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The juxtaposition of a promoter with a locus control region transcriptional domain activates gene expression

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Nonlinear chromatin configurations can juxtapose widely separated elements within a genomic locus; however, it remains unclear how these structures are established and contribute to transcriptional control. A 5′-remote locus control region (LCR) regulates the human growth hormone (*hGH-N*) gene. HSI, a pituitary-specific component of the *hGH* LCR, establishes a domain of polymerase II (PolII) transcription 5′ to *hGH-N*. Repression of this transcriptional domain by HSI deletion or PolII blockade decreases *hGH-N* expression. Here, we show that *hGH-N* activation is accompanied by positioning of the *hGH-N* promoter to this LCR transcriptional domain. Selectively blocking LCR transcription inhibits the formation of this active 'looped' conformation. Thus, HSI is crucial for establishing a domain of noncoding PolII transcription, and this domain is intimately linked with chromatin organization of the active *hGH-N* locus. This integration of LCR transcription with chromatin reconfiguration constitutes a robust pathway for long-range gene activation.

Keywords: epigenetics; locus control region; looping; transcription

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INTRODUCTION

Long-range controls have a crucial and often predominant role in metazoan gene activation (Ho *et al*, 2004; Dean, 2006). Mechanisms of long-range gene activation have been grouped into several structural models (Forsberg & Bresnick, 2001; Ho *et al*, 2004; Dean, 2006). However, the degree to which these models accurately reflect the full array of locus control region (LCR) functions remains to be determined and might vary among loci. The human growth hormone (*hGH*) cluster contains one pituitary-specific *hGH-N* gene and four placenta-specific paralogues (Fig 1A; Chen *et al*, 1989). These five genes are activated by a set of LCR determinants located 15–32 kb upstream of the cluster (Jones *et al*, 1995; Su *et al*, 2000; Ho *et al*, 2002). The positioning of a B-lymphocyte-specific gene, *CD79b*, between the pituitary-specific HSI and the *hGH-N* promoter increases the complexity of the *hGH* gene cluster (Bennani-Baiti *et al*, 1998), as does the location of the more distal HSIII-HSV elements within introns of the striated muscle sodium channel gene, *SCN4A* (Fig 1A). Defining how this complex locus achieves robust and tissue-specific expression of its corresponding genes should determine crucial relationships between chromatin structure and gene regulatory pathways.

Previous studies have shown that the *hGH* LCR and the *hGH-N* promoter are encompassed within a 32 kb domain of acetylated histones H3 and H4 in pituitary chromatin (Elefant *et al*, 2000); this domain is centered at HSI,II. In mouse transgenic models, selective deletion of the pituitary-specific HSI results in the loss of histone acetylation throughout the LCR and a marked decrease in *hGH-N* transcription (Ho *et al*, 2006). HSI is also crucial for establishing a pituitary-specific 'domain of transcription' that encompasses the LCR and the adjacent *CD79b* gene. Repression of transcription in the *CD79b* subregion of this domain, by insertion of a polymerase II (PolII) termination element between HSI and *CD79b,* results in a marked reduction in *hGH-N* expression (Ho *et al*, 2006). The mechanistic link between the HSI-dependent domain of noncoding transcription and enhancement of *hGH-N* expression is investigated further in this paper. A comparison of the higher order chromatin configurations of the active and silent *hGH* loci supports a model in which the promoter activity of *hGH-N* is enhanced by its positioning into the PolII-enriched environment of the LCR transcriptional domain.

RESULTS AND DISCUSSION

Formation of an HSI-dependent chromatin conformation

In contrast to the domain of histone acetylation that directly links the *hGH* LCR with the *hGH-N* promoter, the domain of PolII transcription at the active *hGH* locus, which encompasses the LCR and adjacent *CD79b* region, is separated from the active *hGH-N* gene by a nontranscribed gap (Ho *et al*, 2006). To define further

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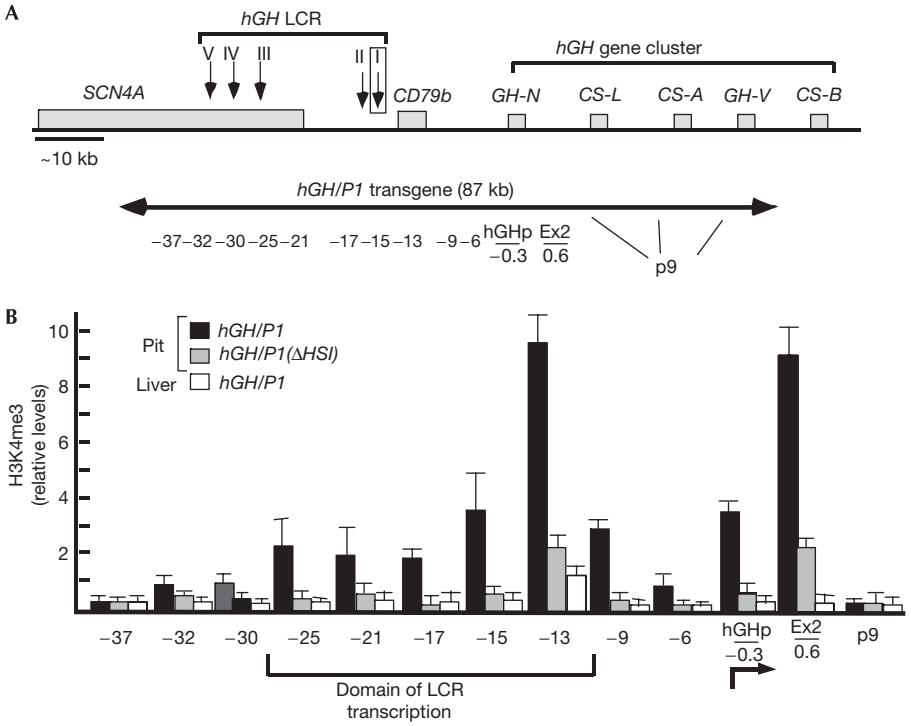

Fig 1 | H3K4me3 modifications that are both pituitary specific and HSI dependent encompass the *hGH* LCR and *hGH-N*. (**A**) Diagram of 100 kb of the human genome on chromosome 17q22 encompassing the *hGH* locus and the *hGH/P1* transgene. The region shown contains the five genes of the *hGH* cluster, the B-lymphocyte-expressed *CD79b* gene and the striated muscle-specific gene *SCN4A* (grey rectangles). All genes are transcribed in the same orientation (from left to right). The five DNase I HS that constitute the *hGH* LCR are indicated by downward arrows. The extent of the 87 kb *hGH/P1* transgene is shown below by the double-headed arrow. The positions of the 13 PCR amplimers used to survey the locus for H3K4me3 modification enrichment are indicated below the arrow as coordinates (kb) relative to the start site of *hGH-N* transcription. The 99 bp deletion in the *hGH/P1(ΔHSI)* transgene that removes two of the three pituitary Pit-1 binding sites is denoted by the boxed HSI. Note that the 'HSI' amplimer (−15) used in these studies detects sequences that are adjacent to this deleted segment in the *hGH/P1(ΔHSI)* transgene. The p9 amplimer corresponds to a conserved sequence located 5′ to each of the four placentally expressed genes in the cluster. (**B**) H3K4me3 modifications in mouse transgenic pituitary (Pit) and liver chromatin and in the absence of HSI. The chromatin immunoprecipitation antibody was specific to the H3K4me3 modification. Each bar represents the mean ± s.d. of four independent assays of the indicated chromatin samples: *hGH/P1* pituitary chromatin (black bars), *hGH/P1(ΔHSI)* pituitary chromatin (grey bars) or *hGH/P1* liver chromatin (white bars). Each histogram bar represents the ratio of the DNA amplification signal from Southern blot detected in the immunoprecipitated sample to that in the corresponding input sample. Each ratio was normalized to the corresponding ratio for a DNA segment within the endogenous skeletal muscle-specific *mMyoD* gene (Weintraub *et al*, 1991; Sawado *et al*, 2003). Each ratio, after normalization to *mMyoD*, is expressed on the *Y* axis in arbitrary units. The amplimer set corresponding to each set of bars is noted below the *X* axis. The site of *hGH-N* transcription initiation is noted below the *X* axis (angled arrow) and the previously defined 'LCR domain of transcription' is indicated by the labeled bracket below the histogram. All comparisons between *hGH/P1* modifications in the pituitary compared with liver are significant ($P<0.001$) except at −37 and p9. Comparisons between *hGH/P1* and *hGH/P1ΔHSI* between −25 and Ex2 inclusive are significant ($P<0.001$). Comparisons between *hGHΔHSI* pituitary and *hGH/P1* liver are significant only at Ex2 ($P<0.01$). H3K4me3, histone H3 lysine 4 trimethylation; *hGH*, human growth hormone; LCR, locus control region.

the structure of the active *hGH* locus, we carried out chromatin immunoprecipitation (ChIP) analysis of histone H3 lysine 4 trimethylation (H3K4-me3) at the *hGH/P1* transgene locus. H3K4-me3 modifications are associated with active chromatin structure and often tightly linked to PolII elongation (Gerber & Shilatifard, 2003; Berger, 2007). The *hGH/P1* transgene extends from −40 to +45 kb relative to the transcriptional start site of *hGH-N* (Fig 1A). The H3K4-me3 modifications at the active *hGH/P1* transgene locus in the mouse pituitary paralleled PolII distribution; they extended through the LCR and adjacent *CD79b* region, and were separated from the *hGH-N* promoter by an intervening gap of unmodified chromatin. The modifications were greatly diminished when HSI was deleted (Fig 1B). The concordant gaps in PolII occupancy and H3K4-me3 modification between the LCR and the *hGH-N* promoter suggested that activation of the *hGH-N* promoter might be mediated by nonlinear interactions with its LCR.

The higher order configuration of the active *hGH-N* locus was explored by using chromosome conformation capture (3C; Cullen *et al*, 1993; Dekker *et al*, 2002; Tolhuis *et al*, 2002). Conditions were established in which the *Bgl*II restriction enzyme effectively and reproducibly digests primary chromatin preparations (Fig 2A; supplementary Fig 1 online). A single nucleotide divergence between the 5′-flanking regions of the *hGH-N* and *hGH-V/hCS*

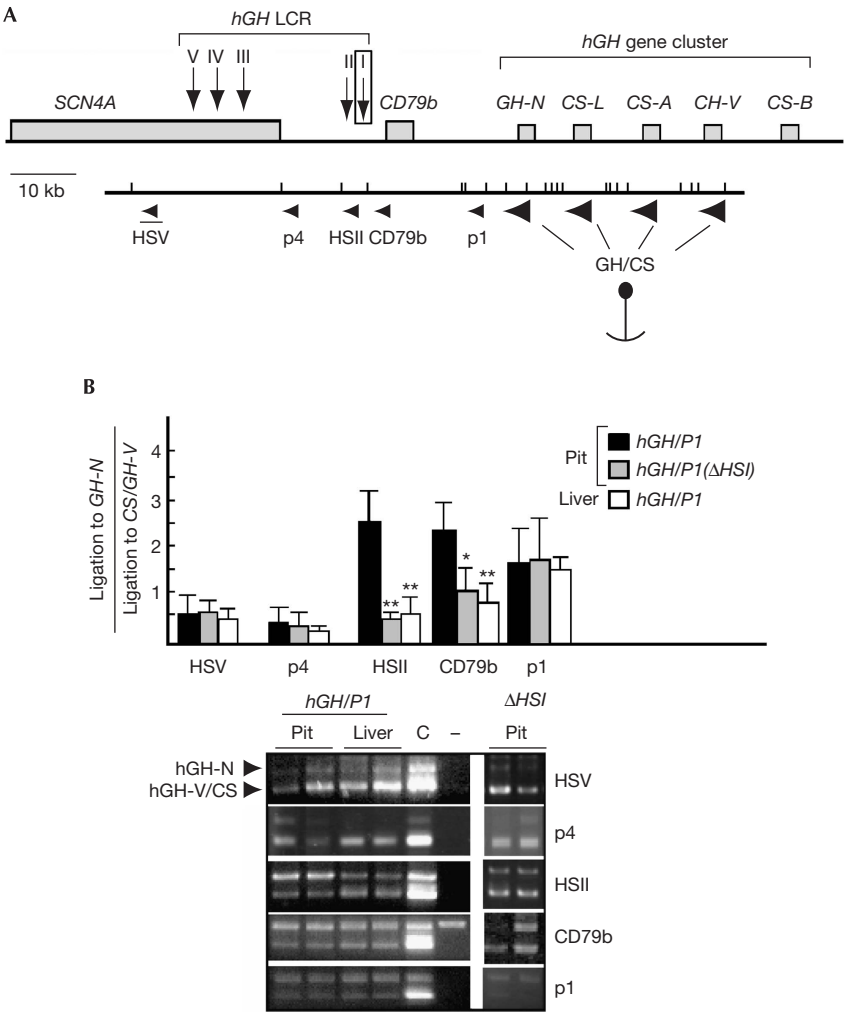

Fig 2 | Interaction between HSI,II and the activated *hGH-N* promoter is HSI dependent and pituitary specific. (**A**) The *Bgl*II map of the *hGH/P1* transgene. Each *Bgl*II site is indicted below the locus map by a tick mark. The position of each of the PCR primers is also shown (arrowheads); the *GH/CS* primer, common to all four *GH*-related genes (large arrowheads), functioned as the anchor primer in these studies. The four upstream primers detect individual *Bgl*II fragments that contain HSV, p4, HSII, CD79b or p1 regions. (**B**) Summary of the 3C analyses in *hGH/P1(TerF)* pituitary (Pit) chromatin. 3C assays were performed on pituitaries (black bars) or livers (white bars) of mice representing two *hGH/P1* transgenic lines and on pituitaries of mice representing two *hGH/P1(ΔHSI)* lines (grey bars). *GH/CS*, the anchor primer for these studies, was paired with each of the four 5′ primers in the PCR analysis of the ligation products. The panels below the histogram show representative ethidium bromide-stained agarose gels containing the indicated PCR amplification products; the distinct bands representing the *hGH-N*- and *hGH-V/hCS*-specific ligation products are indicated by arrowheads at the left of the top panel. The intensities of the bands were quantified. Lane C (control) contains the PCR amplifications of the ligation products of a *Bgl*II-digested *hGH/P1* plasmid DNA. Lane marked − contains the products of the assay performed using the *hGH/P1* pituitary chromatin preparation in the absence of T4 DNA ligase. The band in the CD79b (−) sample represents a background amplification product that is insensitive to *Bgl*II. The histogram values represent ratios of the signal of the *hGH-N*-specific ligation product to the combined placental gene-specific ligation products (supplementary Figs 1,2 online). Each histogram bar represents the mean ± s.d. of four or more independent studies on each of the two transgenic lines. In comparison of the value obtained from *hGH/P1* pituitary chromatin to the indicated value, a single asterisk indicates $P<0.01$ and a double asterisk indicates $P<0.001$. 3C, chromosome conformation capture; *hGH*, human growth hormone; LCR, locus control region.

genes distinguishes *Bgl*II fragments that encompass the *hGH-N* promoter from those encompassing the *hGH-V/hCS* promoters (supplementary Fig 2 online). The initial set of PCR analyses of 3C ligation products used a 'GH/CS' anchor primer that recognizes all of the related *hGH/hCS* genes. *BglII* ligation products containing the *hGH-N* promoter region were distinguished from those containing the placenta-expressed paralogue by size (supplementary Fig 2 online), thereby allowing a direct comparison of the proximity of the *hGH-N* promoter or the promoters of the placenta-expressed paralogue to other defined sites within the locus. The 3C studies represent a minimum of four independent experiments performed on each of two separate *hGH/P1* transgenic lines. Previous studies have shown that the pattern of histone acetylation and the level of *hGH-N* expression are similar in these two lines (Su *et al*, 2000;

Ho *et al*, 2006). The analysis of liver chromatin (nonexpressing cells) from these mice (Fig 2B, white bars) showed that the efficiency of ligation of the *hGH-N* promoter fragment to each of the 5′ restriction fragments decreased as the distance between the GH/CS anchor and upstream primers increased. This relationship is consistent with a linear conformation of the cluster in liver chromatin (Dekker *et al*, 2002). By contrast, the analysis of pituitary chromatin from the same two *hGH/P1* transgenic lines (Fig 2B, black bars) showed that the ratio of ligation products shifted in favour of the fragment containing the *hGH-N* promoter when the anchor GH/CS primer was paired with the primer associated with HSI,II or adjacent *CD79b* restriction fragments. This pattern of ligation frequencies indicated a close positioning between the HSI,II region and the *hGH-N* promoter despite the intervening linear 14.5 kb. The PCR products generated by amplification between the GH/CS primer and the HSII primer were purified and sequenced to confirm their identity. These data support a selective, long-range interaction between the HSI,II region and the *hGH-N* promoter.

To validate the above results, a reciprocal 3C assay was performed using HSII as the anchor primer (Fig 3A). In these assays, the combination of the HSII anchor and *GH/CS* primer generated the expected doublet. Ligations to each of the other primers generated a single, specific PCR product (Fig 3B). For each set of amplifications, the PCR signal was normalized to the random ligation control (Fig 3B, lane C, ligation of *hGH/P1* plasmid DNA) and to ligation fragments generated from the endogenous, universally expressed *ERCC3* locus (Palstra *et al*, 2003; Fig 3C). The high frequency of ligation between the HSII anchor and *CD79b* in both liver and pituitary chromatin samples is consistent with their proximity to the locus in the linear structure (Liu & Garrard, 2005). By contrast, there was a significantly higher ligation frequency between the HSII anchor and the *hGH-N* promoter fragment in pituitary compared with liver chromatin from the same mouse (Fig 3B). The region between *CD79b* and *hGH-N*, represented by the p1 fragment, had a lower ligation frequency to HSII (Fig 3B). The low-level association between the HSII anchor and the p1 fragment is consistent with their linear proximity. The similar ligation frequency between p1 and the HSII anchor fragment in liver chromatin from the same mouse (Figs 2,3) further suggests that these low-level interactions do not contribute significantly to the activating function of the *hGH* LCR. These 3C results confirmed a specific close contact between the HSI,II region and the *hGH-N* promoter in pituitary chromatin.

Next, structural alterations at the active *hGH* locus were assessed for their dependence on HSI. The 3C assay was carried out using pituitary chromatin from two *hGH/P1(ΔHSI)* transgenic mouse lines (Fig 2B, grey bars). PCR analyses showed a pattern of ligation products in the pituitary chromatin of the *hGH/P1(ΔHS)I* mice that was essentially identical to those at the inactive *hGH/P1* locus in liver chromatin (Fig 2B, compare grey with white bars). Thus, looping of HSI,II to the active *hGH-N* promoter in pituitary chromatin is dependent on HSI. The HSI dependency of HSI,II juxtaposition to the *hGH-N* promoter was confirmed by using the HSII anchor primer (Fig 3B, grey bars). This HSI dependency links the specific chromatin conformation at the *hGH* locus in the pituitary to enhancement of *hGH-N* transgene expression (Ho *et al*, 2006).

In addition to the contact between the HSI,II region and the *hGH-N* promoter, 3C analysis also showed that the HSI,II region comes into close proximity with HSIII-HSV; the ligation frequency between HSII and HSIII-HSV fragments is significantly greater in the pituitary than in the liver of the *hGH/P1* mouse (Fig 3B). Of note, this interaction is maintained in the HSI-deleted transgene. Limited functional tests of HSIII-HSV have suggested that this region might have boundary functions (Jones *et al*, 1995), and acetylation of histones H3/H4 at HSV is also unique in its HSI independence (Ho *et al*, 2006). Surprisingly, although the HSI,II fragment crosslinks to both the HSIII-HSV region and the *hGH-N* promoter, there is no evidence for crosslinking between HSIII-HSV and the *hGH-N* promoter. This relationship might reflect dynamic switching of the HSI,II interactions between HSV and the *hGH-N* promoter (Liu & Garrard, 2005). High-resolution 3C analyses of the HSIII-HSV region will be useful in exploring further the configuration of this set of long-range interactions.

The chromatin conformations at the *hGH* transgene locus in pituitary and liver, and at the pituitary locus lacking an active HSI are schematically compared (Fig 3D). The HSI-dependent interaction between the *hGH-N* promoter and the LCR/*CD79b* domain of transcription is specific to the active locus in the pituitary. In the absence of HSI, this interaction is lost, whereas the interaction between HSII and the more 5′ end of the LCR is retained.

Looping is dependent on the LCR domain of transcription

To define further the basis for the interaction between the *hGH-N* promoter and the HSI,II regions, we determined whether looping was dependent on the presence of the LCR noncoding transcriptional domain or, alternatively, whether HSI might be sufficient for this interaction. We have previously shown that insertion of a PolII termination element (*TerF*) 3′ to HSI (Fig 4A, *hGH/P1(TerF)* transgene) selectively interrupts the downstream *CD79b* transcriptional subdomain and results in a six- to eightfold decrease in *hGH-N* transgene expression (Ho *et al*, 2006; Fig 4A, inset). This insertion has no significant impact on HSI formation or on HSI-dependent histone acetylation (Ho *et al*, 2006). 3C assays were performed on pituitary chromatin from two *hGH/P1(TerF)* transgenic mouse lines. The analyses, using both GH/CS and HSII primers as anchors in separate studies (Fig 4B,C), showed that *TerF* insertion effectively blocked looping between HSI,II and the *hGH-N* promoter. The frequency of the crosslinking between HSI,II and the *hGH-N* promoter was reduced to the level seen in the absence of HSI (compare Figs 2–4). Interactions between HSI,II and HSIII-HSV regions were retained in the presence of *TerF* (Fig 4C; data summarized in Fig 4D). We conclude that the full chromatin conformation at the active *hGH* locus is dependent on a functional LCR/*CD79b* domain of transcription. HSI, in the absence of this function, is not sufficient for chromatin reconfiguration and gene activation.

Activation and enhancement of *hGH-N* transcription by its LCR reflects a complex and multifaceted process. The crucial role of HSI in this process is evident from its essential functions in establishing linear domains of histone modification within the locus and in establishing the domain of noncoding PolII enrichment 5′ to the *hGH* cluster. The formation of the HSI-dependent LCR/*CD79b* domain of transcription seems to have a crucial function in the gene activation process; interruption of this domain by insertion of a PolII terminator results in the loss of the

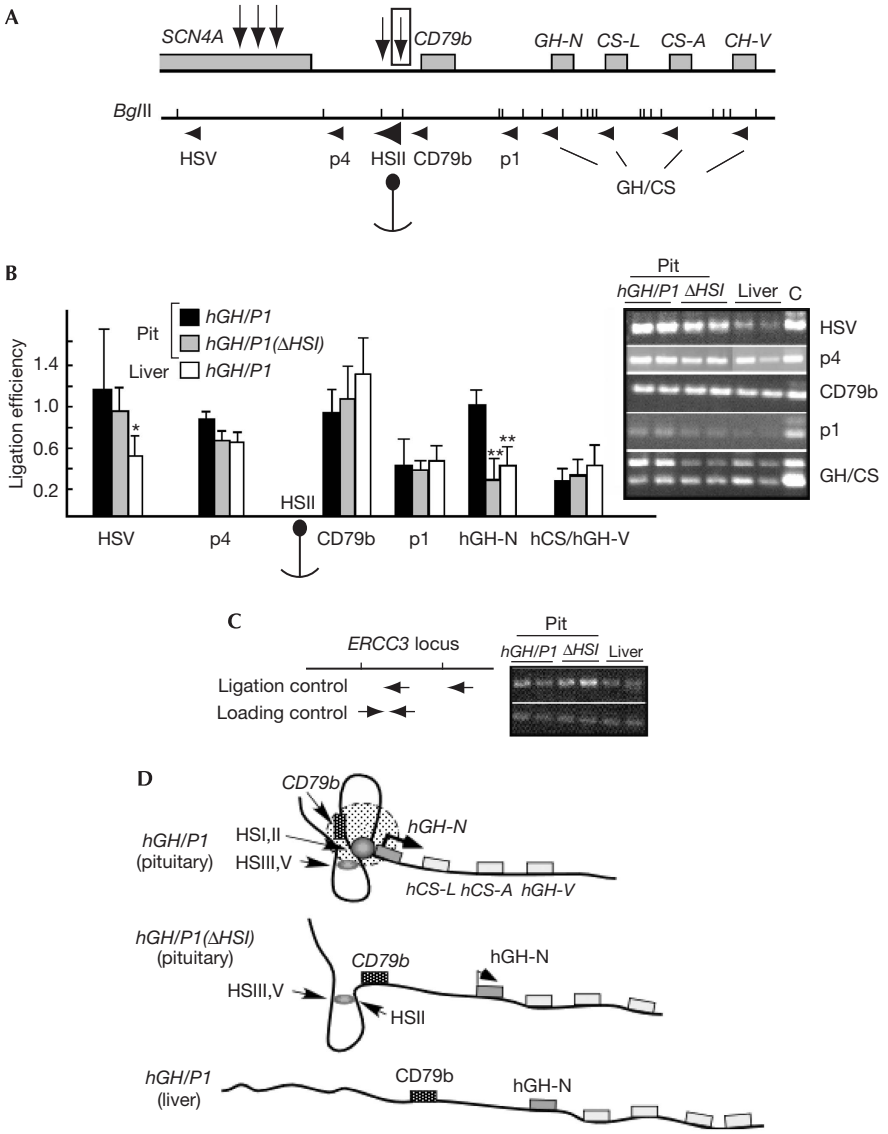

Fig 3 | Reversal of amplification primers in the 3C assay confirms the specific association between *hGH-N* and the HSI,II region. (**A**) Map of primer positions for 3C using the HSII region primer as the anchor. The symbols are as in Fig 2A. (**B**) HSI-dependent association between HSI,II and the *hGH-N* promoter, and evidence for a pituitary (Pit)-specific interaction between HSII and HSIII-HSV regions. The semiquantitative PCR analyses of 3C ligation products were normalized to parallel analyses of the endogenous *ERCC3* locus (**C**). Each bar represents the mean ± s.d. of four independent assays of the indicated chromatin samples: *hGH/P1* pituitary chromatin (black bars), *hGH/P1(ΔHSI)* pituitary chromatin (grey bars) and control liver chromatin (white bars). Lane C contains a PCR analysis of ligation products of *Bgl*II-digested *hGH/P1* plasmid DNA and represents random ligation of equimolar quantities of each of the *Bgl*II fragments in the locus under the conditions of the assay. The ligation efficiency shown on the *Y* axis was calculated using the equation $(\text{signal}_{\text{tissue}}/\text{signal}_{\text{random control}})/(\text{signal}_{ERCC3\ \text{ligation}}/\text{signal}_{ERCC3\ \text{loading}})$. (**C**) 3C analysis of the control *ERCC3* locus. The indicated head-to-head ligation frequency of two adjacent *Bgl*II fragments released from the ubiquitously expressed *ERCC3* locus during the 3C analysis was determined and used as an internal control for each 3C assay. PCR within a unique *Bgl*II fragment was used as the loading control for each analysis. (**D**) A looping model of pituitary-specific *hGH-N* activation. The 3C studies of the *hGH/P1* and *hGH/P1(ΔHSI)* transgene loci in the pituitary and the *hGH/P1* transgene locus in the liver are summarized in a diagrammatic format. 3C, chromosome conformation capture; *hGH*, human growth hormone.

chromatin conformation specific to the active locus and in a corresponding decrease in *hGH-N* expression. Remarkably, disruption of the LCR transcriptional domain does not alter histone acetylation throughout the locus (Ho *et al*, 2006). This suggests that the HSI-dependent histone acetylation at the *hGH* locus is independent of LCR transcription and is, by itself, insufficient for *hGH-N* activation. These relationships support a model in which histone acetylation constitutes an early step in

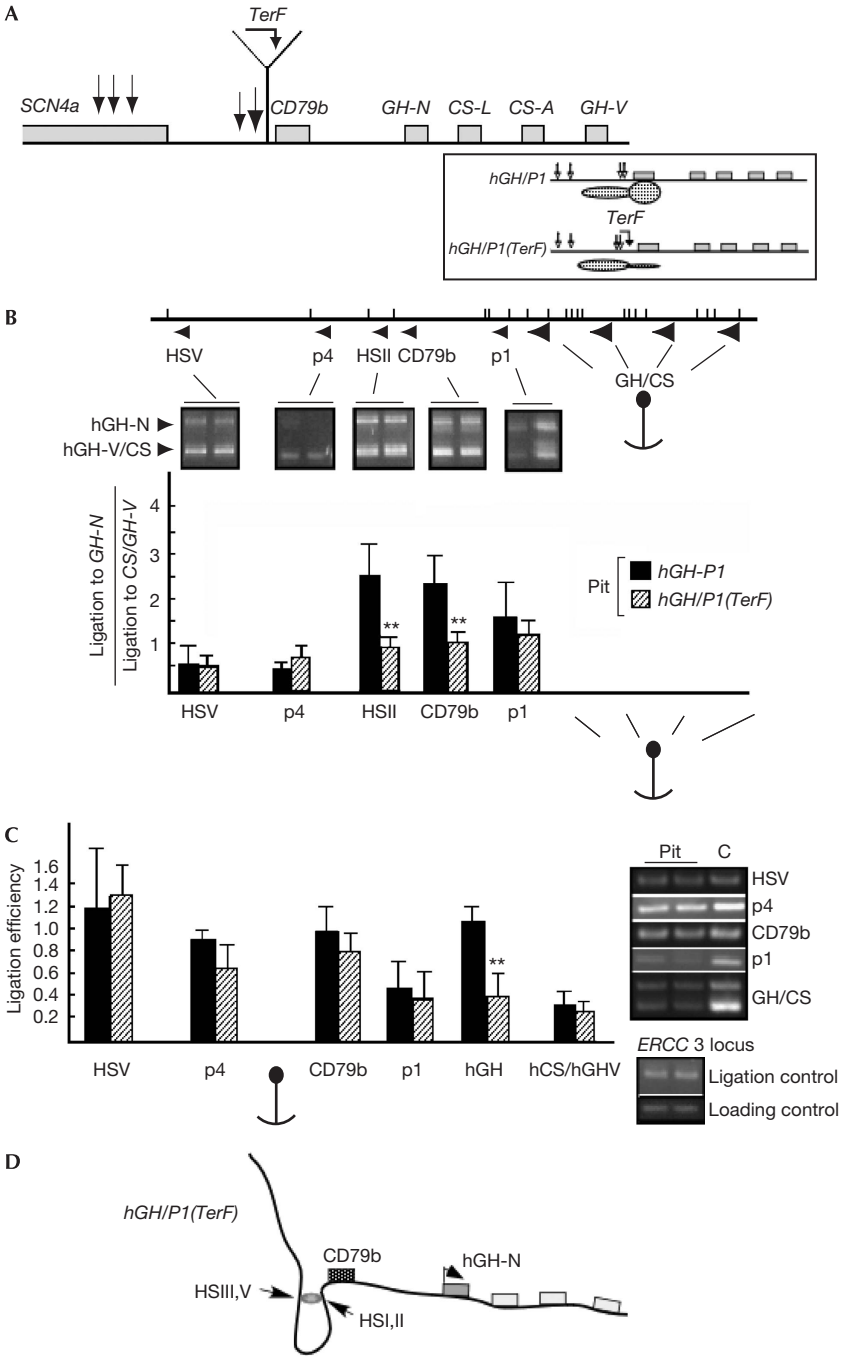

Fig 4 | Interruption of the locus control region domain of transcription by insertion of a polymerase II termination element (*TerF*) destabilizes the active chromatin conformation at the *hGH* locus. (**A**) A map of the *hGH* cluster with the position of the TerF PolII termination element indicated. The angled '*TerF*' arrow indicates the position and orientation of the inserted *TerF* PolII termination element. The diagram in the box shows the impact of the *TerF* mutation in these lines on the levels of noncoding transcripts 5′ and 3′ to the insertion site (Ho *et al*, 2006). The *TerF* insertion selectively represses the transcriptional activity of PolII in the *CD79b* subdomain of the LCR/*CD79b* domain of transcription (stippled ovals). (**B**) Summary of the 3C analysis of the *hGH/P1(TerF)* pituitary (Pit) chromatin using the GH/CS primer as the anchor. The 3C was carried out on pituitaries of mice representing two *hGH/P1(TerF)* transgenic lines. Representative semiquantitative PCR assays are shown below the map. Each histogram bar represents the mean ± s.d. of three separate analyses from each of two lines ($n = 6$). The previous results of the 3C analysis of the *hGH/P1* pituitary chromatin (black bars) are shown to facilitate direct comparison with the *hGH/P1(TerF)* results (stippled bars); $^{**}P < 0.001$. (**C**) Summary of the 3C analysis of the *hGH/P1(TerF)* pituitary chromatin using the HSII primer as an anchor. The 3C was performed as in (**A**); $^{**}P < 0.001$. (**D**) The spatial conformation of *hGH/P1(TerF)* locus in the pituitary in the absence of *CD79b* noncoding transcription. 3C, chromosome conformation capture; *hGH*, human growth hormone; LCR, locus control region.

locus activation. This initial step is followed by the establishment of the LCR/*CD79b* domain of transcription with subsequent reorganization of the chromatin locus and enhancement of *hGH-N* expression.

These studies showed that an active LCR/*CD79b* domain of transcription is essential and a probable prerequisite to looping between HSI,II and the *hGH-N* promoter. These data, along with our previous studies of PIT-1 *trans*-acting factor binding in HSI and the promoter of *hGH-N* (Shewchuk *et al*, 1999; Ho *et al*, 2002), support a model in which occupancy of PIT-1 at both HSI and the *hGH-N* promoter triggers interactions between the two regions that are subsequently stabilized by the noncoding transcriptional activity (Fig 3D). The final chromatin conformation at the active locus places the *hGH-N* promoter in close proximity to the PolII-enriched LCR/*CD79b* transcriptional domain. This relocation of a promoter into an environment rich in elongating PolII is reminiscent of reports of close physical relationships between certain activated genes and PolII-enriched subnuclear 'transcriptional factories' (Iborra *et al*, 1996; Grande *et al*, 1997; Osborne *et al*, 2004; Ragoczy *et al*, 2006). Whether *hGH-N* gene activation is linked to an association with subnuclear transcription factories, or whether the juxtaposition of the promoter with the local accumulation of PolII within its LCR is sufficient will be of interest in future studies. Irrespective of this relationship, the observation that loop formation and expression at the *hGH* locus are both lost when LCR transcription is interrupted suggests that formation of this domain and looping are interdependent and mutually sustaining processes in the pathway of long-range transcriptional control.

METHODS

Transgenic mouse lines. All transgenic mouse lines used in this study were established as described previously; the *hGH/P1* mouse lines were 809F and 811D (copy numbers of transgene are 4 and 5, respectively; Su *et al*, 2000), *hGH/P1(ΔHSI)* lines were 960G and 969E (copy numbers 3 and 4; Ho *et al*, 2002), and *hGH/P1(TerF)* lines were 1301C and 1301G (copy numbers are 3 and 5; Ho *et al*, 2002). ChIP and 3C assays were performed using 3- to 4-month-old compound transgenic mouse lines carrying *hGH/P1*, *hGH/P1(ΔHSI)* or *hGH/P1(TerF)* transgene along with a growth hormone-releasing factor (*hGRF*) transgene under the control of the metallothionine promoter. The *hGRF* transgene stimulates selective expansion of the somatotrope population with consequent pituitary hypertrophy (Mayo *et al*, 1988).

Chromatin immunoprecipitation assays. ChIP was performed as described previously (Ho *et al*, 2006). The sequences of the primers have also been described previously (Ho *et al*, 2006). Each ratio of bound fraction to input was normalized to the corresponding ratio for a DNA segment within the endogenous skeletal muscle-specific *mMyoD* gene (Weintraub *et al*, 1991; Sawado *et al*, 2003).

Chromatin conformation capture assay. The 3C procedure was performed as described previously (Tolhuis *et al*, 2002), but with modifications as described in the supplementary information online.

PCR analysis of chromatin conformation capture ligation products. The semiquantitative PCR analysis is described in the supplementary information online. The ligation efficiency was determined as a ratio of products ligated to the *hGH-N* promoter relative to the *hCS/hGH-V* promoter fragments when the GH/CS primer functioned as the anchor primer (Figs 2B,4B), or relative to an internal control at an endogenous *ercc3* locus when the HSII primer functioned as the anchor primer (Figs 3B,4C). Data were plotted as mean + s.d. and *t*-tests were performed to determine the level of significance in binary comparisons.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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