Highly restricted localization of RNA polymerase II within a locus control region of a tissue-specific chromatin domain

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RNA polymerase II (Pol II) can associate with regulatory elements far from promoters. For the murine \(\beta\)-globin locus, Pol II binds the \(\beta\)-globin locus control region (LCR) far upstream of the \(\beta\)-globin promoters, independent of recruitment to and activation of the \(\beta_{\text{major}}\) promoter. We describe here an analysis of where Pol II resides within the LCR, how it is recruited to the LCR, and the functional consequences of recruitment. High-resolution analysis of the distribution of Pol II revealed that Pol II binding within the LCR is restricted to the hypersensitive sites. Blocking elongation eliminated the synthesis of genic and extragenic transcripts and eliminated Pol II from the \(\beta_{\text{major}}\) open reading frame. However, the elongation blockade did not redistribute Pol II at the hypersensitive sites, suggesting that Pol II is recruited to these sites. The distribution of Pol II did not strictly correlate with the distributions of histone acetylation and methylation. As Pol II associates with histone-modifying enzymes, Pol II tracking might be critical for establishing and maintaining broad histone modification patterns. However, blocking elongation did not disrupt the histone modification pattern of the \(\beta\)-globin locus, indicating that Pol II tracking is not required to maintain the pattern.

Although well-defined basal transcription factors are required for transcription initiation, the assembly of these factors on promoters is highly regulated through transcription factors bound at promoters and at distal enhancers and locus control regions (LCRs) (7, 23). Transcription factors recruit basal factors through protein-protein interactions (47) and indirectly by recruiting chromatin-modifying coactivators, which increase promoter accessibility (5, 32, 60). While it is easy to envision how promoter-bound factors activate transcription, the mechanisms controlling transcription over a long distance on a chromosome are poorly understood. Evidence strongly supports a looping mechanism, in which chromatin structure juxtaposes distal regulatory elements with the gene, thereby allowing contact between factors bound at distant sites (6).

Looping has been implicated in the transcriptional control of the murine \(\beta\)-globin genes (8, 56). The \(\beta\)-globin locus consists of four genes arrayed in the order in which they are expressed developmentally (Fig. 1A). High-level transcription of the \(\beta\)-globin genes requires upstream DNase I-hypersensitive sites (HS1 to HS4) (12, 59) referred to as the LCR (Fig. 1A) (4, 10, 11, 17). As shown for erythroid cells from murine fetal liver, the LCR is positioned close to the active adult \(\beta\)-globin genes but not the inactive embryonic genes (8, 56), despite the fact that the embryonic genes are closer to the LCR linearly on the chromosome. However, determinants for establishing looping have not been defined, nor is it known how looping activates transcription, even though factors that bind the LCR and promoter in vivo are known (14, 25, 33, 52). Looping also occurs within the \(HNF4\alpha\) locus, and a tracking mechanism was proposed to explain loop formation (19). An enhancer-bound complex tracks along ~6.5 kb of chromatin toward the \(HNF4\alpha\) promoter. This process is accompanied by the acetylation of histones in the intervening region through coactivators recruited by the enhancer. In adult erythroid cells, the highly acetylated \(\beta\)-globin LCR and the adult \(\beta\)-globin genes are separated by an ~30-kb hypoacetylated region containing the silent embryonic \(\beta\)-globin genes (15). As the hypoacetylated subdomain is inaccessible to endonucleases and contains regions of high-level CpG dinucleotide methylation (20), the subdomain likely precludes LCR-bound components from accessing active promoters through tracking.

Regardless of how looping occurs, juxtaposition of the LCR with the promoter presumably allows direct access of LCR-bound components to the promoter, thereby resulting in either direct transfer of protein from the LCR to the promoter or alteration of the nucleoprotein structure of the promoter. It has been demonstrated that RNA polymerase II (Pol II) is bound to the LCR in vivo (24, 25). Although the hematopoietic activators GATA-1 and NF-E2 are required for Pol II recruitment to the \(\beta_{\text{major}}\) promoter, Pol II recruitment to the LCR is independent of NF-E2 (24). GATA-1 is necessary for Pol II binding to HS1, HS2, and HS3 but not HS4 (25).

Since Pol II binds the LCR independent of its recruitment to the promoter, we propose a long-range Pol II transfer (LPT) mechanism in which Pol II is first recruited to the LCR, a step which is enhanced by GATA-1. NF-E2 then provides a signal for Pol II relocalization to the promoter, thereby stimulating transcription. Recently, targeted deletion of the LCR in mice revealed that Pol II recruitment to the \(\beta_{\text{major}}\) promoter is only partially dependent upon the LCR under conditions of phynehylhydrazine-induced stress erythropoiesis (51). The LCR
stimulated serine 5 phosphorylation of the carboxy-terminal domain (CTD) of promoter-bound Pol II. Taken together with the finding that LCR-bound Pol II is phosphorylated on serine 5 of the CTD (24), the LCR-dependent appearance of serine 5-phosphorylated Pol II at the promoter might be explained by the transfer of serine 5-phosphorylated Pol II from the LCR to the promoter.

Besides the β-globin locus, Pol II binds distal elements in other loci. Pol II is recruited to the prostate-specific antigen enhancer and tracks ~6 kb downstream to the promoter (37). While tracking through the hypoacetylated subdomain of the β-globin locus is unlikely, for both the β-globin and prostate-specific antigen systems it has been postulated that Pol II bound to a distal element gives rise to promoter-bound Pol II. An alternate model for Pol II function at the β-globin LCR is suggested by the presence of extragenic transcripts at the β-globin locus (2). Pol II transcription through the LCR, originating from an upstream ERV9 long terminal repeat (36, 46) and from HS2 and HS3 in human erythroid cells (31), has been proposed to mediate chromatin modification (16), potentially by mobilizing Pol II-associated chromatin-modifying complexes (64, 65). Extragenic transcripts have also been detected at the major histocompatibility complex class II locus (40) and at the Drosophila bithorax complex (3). Despite these examples, the function of Pol II at distal elements is unclear. At HNF4α, Pol II cross-linking to an upstream enhancer was a consequence of the enhancer-promoter interaction, a finding which illustrates the importance of determining whether Pol II binding to distal elements is independent of promoter binding (19).

Insight into the function of Pol II at distal elements can be obtained by analyzing exactly where Pol II resides within chromatin domains and how it is recruited. We describe here a high-resolution analysis of Pol II binding at the LCR and discriminate between models for how Pol II localizes to and functions at the LCR.

MATERIALS AND METHODS

Cell culture. MEL (43) and CB3 (38) cells were maintained in Dulbecco’s modified Eagle’s medium (Biofluids) containing 1% antibiotic-antimycotic.

FIG. 1. Highly restricted localization of Pol II within the β-globin LCR. (A) Organization of the murine Hbbα β-globin domain. The embryonic (Ey and βH1) and adult (βmajor and βminor) β-globin genes are shown as boxes, and HSs are shown as spheres. The locations of selected real-time PCR amplicons used throughout the study are indicated below the locus. Intervening regions (IVR) represent sequences containing no known functional elements and were analyzed in subsequent ChIP experiments. (B) High-resolution analysis of Pol II binding at the LCR. The relative levels of Pol II binding at 20 sites within the LCR were determined by ChIP with DMSO-induced MEL cells. Black bars represent amplicons within the murine HS cores, as defined by Hardison et al. (18). End points for these amplicons are at positions 258 to 308 (4core), 5700 to 5750 (3core), 11974 to 12046 (2core), and 15551 to 15626 (1core). Stacked gray (or black) and white bars represent the signals obtained with an anti-Pol II antibody and control preimmune serum, respectively. Arrows indicate positions that were analyzed when enrichments for Pol II and preimmune sera did not exceed 0.0002.
(Gibco/BRL) and either 5% colt serum, 5% fetal calf serum (MEL), or 10% fetal calf serum (CB3). Cells were induced to differentiate by incubation with 1.5% dimethyl sulfoxide (DMSO; Sigma) for 4 days. MEL cells were treated with 100 μM 5,6-dichloro-3-thymidine riboside (DRB) (Sigma) for 1 h in growth media.

Differentiation of Primitive ES Cells into EryP colonies. Murine embryonic stem (ES) cells were differentiated into primitive erythroid colonies (EryP colonies) as described previously (26, 27). Briefly, day 4 embryoid bodies were collected, trypsinized, and replated at 10^5 cells/ml in methylcellulose (Sigma) containing 10% plasma-derived serum (Antech, Houston, Tex.), 5% protein-free hybridoma medium (Gibco/BRL), 1-glutamine (2 mM), transferrin (300 μg/ml; Boehringer Mannheim), and monothioglycerol (4.5 × 10^{-3} M), together with 2 U of erythropoietin (Amgen, Thousand Oaks, Calif.)/ml. EryP colonies were collected 4 days later by digestion with cellulase (1 U/ml) at 20 min at 37°C. Cells were resuspended in culture media, cross-linked with 0.4% formaldehyde for 10 min at room temperature, and analyzed by chromatin immunoprecipitation (ChIP).

Quantitative real-time PCR ChIP assay. Quantitative ChIP analysis was performed as described previously (23, 25, 26). Samples were analyzed by quantitaive real-time PCR (ABI Prism 7000) with primers designed by use of Primer Express 1.0 (PE Applied Biosystems) to amplify regions of 50 to 150 bp. Primers were based on Hbb^β promoter sequences (GenBank accession numbers Z13995, X14061, AF126289, and AF133300). The products in 25-μl reaction mixtures were measured on the basis of SYBR green fluorescence, and the approximate products were determined relative to a standard curve generated from a titration of input chromatin. Analysis of denaturation curves postamplification showed that primer pairs generated single products.

Real-time RT-PCR. RNA was prepared from the same cell cultures as those used for ChIP. Total RNA was purified with Trizol (Gibco/BRL). For real-time reverse transcriptase (RT) PCR (RT-PCR), total RNA was purified, and 1 μg of RNA was used to prepare cDNA by annealing with 250 ng of a 5:1 mixture of random and oligo(dT) primers heated at 68°C for 10 min. This step was followed by incubation with RT (Superscript II; Gibco/BRL), 10 mM dithiothreitol (DTT), RNasin (Promega), and 0.5 mM deoxynucleoside triphosphates at 42°C for 1 h. The mixture was diluted to 150 μl and heat inactivated at 95°C for 5 min. The RT-PCR mixture (25 μl) contained 2 μl of cDNA solution and the appropriate primers. Product accumulation was monitored on the basis of SYBR green fluorescence. Relative expression levels were determined from a standard curve.

Antibodies. Rabbit anti-Pol II (N-20; sc-899) was obtained from Santa Cruz Biotechnology. Anti-acetylated histone H3 (H9252) and anti-tetra-acetylated H4 (H9253) antibodies were obtained from Upstate Biotechnology. Rabbit immunoglobulin G (Sigma) and preimmune serum were used as controls.

RESULTS AND DISCUSSION

Pol II exhibits a highly restricted distribution within the LCR. It has been shown that Pol II can be cross-linked to HS1 to HS4 of the β-globin LCR in multiple erythroid cell lines and in fetal liver at 14.5 days postcoitum but not in nonerythroid cells (24, 25). Pol II might be recruited to the LCR as a key step in an LPT mechanism to provide Pol II to the highly active β major promoter. This model assumes that Pol II localizes predominantly to the recruitment sites—the heteromeric protein complexes of the HSs. Alternatively, the presence of Pol II at the LCR might reflect an ongoing process of transcription at the LCR to generate extragenic transcripts (2). This model implies that Pol II resides throughout the LCR.

To test the possibilities outlined above, we used quantitative ChIP to measure Pol II cross-linking to subregions of the LCR in differentiated MEL cells (Fig. 1B). High-resolution measurements were made possible by use of formaldehyde-cross-linked chromatin fragments with an average size of ~400 bp. Pol II bound to HS1 to HS4 cores, with the strongest binding being at the HS2 core. Analysis of eight noncore sequences, within 400 bp of the core amplicons, revealed substantial reductions in Pol II binding. Since the assay resolution depends on the chromatin fragment size, small enrichments at the flanking sequences might reflect Pol II occupancy at the cores. Consistent with this possibility, little or no Pol II could be detected at distances of greater than ~580 bp from the HSs. These results show that Pol II is highly enriched at the HSs, but not throughout the LCR.

The recruitment of Pol II to the LCR in MEL cells and in primary definitive erythroid cells obtained from murine fetal liver (24) might be indicative of a unique function of LCR-bound Pol II at the adult stage of erythropoiesis. As the embryonic β-globin genes are considerably closer to the adult HSs, which are separated from the LCR by the broad hypoacletylated subdomain, the embryonic genes might utilize a distinct mechanism to recruit Pol II. To test this possibility, we examined whether Pol II associates with the LCR in primitive erythroid cells derived from murine ES cells through in vitro differentiation (Fig. 2A). This in vitro differentiation system recapitulates normal erythropoiesis and yields large numbers of homogeneous EryP cells (21, 26, 27, 58, 62), which express high levels of the embryonic globin genes (Fig. 2B).

Pol II bound the Eyr promoter in EryP cells (Fig. 2C), consistent with the high activity of the embryonic β-globin genes in this system. Pol II also bound the HS cores but not intergenic HSs, consistent with the high activity of the embryonic β-globin genes in this system. Pol II also bound the HS cores but not intergenic HSs, consistent with the high activity of the embryonic β-globin genes. The amounts of Pol II at HS2, HS3, and HS4 were comparable throughout the LCR.

The distribution of Pol II at the LCR is not dependent upon transcriptional elongation. The Pol II distribution pattern

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showed in Fig. 1B can be explained by a mechanism in which Pol II is recruited directly to the HSs, but it is instructive to consider other mechanisms. As human HS2 and HS3 function as promoters (31, 50), a small fraction of Pol II, undetectable by ChIP, might engage in extragenic transcription. Alternatively, Pol II might be recruited upstream of the HSs, and the accumulation of Pol II at the HSs might reflect strong pausing of elongating Pol II at the HSs. However, no upstream initiation sites within the murine -globin locus have been described. Pol II pausing is an established mechanism of transcriptional control. For example, Pol II is transcriptionally engaged, but paused, at the 5' end of the inactive hsp70 gene of Drosophila melanogaster (49). Similarly, Pol II is paused on the inactive human c-myc promoter-proximal region (30). Despite examples of Pol II pausing on genic sites, it is unclear what function Pol II pausing would have in extragenic transcription.

If the pattern of Pol II binding within the -globin LCR reflects Pol II processivity and pausing, the elimination of elongation by Pol II should redistribute Pol II at the HSs. To test this possibility, we subjected DMSO-induced MEL cells to a 1-h treatment with the Pol II elongation inhibitor DRB (9). DRB inhibits the kinase activities of P-TEFb and TFIIH, thereby preventing phosphorylation of the Pol II CTD (39). CTD phosphorylation is necessary for elongation but not for Pol II recruitment (34, 44). Treatment of MEL cells with DRB reduced the level of unprocessed primary -major transcripts 68-fold, as determined by quantitative real-time RT-PCR (Fig. 3A). The amount of primary transcripts remaining following DRB treatment was only ~3-fold higher than that in controls lacking RT, indicating that nearly complete repression was achieved. In addition, low-level extragenic transcripts were detected at multiple LCR sites but not at intergenic site IVR3 (Fig. 3A). Like that of -major primary transcripts, the synthesis of extragenic transcripts was eliminated by DRB.

To provide additional evidence that Pol II elongation was eliminated by DRB, we used quantitative ChIP to measure Pol II binding to the -major promoter and to the -major coding region (Fig. 3B). Following DRB treatment, Pol II levels at intron 2 and exon 3 were strongly reduced relative to background levels, while Pol II levels at the promoter were reduced only ~2-fold. Under conditions in which DRB eliminated Pol II binding to the coding region of -major, no reduction in Pol II levels occurred at the HSs (Fig. 3B). Taken together with the highly restricted distribution of Pol II at the HSs (Fig. 1B), these results provide strong evidence against a model in which the majority of LCR-bound Pol II actively tracks throughout the LCR.

Differential distributions of histone modifications and Pol II at the LCR. Recruitment of Pol II to HS1, HS2, and HS3 of the LCR is facilitated by prior GATA-1 binding (25). GATA-1 binding also induces CBP recruitment and increases the acetylation of histones H3 and H4 at HS3 (33). Given that histone tail modifications can serve as recognition sites for protein binding (29, 54), in addition to modulating higher-order chromatin folding (13), we examined whether the histone modification state of the LCR might be a critical determinant of Pol II recruitment to the LCR. Accordingly, the pattern of Pol II binding at the LCR might correlate with the distribution of one or more histone modifications. Alternatively, histone modifications might indirectly increase Pol II access to the LCR by inducing a higher-order chromatin structure transition involving multiple nucleosomes. In this situation, the distributions of specific histone modifications and Pol II binding would not necessarily correlate. Finally, Pol II might localize to the LCR independently of specific histone modifications. As in the second model, the distributions of Pol II binding and histone modifications would not correlate.

To investigate the mechanism of Pol II recruitment, we used antibodies against diacetylated H3 (K9 and K14) (Fig. 4B), H3-meK4 (Fig. 4C), and tetra-acetylated H4 (Fig. 4D) to generate high-resolution maps of these modifications at the ~15-kb LCR. Compared to Pol II binding, which was highest at the HS cores (Fig. 1B), histone modifications were more broadly distributed. Diacetylation of H3 has been linked to the activation of numerous genes (13, 48), and diacetylated H3 has been shown to bind the double bromodomains of TAFII250 at the beta interferon promoter (1). At the -globin promoters, both NF-E2 and GATA-1 are required for the establishment
of high-level H3 acetylation (24, 28) and for Pol II recruitment (25). Within the LCR, H3 acetylation was maximal at or near the HS cores, with similar levels of acetylation at all HSs (Fig. 3A). Unlike Pol II binding (Fig. 1B), H3 acetylation was not necessarily maximal at the cores. With the exception of HS2, the peaks of acetylation occurred at sequences flanking the cores. At HS1 and HS4, the levels of H3 acetylation were 3.5- and 2.6-fold higher, respectively, at sites less than 300 bp downstream from the cores. At locations 650 bp to 2 kb away from the cores, H3 acetylation was considerably reduced, with most sites exhibiting only small amounts of enrichment compared with those for the silent Ey promoter.

Like diacetylated H3, H3-meK4 is enriched in active yeast, chicken, and Tetrahymena chromatin (35, 41, 42, 55). A limited analysis of the distribution of H3-meK4 within the murine β-globin locus (28) revealed a distribution more restricted than that predicted from yeast and chicken studies. H3-meK4 levels were high at HS2 and at the active β-globin genes but were low at intergenic sites adjacent to the active genes in adult erythroid cells. Other sequences within the LCR were not examined previously. The patterns of acetylated H3 (Fig. 4B) and H3-meK4 (Fig. 4C) were similar, with maximal H3-meK4 enrichment at the HSs and smaller amounts of enrichment at external sites. However, the H3-meK4 level was more variable than that of acetylated H3 at the HSs. For example, the H3-meK4 level was higher at HS1 than at other HSs.

Acetylated H4 is a less likely candidate for the recruitment of Pol II than acetylated H3, since NF-E2-dependent activation of the βmajor promoter involves the induction of H3 acetylation at the promoter, with only a small increase in H4 acetylation (24, 28). Moreover, the induction of H4 acetylation at inactive embryonic β-globin genes by trichostatin A-dependent inhibition of histone deacetylase activity failed to stimulate Pol II recruitment and transcriptional activation of these genes (20). Nonetheless, the acetylation of H4 at K5 and K12 is thought to stabilize the binding of TFIID to promoters through interactions with the TAFII250 bromodomains (22). At the LCR, acetylated H4 (Fig. 4D) was more broadly dis-
Superficially, the distributions of Pol II binding and histone modifications are similar, with peaks at or near the HS cores and reduced levels between the cores. However, the differences in their distributions demonstrate that Pol II occupancy does not occur exclusively at sites enriched in the histone modifications examined. A linear regression analysis comparing the levels of H3 acetylation (Fig. 5A), H3 dimethylation (Fig. 5B), and H4 acetylation (Fig. 5C) to the levels of Pol II binding at the sites shown in Fig. 1B confirmed the lack of correlation. A correlation ($R^2$) of 0.741 was observed between the levels of H3 acetylation and H4 acetylation (Fig. 5D), although as noted above, H4 levels were higher than H3 levels at regions between the HSs. While other histone modifications might mediate Pol II recruitment, the restricted distribution of Pol II at the HSs is consistent with the direct recruitment of Pol II to the HSs by DNA-bound factors, rather than attraction through the recognition of acetylated H3 and H4 and H3-meK4.

**Localization of Pol II to the LCR is independent of recruitment to the β-globin promoters.** Chromatin of the murine β-globin locus adopts a looped structure, in which the HSs are in close proximity to the active genes (8, 56). Thus, direct interactions between the LCR and promoters may be required for Pol II binding to the LCR, possibly resulting in the simultaneous cross-linking of factors to both sites. In MEL cells and in murine fetal liver, the adult β-globin genes are active, and Pol II resides at both the LCR and the active adult β-globin promoters. In contrast, in CB3 cells, a MEL cell line lacking the hematopoietic cell-specific subunit of NF-E2, p45/NF-E2 (38), the adult β-globin genes are not expressed and Pol II is absent from the β-major promoter (24). Importantly, Pol II binds the LCR in CB3 cells. In the GATA-1-null cell line G1E (63), Pol II levels are reduced at the β-major promoter and at HS1 to HS3 but are unchanged at HS4, compared to those in G1E cells expressing an activated estrogen receptor fused to GATA-1 (25). These results indicate that Pol II binds the HSs independently of the binding of Pol II to the β-major promoters.

The analysis described above with CB3 cells (24) was carried out with semiquantitative ChIP in which the intensity of ethidium bromide bands was measured densitometrically. This analysis yielded considerably lower signal/noise ratios than our quantitative real-time PCR-based ChIP (20, 21, 28). In addition, Pol II binding was not assessed previously at all promoters of the β-globin locus. Given the major mechanistic implications of Pol II residing at the LCR independently of the promoter, we comprehensively analyzed and quantitated Pol II occupancy at all promoters of the locus and at HS1 to HS4 of the LCR in CB3 cells (Fig. 6). Without p45/NF-E2, little or no Pol II was detected at the embryonic and adult β-globin promoters. The ~2-fold increase in Pol II signals relative to preimmune antibody controls was observed at multiple nonpromoter sites (for example, IVR3 and IVR16). At the LCR, the increases in Pol II signals relative to preimmune controls were 21-fold (HS4), 4.8-fold (HS3), 10.5-fold (HS2), and 17.4-fold (HS1). The lower enrichment at HS3 compared to that seen in MEL cells expressing p45/NF-E2 suggests that the recruitment of Pol II to HS3 requires NF-E2. The results shown in Fig. 6 definitively confirm the previous finding that p45/NF-E2 is not essential for Pol II binding within the LCR (24) and provide

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**FIG. 4.** High-resolution analysis of histone modifications at the LCR. (A) Positions of HS core primers within the murine β-globin domain are shown. (B to D) The relative levels of diacetylated H3 (B), H3-meK4 (C), and multiacetylated H4 (D) within the LCR were determined by ChIP with DMSO-induced MEL cells. The positions examined by ChIP were the same as those examined for Pol II binding in Fig. 1B. As in Fig. 1B, black bars represent amplicons within the murine HS cores. Signals obtained upon ChIP with control preimmune serum are the same as those in Fig. 1B, as these experiments were conducted simultaneously. The graphs show the relative intensities of PCR products from three or more independent experiments.
strong evidence that Pol II recruitment to the LCR does not require concomitant binding to the promoter. Accordingly, cross-linking of Pol II to the LCR does not result from promoter-bound Pol II being brought into close proximity to the LCR, since no Pol II was detected at the promoters in CB3 cells.

In contrast to the results shown in Fig. 6, the activation of HNF4α transcription results in Pol II recruitment to the promoter, prior to detectable enhancer-promoter interactions (19). The subsequent detection of Pol II at the enhancer ~6.5 kb upstream of the promoter was suggested to be a consequence of its simultaneous cross-linking to the two regions. The β-globin and HNF4α systems differ considerably in that the large, multicomponent β-globin LCR is far upstream of the adult β-globin genes, while the small HNF4α enhancer is relatively close to HNF4α. Furthermore, β-globin is considerably more abundant than the activator HNF4α; therefore, the rates of transcription of the two genes are likely to differ greatly, with β-globin transcription being much higher. An additional study found Pol II associated with a distal regulatory element ~2.3 kb upstream of a major histocompatibility complex promoter (40). However, this analysis did not reveal whether Pol II localizes to this distal element independently of promoter binding.

Pol II elongation is not required to maintain the erythroid-specific histone modification pattern of the β-globin locus.

**FIG. 5.** Lack of correlation between the distributions of Pol II binding and specific histone modifications. (A to C) Linear regression analyses of values obtained from Fig. 1B and 4B to D for Pol II binding and either H3 diacetylation (A), H3-K4 dimethylation (B), or multisite H4 acetylation (C) were carried out for the 20 sites within the LCR shown in Fig. 1B. (D) For comparison, a correlation plot is shown for H3 diacetylation and H4 acetylation.

**FIG. 6.** Pol II binding at the LCR is independent of Pol II recruitment to the β-major promoter. Comparison of Pol II binding by ChIP at β-globin gene promoters and HS cores in p45/NF-E2-null CB3 cells. CB3 cells were cultured with 1.5% DMSO for 4 days prior to ChIP analysis. The ~2-fold enrichment of Pol II compared to preimmune serum that was observed at the promoters was also evident at extra-genic sites IVR3 and IVR16 (for the positions of these sites, see Fig. 1A). The graph shows the relative intensities of PCR products from three or more independent experiments (mean and standard error of the mean).
Despite the arguments presented above, one cannot unequivocally rule out the possibility that a small fraction of the Pol II-generating extragenic transcripts functions to establish and/or maintain histone modifications. It has been suggested that Pol II processivity and/or the resulting extragenic transcripts regulate the chromatin structure of tissue-specific loci (2, 16). The activation of β-major transcription correlates with increased acetylated H3 and H3-meK4 levels at the promoter and within the coding region and with a smaller increase in the acetylated H4 level preferentially within the coding region (24, 28). Although we believe that these changes in histone modifications are required for high-level transcription and, as such, precede Pol II recruitment and elongation, this notion has not been proven. Alternatively, histone-modifying enzymes directly interact with the transcriptional machinery, and such enzymes might utilize elongating Pol II to modify histones over a broad region (45, 57). Consequently, the elimination of Pol II elongation should prevent the modifying enzymes from reaching their targets and therefore should change the histone modification pattern.

To determine whether Pol II elongation is required to establish and/or maintain the erythroid-specific composite histone modification pattern of the β-globin locus, we tested whether a 1-h treatment of cells with DRB reconfigures the histone modification pattern. The consequences of the DRB-induced elongation block for the histone modifications of the β-major gene were mild compared to the results seen in the NF-E2-null CB3 cell line, in which β-major is transcriptionally silent. Despite the nearly complete elimination of transcription (Fig. 3A), DRB treatment of induced MEL cells did not change H3 acetylation at the promoter, and only a small reduction (<2-fold) was apparent within β-major (Fig. 7A). In contrast, in a previous study (28), the loss of NF-E2 in CB3 cells reduced H3 acetylation 3-fold at the promoter and 12-fold within exon 3 relative to the results seen in MEL cells. Both acetylation and transcription were rescued upon the expression of p45/NF-E2. H3-meK4 was only slightly affected by DRB treatment (Fig. 7B), despite the 5-fold (promoter) and 31-fold (exon 3) reductions seen in a previous study upon the loss of NF-E2 in CB3 cells (28). H4 acetylation was unchanged following DRB treatment (Fig. 7C), despite the 3.5-fold reduction seen at exon 3 in CB3 cells in a previous study (28). These results indicate that transcriptional elongation is not required to maintain the erythroid-specific composite histone modification pattern at the β-major gene.

Besides the proposed roles for transcription elongation in establishing and maintaining histone modification patterns at genes, Pol II tracking has also been suggested to mediate the remodeling of large extragenic regions (16, 57). Since low levels of DRB-sensitive transcripts were detected at the LCR (Fig. 3A), we tested whether DRB treatment altered histone modifications at the HSs in differentiated MEL cells. Like the results for β-major, no significant reductions in histone acetylation and methylation were detected (Fig. 7). Thus, DRB treatment blocked genic and extragenic transcription of the β-globin locus and abolished Pol II binding downstream of the β-major promoter yet had little or no effect on Pol II binding to the HSs or on histone modifications at β-major and at the HSs. The lack of DRB sensitivity of the modification patterns indicates that elongation is not required to maintain these modifications. The possibility that the histone modifications are sufficiently stable such that preestablished modifications persist during the time course of the experiment is unlikely, given that the turnover of histone acetylation within active chromatin of vertebrate cells involves half-lives of less than 10 min (61).

FIG. 7. Maintenance of the erythroid-specific composite histone modification pattern of the β-globin locus does not require ongoing transcriptional elongation by Pol II. Samples analyzed by ChIP for Pol II binding in Fig. 3 were also used to detect DRB-induced changes in histone acetylation and H3-meK4 at the β-major gene and at the LCR. The graphs show the relative intensities of PCR products from three or four independent experiments (mean and standard error of the mean). PI, rabbit preimmune serum.
Similar kinetics have been observed for the loss of penta-acetylated H4 from transcriptionally competent chromatin in immature chicken erythrocytes expressing adult β-globin (53).

In summary, we have shown that Pol II localizes in a highly restricted manner to the HSs of the LCR, with the highest occupancy being at the HS cores. While the HSs were strongly enriched in acetylated histones H3 and H4 and in H3-meK4, the distributions of the modifications did not strictly correlate with sites of Pol II occupancy. As a blockade of transcriptional elongation did not redistribute Pol II within the LCR or reconfigure the erythroid-specific composite histone modification pattern of the β-globin locus, it is unlikely that Pol II movement pattern. The highly restricted localization of Pol II and the lack of evidence for transcription-dependent histone modification are consistent with a mechanism in which the LCR recruits Pol II, which then undergoes transfer to the promoters in a highly regulated fashion. We speculate that LPT considerably enhances the efficiency of Pol II recruitment achieved by direct recruitment to promoters and therefore would be utilized by loci that exhibit high rates of transcription.

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