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Bystander gene activation by a locus control region

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Random assortment of genes within mammalian genomes establishes the potential for interference between neighboring genes with distinct transcriptional specificities. Long-range transcriptional controls further increase this potential. Exploring this problem is of fundamental importance to understanding gene regulation. In the human genome, the *Igβ* (*CD79b*) gene is situated between the pituitary-specific *human growth hormone* (*hGH*) gene and its locus control region (*hGH* LCR). Igβ protein is considered B-cell specific; its only known role is in B-cell receptor signaling. Unexpectedly, we found that *hIgβ* is transcribed at high levels in the pituitary. This *Igβ* transcription is dependent on pituitary-specific epigenetic modifications generated by the *hGH* LCR. In contrast, expression of *Igβ* at its native site in B cells is independent of *hGH* LCR activity. These studies demonstrated that a gene with tissue-restricted transcriptional determinants (B cell) can be robustly activated in an unrelated tissue (pituitary) due to fortuitous positioning within an active chromatin domain. This 'bystander' gene activation pathway impacts on current concepts of tissue specificity and broadens the importance of critical considerations of active chromatin domains.

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Introduction

Transcriptional controls in higher eukaryotes can be exerted over extended distances (Ptashne, 1986; Bresnick and Tze, 1997). Remote regulatory elements can function by establishing extensive 'activated' chromatin domains that encompass target promoters (Vyas *et al*, 1992; Hebbes *et al*, 1994; Bulger and Groudine, 1999; Elefant *et al*, 2000; Forsberg and Bresnick, 2001; Ho *et al*, 2002). Alternatively, remote regulatory elements can be brought into close juxtaposition with target promoters via long-range 'looping' or 'tracking' mechanisms (Mueller-Storm *et al*, 1989; Tewari *et al*, 1996; McDowell and Dean, 1999; Carter *et al*, 2002; Tolhuis *et al*, 2002). Sets of transcriptional regulatory determinants responsible for long-range gene activation pathways are generally referred to as locus control regions (LCRs) (Forrester *et al*, 1987; Grosveld *et al*, 1987). The identification of LCRs in a wide array of gene systems suggests that long-range transcriptional controls constitute common pathways for gene activation in higher eukaryotes.

The extensive distances over which long-range controls can be exerted, and the close packing and random arrangement of many loci in the mammalian genome, establishes the potential for mutual interference among neighboring genes with distinct developmental or tissue specificities. Understanding how this significant problem in gene regulation is overcome is of fundamental importance. Insulator and boundary elements have been identified that can block a subset of such unwanted interactions (Chung *et al*, 1993; Hebbes *et al*, 1994; Bell and Felsenfeld, 1999). However, simple linear models of insulator/barrier function would be incompatible with effective insulation between loci with overlapping transcriptional control determinants. This suggests that more complex models of gene 'insulation' need to be formulated. Alternatively, certain loci may in fact not be fully insulated from the influence of unrelated transcriptional determinants and chromatin domains in their local environment. In this case, genes may be expressed in 'inappropriate' tissues or developmental settings. Such lack of precision in gene expression may be tolerated by the cell and/or might be compensated by post-transcriptional regulatory pathways.

The *human growth hormone* (*hGH*) cluster contains five genes expressed specifically in the pituitary (*hGH-N*) or in the placenta (*hCS-L*, *hCS-A*, *hGH-V*, and *hCS-B*). The *hGH* LCR in these two tissues is composed of partially overlapping sets of HS. HSI and HSII are specific to pituitary chromatin, HSIV is specific to placental chromatin, while HSIII and HSV are formed in the chromatin of both tissues (Jones *et al*, 1995). The mechanism of long-range activation by the *hGH* LCR has been best defined in the pituitary. The two closely linked pituitary-specific HS, HSI and HSII, are situated 14.5–15.0 kb 5′ to the *hGH-N* promoter. These HS appear to recruit HAT activity with subsequent bidirectional spreading of histone acetylation (Elefant *et al*, 2000; Ho *et al*, 2002). These reactions establish a continuous 32 kb domain of acetylated chromatin that extends to encompass the *hGH-N* promoter. Epigenetic alterations within this domain are required for *trans* factor binding at the *hGH-N* promoter and for effective *hGH-N* transcriptional activation (Ho *et al*, 2002). Targeted inactivation of HSI results in loss of the acetylated histone domain in pituitary chromatin and is accompanied by 90–95% loss of *hGH-N* transgene expression (Ho *et al*, 2002). Thus *hGH-N* transcriptional activation and the establishment of the extensively modified *hGH* LCR chromatin domain are intimately linked processes and both are dependent on the function of HSI.

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The human *CD79b/Igβ* gene (referred to throughout as *hIgβ*) functions specifically in B lymphocytes (Hermanson *et al*, 1988). Igβ proteins form heterodimers with Igα to create the signal transduction subunit of the B-cell receptor (Campbell *et al*, 1991; Clark *et al*, 1992). As such, Igβ plays a critical and nonredundant role in B-cell differentiation and function (Papavasiliou *et al*, 1995). There are no additional functions defined for this protein. Considering this B-cell specificity, it is interesting and potentially problematic that the *hIgβ* gene is situated between HSI of the *hGH* LCR and the *hGH-N* promoter (Bennani-Baïti *et al*, 1998a, b). This organization places the *hIgβ* locus within a hyperacetylated *hGH* LCR chromatin domain in somatotrope nuclei. The close packing of the *hGH-N*, *hIgβ*, and the *hGH* LCR thus presents a complex and potentially informative model to study mechanisms by which genes are shielded from each other's transcriptional control elements.

In the present study, we report the unexpected observation that *hIgβ* is actively transcribed in the pituitary. Interestingly, activation of the *hIgβ* locus in the pituitary occurs in the absence of B-cell-specific transcription factors required for *hIgβ* expression in B cells. This activation of *hIgβ* in the pituitary reflects its fortuitous positioning within the activated chromatin domain generated by the *hGH* LCR. Thus, *hIgβ* is not shielded from the *hGH* LCR as might be expected, but instead is robustly activated by it. This 'bystander' gene activation may represent a relatively common phenomenon. As such it would have important implications for models of developmental and tissue-specific gene regulation within the mammalian genome.

Results

An hIgβ transgene with extensive flanking regions is appropriately expressed in mouse B cells

The five genes within the *hGH* cluster are activated in either pituitary or placenta. In both situations, this activation is dependent on the action of a set of remote 5′ *hGH* LCR elements (Introduction). The B-cell-specific *hIgβ* gene is situated between the *hGH* LCR and the *hGH* cluster. We sought to identify a separate set of chromatin determinants in this region that were responsible for activation of *hIgβ* in B cells. Defining such determinants would allow us to model inter-relationships of transcriptional control elements within this tightly packed region of the genome. As an initial step, we assessed the expression of *hIgβ* from the 87 kb *hGH/P1* transgene (Figure 1A). This previously described transgene (Su *et al*, 2000) contains *hIgβ* along with extensive flanking sequences. The flanking sequences include 31 kb of 5′-flanking sequences encompassing the entire *hGH* LCR and extending into the striated muscle-specific *SCN4A* gene and 51 kb of 3′-flanking sequences that contain *hGH-N* and three of the four placentally expressed genes of the *hGH* gene cluster. The expression of *hIgβ* from the *hGH/P1* transgene was compared to that of the endogenous mouse (m) *Igβ* (Figure 1B). *hIgβ* was robustly expressed in the spleens of four *hGH/P1* transgenic mouse lines. Expression per transgene copy was maintained within a two-fold range, varying from 25 to 42% of endogenous *mIgβ*. Immunofluorescent analysis of transgenic spleens confirmed that hIgβ protein expression was restricted to the B-cell compartment (Figure 1C). B-cell specificity was further substantiated by flow sorting of splenocytes (data not shown). These results indicated that the *hGH/P1* transgene contains determinants sufficient to establish an autonomous, site-of-integration-independent chromatin environment that supports robust expression of *hIgβ* in mouse B cells.

hIgβ transgene is expressed in the mouse pituitary

The fidelity of *hIgβ* expression from the *hGH/P1* transgene was evaluated by a tissue survey (Figure 2A). As expected, the spleen contained high levels of *hIgβ* and *mIgβ* mRNAs. In contrast, these mRNAs were at or below trace levels in the brain, heart, kidney, and liver, and at a slightly higher level in the intestine. Surprisingly, a strong *hIgβ* mRNA signal was detected in the pituitary. While an exact quantitative comparison of hIgβ mRNA levels in B cells and somatotropes is complicated by the presence of mixed cell populations in the spleen and pituitary, it was clear that the hIgβ mRNA level in the pituitary was robust, approximating that in the spleen.

Analysis of all four *hGH/P1* transgenic lines confirmed the robust expression of *hIgβ* mRNA in the pituitary. These studies further revealed that the pituitary expression was copy number dependent, varying by less than three-fold (Figure 2B and real-time RT/PCR analysis, data not shown). Consistent with the expression of the *hIgβ* transgene in the pituitary, we also detected the endogenous *mIgβ* mRNA in this tissue, although at significantly lower levels.

To address the possibility that *hIgβ* mRNA in transgenic mouse pituitary might reflect B-cell contamination, we assayed for *mIgα* mRNA (Figure 2A). *Igβ* and *Igα* are encoded on separate chromosomes in both human and mouse genomes (Campbell *et al*, 1991; Clark *et al*, 1992). As expected, *mIgα* mRNA was easily detected in transgenic mouse splenic RNA. However, *mIgα* mRNA was absent in the pituitary RNA samples, and could not be detected in any of the other surveyed transgenic tissues (Figure 2A). We concluded that the *hIgβ* gene is robustly transcribed in the transgenic mouse pituitary.

The overall structure of hIgβ mRNA in transgenic pituitary and B cells is very similar

The structure of the *hIgβ* transcripts in the pituitary was assessed. We considered two possible origins for the pituitary *hIgβ* transcripts that would differentiate them from authentic (B cell) *Igβ* mRNAs. First, these transcripts could represent noncoding (intergenic) RNAs randomly generated from the active *hGH* LCR chromatin domain. Intergenic transcription has been described throughout the *human β-globin* LCR (Ashe *et al*, 1997; Routledge and Proudfoot, 2002) and it has been implicated in chromatin modification (Gribnau *et al*, 2000). Second, *hIgβ* may be transcribed in the pituitary from an alternative promoter distinct from that used in B cells. To address these possibilities, *hIgβ* mRNA size was analyzed by Northern blotting. These data revealed that *hIgβ* mRNA in transgenic mouse pituitaries was of the same size as in transgenic mouse purified lymphocytes and human B cells (Figure 3A). The normal size of the pituitary *hIgβ* mRNA was incompatible with random transcription within the LCR domain.

To determine whether the pituitary and B-cell *hIgβ* mRNAs begin at different promoters, the structures of their respective 5′ termini were determined by 5′ rapid amplification of cDNA ends (5′ RACE). *hIgβ* mRNAs isolated from the *hGH/P1* transgenic pituitary and human B-cell line revealed multiple

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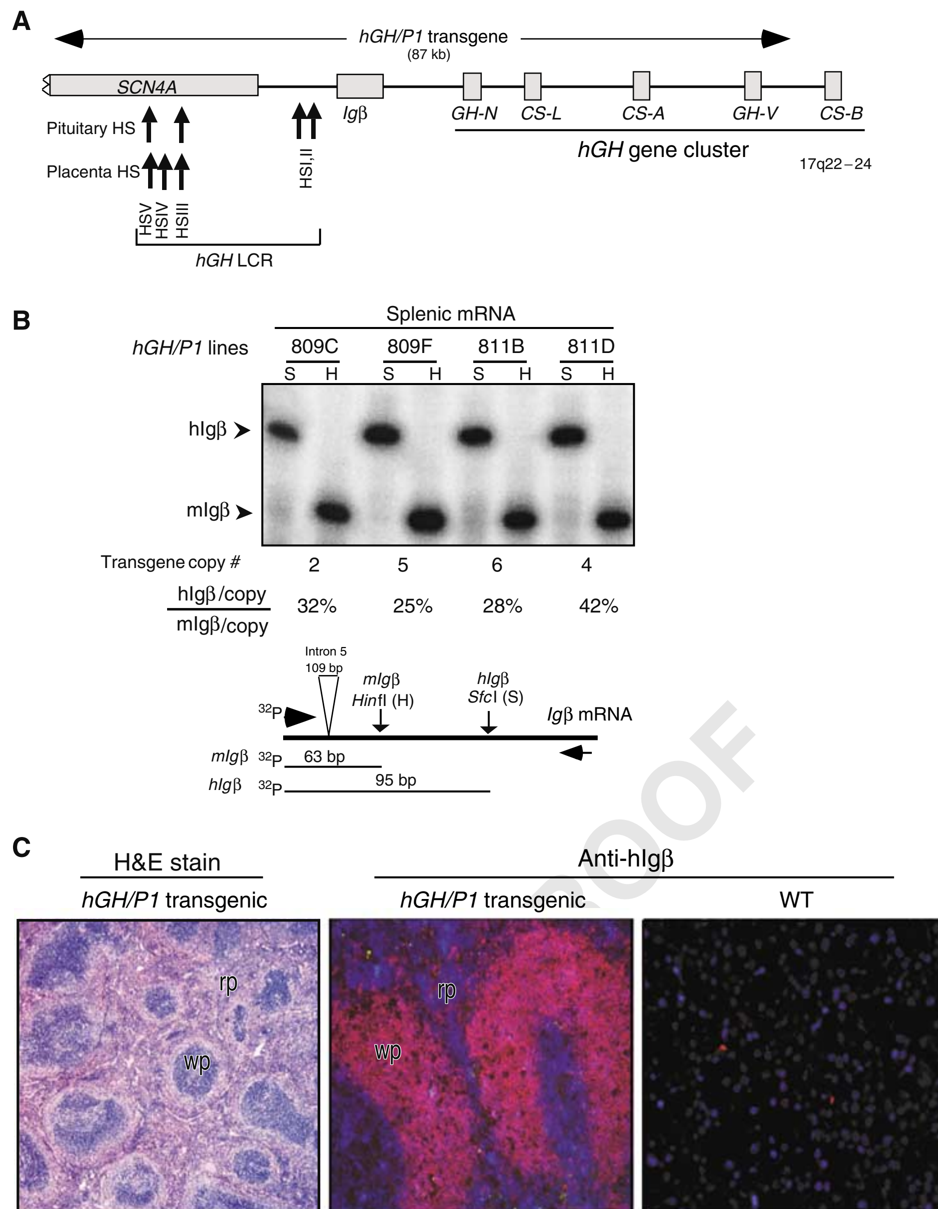

Figure 1 *hIg*β expression in the spleens of *hGH/P1* transgenic mice. (**A**) Map of the *hIg*β*/hGH* locus and the *hGH/P1* transgene. Each gene is represented by a labeled box. *SCN4A*, *hIg*β, and the five genes in the *hGH* cluster are represented. The horizontal arrow above the locus indicates the *hGH/P1* transgene. DNaseI HS of the *hGH* LCR are indicated by upward arrows along with their respective tissue specificities. (**B**) Splenic expression of *hIg*β from the *hGH/P1* transgene is copy number dependent. *hIg*β mRNA and endogenous *mIg*β mRNA were coamplified (RT/PCR) and the cDNAs were distinguished by restriction analysis. A representative RT/PCR analysis is presented in the autoradiograph and a diagram of the assay is shown below. The 5′ primer was ^{32}P-end labeled and the PCR products were digested with *Hin*fI (H) that exclusively digests the *mIg*β PCR product and *Scf*I (S) that exclusively digests the *hIg*β PCR product. The 5′-labeled fragments generated from mouse (63 bp) and human *Ig*β (95 bp) cDNAs are indicated (arrowheads). These signals were quantified from four unique four *hGH/P1* transgenic lines. The transgene copy number for each line is noted below each lane. Transgene expression per transgene copy was normalized to endogenous *mIg*β and values are indicated as percentages below each respective lane. (**C**) *hIg*β transgene is selectively expressed in the white pulp of the mouse spleen. (Left) Hematoxylin and eosin staining of an *hGH/P1* transgenic spleen showing normal architecture of the spleen and indicating the defined areas of red pulp (rp) and white pulp (wp). (Middle) *hGH/P1* transgenic spleen stained with anti-hIgβ (Texas red). The intense hIgβ staining is restricted to white pulp (wp) consistent with the local abundance of B lymphocytes. (Right) Nontransgenic, wild-type (WT) spleen stained with anti-hIgβ. The absence of a signal confirms the species specificity of the anti-hIgβ antibody.

transcription initiation sites distributed across an overlapping 160-nucleotide window (Figure 3B). This scattering of 5′ termini is consistent with a previous study (Thompson *et al*, 1996) and with the TATA-less/GC-rich structure of the *hIg*β promoter (Geng and Johnson, 1993; Donohoe and Blomberg, 1997; Dong *et al*, 2000). Of particular note, the 5′-terminal sequences in the 14 randomly selected pituitary *hIg*β cDNAs were continuous with the genomic sequence encompassing exon 1 of the *hIg*β gene. These data argued against the initiation of transcription from an alternative promoter/exon. The 5′-RACE sequence data also showed that pituitary *hIg*β is normally spliced across intron 1. The correct size of the *hIg*β RT–PCR product extending between exons 5 and 6 (see Figure 1B) confirmed that *hIg*β pituitary mRNA was also

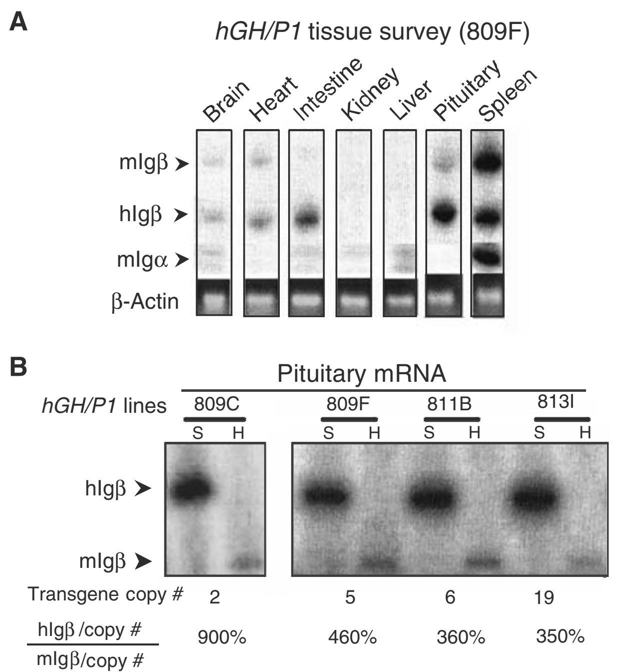

Figure 2 *Ig*β is transcribed from the *hGH/P1* transgene in the mouse pituitary. (**A**) Tissue survey of *hGH/P1* transgenic mice revealed abundant *hIg*β mRNA in the pituitary. A co-RT/PCR endonuclease cleavage assay was used to analyze the tissue specificity of *hIg*β expression in a representative *hGH/P1* transgenic mouse line (line 809F). The RT/PCR assay was identical to Figure 1B with the exception that the 3′ primer is ^{32}P-end labeled. The figure shows the PCR products digested with *Scf*I; the larger band (336 bp) corresponds to *mIg*β and the smaller (250 bp) to *hIg*β (labeled arrows). A separate RT/PCR of *mIg*α mRNA was carried out to monitor B-lymphocyte contamination in each tissue. The levels of hIgβ, mIgβ, and mIgα are shown relative to a β-actin mRNA loading control (ethidium bromide-stained gel). (**B**) Pituitary expression of *hIg*β mRNA from the *hGH/P1* transgene was copy number dependent. Pituitaries from four *hGH/P1* transgenic mouse lines were assayed by RT/PCR for *hIg*β mRNA. The level of *hIg*β mRNA was normalized to endogenous mouse pituitary *Ig*β mRNA and this value is shown as a percentage below each pair of lanes after correction for gene copy number.

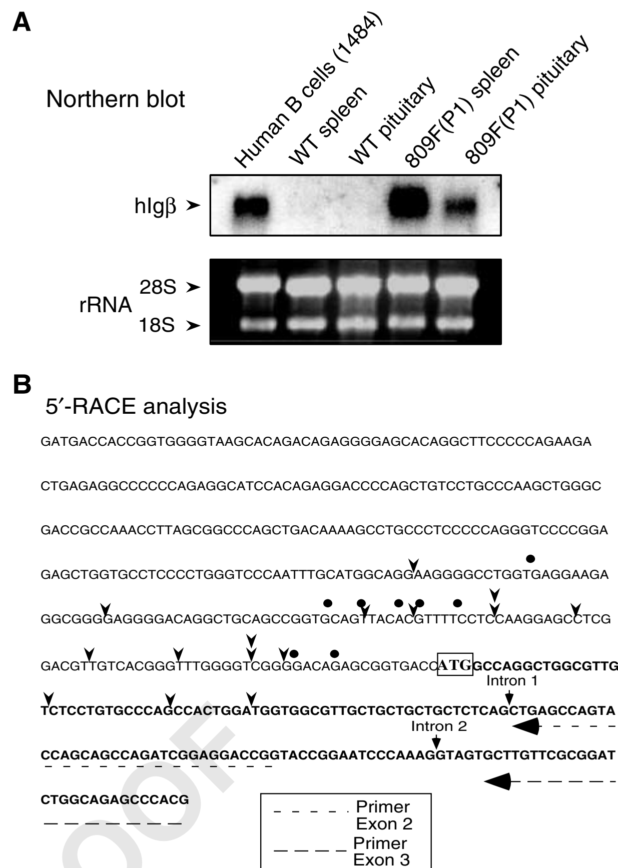

Figure 3 Structural comparison of *hIg*β mRNAs in the transgenic pituitary and B cells. (**A**) Northern blot analysis. Total RNAs from a human B-cell line (1484), from wild-type (WT) mouse lymphocytes (spleen) and pituitary, and lymphocytes (spleen) and pituitary of *hGH/P1* transgenic mouse line 809F were hybridized with a [^{32}P]cDNA *hIg*β cDNA probe. The gel was stained with ethidium bromide to visualize 18S and 28S rRNAs as loading control. The *hIg*β mRNA hybridizing bands comigrated in all tissues. No additional bands were detected. The absence of signal in the lanes containing WT lymphocytes and pituitary RNA confirmed the species specificity of the *hIg*β probe. (**B**) Mapping the 5′ terminus of pituitary and B-cell *Ig*β mRNAs. The sequence of the *hIg*β 5′-flanking region and 5′-terminal transcribed region are shown; the translation start site, *ATG*, is boxed. The 5′ termini of the transcribed mRNAs in B cells and transgenic pituitary were determined by 5′ RACE. The arrowheads indicate transcription start sites of *hIg*β in the *hGH/P1* transgenic mouse pituitary mRNA population. The dots indicate the *hIg*β start sites in human B-cell line 1484. The figure also indicates the locations of the primers used for the 5′-RACE assay (dashed arrows) and the positions of introns 1 and 2.

correctly spliced across intron 5. Thus, on the basis of a number of structural parameters, we concluded that the *hIg*β mRNA in the pituitary is closely related, if not identical to the mature, authentic B-cell *hIg*β mRNA.

*Ig*β *mRNA is expressed in the human pituitary*

We next considered whether the unexpected expression of *hIg*β mRNA in the mouse pituitary reflected a peculiarity of the transgenic model. PolyA-primed cDNA was generated from normal human pituitary and from two human pituitary somatotrope adenomas (Figure 4). Controls for this study included mRNA samples from a transgenic pituitary, from human peripheral blood leukocytes (PBLs), and from a human erythroid cell line (K562). The identity of the pituitary samples was confirmed by the presence of *hGH* mRNA. *hIg*β mRNA was detected in all three human pituitary samples in the absence of *hIg*α mRNA. In contrast, the control PBLs contained both *hIg*β and *hIg*α mRNAs but neither was detected in K562 cells. The relative levels of *hIg*β mRNA and *hGH* mRNA were similar in a comparison of the normal pituitary and pituitary adenoma #361. This comparison, however, revealed a relatively higher level of *hGH* mRNA in adenoma #373. This variation between the two adenomas may reflect the enrichment for somatotropes within the surgical samples or be a peculiarity of the adenoma pathology. This was not further explored. From these data we concluded that the transcription of *hIg*β from the *hGH/P1* transgene in the mouse pituitary faithfully recapitulated the expression of *hIg*β in primary human pituitary tissues.

*hIg*β *transcription in the pituitary is activated by the hGH LCR*

*Ig*β expression in B cells has been shown to reflect the activities of B-cell-specific transcription factors (Omori and

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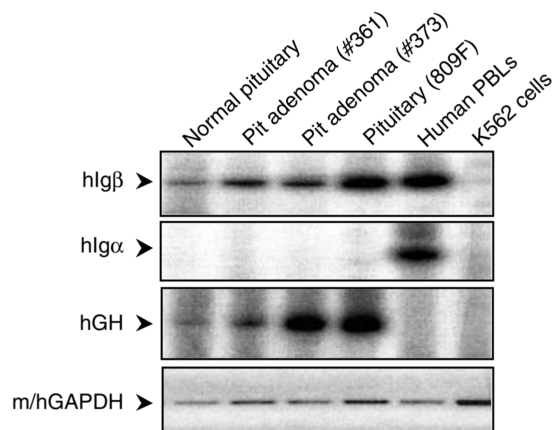

Figure 4 *hIg*β mRNA is expressed in human pituitaries. (Top panel) A normal human pituitary and two human pituitary adenomas (#361 and #373) were evaluated for *hIg*β mRNA by RT–PCR using ^{32}P-labeled RT–PCR primers. Positive controls were human peripheral blood lymphocytes (PBLs), *hGH/P1* transgenic pituitary (809F line), and the negative control was the human erythroid cell line K562. (Second panel) *Ig*α was monitored by RT/PCR with labeled primers. (Third panel) *hGH* RT/PCR with labeled primers identified the human and transgenic mouse pituitaries. (Bottom panel) Detection of *m/hGAPDH* by RT–PCR represented the loading control (ethidium bromide-stained products). All RT/PCR reactions were assayed in the linear range of amplification.

Wall, 1993; Thompson *et al*, 1996; Akerblad *et al*, 1999). Therefore, the expression of *hIg*β in the pituitary is likely to reflect an alternative pathway of gene activation. We speculated that this alternative mode of activation might reflect the localization of the *hIg*β gene within the chromatin domain established in the pituitary by the *hGH* LCR. HSI of the *hGH* LCR has a unique and necessary role in establishing this activated chromatin domain (Introduction). We generated a series of transgenic lines carrying the *hIg*β gene flanked by native sequences that either included (−*8.0Ig*β transgene) or excluded HSI (−*1.3Ig*β transgene and −*0.2Ig*β transgene) (Figure 5A). A semiquantitative co-RT/PCR assay was used to assess *hIg*β mRNA levels in the pituitaries from mice representing three or more independent lines carrying each of the three *hIg*β transgenes. *hIg*β mRNA was robustly expressed from the transgenes containing HSI (−*8.0Ig*β). In contrast, *hIg*β mRNA expression was dramatically lower in mice carrying transgenes truncated 3′ to HSI (−*1.3Ig*β and −*0.2Ig*β) (Figure 5B, left). *hIg*β mRNA levels in representative lines were separately assessed by real-time PCR and normalized to endogenous mouse *GAPDH* mRNA (data not shown, see Materials and methods). The two sets of mRNA assays were in full agreement. These data support the central role of HSI in activating *hIg*β transcription in the pituitary.

The involvement of HSI in the activation of *hIg*β in the pituitary was further tested by a second approach. Deletion of a core 99 bp segment of HSI (*hGH/P1*Δ*HSI* transgene; see Figure 5A) destroys HSI formation, dramatically decreases acetylation throughout the *hGH* LCR, and results in a 20- to 40-fold decrease in *hGH-N* transgene expression (Ho *et al*, 2002). The impact of HSI on *hIg*β transcription was tested by comparing levels of *hIg*β mRNA in the pituitaries of lines carrying the intact *hGH/P1* transgene to lines carrying the inactivated HSI (*hGH/P1*Δ*HSI*). This comparison revealed a mean 47-fold decrease in *hIg*β mRNA in the lines lacking a

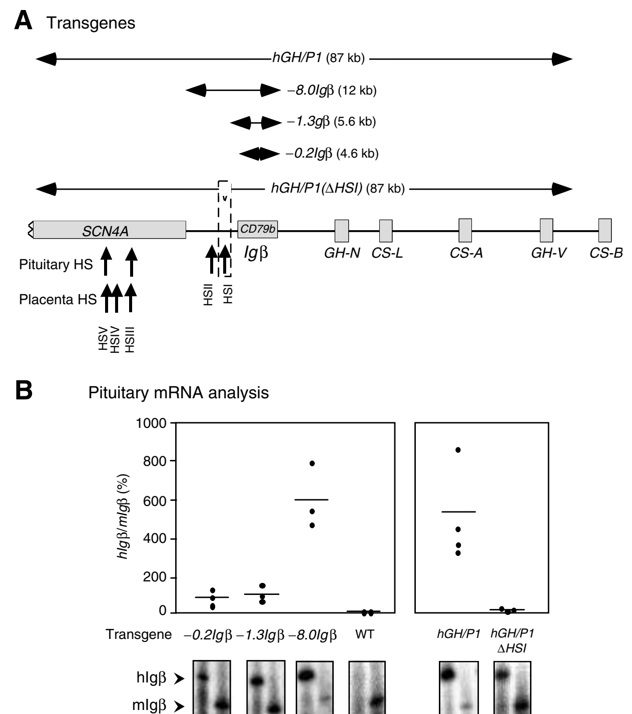

Figure 5 *hIg*β expression in the transgenic pituitary is dependent on the *hGH* LCR. (**A**) Transgene constructs. A map of the *hGH* gene cluster is shown. Horizontal arrows above the map indicate the extent of each transgene. The sizes of the constructs are indicated in parentheses and the designation of each construct indicates the extent of sequences 5′ from the *hIg*β gene promoter. Note that *hGH/P1* and −*8.0Ig*β transgenes include HSI whereas this determinant is excluded from the −*0.2Ig*β and −*1.3Ig*β transgenes. In *hGH/P1(*Δ*HSI)*, a 99 bp segment (dashed box), corresponding to the critical core elements of HSI, has been deleted from the *hGH/P1* transgene (Ho 2002). (**B**) HSI of the *hGH* LCR is a critical determinant of pituitary *hIg*β expression. The ratios of pituitary *hIg*β to *mIg*β mRNAs in the series of *hIg*β transgenic mouse lines (A) are shown. Mice from F1 or later generations and from at least three independent lines (dots) were analyzed (*X*-axis). The *hIg*β to *mIg*β mRNA ratios in each transgenic pituitary were plotted on the *Y*-axis as a percentage. The horizontal lines represent the mean values for each construct in the three or more lines analyzed. Representative co-RT/PCR endonuclease cleavage assays corresponding to each construct are shown below the graph. Wild-type mouse (WT) pituitary RNA was used as negative control. Expression ratios were corrected for transgene copy number.

functional HSI (Figure 5B, right). The data were confirmed by independent real-time PCR analysis of *hIg*β mRNAs in representative lines (data not shown). Thus, the activation of pituitary *hIg*β transcription was dependent on HSI, the major *hGH* LCR determinant in the pituitary.

*Expression of hIg*β *in B cells is independent of hGH LCR action*

The formation of HSI of the *hGH* LCR is specific to the pituitary (Jones *et al*, 1995) and *hGH-N* mRNA is not found in the spleen (Figure 6A). Despite this tissue specificity of *hGH* LCR function, the pronounced effect of HSI on *hIg*β expression in the pituitary prompted us to formally test whether HSI enhances *hIg*β expression in B cells. Comparison of *hIg*β mRNA levels in the spleens of *hGH/P1* and *hGH/P1(*Δ*HSI)* mice revealed equally robust and copy number-dependent levels from both transgenes (Figure 6B;

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compare with Figure 1B). These data are consistent with the tissue specificity of *hGH* LCR function and support the conclusion that HSI activates *hIgβ* transcription in the pituitary but has no appreciable function in the B-cell chromatin context.

hIgβ is not activated by the hGH LCR in the placenta

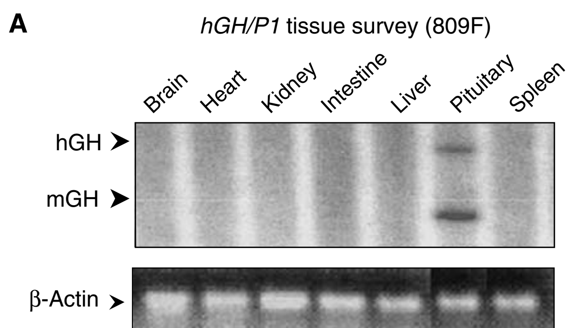
The *hGH* LCR activates expression of pituitary and placental genes from the *hGH* cluster via distinct epigenetic pathways. In the pituitary, the LCR establishes a continuous 32 kb domain of acetylated chromatin (Elefant *et al*, 2000; Ho *et al*, 2002). In contrast, acetylation of the LCR and the *GH* genes in placental chromatin is highly localized to two discontinuous regions, HSV–HSIII region and the region encompassing the four closely linked placental genes within the cluster (Kimura *et al*, 2004). Of particular note, levels of histone H3 and H4 acetylation in the region encompassing *hIgβ* (between HSIII and the *hGH* gene cluster) are quite low in placental chromatin when compared to the pituitary. The dependence of *hIgβ* gene activation in the pituitary on its positioning within a highly acetylated chromatin domain could therefore be further addressed by analyzing *hIgβ* expression in the placenta. In comparison to the robust expression of *hIgβ* mRNA in the transgenic pituitary and human B cells, *hIgβ* mRNA in the placental samples could only be detected at trace levels (Figure 6C and data not shown). These data support the conclusion that activation of *hIgβ* is directly related to its location within the highly acetylated LCR chromatin domain formed in the pituitary.

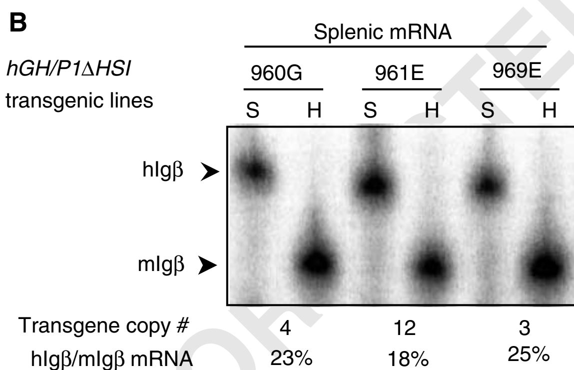
hIgβ mRNA in the pituitary is not effectively expressed at the protein level

The presence of *hIgβ* mRNA in the pituitary suggested that hIgβ protein might be synthesized at this site. hIgβ protein could theoretically contribute to a novel somatotrope signaling complex. hIgβ protein expression was therefore assessed by Western analysis of transgenic pituitary extracts. A strong hIgβ signal was detected in the transgenic spleen extracts and human B-cell extracts. In contrast, the Western blots failed to reveal convincing evidence for hIgβ in the pituitary (Figure 7). Immunofluorescent microscopy was carried out using *hGH/P1* transgenic pituitaries. These studies were essentially negative with only a rare cell showing evidence of positive hIgβ staining (data not shown). We concluded that the abundant *hIgβ* mRNA in the pituitary is either poorly translated or hIgβ protein, once synthesized, is rapidly catabolized due to the lack of a partnering Igα.

Discussion

Regulation of eukaryotic gene transcription reflects complex interactions among transcription factors, cofactor complexes, and epigenetically modified chromatin structures. The present study revealed that the activated chromatin environment established in pituitary somatotropes by the *hGH* LCR triggers





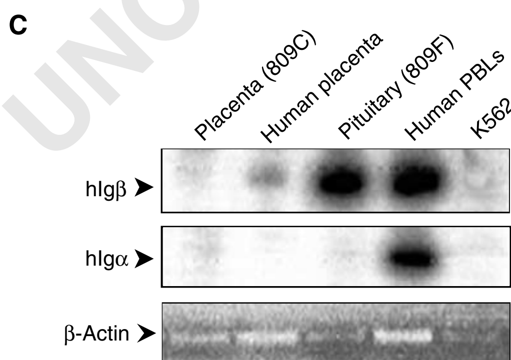


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Figure 6 Activation of *hIgβ* transcription by the *hGH* LCR is limited to the pituitary. (**A**) *hGH-N* mRNA is restricted to the pituitary. *mGH* and *hGH* mRNAs were detected by an RT/PCR endonuclease cleavage assay. This assay, shown below the autoradiograph, was applied to tissues from an *hGH/P1* transgenic mouse. Arrows to the left of the autoradiograph (upper panel) indicate the expected positions of the *mGH* and *hGH* RT/PCR products. In the lower panel, β-*actin* PCR products in each lane are visualized by ethidium bromide staining. (**B**) *hIgβ* transgene expression in the spleen is not linked to HSI activity. Splenic RNA samples from three independent lines of mice carrying the *hGH/P1ΔHSI* construct (lines 960G, 961E, and 969E) were analyzed for *hIgβ* expression by the co-RT/PCR endonuclease cleavage assay (Figure 1B). The level of transgene expression per gene copy was determined. The percentage of *hIgβ* to endogenous *mIgβ* expression is indicated below the autoradiograph. (**C**) hIgβ is not expressed in the placenta. RNA samples from an hGH/P1 placenta (line 809C), a human term placenta, a transgenic pituitary, human PBLs, and an erythroid cell line (K562) were each analyzed for hIgβ expression by RT/PCR. Igα mRNA was assessed to detect potential B-cell contamination of the tissue samples. The lower panel visualizes the products of a β-actin RT/PCR assay by ethidium bromide staining shown as a loading control.

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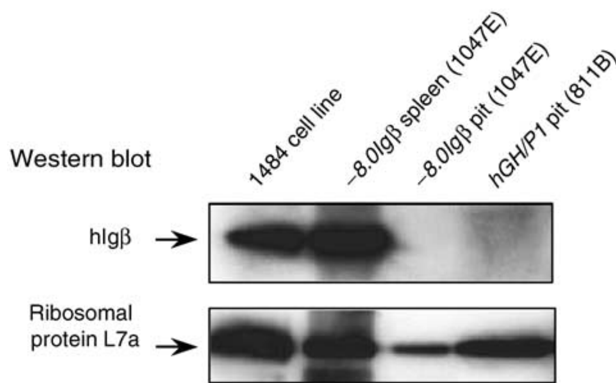

Figure 7 hIgβ protein does not accumulate in *hIg*β transgenic mouse pituitaries. Protein extracts from *−8.0Ig*β and *hGH/P1* transgenic pituitaries (lines 1047E and 811B, respectively), human B-cell line 1484, and a *−8.0Ig*β transgenic spleen (line 1047E) were studied by Western analysis. The top panel was incubated with an antibody to hIgβ and the bottom panel with an antibody to ribosomal protein L7a. Both panels were then developed with secondary antibodies and signals detected by enhanced chemiluminescence. The positions of hIgβ and the ribosomal protein L7 are indicated.

expression of the adjacent B lymphocyte-specific *hIg*β gene. This ectopic activation of *hIg*β reflects its fortuitous positioning within the *hGH* LCR chromatin domain. Activation of the 'bystander' *hIg*β gene by the *hGH* LCR occurs in the absence of B-cell-specific *trans* factors. The *cis*-acting selectivity of this effect is underscored by the observation that *Ig*α, the B-cell-specific partner of *Ig*β, remains unexpressed in the mouse and human pituitaries. Thus, *hIg*β can be activated in two tissues by two distinct mechanisms: it can be driven in the spleen by a pathway controlled by B-cell-specific transcription factors and it can be driven in the pituitary by the epigenetic influence of an encompassing somatotrope-specific chromatin domain. The latter 'bystander' gene activation pathway, which is unlikely to be unique, adds to the complexity of gene regulation and tissue specification models.

The mechanistic basis for *hIg*β expression in pituitary was initially explored by defining the structure of the *hIg*β transcripts. Northern blot analysis, RT/PCR across splice junctions, and 5′ RACE indicated that pituitary *hIg*β transcripts corresponded to full-length, polyadenylated *hIg*β mRNAs (Figures 1B, 3A and B). 5′-RACE analysis further revealed a heterogeneity of the 5′ ends of *hIg*β in both B cells and pituitary that is consistent with the lack of a TATA motif and the GC-rich structure of the *hIg*β promoter (Geng and Johnson, 1993; Donohoe and Blomberg, 1997; Dong *et al*, 2000). Thus, the *hIg*β gene, although considered B-cell specific, appears to be robustly expressed as a normally structured mRNA in the pituitary.

Although the structures of the *Ig*β mRNAs in B cells and in the pituitary are remarkably similar, subtle differences were noted. Northern analyses (Figure 3A and data not shown) revealed a more sharply focused *Ig*β mRNA band in B cells than in the pituitary and the 5′-RACE analysis revealed that the 5′ termini of the *Ig*β mRNA in B cells were more tightly grouped than in the pituitary. These observations may reflect qualitative differences in the accuracy of transcriptional initiation in B cells and by the 'bystander' pathway of *Ig*β activation in the pituitary.

It is of note that endogenous *mIg*β mRNA is also detected in mouse pituitaries. However its levels, when corrected for gene copy number, are substantially lower than *hIg*βmRNA expression from the *hGH/P1* transgene (Figure 2A and B). This could reflect a difference in the chromatin environment and transcriptional control of the human and murine *GH* loci. Whereas primates have a five-gene *GH* cluster, rodents have a single, isolated *GH* gene (Barsh *et al*, 1983; Chen *et al*, 1989). Whether the single *mGH* gene is under LCR control has not been determined. Computer searches reveal a conserved noncoding region in the mouse genome 5′ to the *mIg*β gene that can be aligned with HSI of the hGH LCR. However, it is not known if this sequence represents a functional correlate of the hGH LCR. A definitive comparison should emerge once detailed epigenetic and functional analyses of the *mGH* locus are established.

The 'bystander' effect exerted by the *hGH* LCR on *hIg*β in the pituitary raised the question of whether *hIg*β exerts a reciprocal activation of *hGH* in B lymphocytes. Analysis of *hGH/P1* transgenic mice failed to reveal *hGH-N* mRNA in spleen and analysis of human B cells was similarly negative for *hGH-N* mRNA (Figure 6A). These results suggest that the *hIg*β gene is activated by localized chromatin modifications that do not extend downstream to encompass the *hGH-N* promoter. This model can now be tested by defining the epigenetic modifications linked to *hIg*β expression in B cells.

The observation that genes with distinct expression patterns can be located in close proximity has led to the concept that these genes must be functionally shielded from one another (Dillon and Sabbattini, 2000). Such shielding may be mediated by boundary or insulator elements that protect adjacent genes from mutual positive or negative interference. This model has been most actively supported in studies of the chicken (c) β-*globin* locus (Chung *et al*, 1993, 1997; Hebbes *et al*, 1994). A constitutive HS located at the 5′ border of the hyperacetylated cβ-*globin* cluster defines the active domain and separates it from an adjacent heterchromatic region (Prioleau *et al*, 1999). Although such boundaries may be present in certain genomic regions, their function(s) have yet to be extensively tested in their native contexts. The present finding suggests that this model is not universal and that gene expression in higher eukaryotes may not be so neatly controlled in all cases. Q2

Activation of the *hIg*β gene in the pituitary may be difficult to explain solely on the basis of its presence within an active chromatin domain. For example, the *h*α-*globin* genes are situated within a region of constitutively open chromatin and yet their expression is erythroid specific (Vyas *et al*, 1992). Therefore, an open chromatin environment does not guarantee transcription activation. While the activated chromatin context is an essential prerequisite for activation, transcription factor association is certainly involved in this process. There are interesting relationships between B lymphocyte and somatotrope transcription factors that may be relevant to *hIg*β activation in the pituitary. Motifs involved in *Ig*β expression bind factors such as early B-cell factor (EBF), Oct-2, LyF1/μB, and PU.1 (Omori and Wall, 1993; Thompson *et al*, 1996; Akerblad *et al*, 1999). The octamer motif, bound by Oct-2 and the ubiquitous Oct-1, is a major determinant of *Ig*β promoter activity. Oct-1, Oct-2, and Pit-1 belong to the family of POU homeodomain transcription factors. Pit-1 is abundantly expressed in pituitary cells (Ingraham *et al*, 1988;

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Asa *et al*, 1993) and is critical to *hGH-N* activation (Shewchuk *et al*, 1999, 2002). Crossbinding of Pit-1 and the Oct factors to corresponding *cis* elements has been demonstrated *in vitro*, although it has not been possible to confirm actual cross-activation using cell transfection models (Elsholtz *et al*, 1990). It is possible that the *hIg*β promoter, when in the activated *hGH* LCR chromatin domain, is permissive to binding by abundant and closely related factors such as Pit-1. Alternatively, the active chromatin conformation within the *hGH* LCR may promote direct association of the basal transcriptional complex. This later scenario might predict a slightly less constrained site of transcription initiation of *hIg*β in the pituitary relative to B cells.

'Bystander' activation of *hIg*β, while secondary to the effects of the *hGH* LCR, may have a subsequent impact on *hGH* expression itself. Studies in the β-*globin* locus have demonstrated that two genes, when under the control of a common LCR, can alter each other's expression via transcriptional competition (Giglioni *et al*, 1984; Dillon *et al*, 1997). Prior studies of the *hGH* cluster revealed that direct linkage of HSI,II to the *hGH-N* transgene results in overexpression of serum hGH and a giant phenotype in transgenic mice (Jones *et al*, 1995). This superinduction of *hGH* could reflect the artificial proximity of HSI,II to the *hGH* promoter in the transgene construct, or alternatively, it might reflect the exclusion of a competing *hIg*β transcriptional unit. Weighing against this second possibility is the observation that mice homozygous for deletion of the mIgβ gene promoter lack evidence of growth abnormalities (Gong and Nussenzweig, 1996; Nussensweig, personal communication). Thus transcriptional inactivation of Igβ, at least in the mouse, fails to significantly impact on mGH expression. Whether bystander activation of a gene can have a secondary impact on its local environment can now be explored in greater detail.

The present data suggest that genes and their *cis*-acting sequences are not necessarily organized into discrete and functionally insulated chromosomal domains. The prevalence of long-range transcriptional controls in higher eukaryotes and the presence of extensive domains of modified chromatin make it likely that transcriptional interactions among closely packed genes may be the rule rather than the exception. Such 'bystander' effects impact on current concepts regarding tissue specificity, developmental controls, and widen the considerations of how gene control circuits are organized. Together, these observations add another layer of complexity to our understanding of gene activation mechanisms.

Materials and methods

Cell lines and primary cells

Human K562 erythroid cell line and human lymphoblastoid cell line CRL-1484 were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin. Peripheral blood lymphocytes (PBLs) from a normal donor were purified from fresh, heparinized whole-blood samples on Ficoll–Paque gradients (Amersham Biosciences). Studies of human samples were approved by the University of Pennsylvania Institutional Review Board.

Human pituitary and placenta samples

A preparation of polyA-primed cDNA from normal human pituitaries was a gift of Dr Roman Perez-Fernandez (Compostela University). Pituitary GH-secreting adenomas were donated by Dr Peter J Snyder (University of Pennsylvania). Portions of human full-term placentas were obtained from the Obstetrics service at the University of Pennsylvania.

RT/PCR analyses

In all cases, 0.5 μg of total RNA extracted from tissues or cell lines was reverse transcribed with an oligo-dT primer in the presence of AMV reverse transcriptase, then coamplified using a primer set corresponding to regions conserved between human and mouse (Table I). Either the 5′ or 3′ primer was end-labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase. This primer set spans intron 5 in order to distinguish cDNA from amplified genomic DNA by fragment sizes. PCR products were digested with a restriction enzyme; *mIg*β cDNA is specifically cleaved by *Hin*fI, *hIg*β cDNA by *Sfc*I, and *hGH-N* by *Bst*NI. Fragments were separated on 6% polyacrylamide/denaturing gels, and bands were quantified on a Storm 840 PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The ratios of digested cDNA fragments were normalized to transgene copy number. β-*actin* and *GAPDH* cDNAs were amplified for 24 cycles with unlabeled primers and analyzed on agarose gels. All PCR results were determined to be within the linear range of amplification by analysis of serial cycles.

RNA quantification by real-time RT/PCR

Pituitary RNA samples were reverse transcribed with random hexamers in the presence of MMLV reverse transcriptase. The cDNA was diluted to 10 ng/μl and was used for real-time PCR using TaqMan reagents on an ABI Prism 7700 Sequence Detector (Applied Biosystems). All samples were run in duplicate. Amplification of the hIgβ mRNA was normalized with a reference mGAPDH mRNA probe. All primers and probes for real-time analysis of hIgβ and mGAPDH mRNAs were standardized reagents purchased from Applied Biosystems.

Northern analyses

Total RNA was extracted from lymphocytes (spleen), pituitaries, and cell lines with RNA-Bee (Tel-Test, TX). A 20 μg portion of each RNA sample was separated on a 1.2% agarose gel containing 2.2 M formaldehyde in 1 × MOPS, and transferred to a Zeta-Probe membrane (Bio-Rad). The membrane was probed with ^{32}P-labeled *hIg*β cDNA probe (Table I; Northern probe) at 65°C in PerfectHyb buffer (SigmaAldrich). The membrane was then washed (1 × SSC, 0.1% SDS, 0.5 × SSC, 0.1% SDS, and finally 0.1 × SSC, 0.1% SDS) at room temperature to 65°C and signals were detected by exposure to a phosphorimager screen.

5′ rapid amplification of cDNA ends

mRNAs isolated from B-cell line 1484 and hGH/P1 transgenic pituitaries were reverse transcribed using an antisense hIgβ-specific primer corresponding to a site in exon 6 of hIgβ (5′TCATGGGGCGACCTGGCTC3′). The RT product was ethanol precipitated in the presence of carrier (glycoblue) and resuspended in water. A polyA tail was added to the 3′ end of the transcribed cDNA using terminal dinucleotidyl transferase (Promega). An initial PCR was run using the oligo-dT-adaptor primer (Table I) and an hIgβ-specific antisense primer on exon 3 (Table I). A subsequent nested PCR was run using the same oligo-dT-adaptor primer and hIgβ-specific antisense primer corresponding to a region of exon 2 (Table I). The products were cloned into the pGEMT vector system I (Promega) and sequenced by chain termination.

*FACS analysis of mouse spleen B cells for hIg*β *protein*

Spleen cells were harvested, suspended in PBS, and lymphocytes were isolated on Ficoll gradients. Cells were stained for 30 min using a panel of monoclonal antibodies conjugated to fluorescent dyes: anti-mouse B220-APC (B-cell surface marker) (Pharmingen, catalog number 01129A), anti-mouse Igβ FITC (Southern Biotechnology Associates, catalog number 1830–02), and anti-human Igβ PE (Pharmingen, catalog number 555679). Isotype-matched monoclonal controls were labeled with FITC, PE, and APC. Using a FACSCalibur flow cytometer (BD Immunocytometry systems) 10 000–20 000 cell events were acquired. The lymphocyte population to be analyzed was selected by side scatter and forward scatter. Analyses of the stained cells were performed using Cell Quest Software.

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Table I Primers and probes used in the experiments

	Sequences (5′ to 3′)
PCR primers	
mhIgβ5′	5′GGAGGAAGATCACACCT3′
mhIgβ3′	5′ATCCCCAGAGAACTCC3′
mhIgα5′	5′GTTCAGGAAACGATGGCAGA3′
mhIgα3′	5′TCACTAAGTGGCCCTGACAGA3′
mhGH5′	5′GCCTGCTCTGCCTGC3′
mhGH3′	5′GACTGGATGAGCAGCAG3′
mh β-Actin 5′	5′TGTGATGGTGGGAATGGGTCAG3′
mh β-Actin 3′	5′TCGGTGAGCAAGCACAGGGTG3′
mhGAPDH 5′	5′GCCAAAAGGGTCATCATCTC3′
mhGAPDH 3′	5′CTGCTTCACCACCTTCTTGA3′
5′-RACE primers	
Exon 6 hIgβ 3′	5′TCATGGGGCGACCTGGCTC3′
Oligo-dT-adaptor primer 5′	5′GACTCGAGTCGACATCGAT(17)3′
Exon 3 hIgβ 3′	5′CGTGGGCTCTGCCAGATCCGCGAACAAGC3′
Exon 2 hIgβ 3′	5′CGGTCCTCCGATCTGGCTGCTGGTACTGGCTCAG3′
Probes	
5′ primer for dot blot probe	5′TCATCCTCTTCATCATCGTGCC3′
3′ primer for dot blot probe	5′TGCTGCCCTTGTCCTTCTAC3′

Table II Transgenes, line designations, construct sizes, and copy numbers

Transgene	Construct size (kb)	Line designation	Copy number
−0.2Igβ	4.6	1008A	2
		1007A	2
		1096C	4
−1.3Igβ	5.6	1014A	3
		1014A	2
		1092Fa	3
−8.0Igβ	12	989D	7
		1002D	10
		1047C	3
hGH/P1	87	809C	2
		809F	5
		811B	6
		811D	4
		813I	19
hGH/P1(ΔHSI)	87	960G	4
		961E	12
		969E	3

Protein extraction and Western blotting

Proteins from tissues and cell lines were purified in proteinase inhibitor buffer (1 mM potassium acetate, 1.5 mM magnesium acetate, 2 mM dithiothreitol (DTT), 10 mM Tris–HCl (pH 7.4), 100 mg of phenylmethylsulfonyl fluoride (PMSF)/ml, 2 mg of aprotinin/ml, 2 mg of pepstatin A/ml) by repeated pipetting. After clarification, the supernatants were separated and transferred using NuPage Bis–Tris gels system (Invitrogen). Signals were detected using monoclonal mouse anti-human Igβ (Pharmingen) and polyclonal anti-L7a rabbit serum (Zimiecki, 1990; Ji *et al*, 2003). Sheep anti-mouse IgG-HRP (Santa Cruz) and donkey anti-rabbit IgG-HRP (Amersham) were used as secondary antibodies.

Immunofluorescent microscopy

Polyclonal goat anti-hIgβ antibodies (Santa Cruz Biotechnologies) and anti-hGH monoclonal antibodies (mAb 9) (Bennani-Baïti *et al*, 1998a, b) were used at a 1:1000 dilution. As secondary antibodies, donkey anti-goat Cy3 (Vector) and donkey anti-mouse Cy2 (Vector) were used at 1:200. Immunohistochemistry was performed on fresh frozen mouse spleen and pituitary sections. Defrosted slides were placed in buffered neutralized formalin for 5 min, transferred to distilled water, blocked with protein blocking agent (Immunotech) for 10 min and incubated overnight with the primary antibody at 4°C. The next day the slides were washed and incubated for 30 min at 37°C with the secondary antibodies. Finally, the slides were incubated for 10 s with 4′,6-diamidino-2 phenylindole, dihydrochloride (DAPI), covered with mounting medium and observed under a fluorescence microscope.

Transgenic constructs and generation of transgenic mouse lines

hGH/P1 wild-type and *hGH/P1(ΔHSI)* deletion lines have been previously reported (Su *et al*, 2000; Ho *et al*, 2002) (Table II). *−8.0Igβ* contains a 12 kb genomic fragment derived by *Eco*RI digestion of P1 6057 that includes 8.0 kb 5′ to the *hIgβ* gene and 975 bp 3′ to the *hIgβ* polyA addition site. *−1.3Igβ* contains a 5.6 kb genomic fragment generated by *Bgl*II and *Eco*RI digestions with 1.3 kb of 5′-flanking sequences. *−0.2Igβ* contains a 4.6 kb genomic fragment generated by *Bsi*EI and *Eco*RI digestions that contains 200 bp of 5′-flanking region. Each construct was microinjected into fertilized mouse oocytes (C57BL/6 × SJL) to generate the transgenic lines (Transgenic & Chimeric Mouse Core, University of Pennsylvania). Founders were identified by dot-blot analyses of tail DNA using a PCR-generated, 740 bp probe, corresponding to a region between exons 4 and 6 of the *hIgβ* gene (Table I). Transgenic lines used in the present study are listed in Table II.

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