Molecular Cell *23*, 365–375, August 4, 2006 ©2006 Elsevier Inc. DOI 10.1016/j.molcel.2006.05.041

Locus Control Region Transcription Plays an Active Role in Long-Range Gene Activation

Yugong Ho,[1] Felice Elefant,[1,3] Stephen A. Liebhaber,[1,2] and Nancy E. Cooke[1,2,*]
[1] Department of Genetics
[2] Department of Medicine
University of Pennsylvania School of Medicine
Philadelphia, Pennsylvania 19104

Summary

Activation of eukaryotic genes often relies on remote chromatin determinants. How these determinants function remains poorly understood. The *hGH* gene is activated by a 5′-remote locus control region (LCR). Pituitary-specific DNase I hypersensitive site I (HSI), the dominant *hGH* LCR element, is separated from the *hGH-N* promoter by a 14.5 kb span that encompasses the B-lymphocyte-specific *CD79b* gene. Here, we describe a domain of noncoding Pol II transcription in pituitary somatotropes that includes the *hGH* LCR and adjacent *CD79b* locus. This entire "LCR domain of transcription" is HIS dependent and terminates 3′ to *CD79b*, leaving a gap in transcription between this domain and the target *hGH-N* promoter. Insertion of a Pol II terminator within the LCR blocks *CD79b* transcription and represses *hGH-N* expression. These data document an essential role for LCR transcription in long-range control, link "bystander" *CD79b* transcription to this process, and support a unique model for locus activation.

Introduction

Long-range gene activation plays a prominent role in metazoan gene expression (reviewed in Dean [2006]). Transcription determinants can be separated from their target promoters by thousands of base pairs. In some cases, these distal regulatory elements are involved in the activation of multiple promoters within a gene cluster, switching from one to another in a developmentally controlled or tissue-specific manner (Li et al., 2002; Ho et al., 2004). Defining the mechanisms mediating these long-range interactions remains a major challenge in the field of eukaryotic gene expression.

Locus control regions (LCRs) encompass an intensively characterized group of long-range transcriptional control determinants. An LCR is defined as a set of elements sufficient to generate an autonomous chromatin environment (Grosveld et al., 1987; Festenstein et al., 1996). Characteristically, LCR components colocalize with DNase I hypersensitive sites (HS) in the chromatin of expressing cells. The mechanisms of LCR action at different loci appear to be quite diverse (Dean, 2006). Understanding how these determinants activate gene expression over extensive distances is clearly central to defining critical pathways of transcriptional regulation.

Mammalian nuclei contain thousands of transcripts that do not correspond to known structural genes or to RNAs with defined functions. The vast majority of these intergenic transcripts are likely to constitute background "noise" in the transcriptome. However, accumulating evidence indicates that in particular situations these transcripts have essential functions in gene expression (Plath et al., 2002). In some cases the process of intergenic transcription, rather than its RNA product, may directly impact on chromatin structure (Schmitt et al., 2005). The importance of noncoding transcription in gene regulation is supported by reports of transcription through regions encompassing a number of LCRs (Masternak et al., 2003; Ashe et al., 1997; Routledge and Proudfoot 2002). Although transcription through regulatory domains may correlate with expression of linked genes (Gribnau et al., 2000), it remains unclear whether this process represents an active role in gene regulation or, alternatively, reflects promiscuous transcription through already "open" chromatin regions.

The *human growth hormone* (*hGH*) cluster contains five highly conserved genes (Chen et al., 1989) (Figure 1A, top). *hGH-N* is specifically expressed in pituitary somatotropes while expression of *hCS-L*, *hCS-A*, *hGH-V*, and *hCS-B* is restricted to placental syncytiotrophoblasts. This cluster is controlled by a remote LCR (*hGH* LCR) comprising five DNase I HS located −14.5 to −32 kb 5′ to the *hGH-N* gene (Jones et al., 1995; Su et al., 2000). Closely linked HSI and HSII are pituitary specific, HSIV is placental specific, and HSIII and HSV are present in both tissues. The *hGH* LCR and the *hGH-N* promoter are encompassed by a continuous 32 kb pituitary-specific domain of acetylated histones H3 and H4 with a central peak located at HSI (Elefant et al., 2000). Site-specific inactivation of HSI results in loss of acetylation throughout this domain, loss of critical *trans*factor occupancy at the *hGH-N* promoter, and a 20-fold reduction in *hGH-N* expression (Ho et al., 2002). Thus, HSI plays an essential role in the establishment of the acetylated domain and in activation of *hGH-N* transcription in the pituitary. Whether the long-range activating capacity of HSI is limited to its acetylating function or reflects a more complex mechanism is currently unclear.

Here we report that HSI has a second and apparently independent role in *hGH-N* activation. The data revealed that HSI plays an essential role in the establishment of a complex domain of intergenic transcription 5′ to the *hGH* cluster. Insertion of an exogenous transcriptional terminator within this domain selectively blocked a subset of downstream LCR transcripts and repressed *hGH-N* transcription. These changes occurred in the absence of appreciable alterations in histone acetylation within the *hGH* locus. These data lead us to conclude that the remote transcriptional domain plays a direct role in LCR-mediated, long-range gene activation.

*Correspondence: necooke@mail.med.upenn.edu
[3] Present address: Department of Bioscience and Biotechnology, Drexel University, Philadelphia, Pennsylvania 19104.

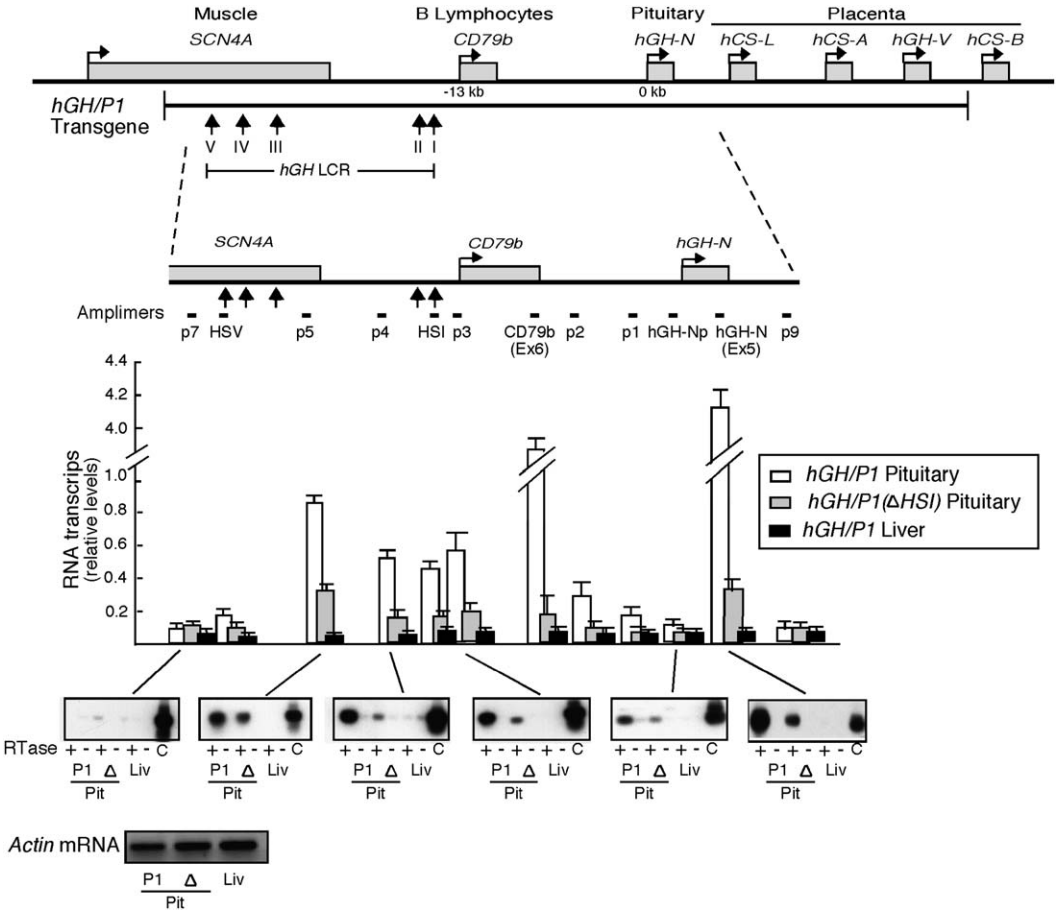

Figure 1. Pituitary-Specific Transcription of the *hGH* LCR

(Top) Map of the *hGH* locus and the *hGH/P1* transgene. A 100 kb region of the human genome on chromosome 17q22 is shown. Each structural gene is shown as a rectangle with its corresponding transcriptional orientation indicated by an angled arrow. The five DNase I HS that constitute the *hGH* LCR are indicated (upward arrows with Roman numerals), as is the extent of the *hGH/P1* transgene (bracketed line below the map). A slightly expanded view of the region encompassing the *hGH-N* gene is shown with the relative positions of the 12 RT-PCR amplimers (short bars) used to detect transcripts. Note that the HSI amplimer used in these studies detects sequences that are adjacent to, but not included within, the 99 bp HSI deletion site in the *hGH/P1(ΔHSI)* transgene. Also, the p9 amplimer corresponds to a common sequence present 5′ to each of the four placentally expressed genes in the cluster. (Bottom) Transcripts are present throughout the *hGH* LCR and are both pituitary specific and HSI dependent. The histogram summarizes relative levels of transcripts (y axis) at 12 sites throughout the *hGH* locus and are plotted in alignment with the corresponding amplimer sets (top). The transcripts were detected by a semiquantitative PCR survey of cDNA libraries generated from randomly primed total cellular RNA samples. These values, represented in arbitrary units, were calculated as a ratio between the RT/PCR amplification at each site and the corresponding amplification from the plasmid control template (*hGH/P1*). The autoradiograph insets show representative studies corresponding to six of the sites. A line connects each representative autoradiograph to its relevant three bar set of histograms. The PCR products were quantified by phosphorimager analysis and normalized to signals of the PCR products from 0.007 ng of control *hGH/P1* plasmid DNA (C) using the same set of PCR primers. The PCR products represent analyses of pituitary RNA (Pit) from *hGH/P1* (P1, white bars) and *hGH/P1(ΔHSI)* (Δ, gray bars) lines, and hepatic RNA from an *hGH/P1* mouse (Liv, black bars). Two *hGH/P1* lines and two *hGH/P1(ΔHSI)* lines were studied in each of two independent assays. Each histogram bar represents the average ±SD from these four independent assays. The values for liver RNAs reflect an average value from independent analyses of liver RNA isolated from two mice from each of two *hGH/P1* transgenic lines. RT-PCR analyses for *β-actin* mRNA (inset at the bottom of the figure) were carried out on each sample to control for adequacy of the respective RNA preparation.

Results

The *hGH* LCR Is Transcribed in a Pituitary-Specific and HSI-Dependent Manner

The *hGH/P1* transgene contains the entire *hGH* LCR and first four genes of the *hGH* gene cluster (Figure 1) (Su et al., 2000). In the *hGH/P1* transgenic mouse pituitary, a 32 kb acetylated chromatin domain extends from a 5′ boundary at HSV to a 3′ terminus that encompasses the *hGH-N* promoter (Elefant et al., 2000). This chromatin structure at the *hGH* transgene accurately replicates chromatin modifications detected in the human pituitary (Elefant et al., 2000). Inactivation of HSI in the *hGH/P1* transgene results in loss of LCR acetylation and repression of *hGH-N* transgene expression (Ho et al., 2002). These data suggested a model in which HAT complexes are recruited to HSI and subsequently spread bidirectionally to establish the modified chromatin domain and activate *hGH-N* (Ho et al., 2002). Studies documenting association of HAT complexes with Pol II (Wittschieben et al., 1999) suggest that polymerase may serve as a "motor" for spreading chromatin-modifying complexes.

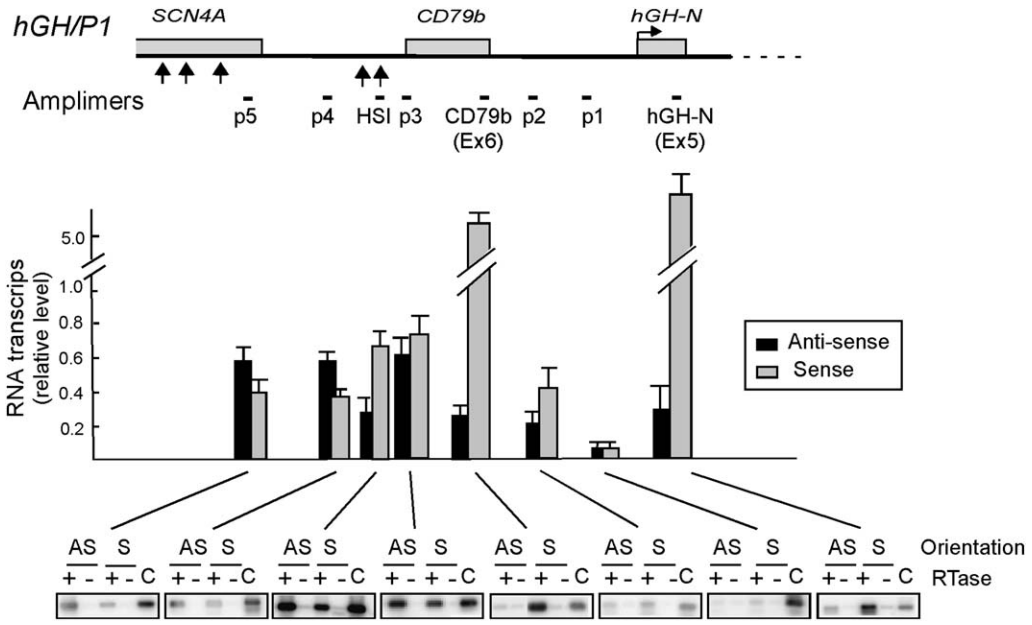

Figure 2. Transcription within the *hGH* LCR Is Bidirectional

(Top) Map of the *hGH* locus indicating sites assayed for transcript orientation. The RT-PCR amplimer sets used in the analysis are shown below the locus diagram (as in Figure 1). Each RT reaction was performed using a sense or antisense specific primer from the relevant amplimer set. (Bottom) Relative levels of antisense and sense transcripts within the *hGH* LCR. Strand-specific RT reactions were followed by PCR reactions corresponding to the amplimer sites indicated above. The results were quantified by Southern blot hybridization with unique sequence probes and calculated (as in Figure 1). Each bar represents the average ±SD value from four independent studies. A representative data set is displayed below the histogram. Amplification products are shown in the presence and absence of reverse transcriptase (RTase), and the polarity of the initial strand-specific RT primer, antisense (AS), or sense (S) is indicated. The control PCR used 0.007 ng *hGH/P1* plasmid DNA as template (C), a value demonstrated by serial dilutions to be in the linear range of detection (data not shown).

Such a model would predict a tight linkage between histone acetylation and intergenic transcription through the *hGH* LCR.

To determine whether polymerase tracks through the *hGH* LCR, this region was probed for corresponding transcripts. RNA isolated from somatotrope-enriched pituitaries of *hGH/P1* transgenic mice was randomly primed to generate a cDNA library. This library was surveyed with a series of site-specific PCR amplimers (Figure 1 and see Table S1 in the Supplemental Data available with this article online). Relative levels of transcripts, normalized to internal controls, were determined across the locus. Transcripts were detected at sites extending from −21 kb relative to the transcriptional start site of *hGH-N* through the *CD79b* region (Figure 1 histogram, white bars, from amplimers p5 through *CD79b*[Ex6]). Transcript levels within the LCR (amplimers p5, p4, HSI, and p3) ranged from 10% to 20% that of the *hGH-N* mRNA. Regions between HSV and p5 could not be accurately assessed due to the high levels of sequence conservation between the human and mouse *SCN4a* gene exons. There was minimal evidence of transcripts at the 5′ boundary of the LCR (amplimer HSV) or 3.6 kb further 5′ (amplimer p7). A high density of transcripts was detected immediately 3′ to HSI in the region corresponding to *CD79b*. This transcription across the *CD79b* gene was comparable to that at *hGH-N* (compare *CD79b*[Ex6] with *hGH-N*[Ex5]) and was in agreement with our prior report of robust *CD79b* bystander transcription in the pituitary (Cajiao et al., 2004). Transcript levels fall abruptly 3′ of *CD79b*, consistent with the action of *CD79b* 3′ processing signals. As expected, *hGH-N* was itself robustly transcribed and transcription returned to background levels within the region of the cluster containing the placentally expressed genes (p9 amplimer). Analysis of mice carrying *hGH/P1* transgene with a 99 bp deletion that removes critical HSI determinants *(hGH/P1[ΔHIS])*; Ho et al., 2002) reveals a marked repression of transcription throughout the *hGH* LCR and adjacent *CD79b* (Figure 1 histogram, gray bars). The entire *hGH* locus was transcriptionally inactive in hepatic RNA of an *hGH/P1* mouse (Figure 1, black bars). In summary, these data revealed that the *hGH* LCR is transcribed, that this domain of transcription extends to include the contiguous *CD79b* gene, and that transcription of this entire region is pituitary specific and HSI dependent. Because of the shared HSI dependence of intergenic transcription within the LCR (5′ to HSI) and through the contiguous *CD79b* (3′ to HSI), we refer to these two regions collectively as the LCR domain of transcription.

Transcript orientations throughout the *hGH/P1* transgene locus were assessed by strand-specific RT/PCR (Figure 2). The strand specificity of this assay was validated by the 15-fold predominance of sense transcripts at *hGH-N*. We found a similar predominance of sense-oriented transcripts at *CD79b*. In contrast, transcription of the LCR 5′ to *CD79b* was bidirectional with no more than a 2-fold excess of one orientation over another. In summary, these data revealed that the robust, HSI-dependent LCR domain of transcription is

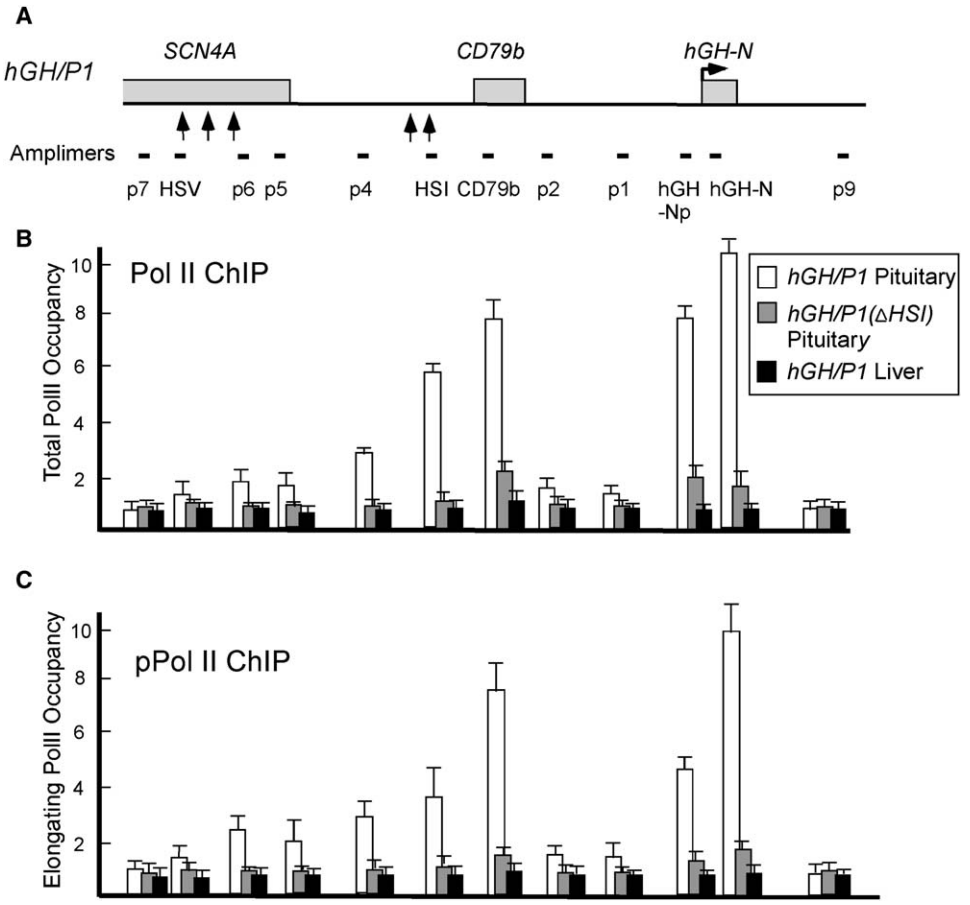

Figure 3. The *hGH* LCR Is Traversed by Elongating RNA Pol II

(A) Sites of the amplimers used to detect DNA in the ChIP assays. Each PCR amplimer, shown below the locus map, detects a unique sequence. The exact position of each primer set is listed (see Table S2). The data in (B) and (C) are aligned below the corresponding amplimer sets.

(B) ChIP analyses for total Pol II in transgenic pituitary and liver chromatin. The antibody used in this ChIP assay is specific to the N terminus of Pol II and recognizes all isoforms. Each bar represents the average ±SD of six independent assays of the indicated chromatin samples: *hGH/P1* pituitary chromatin (white bars), *hGH/P1(ΔHSI)* pituitary chromatin (gray bars), or control *hGH/P1* liver chromatin (black bars). The values represent a ratio of the DNA signal detected in the immunoprecipitated sample to that in the corresponding input sample. The "Total Pol II Occupancy" values on the y axis are in arbitrary units.

(C) ChIP analysis of elongating pPol II in transgenic pituitary and liver chromatin. The antibody used for these ChIP studies recognizes the Ser5-phosphorylated residues in the C-terminal domain of Pol II (pPol II). The data, generated as in (B), represent the average ±SD from four independent assays of each indicated chromatin sample. Each set of histogram bars represents occupancy in the *hGH/P1* pituitary, *hGH/P1(ΔHSI)* pituitary, and *hGH/P1* liver, as in (B).

complex—bidirectional 5′ of *CD79b*, but transcribed in the "sense" orientation through *CD79b*.

The *hGH* LCR Is Transcribed by RNA Polymerase II

Transcription through the *hGH* locus was further characterized by Pol II chromatin immunoprecipitation (ChIP) (Figure 3). The amplimers used in this study correspond to a set of unique sequences across the *hGH* locus (Figure 3A; Table S2). The ratio of DNA in the bound versus input sample at each position was assessed across the locus by semiquantitative PCR (Ho et al., 2002). The endogenous *myoD* gene, expressed specifically in skeletal muscle, served as the normalization control for the ChIP studies (Sawado et al., 2003). The initial ChIP was carried out with an antibody to the N terminus of Pol II that recognizes all RNA Pol II isoforms (Figure 3B). Each study was performed twice on tissue samples isolated from each of two *hGH/P1* lines (809F and 811D; Su et al., 2000). The data revealed that levels of Pol II increased from very low abundance at the 5′ end of the LCR to substantial levels more central to the LCR and at *CD79b* (Figure 3B, white bars). Pol II occupancy dropped off 3′ of *CD79b* and did not become significant again until the *hGH-N* promoter was encountered. The *hGH-N* promoter and coding region had high Pol II concentrations, and Pol II occupancy dropped to minimal levels within the placentally expressed genes of the cluster (at amplimer p9).

The relationship between Pol II activity and LCR function was assessed by ChIP analysis of two *hGH/P1(ΔHSI)* lines (960G and 969E; Ho et al., 2002). Analysis of pituitary chromatin from mice with the HSI deletion demonstrated levels of Pol II occupancy throughout the LCR domain of transcription that were substantially lower than those seen in the *hGH/P1* transgene (Figure 3B, gray bars). Pol II occupancy in hepatic chromatin

from the *hGH/P1* transgenic lines was at background levels (Figure 3B, black bars). Comparison of the *hGH/P1(ΔHSI)* pituitary chromatin ChIP analyses with that of the *hGH/P1* hepatic chromatin revealed that the *CD79b* and *hGH-N* loci retained low but significant levels of Pol II in pituitary chromatin. These residual levels of Pol II in the absence of HSI are consistent with the 5% residual level of pituitary *hGH-N* transcription from the *hGH/P1(ΔHSI)* transgene (Ho et al., 2002). From these studies, we conclude that Pol II is recruited to the *hGH* LCR and that this recruitment is pituitary specific and HSI dependent.

A second series of Pol II ChIPs was carried out using an antibody specific for the elongating isoform of Pol II (Ser-5 phosphorylated C-terminal domain [CTD]; pPol II) (Orphanides and Reinberg, 2002) (Figure 3C). The distribution of elongating pPol II at the *hGH* locus in pituitary chromatin was similar to that of total Pol II (compare Figures 3B and 3C, white bars). Of note, the *hGH-N* promoter was selectively enriched for nonphosphorylated Pol II when compared to the structural gene. This is consistent with direct recruitment of unphosphorylated Pol II to the *hGH-N* promoter and its subsequent conversion to the CTD-phosphorylated pPol II form as it is released and initiates elongation (Dahmus, 1996). Deletion of HSI resulted in a marked loss of pPol II throughout the locus (Figure 3C, gray bars), and there was no appreciable pPol II occupancy at the *hGH/P1* transgene locus in hepatic chromatin (Figure 3C, black bars). The parallel between the distributions of elongating pPol II and the intergenic RNA transcripts, and their shared dependence on HSI activity, leads us to conclude that the LCR domain of transcription in the pituitary is generated by Pol II tracking through this region.

The *hGH* LCR Domain of Transcription Plays an Essential Role in Long-Range *hGH-N* Activation

To test whether Pol II tracking plays a direct role in *hGH* LCR action, an exogenous Pol II termination element was inserted within the LCR domain of transcription (Figure 4 top; TerF). The 2.2 kb "*Ter F*" fragment corresponds to a segment of the human *β-globin* gene extending from exon 2 through its 3′-flanking region (Dye and Proudfoot, 1999, 2001) (see Figure S1). *TerF* targets efficient 3′ processing of nascent Pol II transcripts and triggers subsequent dissociation of Pol II from the DNA template at the cotranscriptional cleavage site (CoTC) (West et al., 2004; Teixeira et al., 2004). *TerF* was inserted in the sense orientation relative to the *hGH* cluster at a site 1.5 kb 3′ of HSI. The goal of this insertion was to selectively block HSI-dependent transcription through *CD79b*. Five mouse lines carrying the *hGH/P1(TerF)* transgene were generated, and the transgene copy number of each line was determined by Southern blot analysis (Su et al., 2000) (data not shown). Analysis of pituitary transcripts from mice representing each of the five lines revealed a mean 5-fold drop in the levels of transcripts from a site 5′ of the CoTC to a site 3′ of the CoTC region (see Figure S1). This drop was consistent with the CoTC termination function (Dye and Proudfoot, 2001).

Having established that the *TerF* element was functioning in vivo to terminate transcription at a site 3′ to HSI, we tested the model that Pol II tracking is directly related to establishment of histone H3/H4 acetylation within the *hGH* locus. Pituitary chromatin samples isolated from mice carrying *hGH/P1(TerF)* (lines 1301C and 1301G) were analyzed by ChIP assays using antibodies specific for acetylated histones H3 and H4 (Figure 4). Comparative ChIP assays were performed in parallel on pituitary chromatin from *hGH/P1* and *hGH/P1(ΔHSI)* mouse lines. Analysis of the *hGH/P1* transgenic pituitary chromatin confirmed the previously reported hyperacetylation of the *hGH* LCR and the *hGH-N* promoter (Elefant et al., 2000; Ho et al., 2002). The dramatic loss of acetylation across this region in *hGH/P1(ΔHSI)* transgenic pituitary chromatin confirmed the dependence of these modifications on HSI activity (Figure 4) (Ho et al., 2002). Remarkably, the analysis of the *hGH/P1(TerF)* lines revealed that insertion of *TerF* within the LCR domain of transcription had no appreciable impact on histone H3/H4 acetylation either 5′ or 3′ of the insertion site (Figure 4). Of particular note, full levels of core histone H3/H4 acetylation were maintained at the *hGH-N* promoter. These data suggest that the establishment and/or maintenance of the extensive and continuous domain of histone acetylation between the *hGH* LCR and the *hGH-N* promoter is not linked to Pol II tracking through this region.

In light of the observation that *TerF* insertion within the domain of LCR transcription had no apparent effect on histone H3/H4 acetylation (Figure 4), we next directly assessed the impact of the *TerF* insertion on transcription through the locus. Transcript levels were assayed at two sites 5′ of the *TerF* insertion and at a site 3′ of its insertion (Figure 5A). These values were normalized to *actin* mRNA levels and compared to levels at the corresponding sites in two representative *hGH/P1* lines. Although the *TerF* insertion had no significant impact on transcripts 5′ to its insertion site, it resulted in a 5-fold mean decrease in levels of transcripts 3′ to its insertion. Thus, the *TerF* insertion resulted in a unilateral, downstream repression of transcripts within the LCR domain of transcription. This selective impact within the transcription domain is consistent with the orientation and position of the *TerF* insertion.

To address whether LCR transcription plays a direct role in *hGH-N* gene expression, the impact of the *TerF* insertion on *hGH-N* transcription was determined in each of the five *hGH/P1(TerF)* lines and compared to two *hGH/P1* lines (Figure 5B). *hGH-N* mRNA in each transgenic pituitary was quantified relative to endogenous *mGH* mRNA and normalized to transgene copy number. The normalized transgene expression levels among the five *hGH/P1(TerF)* lines were highly consistent with less than a 2-fold variation between the highest and lowest values. The mean *hGH-N* expression in these lines was 6-fold below that in two *hGH/P1* lines. These data lead us to conclude that Pol II tracking within the LCR domain of transcription is integral to long-range control of the *hGH-N* promoter.

Discussion

The *hGH* LCR Establishes a Domain of Pol II Transcription 5′ to the *hGH-N* Gene

The *hGH* gene cluster presents a challenging and potentially informative model of long-range control.

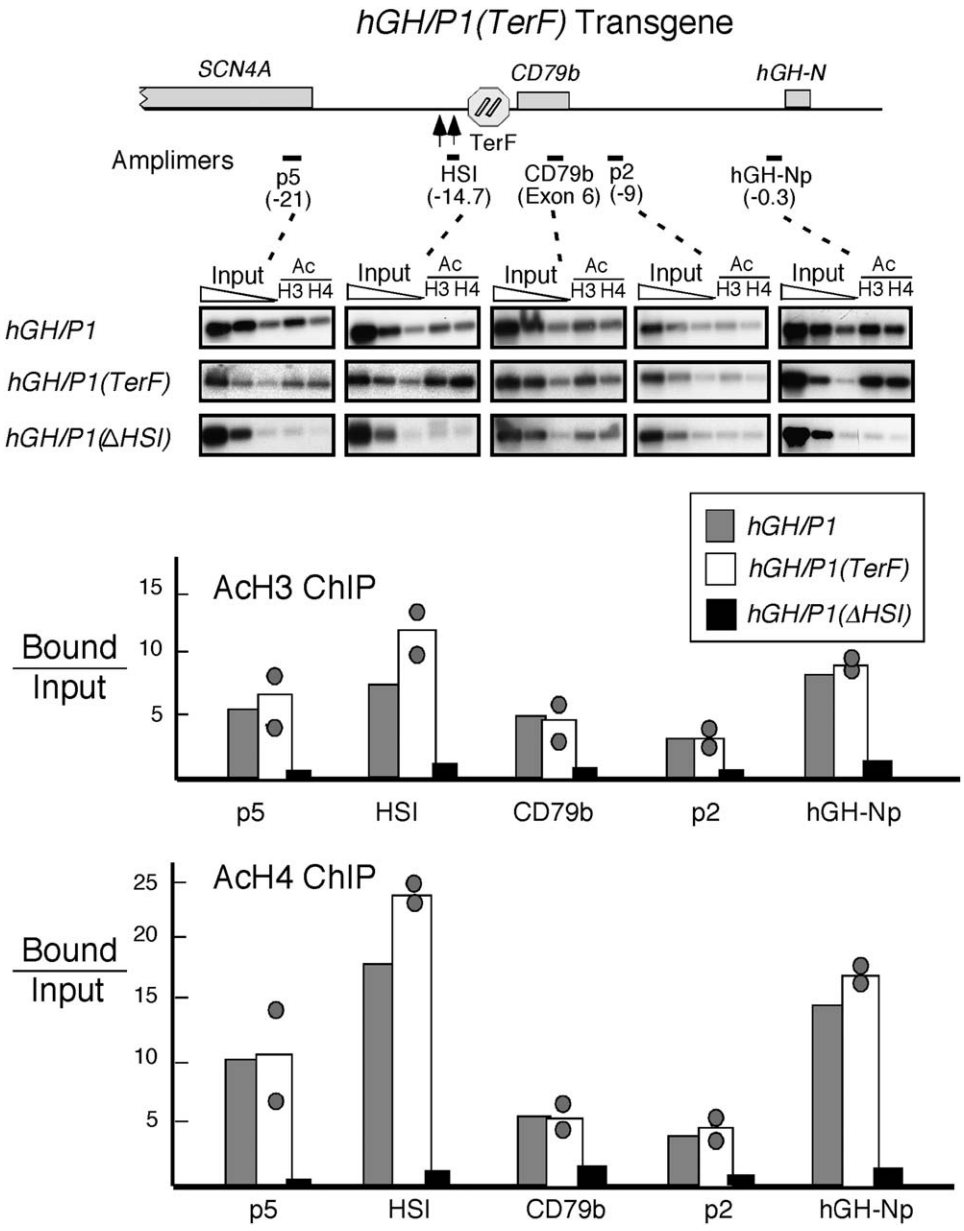

Figure 4. Insertion of a Pol II Terminator 3′ to HSI Does Not Affect Histone Acetylation in the *hGH* Locus

A map of the relevant region of the *hGH/P1(TerF)* transgene and the positions of the PCR amplimers used to assess the impact of the *TerF* insertion on histone H3/H4 acetylation are shown. Amplimers p5 (map coordinate −21) and HSI (−14.7) are both located 5′ of the *TerF* insertion. Amplimers *CD79b*(exon 6), and p2(−9), *hGH-Np* (−0.3) are located 3′ of the insertion. Sequential dilutions of the input chromatin sample are shown along with a single linear-range dilution of the bound fraction. The ratio of bound/input was normalized to the corresponding ratio of bound/input chromatin at the *MyoD* locus. The value on the y axis reflects the final normalized ratio. The values for *hGH/P1(TerF)* (white bars) represent the average for two lines analyzed (1301C and 1301G).

Its activation in the pituitary and placenta is dependent on overlapping sets of LCR determinants. These LCR determinants mediate distinct patterns of chromatin modifications and activate different subsets of genes from the *hGH* gene cluster in these two tissues (Ho et al., 2002; Kimura et al., 2004). Additional complexity at this locus is introduced by the B-lymphocyte-specific gene, *CD79b*, which is interposed between the *hGH* LCR and the *hGH* cluster (Bennani-Baïti et al., 1998), and by the location of HSIII-HSV within the adjacent striated muscle sodium channel gene, *SCN4A* (Figure 1A). Defining how the *hGH* LCR functions in this environment tightly packed with tissue-specific genes and how each of these genes selectively expresses its protein product only in its corresponding tissue should yield significant insights into these complex processes.

The current study focuses on LCR-mediated activation of *hGH-N* in the pituitary. The *hGH* LCR and the *hGH-N* promoter are located in a continuous, HSI-dependent domain of acetylated chromatin in primary human pituitary tissue and in the pituitaries of mice carrying the *hGH* transgene locus (Elefant et al., 2000;

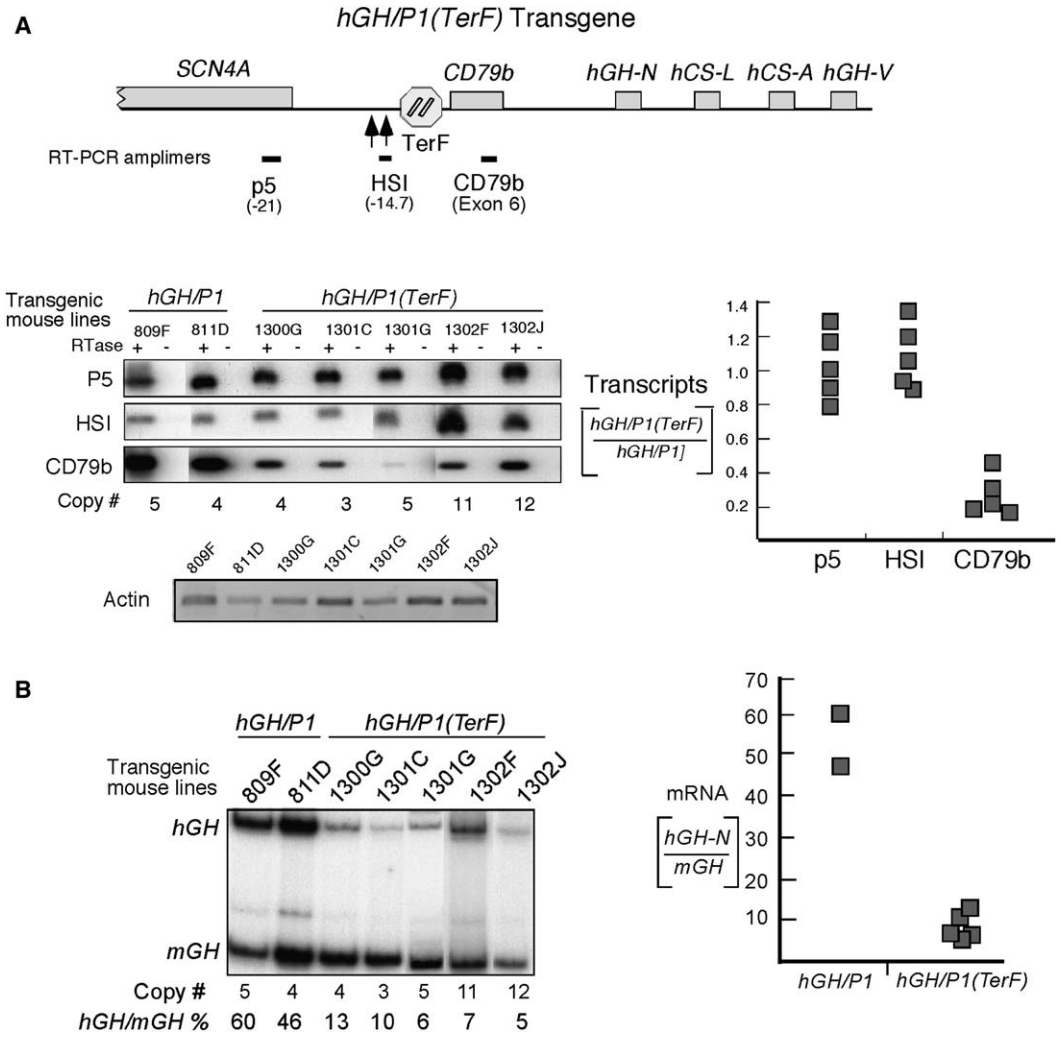

Figure 5. Pol II Tracking Is Required for Full *hGH* LCR Activity in Pituitary

(A) Insertion of a Pol II terminator 3′ to HSI results in a unilateral loss of transcripts from the LCR domain of transcription. RNA from the pituitaries of *hGH/P1* and *hGH/P1(TerF)* transgenic mice was assayed by semiquantitative RT-PCR using the indicated p5, HSI, and *CD79b*(exon 6) primer sets followed by gel analysis and Southern hybridization. A parallel RT-PCR assay for β-*actin* mRNA from each sample was used to control for the quality of the RNA preparations. Presence (+) or absence (−) of RTase is indicated above relevant lanes. The corresponding transgene copy number for each line is indicated below each data set. Transcript signals detected by RT-PCR with each of the three primer sets were normalized to the β-*actin* mRNA amplification signal and to the transgene copy number of the corresponding line. This value was then divided by the average normalized values from the two *hGH/P1* lines. The final value on the y axis reflects the ratio of transcription at each site in each *hGH/P1(TerF)* line with that in the *hGH/P1* transgenic lines. The value for each of the five *hGH/P1(TerF)* lines at each of the three positions is plotted (shaded squares). (B) *TerF* insertion 3′ of HSI represses *hGH-N* transgene expression. The left panel shows a representative autoradiograph of a coRT-PCR quantification of *hGH-N* mRNA relative to endogenous *mGH* mRNA in *hGH/P1* and *hGH/P1(TerF)* transgenic mouse pituitaries. The *hGH-N/mGH* mRNA ratios were corrected for transgene copy number, and the resultant relative expression levels are shown below each respective lane. The right panel shows the relative level of *hGH-N* expression in pituitaries of *hGH/P1(TerF)* and *hGH/P1* transgenic mice normalized to levels of endogenous *mGH* mRNA and transgene copy number. The normalized levels of *hGH-N* mRNA expression in each line are plotted (shaded boxes). The *TerF* insertion resulted in a mean 6-fold decrease in pituitary *hGH-N* transgene expression.

Ho et al., 2002). These data support a model in which histone acetyltransferase (HAT) complexes are recruited to HSI and subsequently spread bidirectionally, 5′ to a boundary at HSV and 3′ to encompass and activate the *hGH-N* promoter. It has been further proposed that Pol II powers this extension of HAT activity. However, two findings in the present study specifically argue against this simple model. The first is that the LCR transcripts 5′ to HSI are bidirectional, suggesting that they originate at multiple sites rather than uniquely at HSI. The second, and more direct, point is that insertion of an exogenous Pol II termination element within the LCR domain of transcription, between HSI and *hGH-N*, had no significant impact on histone H3/H4 acetylation within the *hGH* locus or at the *hGH-N* promoter. These findings suggest that the roles of HSI in targeting histone acetylation to the *hGH* locus and in stimulating Pol II tracking through the *hGH* LCR are not directly linked.

Transcription of LCRs and enhancer elements has been reported in several systems. Intergenic transcripts are generated from the β-*globin* LCR (Ashe et al., 1997; Plant et al., 2001; Gribnau et al., 2000). Interestingly, intergenic transcripts in the human β-*globin* LCR, as in the present report, do not correlate with histone

acetylation pattern (Haussecker and Proudfoot 2005). Furthermore, global inhibition of transcriptional elongation fails to redistribute Pol II at the HS core sites or alter histone modifications in the murine *β-globin* LCR. It is postulated that Pol II in the latter system is directly transferred from LCR sites of recruitment to the target *β-globin* promoter (Johnson et al., 2003). Extragenic transcripts have also been identified within the LCR of the *MHC class II* gene (Masternak et al., 2003). The similar patterns of histone acetylation and Pol II distribution suggest that in this model system Pol II may distribute HAT activity via a "piggyback" mechanism through the *MHC class II* locus. Recent studies have revealed that androgen receptor (AR) regulation of *prostate-specific antigen* (*PSA*) requires both promoter-proximal sequences as well as an upstream enhancer; recruitment of AR and cofactors to both sites creates a loop that facilitates Pol II tracking from the enhancer to the promoter (Wang et al., 2005). These studies indicate that the mechanisms underlying long-range controls vary among systems. It is noteworthy that in none of these systems has a site-specific blockade of Pol II been used to establish a direct functional link between intergenic transcription and target gene activation.

Pol II Tracking Is Essential to Full *hGH* LCR Activity

In the present study, we observed that insertion of an exogenous Pol II terminator within the LCR domain of transcription represses expression of the remote *hGH-N* gene. These results, along with those previously reported (Ho et al., 2002), suggest significant complexity in HSI action as an LCR component. The establishment of a broad domain of histone acetylation in the LCR and the formation of an LCR domain of transcription are both HSI dependent. However, the results of the current study suggest that acetylation of the locus is not dependent on LCR transcription. The insertion of the *TerF* element significantly represses Pol II tracking 3′ to the insertion site but has no apparent effect on histone H3/H4 acetylation in this region. These results suggest that the two HSI-dependent activities, Pol II tracking and histone acetylation, are not mechanistically linked. Thus, these results demonstrate an essential role for LCR transcription in the control of *hGH-N* gene activity, but they do not address the direct role of histone acetylation in this process.

The fact that *TerF* insertion results in a unilateral (3′) repression of transcription and has no appreciable impact on the level of LCR transcription 5′ to the insertion site is important when considering its quantitative impact on *hGH-N* expression. While *TerF* insertion results in a mean 6-fold decrease in *hGH-N* expression (Figure 5), HSI inactivation represses expression of the linked *hGH-N* by 20-fold (Ho et al., 2002). This difference in the extent of *hGH-N* repression may be directly related to corresponding effects on LCR transcription. While HSI inactivation results in a global loss of transcripts throughout the LCR domain (Figures 1 and 3), the *TerF* insertion selectively eliminates transcription 3′ to the insertion site, namely across the *CD79b* gene (Figure 5A). These data (summarized in Figure 6) lead us to propose that Pol II tracking within the *hGH* LCR domain of transcription acts in an additive fashion; the selective retention of transcription in the LCR 5′ of the *TerF* insertion may contribute independently to this process and maintain a moderate residual level of *hGH-N* expression. Loss of the transcription throughout the entire LCR domain secondary to HSI deletion results in a further incremental loss of *hGH-N* function. Thus, in the comparison of the *hGH/P1*, *hGH/P1(TerF)*, and *hGH/P1(ΔHSI)* transgenes, the levels of *hGH-N* expression appear to be directly related to the overall levels of LCR transcription (Figure 6).

How does the LCR domain of transcription contribute to long-range gene activation? The gap between the LCR domain of transcription and the *hGH-N* gene makes it unlikely that the mechanism represents direct linear delivery of polymerase or coactivator complexes from the LCR to the *hGH-N* promoter. It seems more likely that the act of transcription has a direct effect(s) on LCR structure and that this altered structure triggers subsequent steps in the activation pathway. Pol II tracking can deplete local H2A and H2B histone content (Nacheva et al., 1989) and trigger replacement of standard histones with histone variants (Mito et al., 2005; Wirbelauer et al., 2005). In addition, it has been shown that complexes containing H3 lysine 4 (H3K4) methyltransferase associate with the elongating (Ser5 phosphorylated) Pol II (Bernstein et al., 2002; Ng et al., 2003; Santos-Rosa et al., 2002). H3K4 trimethylation is a highly conserved component of an active chromatin environment (Berger, 2002); chromodomain proteins are targeted to such H3K4 methylated histones (Flanagan et al., 2005), and their local action may be critical to LCR function. Thus, by a number of mechanisms, Pol II tracking might create a modified and accessible chromatin conformation that would facilitate subsequent recruitment of coregulators. These effects may enhance direct interactions between one or more LCR determinants and the *hGH-N* promoter, possibly via looping mechanisms (Tolhuis et al., 2002). Tracking of Pol II within the LCR domain may also anchor the *hGH-N* locus to an established nuclear subcompartment in which the *hGH-N* promoter would have access to enriched sources of Pol II and other transcription factors (Osborne et al., 2004).

The role of *CD79b* transcription in *hGH* LCR action is of particular interest. The initial observation that the B-lymphocyte-specific *CD79b* gene is robustly transcribed in the pituitary was unexpected (Cajiao et al., 2004). Although our initial impression was that activation of *CD79b* in the pituitary reflected a passive response of this gene to the encompassing activated *hGH* LCR chromatin milieu, its robust nature suggested that it might play an active role in *hGH* LCR function. The present data (summarized in Figure 6) support this active model. Selective elimination of *CD79b* transcription in pituitary chromatin by the *TerF* insertion in the *hGH* LCR is paralleled by a marked repression in *hGH-N* expression. This observation suggests that the bystander transcription at the *CD79b* locus in the pituitary, once triggered by HSI action, goes on to enhance LCR function by magnifying the overall level and density of Pol II tracking in the region. This apparent appropriation of the *CD79b* transcription unit for the purpose of amplifying LCR function may reflect an evolutionary adaptation to its positioning within the *hGH* locus. The fact that the abundant *CD79b* mRNA does not encode detectable levels of protein in the pituitary (Cajiao et al., 2004) further suggests that

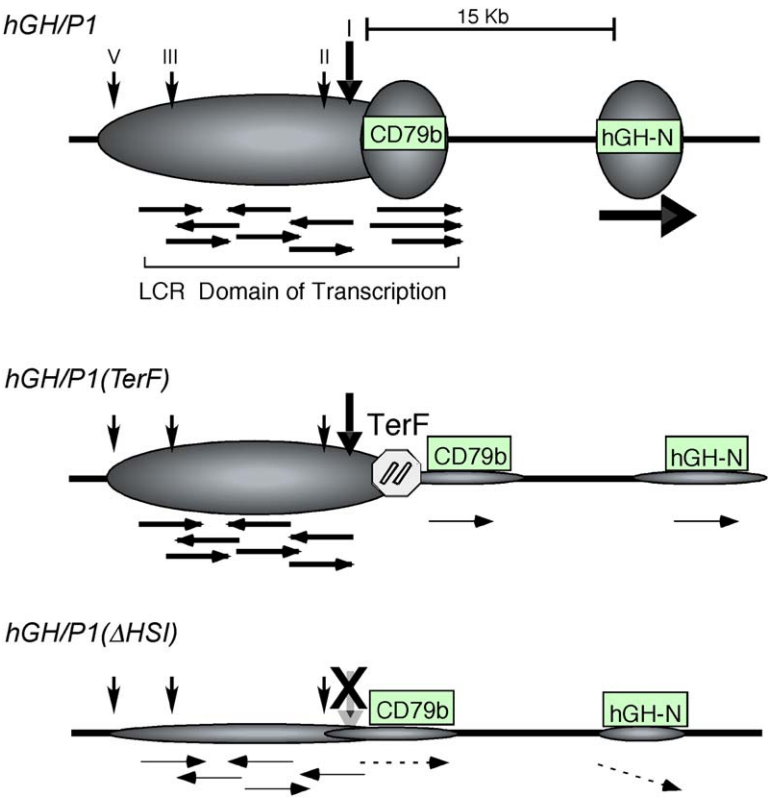

Figure 6. Evidence for a Functional Link between LCR Transcription and Long-Range Gene Activation at the *CD79b/hGH* Locus

(Top) Transcriptional configuration of the wild-type *hGH* transgene locus. The *hGH* transgene locus, encompassing the *hGH* LCR, *CD79b* gene, and *hGH-N* gene, is drawn in a simplified format. Each HS formed in pituitary chromatin is shown (vertical arrows). HSI, the major pituitary-specific LCR element (heavy vertical arrow), and the 15 kb distance from HSI to the target *hGH-N* promoter are indicated. The gray ovals represent regions enriched for transcripts and for pPol II. The HSI-dependent LCR domain of transcription (bracketed) is comprised of the *hGH* LCR and contiguous *CD79b* transcription unit. The density and direction of the horizontal arrows below the diagram indicate the polarity and strength of the transcription, respectively. (Middle) The impact of the TerF Pol II terminator insertion on LCR transcription and long-range activation of *hGH-N*. The *TerF* terminator element is represented (hexagon with a double bar). The unilateral repression of Pol II transcription within the *CD79b* component of the *hGH* LCR transcription domain and the 6-fold reduction in transcription from the remote *hGH-N* promoter are indicated (light horizontal arrows). (Bottom) The impact of HSI deletion on LCR transcription and *hGH-N* expression. Deletion of HSI from the *hGH/P1* transgene (bold X) results in a loss of pPol II tracking throughout the LCR domain of transcription both 5′ and 3′ of the HSI deletion site. This results in a 20-fold repression of *CD79b* (Cajiao et al., 2004) and *hGH-N* transcription (Ho et al., 2002) (dashed arrows).

the somatotrope has developed a countervailing mechanism to block the ectopic expression of the potentially toxic *CD79b* protein product, Igβ.

Other mechanistic possibilities that link Pol II tracking with LCR function cannot be eliminated at this time. It remains possible, for example, that LCR transcripts may themselves play a direct role in activation and maintenance of *hGH-N* expression by recruiting *trans*-activators to the chromatin sites (Sleutels et al., 2002; Akhtar et al., 2000; Sanchez-Elsner et al., 2006). By directly targeting these transcripts without concurrently releasing Pol II from the chromatin template, it should be possible to gain further insights into these important distinctions. Such studies will contribute to a more comprehensive understanding of how Pol II tracking and intergenic transcription contribute to long-range control in the mammalian genome.

Experimental Procedures

Detection of Transcripts Emanating from the *hGH* Locus

To generate hyperplastic, somatotrope-enriched pituitaries, each of two *hGH/P1* transgenic line (lines 809F and 811D; Su et al., 2000) were crossed with mice carrying a human growth hormone-releasing factor (*hGRF*) transgene (Mayo et al., 1988). Pituitary and liver RNAs were prepared from these 3-month-old compound transgenic mice. RNAs from five pituitaries were pooled for each set of assays. RNA was treated with RQ1 DNase (Promega, Madison, Wisconsin) to eliminate DNA contamination. For the analysis of total transcripts at each site along the locus, a cDNA library was first generated. Five micrograms of total RNA was incubated at 42°C for 50 min with reverse transcriptase (RT) along with 300 ng of a random hexamer per 20 μl reaction. RT was heat inactivated, and 2 μl of the treated reactions was used for subsequent sequence-specific PCR. To control for amplification of contaminant DNA, a parallel library was generated in the absence of RT. The RT(+) and RT(−) libraries were assayed in parallel and within the linear range of amplification for the presence of cDNAs corresponding to defined positions across the *hGH* locus (Figure 1). The primers used to detect cDNAs at each position can be found in Table S1. To define the orientation of the noncoding transcripts, 0.5–1 μg of pituitary RNA was reverse transcribed with strand-specific primers prior to PCR amplification with site-specific amplimers (Table S1). PCR-generated cDNAs from the library analysis or from the strand-specific analysis were separated on 1.5% agarose gels and Southern hybridized with ^{32}P-labeled probes generated by the same sets of PCR primers. Each band was quantified by phosphorimager analyses (Molecular Dynamics, Sunnyvale, California). *hGH/P1* plasmid DNA was used as the control template to assess the PCR efficiency of each primer set. Serial dilutions of the *hGH/P1* plasmid DNA (7–0.007 ng) were assayed to determine the linear range for the PCR amplification (data not shown). In all cases, 0.007 ng fell within the linear range of the signals obtained from the RT-PCR. The RT-PCR signal levels were normalized to the level of PCR signals from 0.007 ng of *hGH/P1* plasmid DNA generated with the same set of primers (C; control) and was calculated in arbitrary units.

To quantify transcripts bracketing the *TerF* element, RT-PCR was performed at sites corresponding to p5, HSI, and exon 6 of *CD79b* (Figure 5). In each case, the RT-PCR signal was determined to be in the linear range and its value was normalized to the *β-actin* RT-PCR cDNA product from the same RNA sample to correct for the quantity and quality of the input RNA. The normalized value from each of the five *hGH/P1(TerF)* pituitary RNA samples was

divided by the average of the normalized RT-PCR values at the corresponding site in two representative *hGH/P1* transgenic lines.

CoRT-PCR Assay for *hGH-N* Expression

Pituitary RNA from transgenic mice carrying *hGH/P1* or the *hGH/P1(TerF)* transgene was extracted from a single pituitary of each line. The RT-PCR assays were performed as described previously (Jones et al., 1995, Ho et al., 2002). The resultant *hGH-N* to *mGH* RNA ratios were divided by the relevant transgene copy number to establish transgene expression per copy.

Chromatin Immunoprecipitation Assays

ChIP assays were performed as previously described on compound transgenic (GRF) mice (Ho et al., 2002). The antibodies recognized the N-terminal sequences of Pol II (Santa Cruz Biotechnology Santa Cruz, California [sc-899]) or the Ser-5-phosphorylated Pol II C-terminal domain (pPol II) (Covance Research Products, Cumberland [VA (H-14, MMS-134R)]). For acetylated H3 and H4 ChIP, the antibodies used recognize diacetylated H3 (06-599) and tetraacetylated H4 (06-598) (Upstate Cell Signaling Solutions, Charlottesville, Virginia). For ChIPs using the pPol II antibody, phosphatase inhibitors (0.5 mM sodium orthovanadate, 10 mM sodium molybdate, and 40 mM β-glycerol phosphate) were included in buffers. To precipitate IgM-containing immune complexes, rabbit anti-mouse IgM (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pennsylvania) was prebound to the protein A-agarose (2 mg/ml) for 3 hr. Normal rabbit serum (Santa Cruz) was used as control for the ChIP assays. The input and bound DNA were amplified by PCR. The sequences and position of the primers used for PCR are summarized in Table S2. PCR products were quantified by Southern blotting as described above. A series of dilutions of the chromatin input fraction was used to determine the linear region for the PCR amplification at each site as described (Ho et al., 2002) (Figure 5 and data not shown). The signals of bound fractions were normalized to the signals of 0.03% of the input. The ratio of signal from the bound to input samples was normalized to the ratio of bound to input obtained from the mouse endogenous *MyoD* gene. The sequences of the primers for *MyoD* are as previously reported (Sawado et al., 2003).

Insertion of the Transcriptional Terminator Element into the *hGH* Locus

The insertion of the human *β-globin* transcriptional terminator element (*TerF*) into the *hGH/P1* clone was performed by RecA-assisted homologous recombination (Yang et al., 1997; Ho et al., 2002). *TerF*, a 2.2 kb DNA fragment from the human *β-globin* gene (*pβΔ5-7*; gift of N. Proudfoot, Oxford University; Dye and Proudfoot, 2001), was inserted between the *hGH* LCR and *hGH-N* (coordinate −13,660 bp relative to the *hGH-N* transcription start site). This *TerF* was inserted in the sense orientation relative to *hGH-N*. Accurate insertion in the resultant *hGH/P1(TerF)* transgene was confirmed by DNA sequencing. A "fingerprinting" approach (Ho et al., 2002) was carried out to confirm that there were no unexpected alterations in the *hGH/P1(TerF)* DNA (data not shown).

Generation of Transgenic Mouse Lines

The *hGH/P1(TerF)* DNA was linearized with NotI and purified through an Elutip (Schleicher and Schuell Bioscience, Keene, New Hampshire). The DNA was diluted to 2 ng/μl in 10 mM Tris-HCl (pH 7.5) and 0.1 mM Na_2EDTA and was microinjected into the pro-nucleus of C57Bl/6J X SJL mouse zygotes. The integrity and copy number of each transgene were determined as described (Su et al., 2000, Ho et al., 2002).

Supplemental Data

Supplemental Data include one figure, two tables, and Supplemental Experimental Procedures and can be found with this article online at http://www.molecule.org/cgi/content/full/23/3/365/DC1/.

Acknowledgments

This study was supported by NIH K01 DK064011 (Y.H.) and R01 HD25147 (N.E.C. and S.A.L). The authors thank Dr. Jean Richa and the Transgenic and Chimeric Mouse Facility of the University of Pennsylvania (NIH P30 CA016520, P30 DK050306, and P30 DK019525) for generating the transgenic mice and Dr. Tom Kadesch for critical review of the manuscript. The authors gratefully acknowledge the gift of the *TerF* construct from Dr. N. Proudfoot, Oxford University.

Received: March 1, 2006
Revised: May 9, 2006
Accepted: May 31, 2006
Published: August 3, 2006

References

Akhtar, A., Zink, D., and Becker, P.B. (2000). Chromodomains are protein-RNA interaction modules. Nature *407*, 405–409.

Ashe, H.L., Monks, J., Wijgerde, M., Fraser, P., and Proudfoot, N.J. (1997). Intergenic transcription and transinduction of the human beta-globin locus. Genes Dev. *11*, 2494–2509.

Bennani-Baïti, I.M., Cooke, N.E., and Liebhaber, S.A. (1998). Physical linkage of the human growth hormone gene cluster and the CD79b (Ig beta/B29) gene. Genomics *48*, 258–264.

Berger, S.L. (2002). Histone modifications in transcriptional regulation. Curr. Opin. Genet. Dev. *12*, 142–148.

Bernstein, B.E., Humphrey, E.L., Erlich, R.L., Schneider, R., Bouman, P., Liu, J.S., Kouzarides, T., and Schreiber, S.L. (2002). Methylation of histone H3 Lys 4 in coding regions of active genes. Proc. Natl. Acad. Sci. USA *99*, 8695–8700.

Cajiao, I., Zhang, A., Yoo, E.J., Cooke, N.E., and Liebhaber, S.A. (2004). Bystander gene activation by a locus control region. EMBO J. *23*, 3854–3863.

Chen, E.Y., Liao, Y.C., Smith, D.H., Barrera-Saldaña, H.A., Gelinas, R.E., and Seeburg, P.H. (1989). The human growth hormone locus: nucleotide sequence, biology, and evolution. Genomics *4*, 479–497.

Dahmus, M.E. (1996). Reversible phosphorylation of the C-terminal domain of RNA polymerase II. J. Biol. Chem. *271*, 19009–19012.

Dean, A. (2006). On a chromosome far, far away: LCRs and gene expression. Trends Genet. *22*, 38–45.

Dye, M.J., and Proudfoot, N.J. (1999). Terminal exon definition occurs cotranscriptionally and promotes termination of RNA polymerase II. Mol. Cell *3*, 371–378.

Dye, M.J., and Proudfoot, N.J. (2001). Multiple transcript cleavage precedes polymerase release in termination by RNA polymerase II. Cell *105*, 669–681.

Elefant, F., Cooke, N.E., and Liebhaber, S.A. (2000). Targeted recruitment of histone acetyltransferase activity to a locus control region. J. Biol. Chem. *275*, 13827–13834.

Festenstein, R., Tolaini, M., Corbella, P., Mamalaki, C., Parrington, J., Fox, M., Miliou, A., Jones, M., and Kioussis, D. (1996). Locus control region function and heterochromatin-induced position effect variegation. Science *271*, 1123–1125.

Flanagan, J.F., Mi, L.Z., Chruszcz, M., Cymborowski, M., Clines, K.L., Kim, Y., Minor, W., Rastinejad, F., and Khorasanizadeh, S. (2005). Double chromodomains cooperate to recognize the methylated histone H3 tail. Nature *438*, 1181–1185.

Gribnau, J., Diderich, K., Pruzina, S., Calzolari, R., and Fraser, P. (2000). Intergenic transcription and developmental remodeling of chromatin subdomains in the human beta-globin locus. Mol. Cell *5*, 377–386.

Grosveld, F., van Assendelft, G.B., Greaves, D.R., and Kollias, G. (1987). Position-independent, high-level expression of the human beta-globin gene in transgenic mice. Cell *51*, 975–985.

Haussecker, D., and Proudfoot, J.N. (2005). Dicer-dependent turnover of intergenic transcripts from the human β-globin gene cluster. Mol. Cell. Biol. *25*, 9724–9733.

Ho, Y., Elefant, F., Cooke, N.E., and Liebhaber, S.A. (2002). A defined locus control region determinant links chromatin domain acetylation with long-range gene activation. Mol. Cell *9*, 291–302.

Ho, Y., Liebhaber, S.A., and Cooke, N.E. (2004). Activation of the human GH gene cluster: roles for targeted chromatin modification. Trends Endocrinol. Metab. *15*, 40–45.

Johnson, K.D., Grass, J.A., Park, C., Im, H., Choi, K., and Bresnick, E.H. (2003). Highly restricted localization of RNA polymerase II within a locus control region of a tissue-specific chromatin domain. Mol. Cell. Biol. *23*, 6484–6493.

Jones, B.K., Monks, B.R., Liebhaber, S.A., and Cooke, N.E. (1995). The human growth hormone gene is regulated by a multicomponent locus control region. Mol. Cell. Biol. *15*, 7010–7021.

Kimura, A.P., Liebhaber, S.A., and Cooke, N.E. (2004). Epigenetic modifications at the human growth hormone locus predict distinct roles for histone acetylation and methylation in placental gene activation. Mol. Endocrinol. *18*, 1018–1032.

Li, Q., Peterson, K.R., Fang, X., and Stamatoyannopoulos, G. (2002). Locus control regions. Blood *100*, 3077–3086.

Masternak, K., Peyraud, N., Krawczyk, M., Barras, E., and Reith, W. (2003). Chromatin remodeling and extragenic transcription at the MHC class II locus control region. Nat. Immunol. *4*, 132–137.

Mayo, K.E., Hammer, R.E., Swanson, L.W., Brinster, R.L., Rosenfeld, M.G., and Evans, R.M. (1988). Dramatic pituitary hyperplasia in transgenic mice expressing a human growth hormone-releasing factor gene. Mol. Endocrinol. *2*, 606–612.

Mito, Y., Henikoff, J.G., and Henikoff, S. (2005). Genome-scale profiling of histone H3.3 replacement patterns. Nat. Genet. *37*, 1090–1097.

Nacheva, G.A., Guschin, D.Y., Preobrazhenskaya, O.V., Karpov, V.L., Ebralidse, K.K., and Mirzabekov, A.D. (1989). Change in the pattern of histone binding to DNA upon transcriptional activation. Cell *58*, 27–36.

Ng, H.H., Robert, F., Young, R.A., and Struhl, K. (2003). Targeted recruitment of Set1 histone methylase by elongating Pol II provides a localized mark and memory of recent transcriptional activity. Mol. Cell *11*, 709–719.

Orphanides, G., and Reinberg, D. (2002). A unified theory of gene expression. Cell *108*, 439–451.

Osborne, C.S., Chakalova, L., Brown, K.E., Carter, D., Horton, A., Debrand, E., Goyenechea, B., Mitchell, J.A., Lopes, S., Reik, W., and Fraser, P. (2004). Active genes dynamically colocalize to shared sites of ongoing transcription. Nat. Genet. *36*, 1065–1071.

Plant, K.E., Routledge, S.J., and Proudfood, N.J. (2001). Intergenic transcription in the human beta-globin gene cluster. Mol. Cell. Biol. *21*, 6507–6514.

Plath, K., Mlynarczyk-Evans, S., Nusinow, D.A., and Panning, B. (2002). Xist RNA and the mechanism of X chromosome inactivation. Annu. Rev. Genet. *36*, 233–278.

Routledge, S.J., and Proudfoot, N.J. (2002). Definition of transcriptional promoters in the human beta globin locus control region. J. Mol. Biol. *323*, 601–611.

Sanchez-Elsner, T., Gou, D., Kremmer, E., and Sauer, F. (2006). Noncoding RNAs of trithorax response elements recruit Drosophila Ash1 to ultrabithroax. Science *311*, 1118–1123.

Santos-Rosa, H., Schneider, R., Bannister, A.J., Sherriff, J., Bernstein, B.E., Emre, N.C., Schreiber, S.L., Mellor, J., and Kouzarides, T. (2002). Active genes are tri-methylated at K4 of histone H3. Nature *419*, 407–411.

Sawado, T., Halow, J., Bender, M.A., and Groudine, M. (2003). The beta-globin locus control region (LCR) functions primarily by enhancing the transition from transcription initiation to elongation. Genes Dev. *17*, 1009–1018.

Schmitt, S., Prestel, M., and Paro, R. (2005). Intergenic transcription through a polycomb group response element counteracts silencing. Genes Dev. *19*, 697–708.

Sleutels, F., Zwart, R., and Barlow, D.P. (2002). The non-coding Air RNA is required for silencing autosomal imprinted genes. Nature *415*, 810–813.

Su, Y., Liebhaber, S.A., and Cooke, N.E. (2000). The human growth hormone gene cluster locus control region supports position-independent pituitary- and placenta-specific expression in the transgenic mouse. J. Biol. Chem. *275*, 7902–7909.

Teixeira, A., Tahiri-Alaoui, A., West, S., Thomas, B., Ramadass, A., Martianov, I., Dye, M., James, W., Proudfoot, N.J., and Akoulitchev, A. (2004). Autocatalytic RNA cleavage in the human beta-globin premRNA promotes transcription termination. Nature *432*, 526–530.

Tolhuis, B., Palstra, R.J., Splinter, E., Grosveld, F., and de Laat, W. (2002). Looping and interaction between hypersensitive sites in the active beta-globin locus. Mol. Cell *10*, 1453–1465.

Wang, Q., Carroll, J.S., and Brown, M. (2005). Spatial and temporal recruitment of androgen receptor and its coactivators involves chromosomal looping and polymerase tracking. Mol. Cell *19*, 631–642.

West, S., Gromak, N., and Proudfoot, N.J. (2004). Human $5' \rightarrow 3'$ exonuclease Xrn2 promotes transcription termination at co-transcriptional cleavage sites. Nature *432*, 522–525.

Wirbelauer, C., Bell, O., and Schubeler, D. (2005). Variant histone H3.3 is deposited at sites of nucleosomal displacement throughout transcribed genes while active histone modifications show a promoter-proximal bias. Genes Dev. *19*, 1761–1766.

Wittschieben, B.O., Otero, G., de Bizemont, T., Fellows, J., Erdjument-Bromage, H., Ohba, R., Li, Y., Allis, C.D., Tempst, P., and Svejstrup, J.Q. (1999). A novel histone acetyltransferase is an integral subunit of elongating RNA polymerase II holoenzyme. Mol. Cell *4*, 123–128.

Yang, X.W., Model, P., and Heintz, N. (1997). Homologous recombination based modification in Escherichia coli and germline transmission in transgenic mice of a bacterial artificial chromosome. Nat. Biotechnol. *15*, 859–865.