted at DNase I HS sites, and we have shown this to be the case at the human and

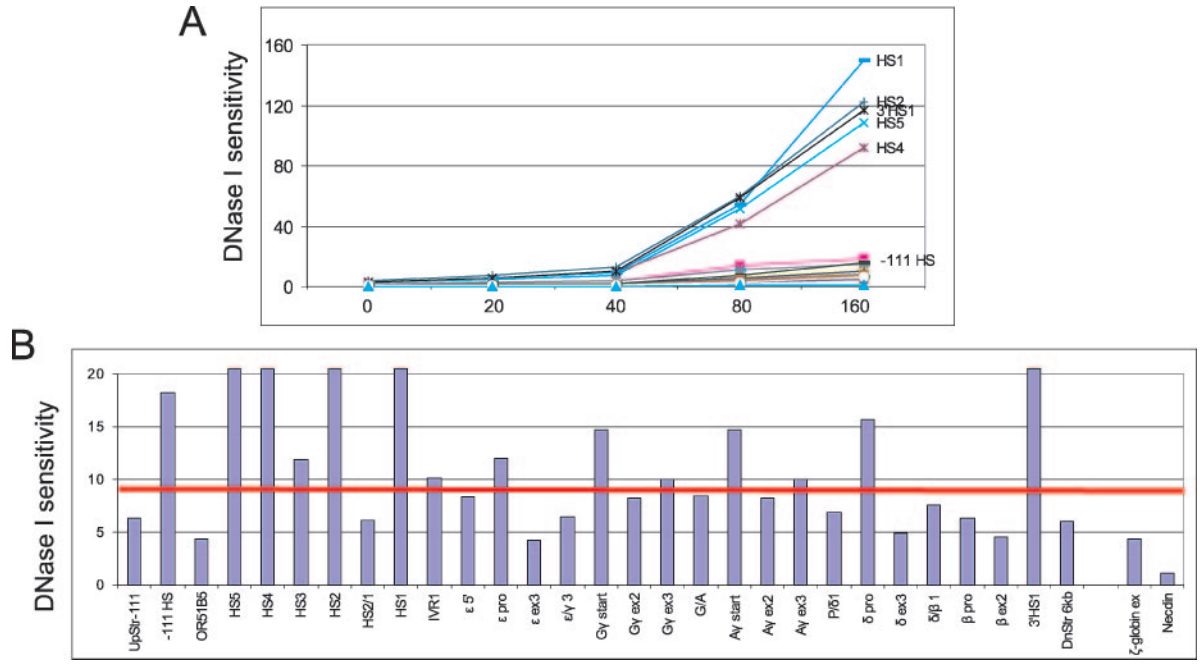

FIG. 6. Analysis of the human β-globin chromatin domain. (A) DNase I sensitivity was analyzed using qPCR with amplicons across the globin gene region as shown in Fig. 1A. The *x* axis indicates increasing concentrations of DNase I (20, 40, 80, and 160 U), and the *y* axis indicates the average level of DNase I sensitivity observed at each of the amplicons compared to a mock-treated DNA control ($n = 3$). The sites most sensitive to DNase I are labeled. (B) The data for digestion with 80 U of DNase I are plotted to reveal the differences in sensitivity among other sites in the globin locus and flanking regions. The horizontal red line divides the sites analyzed into two arbitrary groups based on sensitivity.

mouse LCR HS2 sites (29). Using an antibody to the C terminus of H3, we established a nucleosome occupancy profile across the region of chromosome 11 containing the globin locus. H3 is depleted at the LCR HS sites (except HS3, see below) and at the highly transcribed γ-globin gene promoters compared to that of the rest of the locus (Fig. 4). The region as a whole is somewhat depleted of H3 relative to the inactive necdin gene (see Fig. S2D in the supplemental material). Notably, there is a complete correspondence between the absence of the K36 trimethyl marks and the depletion of nucleosomes. When corrected for H3 loss, the reciprocal detection of monomethylated and hypermethylated marks across the γ-globin genes is reinforced; at the γ-globin promoter and start site, the levels of H3K4me2 are increased and become fairly uniform across the gene (see Fig. S3 in the supplemental material). These results are consistent with transcription-coupled methylation resulting in the accumulation of the trimethylated states of H3K4, K9, and K36 in highly transcribed genes.

Chromatin organization in the human β-globin domain. To integrate the histone methylation signatures we observed into the context of the human β-globin domain more generally, we undertook further epigenetic and chromatin studies. In mouse fetal liver cells which express the adult globin genes, subdomains of H3 diacetylation (K9, K14) and H3K4 dimethylation are seen over the LCR and each of the adult β-globin genes: the intervening, inactive embryonic genes lack these modifications (8, 18). In a pattern complementary to that in the adult mouse locus, in embryonic/fetal K562 cells the LCR and the γ-globin genes contain these modifications and the adult genes do not (Fig. 5A). These modifications are not detected upstream of the LCR at HS5 or downstream of the highly transcribed γ-globin genes, although some modification was noted at the δ-globin promoter which produces a trace level of transcripts. A relatively low level of modification was observed over the ε-globin gene, which may reflect the lower level of transcription of this gene in these K562 cells than those of γ genes.

Additional ChIP experiments using an antibody to H3 acetylated at K9 (data not shown) gave a pattern indistinguishable from that in Fig. 5A with the diacetylated (K9 and K14) antibody, consistent with reports that the latter antibody is primarily against the K9 modification (15). Thus, K9 acetylation and trimethylation (Fig. 3B) were observed together in the highly transcribed γ-globin genes. It has been suggested that these modifications are mutually exclusive and that a reciprocal relationship exists in euchromatin between H3K9ac and K9me which corresponds to either gene activity (K9ac) or gene repression (K9me) (45, 58). The role of H3K9me3 in active genes remains to be further explored. The patterns of H3K9 acetylation and trimethylation appear to be resolvable in larger genes with K9 methylation more prominent in 3′ sequences (64).

H3 diacetylation and H3K4me2 in chicken erythroid cells terminate at the locus flanking 5′ HS4 and 3′ HS1, which are sites of in vivo binding of the insulator factor CTCF. In mouse erythroid cells, the corresponding 5′ HS5 and 3′ HS1 sites and a more distant 5′ HS-60/62 site bind CTCF in vivo (8) and these three sites participate in the formation of the globin chromatin hub (61). The analogous hub sites in the human locus, HS-111, HS5, and 3′ HS1 (46), contain CTCF motifs and bind CTCF in vitro (16). Figure 5B shows, using ChIP, that these sites also bind CTCF in vivo and that no other sites of CTCF interaction are detected in other regions of the locus.

Thus, the 3′ termination of acetyl histone H3 and H3K4me2 in the human globin locus does not correspond to a CTCF interaction site. The CTCF interaction sites do not correspond to transitions in H3K9 or K36 methylation patterns (see Fig. 3).

DNase I sensitivity extends beyond the human β-globin domain. In mouse fetal liver cells, the domain of general DNase I sensitivity in the globin locus extends beyond HS-60/62 and 3′ HS1 sites (8). We used a sensitive and quantitative PCR approach to survey DNase I sensitivity across the human β-globin locus and flanking sequences (41). K562 cell nuclei were digested with various concentrations of DNase I, and the purified DNA was amplified by real-time qPCR. The LCR HS sites, except for HS3, which is a weak HS site in K562 cells (A. Dean, unpublished data), were the most sensitive to digestion, along with 3′ HS1 and the distant HS-111, as expected (Fig. 6A). The data in Fig. 6B, at an expanded scale, show the results for the 80-U DNase I digestion. The second most sensitive group of sites included the active globin gene promoters, regardless of the level at which they are transcribed (ε, γ, and δ), and LCR HS3. Lower sensitivity was observed in the exons of these genes and at the silent β gene. DNase I sensitivity in the ORG clusters upstream of HS5 and HS-111 and downstream of 3′ HS1 were indistinguishable from sensitivity at several positions within the globin locus. We conclude that there is no distinguishing general sensitivity to DNase I within the globin locus compared to that of the ORG clusters that flank it. Interestingly, there was a very close inverse correlation between DNase sensitivity and the presence of H3 on the chromatin (see Fig. S4 in the supplemental material).

These results indicate that, similar to those of the mouse locus, the human β-globin locus CTCF sites do not correspond to transitions in DNase I sensitivity. Nor do the CTCF sites correspond to transitions in nucleosome density as judged by H3 enrichment. We did not observe a transition to DNase I-resistant chromatin or altered nucleosome density, as judged in comparison to the inactive, brain-specific necdin gene, within more than 20 kb upstream of HS-111 or 6 kb downstream of 3′ HS1, even though the ORG cluster genes in these regions remain repressed. We conclude that the globin locus flanking HS-111 and 3′ HS1 that engage in chromatin loop formation correlate with CTCF interaction but not with transitions in epigenetic marks or chromatin structure. Recent data call into question the role of loop formation in the regulation of transcription in the mouse globin locus (4, 56).

We documented that H3 hyperacetylation was limited to a subdomain of the globin locus in K562 cells. However, extragenic transcription, pol II localization and H3K36me3 extended far upstream. In transcribed genes in yeast, histone deacetylases are recruited by the H3K36me3 mark in a process that ensures that coding sequences, which have been acetylated during transcription, are returned to a deacetylated state to suppress intragenic transcription initiation (10, 27, 28). The functional role of the extensive H3K36 trimethyation in transcribed extragenic sequences upstream of and also within the β-globin locus is intriguing and remains to be further explored.

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