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An RNA-Independent Linkage of Noncoding Transcription to Long-Range Enhancer Function

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The detection of noncoding transcription at multiple enhancers within the mammalian genome raises critical questions regarding whether and how this activity contributes to enhancer function. Here, using *in vivo* analysis of a human growth hormone (*hGH*) transgene locus, we report that activation of a domain of noncoding transcription adjacent to the long-range *hGH-N* enhancer, HSI, is established by the enhancer independent of any interactions with its target promoter. We further demonstrate that the appearance of this enhancer-linked noncoding transcription is temporally and spatially concordant with induction of *hGH-N* in the embryonic pituitary. Finally, we show that the level of transcriptional enhancement of *hGH-N* by HSI is directly related to the intensity of HSI-dependent noncoding transcription and is fully independent of the structure of the locally transcribed RNA. These data extend our understanding of the relationship of long-range enhancer activity to enhancer-dependent noncoding transcription and establish a model that may be of general relevance to additional mammalian loci.

Alterations in the cellular transcriptome drive critical developmental pathways. These changes in mRNA representation are heavily dependent on selective controls of gene transcription. Whereas the accuracy of transcriptional initiation is established by promoter elements, the timing and levels of gene expression are often controlled by regulatory determinants that are remotely situated from their target promoters (10, 45). The activities of these remote determinants track with alterations in the structure and higher-order configuration of defined chromatin domains (22, 38, 48). What remains unclear is how these remote elements are themselves activated and organized and how they impact target promoters.

Mechanisms of enhancer function are intimately linked to the recruitment of macromolecular complexes that impart covalent and higher-order alterations in chromatin structure (4, 19, 20). These epigenetic modifications can be confined to *trans*-factor recruitment sites or they can be quite extensive. A number of models link specific subsets of structural alterations at enhancers to the transcriptional activation (13, 34). The way in which distal enhancers impact their target promoters is of current interest. One model invokes a linear progression ("tracking") of factors and/or polymerase II (PolII) from the enhancer recruitment site to the target promoter (17, 51). In another model, the enhancer-promoter interactions are mediated via long-range "looping" that brings the corresponding sites into direct contiguity (6, 35, 47). The extensive use of technologies designed to capture native chromatin conformations has revealed that major alterations in higher-order chromatin structure may be a common occurrence in both the activation and the repression of target genes by remote regulatory elements (11, 26, 28). Finally, alterations in the composition and configuration of a locus can be linked to reorganization of the nuclear architecture, localizing a gene within regions conducive to either transcriptional enhancement or to solidification of gene silencing (36).

Recent studies indicate that the function of an enhancer may be accompanied by its own transcription (12, 16, 33). A role for this enhancer-linked "noncoding" transcription in gene regulation has been inferred from genome-wide surveys (30, 31). Such observations, while intriguing, are essentially descriptive. Mechanistic links between enhancer transcription and promoter activity need to be more fully explored. The structures of these noncoding transcription units are in most cases undefined, and whether their establishment is enhancer autonomous or dependent on cooperative interactions between the enhancer and its target promoter has been only minimally explored. Finally, it remains to be established whether noncoding transcriptional activity at enhancer elements plays an essential role in gene expression and whether such activity relates to transcription *per se* or is dependent on actions of the enhancer-encoded RNAs. It is clear from recent studies that maximally informative experimental approaches to these complex problems necessitate the exploration of transcriptional regulatory circuits in intact, physiologically relevant settings (43).

The mammalian growth hormone gene (*GH*) gene is selectively and robustly expressed in somatotrope cells of the anterior pituitary. In the case of the human GH locus (*hGH*), this expression is under the control of a set of remote regulatory elements that constitute the *hGH* locus control region (*hGH* LCR) (27). The *hGH* LCR encompasses four DNase I hypersensitive sites (HS) in pituitary chromatin located 14.5 to 32 kb 5′ to the *hGH-N* transcription start site (27) (Fig. 1A). These elements are collectively sufficient to establish an autonomous chromatin domain that supports robust, pituitary-specific, and developmentally appropriate expression of an *hGH* transgene irrespective of its site of integration in the mouse genome (2, 27, 44). A single, defined pituitary-specific component of this LCR, HSI, located 14.5 kb 5′ to the *hGH* gene promoter, serves an essential function in the *hGH-N* transcriptional enhancement (22). Site-specific inactivation of critical *trans*-acting factor binding sites within HSI results in a 20-fold decrease in *hGH-N* expression *in vivo* (22). Thus, HSI

Received 1 December 2011 **Returned for modification** 27 December 2011
Accepted 6 March 2012
Published ahead of print 19 March 2012
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doi:10.1128/MCB.06650-11

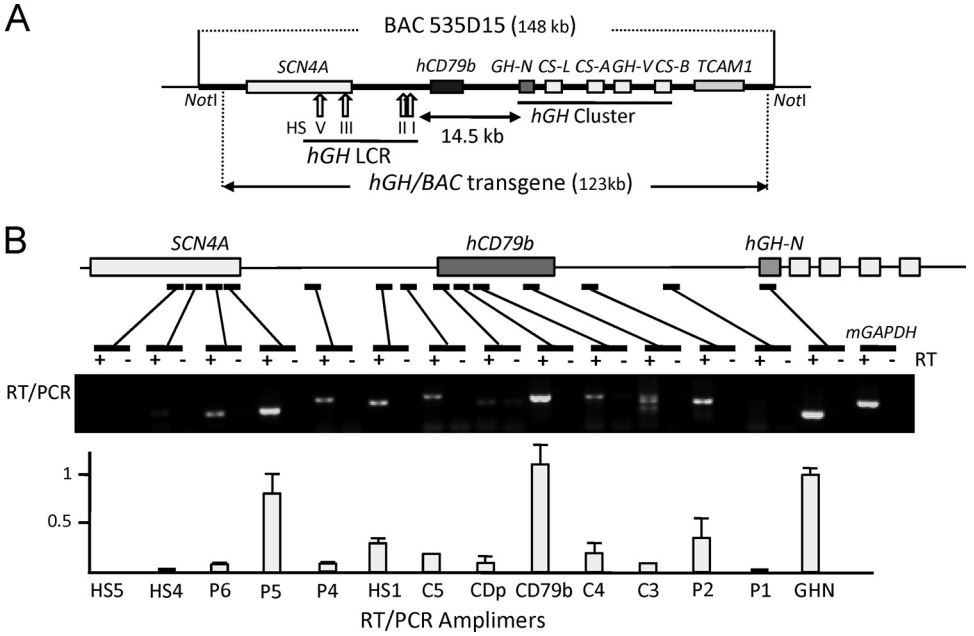


FIG 1 Transcript mapping across the *hGH/BAC* transgene in the mouse pituitary revealed a peak of transcriptional activity across the *hCD79b* region. (A) Map of the *hGH/BAC* transgene. The 123-kb *hGH/BAC* transgene, released from the originating BAC clone by NotI digestion, was used to generate the *hGH/BAC* transgenic mouse lines (49). Each structural gene is indicated by a labeled box (exonic substructures are not shown). The vertical arrows labeled with roman numerals indicate the positions of DNase I hypersensitive sites (HS) that form in pituitary chromatin and constitute the *hGH* LCR. HSI,II are pituitary-specific DNase I HS of the *hGH* LCR. (B) Transcriptional profile across the *hGH/BAC* transgene. The presence of transcripts corresponding to each of 14 sites across the *hGH/BAC* transgene locus was determined by RT-PCR of transgenic mouse pituitary RNA. The position of each amplimer is indicated on the below the map by a bar and the sequences are listed on Table 1. Reactions were carried out in the presence or absence of reverse transcriptase (+ and −, respectively). Amplified cDNAs were analyzed on a 1% agarose gel. The concentration of each amplified RNA segment, shown in the histogram, was determined by PhosphorImager quantification of corresponding [α-^{32}P]dCTP-labeled RT-PCR products generated by a separate set of reactions. Each value was normalized to a parallel amplification of genomic DNA to adjust for minor differences in amplification efficiency. The data are normalized to an arbitrary value of 1.0 for *hGH-N* mRNA. A robust transcriptional domain was detected over *hCD79b*, located between HSI and its target *hGH-N*.

constitutes a model of a potent long-range enhancer of *in vivo* gene expression.

The *hGH* LCR, extending 5′ from HSI to HSV, is itself bidirectionally transcribed by PolII in the pituitary, and this transcriptional activity is HSI dependent (23). Remarkably, a gene encoding a B-cell-specific, transmembrane receptor protein is situated immediately 3′ to HSI, between HSI and its target *hGH-N* promoter (3) (Fig. 1A). This *hCD79b* locus is robustly transcribed in the pituitary, as well as in B cells, although Igβ, the encoded protein, is only produced in B cells (5). Site-specific inactivation of HSI results in a loss of *hCD79b* transcription in the pituitary with a corresponding loss of *hGH-N* gene expression (5). This same mutation has no adverse effect on *hCD79b* in B cells (5). Remarkably, insertion of a PolII termination element between HSI and *hCD79b* represses *hCD79b* transcription in the pituitary with a comparable loss in *hGH-N* expression. This PolII terminator insertion has no effect on the formation of HSI itself, nor does it repress the bidirectional transcription between HSI and HSV (23). These data support a model in which noncoding transcription across the *hCD79* region, immediately 3′ to HSI, plays an essential and specific role in HSI-mediated long-range enhancement of *hGH-N* gene transcription. In the present study, we explore the mechanistic basis for the activation and function of this domain of noncoding transcription. The data revealed a quantitative relationship between noncoding transcription 3′ to HSI and the enhancement of *hGH-N* transcription. These data further demonstrated that the HSI enhancer activity is a direct effect of noncoding transcription *per se* and is fully independent of the structure of this encoded RNA.

MATERIALS AND METHODS

BAC transgene modifications and generation of transgenic mouse lines. Modifications were introduced in the *hGH/BAC* transgene according to a published protocol (15). The primer sets for constructing shuttle vectors are shown in Table 1. Modified BAC DNAs were linearized with NotI prior to microinjection. The released 123-kb DNA fragment was microinjected into fertilized mouse oocytes (C57BL/6×SJL) to generate the transgenic lines. The University of Pennsylvania Transgenic & Chimeric Mouse Core carried out all microinjection procedures under IACUC approved protocols. Transgenic founders were identified by PCR and Southern blot analysis of tail genomic DNA. The *−8.0CD79b* and *−1.3CD79b* transgenic lines had been previously described (5).

RNA isolation and Northern blot analysis. Total RNA was extracted from tissue samples with RNA-Bee (Tel-Test) according to the manufacturer's procedure. A 10-μg portion of each RNA sample was separated on a 1.2% agarose gel containing 2.2 M formaldehyde in 1× morpholinepropanesulfonic acid and transferred to a Zeta-probe membrane (Bio-Rad). The membrane was incubated with ^{32}P-labeled probes in hybridization buffer at 65°C overnight and washed (2× SSC–0.1% sodium dodecyl sulfate [SDS] and 1× SSC–0.1% SDS) at 65°C, and signals were detected by exposure to X-ray film (Kodak).

Reverse transcription-PCR (RT-PCR) and quantitative RT-PCR (qPCR). Isolated total RNAs were treated with RQ DNase (Promega) and purified using an RNeasy minikit (Qiagen). In all cases, 1 μg of purified RNA was reverse transcribed by SuperScript III reverse transcriptase (In-

TABLE 1 Oligonucleotides used in this study

Method and oligonucleotide	Sequence (5′–3′)
hGH/BAC transgene modification	
(*CD*Δ*1.6*)*hGH/BAC*	
L-arm-5′	CAGGGCGCGCCGACGATTTAGCATCTCTTCC TCTCCTGGG
L-arm-3′	GTGGAATTCACTGGGAGAAGATTCAGTCCA GGTC
R-arm-5′	GGGCGAATTCTGTGCGACTATGTCCTGTGTCC
R-arm-3′	GCCATTAATTAATATGCCCATTACAACAGCCT
(Δ*CD*λ)*hGH/BAC*	
L-arm-5′	Same as with (*CD*Δ*1.6*)*hGH/BAC*
L-arm-3′	CCACCTCGAGAAGATTCAGTCCAGGTC
R-arm-5′	AGGAGTCATCTCGAGGTCGCCCCATGACCT GGGTGCAG
R-arm-3′	CCCACTTAATTAAGGGCTCACAGATGCCACA TTC
λ-5′	CAATCTCGAGCCGGATTCGGTATGGCTG
λ-3′	GCTTCTCGAGACACCTTATGTTCTATAC
RT-PCR	
HSV-5′	CTTGCCCAGTCCTCACACTT
HSV-3′	CTGAGGCTTCTGTCCTCCTT
HSIV-5′	TGCCTCTACGTGGACATCTC
HSIV-3′	TATCAGCAGAGAGTGCACAA
P6-5′	CTGGGTGGCGTAGAGATG
P6-3′	GACCCACGTTGTCGTAGTTG
P5-5′	GCCTCAAAACCTGATTGG
P5-3′	GGAGATCTCTGAGGCTGG
P4-5′	GCTGTATTCTTCCAGACAAG
P4-3′	GAGCTAAGCTATGAGGATGC
HS1-5′	CCAAGCCTTTCCCAGTTATAC
HS1-3′	GATCTTGGCCTAGGCCTC
P3-5′	CTTCCCCCAGAAGACTGA
P3-3′	GGAGGAAAACGTGTAACTGC
C5-5′	AGACCCTCTGCCTTCCAACCATGGCAT
C5-3′	CACTGGGAGAGATTCAGTCCAGGTC
CDp-5′	GGACAGGTGCCTATTTCGCTC
CDp-3′	GACCCCAAACCCGTGACAAC
CD79b-5′′	GACCATGGCCAGGCTGGCGTTGTCTCCTGT
CD79b-3′	AGCGTCTGGATCATGATGATACCATCCTTC
C4-5′	GGAGGAAGATCACACCT
C4-3′	ATCCCCAGAGAACTCCC
C3-5′	GTTCTCTGGGGATGGACGGGACCCAGCC
C3-3′	TGGCATGCAGCCCCGTTCCAG
P2-5′	TGCTCAGACCAGCCTATGCA
P2-3′	TCAACAGGAAGTGGAGCACA
P1-5′	GATTACAAGCGCCCACTACC
P1-3′	GAGAGAATAAGCCAGGAGGTG
GHN-5′	TTTGACAACGCTATGCTCCG
GHN-3′	GCCAAAAGGGTCATCATCTC
mGAPDH-5′	GCCAAAAGGGTCATCATCTC
mGAPDH-3′	CTGCTTCACCACCTTCTTGA
λ5-5′	CGCTTATGCGGATTATTGCCGTAG
λ5-3′	ACCTCTCTGCCTGCGATGGTTGGAG
λm-5′	TACCGCAAGCAGCTTGGCCTG
λm-3′	AGCATCAGCTAACTCCTTCG
λ4-5′	GTCAGTCAGTGCGTGAAGCC
λ4-3′	CTACTCGCTACTGCGCTGGC
λ3-5′	GGACGTTTCTATAAGATGCGTG
λ3-3′	GTCGGTTGTATTTCCCTCCAG

vitrogen) with random primers, amplified using a primer set (Table 1), and separated on 1% agarose gel. For qPCR analyses, 1 μg of purified RNA was reverse transcribed into cDNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems). The levels of *hGH-N* (Hs00236859_m1), *hCD79b* (Hs00236881_m1), λ, and *mGAPDH* (Mm99999915_g1) RNAs were assessed by using TaqMan Universal PCR Master mix (Applied Biosystems), and real-time PCRs were performed by a 7900HT machine and analyzed using SDS2.2 software.

DIG-labeled RNA probe synthesis, embryo sectioning, and *in situ* hybridization. 0.5 kb cDNA of each gene (*hGH-N*, *hCD79b*, *Pit-1*, *Prop-1*, *Shh*, and *Pitx1*) in the pGEM-T Easy vector (Promega) was transcribed *in vitro* to generate digoxigenin (DIG)-labeled antisense RNA probes. Embryos were collected at specified days postcoitus and fixed overnight at 4°C in 4% paraformaldehyde. The fixed embryos were then washed in phosphate-buffered saline (PBS), immersed in 30% sucrose in PBS overnight at 4°C, embedded in OCT (Sakura), quick frozen on dry ice, and cryosectioned at 20 μm. Sectioning and *in situ* hybridizations were performed as described previously (37). Briefly, sections were washed in PBS, fixed in 4% paraformaldehyde for 10 min, washed in PBT (1× PBS with 0.1% Tween 20), and permeabilized with 1.0 μg of proteinase K/ml for 10 min. Sections were then washed in PBT, fixed in 4% paraformaldehyde for 5 min, acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min, washed in PBT, and air dried. The sections were then incubated with RNA probes in 150 μl of hybridization buffer (10 mM Tris [pH 7.5], 600 mM NaCl, 1 mM EDTA, 0.25% SDS, 10% dextran sulfate, 1× Denhardt solution, 200 μg of yeast tRNA/ml, 50% formamide), covered with Hybri-slips (Sigma), and incubated overnight at 65°C in a humidified box. The next day, coverslips were removed and washed for 30 min in 1× SSC–50% formamide at 65°C. The slides were then transferred to TNE (10 mM Tris [pH 7.5], 500 mM NaCl, 1 mM EDTA) at 37°C for 10 min, incubated in RNase A in TNE for 30 min at 37°C, and washed in TNE for 10 min. Sections were washed in 2× SSC for 20 min at 65°C, washed twice in 0.2× SSC for 20 min at 65°C, and transferred into MABT (100 mM maleic acid, 150 mM NaCl, and 0.1% Tween 20 [pH 7.5] with NaOH). The slides were blocked in 2% blocking reagent (Roche) in MABT with 20% heat-inactivated goat serum (Sigma) for at least 1 h and then incubated overnight at 4°C with anti-DIG alkaline phosphatase-conjugated antibodies (Roche) in MABT blocking buffer with 5% serum. Slides were washed in MABT and equilibrated in NTM (100 mM Tris [pH 9.5], 100 mM NaCl, 50 mM $MgCl_2$) for 10 min. Color detection was performed using BM purple (Roche) in a humidified box at room temperature for 4 to 12 h.

RESULTS

Transcription within the *hGH* transgene locus 3′ to HSI is robust and noncontiguous with the *hGH-N* promoter. We previously mapped the location of the B-cell-specific gene, *hCD79b*, to a site immediately 3′ to HSI, between the *hGH* LCR and *hGH-N* (3, 5) (Fig. 1A). Although *hCD79b* is transcribed in the pituitary, its encoded product, the immunoglobulin receptor Igβ subunit, remains B cell restricted (5). This noncoding transcription of *hCD79b* in the pituitary is dependent on the activity of HSI, whereas the transcription of *hCD79b* in B cells is controlled by a distinct set of B-cell-specific promoter-proximal determinants (1, 39, 46). Our studies initially suggested a model in which *hCD79b* transcription represented a functionally neutral "bystander" event reflecting its fortuitous location in an "activated" chromatin environment between *hGH* and its LCR in pituitary (5). This proposed functional neutrality was subsequently brought into question by the observation that targeted repression of *hCD79b* transcription in the pituitary resulted in a dramatic loss of *hGH-N* expression (23). These data led us to conclude that noncoding transcription across the *hCD79b* region plays an essential role in the HSI-dependent enhancement of *hGH-N* expression.

Prior to exploring the basis for *hCD79b* transcription in the pituitary and its mechanistic relationship to HSI enhancer function, we mapped the transcriptional architecture across an intact

hGH locus in the pituitary of an *hGH/BAC* transgenic mouse. The *hGH/BAC* 123-kb transgene encompasses the full *hGH* LCR, the five-gene *hGH* cluster, and the intervening *hCD79b* (Fig. 1A). Analysis of pituitary RNA from an *hGH/BAC* transgenic mouse revealed various levels of transcription throughout the LCR and a discrete robust peak of transcription coincident with *hCD79b*. The level of *hCD79b* transcription was equivalent to that at the *hGH-N* gene, as assessed by mRNA abundance (Fig. 1B). Of note, there was a gap in transcription between the 3′ end of *hCD79b* and the *hGH-N* promoter ("P1" amplimer). This gap is concordant with a corresponding gap of PolII occupancy in this region (23). These data led us to conclude that transcription across *hCD79b* is robust and circumscribed and that PolII does not linearly track between *hCD79b* and *hGH-N*.

The domain of noncoding transcription 3′ to HSI can be established in the pituitary independent of the *hCD79b* promoter. *hCD79b* transcription in pituitary is HSI dependent (5). The basis for this transcriptional activation is unclear. We sought to determine whether the *hCD79b* promoter, as previously defined in B-cell-expression studies (1, 21, 39, 46), is necessary for this activity. The promoter and contiguous 5′ end of the *hCD79b* gene (extending through exon 2) was deleted from the *hGH/BAC* transgene (Fig. 2A). Six corresponding (*CDΔ1.6*)*hGH/BAC* transgenic mouse lines were established. The structure of the (*CDΔ1.6*)*hGH/BAC* transgene was validated by detailed mapping of the corresponding mouse genome (data not shown). Remarkably, the expression of *hCD79b* mRNA in the mouse pituitary was unaltered by this extensive 5′ deletion. In contrast, this same 5′-terminal deletion resulted in complete loss of *hCD79b* expression in the B cells of the (*CDΔ1.6*)*hGH/BAC* mice (Fig. 2B). It was of note that the amount and size of the *hCD79b* mRNA, as assessed by Northern analysis of each of the (*CDΔ1.6*)*hGH/BAC* mouse pituitaries, was essentially the same as in the pituitary of the mouse carrying the intact *hGH/BAC* transgene (Fig. 2B and data not shown). The lack of any apparent change in size of the *hCD79b* mRNA generated from the *CDΔ1.6*)*hGH/BAC* transgene most likely reflects the small sizes of exon 1 (76 bp) and exon 2 (51 bp). We further demonstrated that the mRNA generated from the 5′-deleted *hCD79b* gene [(*CDΔ1.6*)*hGH/BAC*] was initiated within intron 2 (5′ rapid amplification of cDNA ends assay; unpublished data). The position of this new intronic transcription start site was fully consistent with the size of the resultant *hCD79b* mRNA in the pituitary, as detected on Northern analysis. These data led us to conclude that *hCD79b* transcription in the pituitary is not dependent on the *hCD79b* promoter/enhancer elements that had been previously defined to operate in B cells.

Enhancer action of the noncoding transcription domain 3′ to HSI is independent of RNA structure. The key role played by *hCD79b* transcription in HSI enhancer function could reflect the structure of the encoded *hCD79b* RNA. Alternatively, it could reflect transcriptional activity through this region in a manner that is independent of *hCD79b* RNA sequence or structure. To distinguish between these two models, we replaced the *hCD79b* gene with an identically sized fragment of bacteriophage λ DNA [(Δ*CD*λ)*hGH/BAC*; Fig. 3A]. The replaced *hCD79b* segment extended from bp −500 (relative to the native *hCD79b* transcription start site) through the coding region, preserving only the 3′-processing signals. Six (Δ*CD*λ)*hGH/BAC* transgenic mouse lines were generated and studied. Remarkably, a prominent 3.3-kb λ RNA band was observed by Northern blotting of the pituitaries

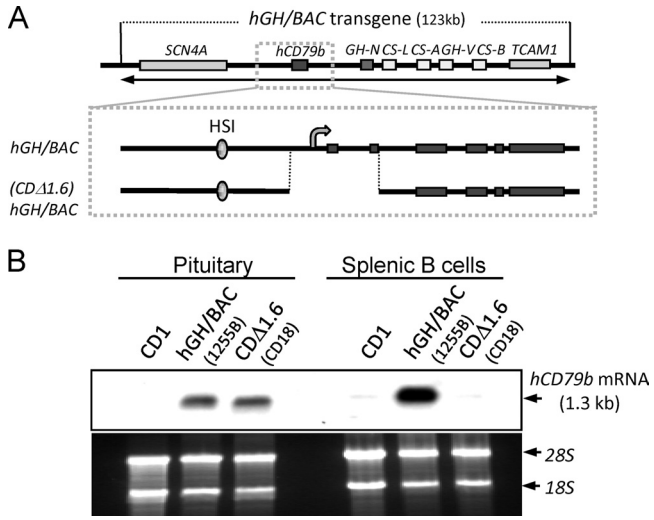


FIG 2 *hCD79b* transcription can be activated in the pituitary in the absence of the *hCD79b* promoter. (A) Diagram of the *hGH/BAC* transgene and a derivative transgene lacking the 5′ terminus of *hCD79b*. A 1.6-kb deletion from the *hGH/BAC* encompassing the promoter and first two exons was created by homologous recombination in *E. coli* to generate (*CDΔ1.6*)*hGH/BAC*. The site of transcription initiation (promoter) encompassed by the deletion in (*CDΔ1.6*)*hGH/BAC* is indicated by a bent arrow. The structure of the transgene was confirmed by restriction mapping, targeted amplification, and sequencing across critical regions. The modified BAC DNA was released from the plasmid by NotI digestion, purified, and injected into fertilized mouse oocytes to generate transgenic mouse lines. (B) Deletion of the promoter and 5′ end of *hCD79b* [(*CDΔ1.6*)*hGH/BAC* transgene] selectively ablates *hCD79b* mRNA expression in B cells but not in the pituitary. Pituitary and B-cell RNAs from a mouse transgenic for the intact *hGH/BAC* (line 1255B) and the (*CDΔ1.6*)*hGH/BAC* line (CD18) were analyzed by Northern hybridization, along with a sample from a nontransgenic mouse (CD1) as a negative control. Membranes were probed with a segment of the *hCD79b* cDNA. The position of the *hCD79b* mRNA (~1.3 kb) is indicated to the right of the autoradiograph. Visualization of 28S and 18S rRNA (lower panel) was used to control for gel loading and RNA quality. The data reveal that the promoterless transgene was expressed in pituitary, but not in splenic B cells of the same (*CDΔ1.6*)*hGH/BAC* mouse.

isolated from all but one of these lines (Fig. 3B and E). The expression of this "λ RNA" was pituitary specific (Fig. 3C). Based on the size of this RNA and the position of the retained *hCD79b* 3′-processing signal, the initiation of λ RNA transcription was mapped to a site located 0.7 kb within the λ DNA replacement cassette, ~1.4 kb 3′ to HSI. These data led us to conclude that transcription can be initiated 3′ to HSI in a manner independent of native promoter elements. This conclusion was fully consistent with the analysis of the (*CDΔ1.6*)*hGH/BAC* transgene (Fig. 2).

To further explore the relationship between the noncoding transcription 3′ to HSI and the activity of the *hGH-N* gene, we assessed the distribution of transcription in the pituitary across the (Δ*CD*λ)*hGH/BAC* transgene locus. The study revealed a peak of transcription coinciding with the λ DNA insert, followed by a gap in transcripts between the (λ) insert 3′ terminus containing the *hCD79b* 3′ processing signals and the *hGH-N* promoter (Fig. 3D). This transcriptional profile was remarkably consistent with that observed at the native locus (Fig. 1B); both studies demonstrated variable transcription at sites within the LCR region and robust transcription 3′ to HSI. Further, these studies revealed the same discontinuity between the HSI-dependent noncoding transcription domain (*hCD79b* or λ) and the *hGH-N* promoter. These

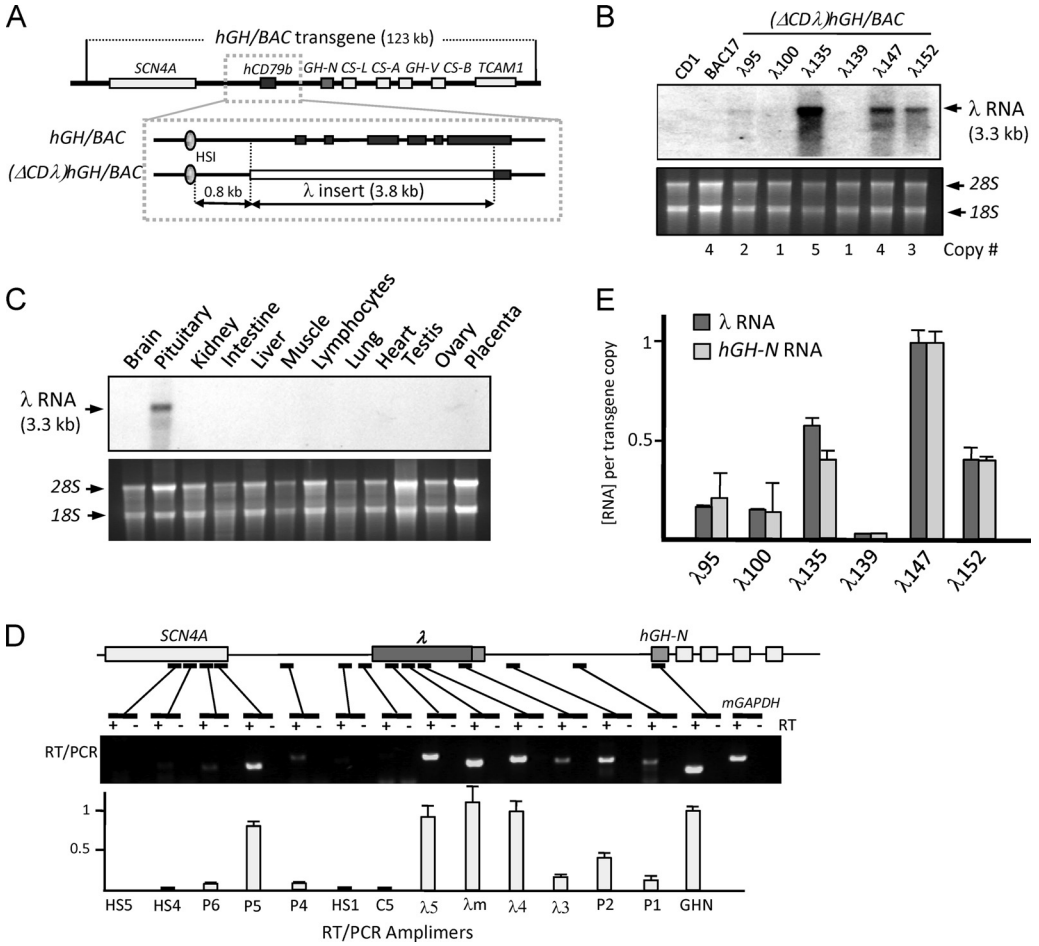


FIG 3 The intensity of transcription 3′ to HSI is a direct, quantitative, and RNA-independent determinant *hGH-N* expression. (A) Replacement of the *hCD79b* gene in the *hGH/BAC* transgenes by a segment of λ phage DNA. An expanded view of the HSI/*hCD79b* region of the *hGH/BAC* is shown below the map of the *hGH/BAC* transgene. The *hCD79b* gene (exons; dark rectangles), extending from −0.5 kb to the middle of exon 6, was substituted with an identically sized fragment of bacteriophage λ DNA (3.8 kb, open rectangle). The segment of the *hCD79b* exon 6 containing its 3′ processing determinant was retained 3′ to the λ DNA insert. The resultant (Δ*CD*λ)*hGH/BAC* transgene was released from vector sequences and microinjected into fertilized mouse oocytes. Six transgenic lines were established and studied. (B) Northern blot analysis of pituitary RNA from (Δ*CD*λ)*hGH/BAC* transgenic mice reveals the expression of λ RNA. Total pituitary RNA from each of the six (Δ*CD*λ)*hGH/BAC* lines, from a nontransgenic control mouse (CD1), and from a mouse line carrying the intact *hGH/BAC* (line BAC17) were resolved on a 1.2% denaturing agarose gel, and Northern blot hybridization was performed using a λ DNA probe. Ethidium bromide-stained rRNA on the gel was used to assess equivalent RNA loading and quality control. The transgene copy number of each line is indicated below the gel. Five of the six (Δ*CD*λ)*hGH/BAC* lines expressed a 3.3-kb λ RNA. The levels of λ RNA varied among the lines (see also panel E). (C) Expression of the λ RNA from the (Δ*CD*λ)*hGH/BAC* transgene is restricted to the pituitary. A Northern blot of RNAs isolated from the indicated tissues of a (Δ*CD*λ)*hGH/BAC* transgenic mouse (λ135) was probed with a ^{32}P-labeled λ DNA segment. rRNA controls are as described in panel B. The 3.3-kb λ transcript was expressed only in pituitary. (D) Transcription mapping across the (Δ*CD*λ)*hGH/BAC* transgene in the pituitary. Transgenic (Δ*CD*λ)*hGH/BAC* pituitary RNA was subjected to RT-PCR using the 14 primer sets (Table 1) shown below the transgene map. Amplification of *mGAPDH* mRNA served as a positive control. RT-PCR products were assayed on an ethidium bromide-stained agarose gel. The concentration of each amplified segment shown in the histogram was measured by PhosphorImager quantification of corresponding [α^{32}P]dCTP-labeled RT-PCR. Each value was normalized to a parallel amplification of genomic DNA to adjust for minor differences in amplification efficiency. The peak of transcription 3′ to HSI (corresponding to the λ DNA insert) and the gap of transcription between this region and the *hGH-N* promoter from the (Δ*CD*λ)*hGH/BAC* transgene demonstrated a remarkable conservation with the pattern of transcription across the native locus (compare to Fig. 1). (E) Direct and quantitative relationship between λ RNA levels and *hGH-N* mRNA levels in the pituitaries of six (Δ*CD*λ)*hGH/BAC* transgenic lines. The histogram shows λ RNA levels and *hGH-N* mRNA levels as determined by real-time PCR. The results (± the standard deviation) were from averages of four independent experiments. Each value was normalized to the corresponding transgene copy number and was related to the level of the highest expressing line (λ147; defined as 1.0).

two concordant sets of data led us to conclude that transcription does not track in a linear fashion from the HS1-dependent transcriptional domain to the *hGH-N* promoter.

The levels of λ RNA were next quantified in each of the six (Δ*CD*λ)*hGH/BAC* lines and compared to the corresponding levels of *hGH-N* mRNA. Each value, normalized to transgene copy, was plotted in arbitrary units (Fig. 3E). This analysis revealed a direct and quantitative relationship between the level of noncoding transcription 3′ to HSI and the level of *hGH-N* expression (Pearson $r = 0.97$). This quantitative relationship between *hGH-N* expression and λ RNA levels further allowed us to conclude that the enhancing activity of HSI is directly linked to the act of transcription 3′ to HSI rather than to the structure of the locally generated *hCD79b* transcripts.

The initiation of transcription across *hCD79b* parallels the activation of *hGH-N* during pituitary development. The observation that transcription of *hCD79b* is tightly correlated with, and essential to, the activation of *hGH-N* led us to question whether *hCD79b* transcription was activated with the same developmental specificity as *hGH-N*. To test this, sagittal sections of the anterior region of *hGH/BAC* embryos 8.5 to 17.5 days postcoitus (dpc) were probed with DIG-labeled synthetic antisense RNAs. A series of well-established controls for pituitary developmental were used to validate embryo staging of each sample (Fig. 4A and B). Consistent with expectations, Pit-1, the POU-homeodomain protein essential to *GH* gene expression (52), was activated at 14.5 days within the nascent pituitary. This was followed 2 days later by the appearance of robust signals corresponding to *hGH-N* mRNA and *hCD79b* transcripts, as well as the endogenous *mGH* mRNA (Fig. 4C). Both *hGH-N* and *hCD79b* mRNAs appear within the same time window, between embryonic day 15.5 (e15.5) and e16.5. The coexpression of *hGH-N* and *hCD79b* RNAs was subsequently maintained through gestation (Fig. 4C) and into the postnatal period (data not shown). *hGH-N* activation was also observed to occur between e15.5 and e16.5 in an *in situ* hybridization analysis of an (Δ*CD*λ)*hGH/BAC* line (data not shown). These combined data support a functional linkage between the act of noncoding transcription 3′ to HSI (either *hCD79b* or λ) and *hGH-N* gene activation during pituitary development.

Transcription across *hCD79b* is established independent of interactions with the *hGH-N* promoter. Recent studies have suggested that activation of enhancer-associated, noncoding transcription, generating "enhancer RNAs," is dependent upon cooperative interactions between the enhancer and its target promoter (30). To test the relevance of this model to the *hGH* locus, we compared *hCD79b* transcription in the presence or absence of *hGH-N* (Fig. 5A). The *−8.0CD79b* transgene isolates the intact HSI-*hCD79b* region from the downstream *hGH-N* (5). Analysis of *hCD79b* mRNA expression in the *−8.0CD79b* transgenic pituitaries revealed that it was indistinguishable from the intact *hGH/BAC* transgene (Fig. 5B). In contrast, a more extensive deletion that also removes HSI (*−1.3CD79b* transgene; Fig. 5A) ablated *hCD79b* transcription in pituitary (Fig. 5B). These data confirmed that the domain of noncoding transcription across *hCD79b* is HSI-dependent and demonstrated that it can be fully activated in the pituitary in the absence of interactions with the target *hGH-N* promoter. This observation, along with the previous demonstration that transcription in this region is independent of the *hCD79b* promoter (Fig. 2), support a model in which HSI has a direct and possibly sufficient role in the activation of transcription across the *hCD79b* noncoding domain in the pituitary.

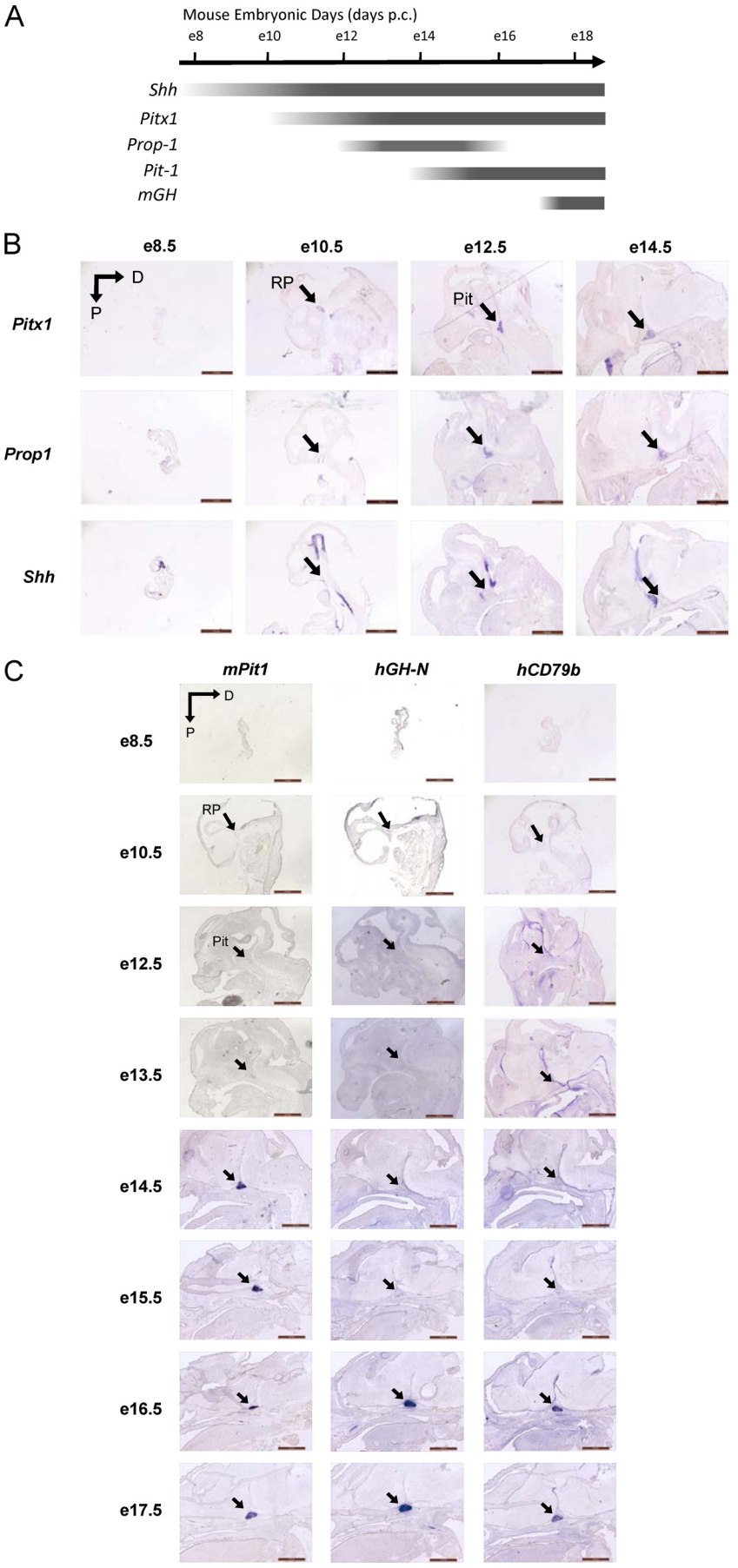
DISCUSSION

Transcriptional regulation in higher organisms depends on an array of long-range regulatory elements. These elements can mediate locus insulation, transcriptional enhancement, and/or sublocalization of the region within the nucleus (4, 10). Communications between enhancers and their target promoters are of particular importance for control of tissue-specific gene expression as well as for establishing patterns of temporally restricted expression (20, 32, 50). Recent genome-wide studies have revealed that many transcriptional enhancers are transcribed and generate a variety of noncoding RNAs (30). The presence of transcription coincident with, or closely linked to, an enhancer determinant has been shown in some cases to be concordant with the activity of target genes (30). Of significant interest, these and related studies have yet to delineate critical relationships between enhancer-linked transcription and target gene expression. Outstanding questions include: how are these enhancer-linked transcriptional units established, are they temporally linked to and/or dependent upon cooperative interactions with their target promoters, and is their impact on target gene expression dependent on sequences and/or structures of the locally generated noncoding transcripts?

In the present study, we focused our analyses on the control of the human growth hormone gene (*hGH-N*) by its remote enhancer, HSI. Our previous observations revealed that a domain of noncoding transcription located immediately 3′ to HSI, encompassing *hCD79b*, plays an essential role in HSI-mediated enhancement of *hGH-N* expression in the pituitary (23). Selective repression of transcription through this region in transgenic mouse pituitary, by insertion of a PolII terminator immediately 3′ to HSI, results in a major loss of *hGH-N* expression. Importantly, the insertion of this transcription termination element does not alter the formation of HSI itself, nor does it impact on noncoding transcription within the regions of the LCR 5′ to HSI (23). These observations support a direct role for transcription through the *hCD79b* region in *hGH-N* gene activation.

Noncoding RNAs have been studied as key players in imprinting and gene dosage compensation (7, 18, 29). More recently, it has been shown that depletion of particular long noncoding RNAs can result in repression of neighbor coding genes (40). This suggests that at least in some situations noncoding RNAs may contribute to the enhancement of target gene expression (8, 25). This enhancement might reflect RNA-mediated recruitment of basal transcription factors, transcriptional activators, chromatin remodeling factors, and/or displacement of transcriptional repressors (40). Our present studies demonstrated that noncoding transcription may also work in an RNA-independent manner. Of particular note is the observation that the HSI-dependent enhancement of *hGH-N* expression is fully maintained when the structure of the noncoding transcription domain 3′ to HSI is switched from the native *hCD79b* sequence to a comparably sized segment of bacteriophage λ DNA [Fig. 3A, (Δ*CD*λ)*hGH/BAC*]. A quantitative analysis of six genetically distinct (Δ*CD*λ)*hGH/BAC* transgenic lines further revealed a positive concordance between the levels of λ DNA transcription and *hGH-N* expression (Fig. 3E). These results indicated that enhancement of *hGH-N* expression is directly and quantitatively related to the intensity of the noncoding transcriptional activity 3′ to HSI. The data further revealed that this enhancing activity is independent of the locally generated RNA transcripts. From these data, we conclude that the mechanistic linkage between the HSI-dependent domain of transcription and *hGH-N* expression reflects noncoding transcription *per se* and not some attribute of the locally generated RNA transcript sequence or structure.

The dependence of *hGH-N* expression on HSI activity, and the mechanistic linkage between HSI-enhancing activity and transcription across *hCD79b*, predicts that *hCD79b* transcripts should appear in the embryonic pituitary concordant with, or preceding, the appearance of *hGH-N* mRNA. This prediction was confirmed by *in situ* hybridization studies. The *hCD79b* and *hGH-N* transcripts were both initially detected between 15.5 and 16.5 dpc in the developing pituitary and were subsequently maintained into postnatal life (Fig. 4C). This coordinate spatial and temporal spec-


A
Mouse Embryonic Days (days p.c.)
e8
e10
e12
e14
e16
e18
Shh
Pitx1
Prop-1
Pit-1
mGH
B
e8.5
e10.5
e12.5
e14.5
D
P
RP
Pit
Pitx1
Prop1
Shh
C
mPit1
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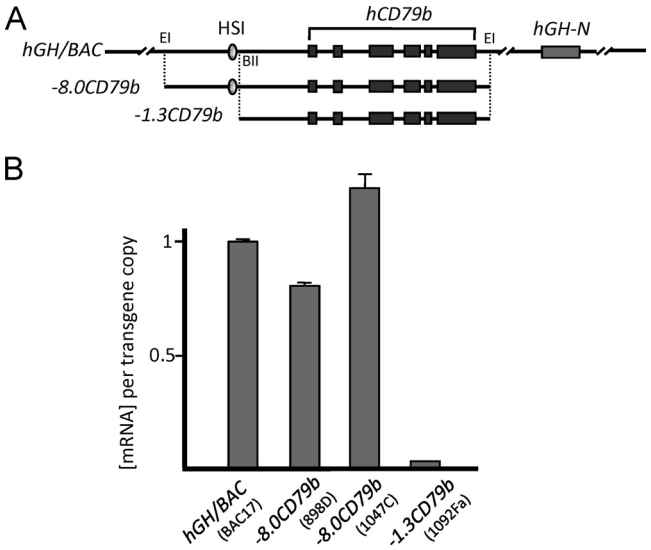

FIG 5 Transcription of the *hCD79b* domain in the pituitary is HSI dependent and established independent of the target *hGH-N* promoter. (A) Map of the *hGH/BAC* transgene and two derived transgenes lacking *hGH-N*. The *−8.0CD79b* transgene is a 12-kb genomic fragment of the *hGH/BAC* that encompasses HSI of the *hGH* LCR and the full *hCD79b* gene. The *−1.3CD79b* transgene is a 5.6-kb genomic subfragment that contains the full *hCD79b* but excludes the HSI region. Abbreviations: EI, EcoRI; BII, BglII. (B) Quantitative determination of *hCD79b* mRNA in transgenic pituitaries. Pituitary RNAs from mice carrying the *hGH/BAC*, *−0.8CD79b*, and *−1.3CD79b* transgenes were assessed for levels of *hCD79b* mRNA in the pituitary by qPCR. Each result was normalized to the corresponding transgene copy number and is represented as a value relative to the expression of the intact *hGH/BAC* transgene (defined as 1.0). Each value and standard deviation on the histogram is derived from three separate studies. The transgene name and specific line designation are shown below the respective bars. Note that pituitary *hCD79b* expression requires HSI, but not the target *hGH-N* promoter.

ificity of transcription support the mechanistic linkage between the HSI-dependent noncoding transcription and *hGH-N* gene activation.

The present study also addresses the questions of how transcription through the *hCD79b* region is activated in the pituitary and how this might relate to *hGH-N* activation. Kim et al. recently published a model of enhancer and target gene relations based on a tissue-specific enhancer in neuronal tissue (30). These researchers concluded that enhancer transcription is dependent on interaction of the enhancer with its target promoter. In the context of the *hGH* locus, this appears not to be the case. Analysis of a series of transgenic models revealed that transcription 3′ to HSI is dependent on HSI activity and independent of the target promoter. Several lines of evidence support this conclusion. The first was the observation that a transgene encompassing the HSI-*hCD79b* region could fully activate *hCD79b* transcription in the pituitary independent of the target *hGH-N* promoter (Fig. 5). Second, we found that activation of the *hCD79b* region was maintained in a transgene lacking the *hCD79b* promoter. This same deletion of the 5′ terminus of *hCD79b* effectively eliminated its transcription in B cells (5). Finally, transcription in this region was supported when the native sequences were replaced by a segment of λ DNA (Fig. 3). Based on these data we propose that this transcriptional domain is established in an HSI-dependent manner and does not require the presence or actions of the *hGH-N* target promoter. The findings lead us to conclude that the establishment of this domain of transcription serves as an initiating event in the pathway of *hGH-N* activation (Fig. 6).

In prior studies we had demonstrated that the chromatin configuration of the *hGH* locus in the adult pituitary places the *hGH-N* promoter in close proximity to HSI (24). This configuration is not present in the chromatin of nonexpressing tissues. In addition, we have previously shown that the pituitary-specific POU-homeodomain transcription factor Pit-1, a protein essential for *hGH-N* gene expression, occupies an array of *cis*-acting binding sites within HSI prior to binding its cognate sites within the *hGH-N* promoter (41, 42). The sequential assembly of the Pit-1 complexes at HSI and then the *hGH-N* promoter, separated as they are by 14.5 kb, may play a key role in establishing the "looping" structure between *HSI/hCD79b* region and *hGH-N* promoter in definitive somatotropes (24). This looping would position the intensely transcribed *hCD79b* domain, or the surrogate transcribed λ DNA domain, in close proximity to the *hGH-N* promoter (Fig. 6). Thus, the impact of the domain of noncoding transcription on *hGH-N* promoter activity may reflect the juxtaposing of a *cis*-acting "mini-PolII factory" established 3′ to the HSI enhancer element in close proximity to the *hGH-N* promoter. This model would be consistent with the direct impact of the transcription *per se* through this region, rather than a function of the encoded RNA transcripts, on enhancer function.

Altogether, our studies lead us to propose a model in which HSI-linked activation of the *hCD79b* domain of noncoding transcription may constitute an initial and critical step in the developmental pathway of the anterior pituitary. The present data suggest that the higher-order chromatin configuration of the *hGH* locus in the pituitary, in concert with the intense transcription immediately 3′ to the long-range enhancer, may activate high levels of *hGH-N* expression and sustain this robust activity throughout adult life. However, this model does not exclude roles for additional factors and interactions that solidify the connections be-

FIG 4 Transcriptional activation of the *hCD79b* domain and activation of *hGH-N* expression are temporally concordant during pituitary development. (A) Timing of pituitary marker gene expression during mouse embryonic development. The approximate timing of mRNA expression of pituitary marker genes shown here is adapted and modified from Dattani and Preece (9). (B) Developmental time course of expression of *Pitx1*, *Prop-1*, and *sonic hedgehog* (*Shh*). *In situ* hybridization signals in the e8.5 through e14.5 mouse embryo heads of *hGH/BAC* (line BAC17) are shown. The transcription factor *Pitx1* is an early pituitary-restricted maker initially expressed in Rathke's pouch at e9.5 (14, 52). This is followed by sequential activation of the genes encoding the transcription factors *Prop-1* and *Pit-1*, at 12.5 and 13.5 dpc, respectively. *Shh* is expressed throughout the oral ectoderm except in the Rathke's pouch, creating a boundary between two ectodermal domains of *Shh*-expressing and -nonexpressing cells. Abbreviations: RP, Rathke's pouch; Pit, pituitary; D, dorsal; P, posterior. Scale bars, 1 mm. This study confirmed the embryonic dates of the samples as estimated from copulation timing. (C) Developmental time course of *Pit1*, *hGH-N*, and *hCD79b*. The transcriptional activation of *Pit-1*, *hGH-N*, and *hCD79b* was studied by *in situ* hybridization on *hGH/BAC* transgenic embryos (line BAC17) from e8.5 through e17.5, as indicated. Pit-1 mRNA signal was first detected at e14.5 (1). Its positioning is within the caudomedial region of the pituitary gland, a region that ultimately gives rise to somatotropes, lactotropes, and thyrotropes. Signals corresponding to *hGH-N* and *hCD79b* RNA first appear in the pituitary between e15.5 and e16.5. The timing of *hGH-N* activation was also assessed in (*CDΔλ*)*hGH/BAC* transgenic embryos; *hGH-N* mRNA was found to appear within the same time window and with the same pituitary-specific distribution as observed in the *hGH/BAC* embryos (data not shown).

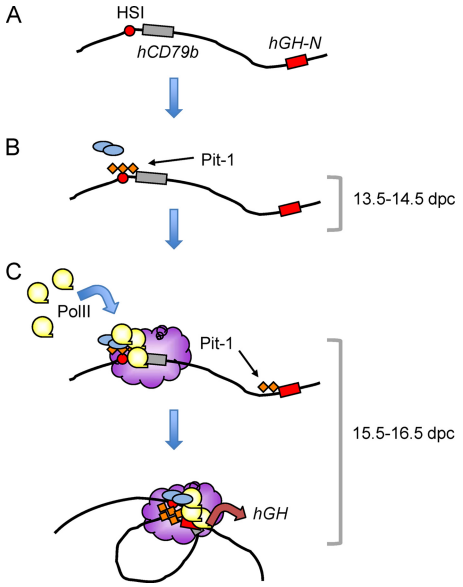

FIG 6 Model of HSI-mediated long-range enhancer action. (A) The inactive *hGH* chromatin locus lacks a specific higher-order chromatin conformation (24). (B) The initial step in *hGH-N* LCR activation is triggered at e13.5 to e14.5 by binding of Pit-1 (orange diamonds) to an array of cognate binding sites at HSI. Pit-1 recruits additional transcriptional complexes (blue ovals), including HAT complexes that extend acetylation to, and increase the exposure of, the chromatin-embedded *hGH-N* promoter (53). (C) A domain of intense PolII activity is established by HSI over the *hCD79b* region and is brought into contiguity with the *hGH-N* promoter between e15.5 and e16.5 via a chromatin "looping" configuration. The formation of a localized region of intense transcriptional activity (purple cloud) occurs as an autonomous function of HSI, being independent of both *hCD79b* and *hGH-N* promoters (Fig. 2 and 3). Pit-1 is recruited to the array of cognate sites at the *hGH-N* promoter during this developmental window. Interactions between the HSI/*hCD79b* region and the *hGH-N* promoter are stabilized. This "looping" within the locus, possibly mediated by Pit-1 dimerization (24, 41), brings the HSI-generated "transcriptional factory" into close contact with the *hGH-N* promoter. This conformation supports stable and robust expression of *hGH-N* in the adult pituitary somatotrope.

tween the enhancer-linked domain of noncoding transcription and the target *hGH-N* promoter. The identification and characterization of these additional determinants of long-range enhancer function present critical challenges for future studies.

ACKNOWLEDGMENTS

We thank the University of Pennsylvania Transgenic and Chimeric Mouse Facility (supported by National Institutes of Health [NIH] P30 grants DK019525, DK050306, and CA016520) for generation of the transgenic mice. We also thank Douglas Epstein, University of Pennsylvania, for critical review and comments, and Diane Dolson and Staci Rakowiecki for help with the *in situ* hybridization.

This study was supported by NIH grants R01 HD/DK25147 and R01 HD/DK046737 (to N.E.C. and S.A.L.).

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