

Patterns of histone acetylation suggest dual pathways for gene activation by a bifunctional locus control region

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The five genes of the human growth hormone (hGH) cluster are expressed in either the pituitary or placenta. Activation of the cluster is dependent on a locus control region (LCR) comprising pituitary-specific (HSI,II, –15 kb), placenta-specific (HSIV, –30 kb) and shared (HSIII, –28 kb; HSV, –32 kb) DNase I hypersensitive sites. Gene activation in the pituitary is paralleled by acetylation of a 32 kb chromatin domain 5' to the cluster centered at HSI,II. In the present study we observed that acetylation of this region in placental chromatin was discretely limited to shared HSIII and HSV. Transgenic studies revealed placenta-specific activation of linked genes by a determinant (P-element) located 2 kb 5' to each of the four placentally expressed genes. A localized peak of histone acetylation was observed at these P-elements in placenta but not pituitary. These data support a model for bifunctional action of the hGH LCR in which separate positive determinants, HSI,II and the P-elements, activate their respective target genes by tissue-specific recruitment of distinctly regulated histone acetyl transferase activities.

Keywords: chromatin/gene expression/histone acetylation/human growth hormone/locus control region

Introduction

In higher eukaryotes, the majority of DNA is packaged into a compact and repressive chromatin structure. This permits only a small portion of the genome to be expressed in any given cell or tissue type. Dominant control elements termed locus control regions (LCRs) may contribute to tissue-specific gene expression via localized alterations in chromatin structures (W.C. Forrester *et al.*, 1987; Felsenfeld, 1996; Felsenfeld *et al.*, 1996). LCR determinants can be detected and mapped by their ability to establish DNase I hypersensitive sites (HS) in the chromatin of expressing cells. LCRs are operationally defined by their ability to confer tissue-specific, copy-number dependent, high-level expression on linked transgenes irrespective of their sites of integration in the host genome (Grosveld *et al.*, 1987; Festenstein *et al.*,

1996; Li *et al.*, 1999). Although the mechanism(s) underlying LCR function remains unclear, extensive histone acetylation of the chromatin encompassing LCRs has been associated with their activity in systems where this has been studied (Hebbes *et al.*, 1994; Elefant *et al.*, 2000; Schubeler *et al.*, 2000). Since histone acetylation promotes chromatin decondensation and increases accessibility to *trans*-factor binding (Vettese-Dadey *et al.*, 1994; Brownell and Allis, 1996; Gregory *et al.*, 1998; Luger and Richmond, 1998), this modification provides a mechanism linking LCR function to chromatin opening and subsequent transcriptional activation.

The *hGH* cluster contains five closely linked genes spanning 48 kb on chromosome 17q22–24. These genes share >94% sequence identity and are arranged in a uniform transcriptional orientation: 5'-*hGH-N*, *hCS-L*, *hCS-A*, *hGH-V*, *hCS-B*-3'. This organization and structure suggests that the *hGH* cluster arose through a series of relatively recent gene duplication events (Chen *et al.*, 1989). Despite the high level of sequence identity among these genes and their respective promoter elements, they exhibit mutually exclusive patterns of expression. *hGH-N* is selectively expressed in the somatotrope and somatolactotrope cells of the anterior pituitary (Bennani-Baiti *et al.*, 1998a), whereas expression of the remaining four genes (collectively referred to as *hCS*) is restricted to the syncytiotrophoblast layer of the mid-to-late gestation placental villi (Liebhaber *et al.*, 1989; Walker *et al.*, 1991; MacLeod *et al.*, 1992). The *hCS-A* and *hCS-B* genes are expressed at very high levels by term gestation whereas *hCS-L* and *hGH-V* are expressed only at trace levels. The mechanism(s) underlying this tissue specificity and the expression levels of these genes remains unclear.

The expression of the *hGH* cluster in both pituitary and placenta is under the control of its LCR. When introduced into the mouse genome on their own, *hGH-N* or *hCS-A*, even with extensive segments of contiguous flanking sequences, are either not expressed or expressed at low and sporadic levels (Jones *et al.*, 1995). The *hGH* LCR is marked by a set of tissue-specific HS: HSI,II (because of their very close linkage, HSI and HSII are dealt with as a single entity), HSIII and HSV in pituitary nuclear chromatin, and HSIII, HSIV and HSV in the chromatin of placental syncytiotrophoblast nuclei. Linkage of the full set of HS to an *hGH-N* transgene selectively drives expression in somatotropes in a robust copy-number manner (Jones *et al.*, 1995; Bennani-Baiti *et al.*, 1998a; Shewchuk *et al.*, 1999). Similarly, *hCS*, when linked to the LCR, is expressed selectively in the mouse placenta in a consistent, copy-number-dependent fashion (Su *et al.*, 2000). These studies support a bifunctional role for the *hGH* LCR and demonstrate that the transgenic mouse model is a reliable system to study its action.

Distinct regulatory elements within the hGH LCR appear to be involved in promoting *hGH-N* and *hCS* expression and specification. HSI,II, the pituitary-specific set of HS, is fully sufficient to confer high-level, somatotrope-specific, position-independent expression on a linked *hGH-N* transgene (Jones *et al.*, 1995; Bennani-Baiti *et al.*, 1998a; Shewchuk *et al.*, 1999). Of note, this expression is much higher than that of the endogenous *mGH* and is not copy-number dependent (Jones *et al.*, 1995). These data suggest that HSI,II is the major positive regulator of LCR action in the pituitary but must be linked to other LCR determinants to establish a fully insulated and autonomously controlled locus. The basis for placenta-specific expression of the remaining genes in the cluster is less well understood. Cell culture-based studies by others (Nachtigal *et al.*, 1993) have suggested that the specificity of *hCS* expression in the placenta reflects repression of its expression in the pituitary. Cell transfection studies have mapped a repressive activity to a 263 bp conserved element (P-element) located 2 kb 5' to each of the placentally expressed genes. This element is embedded in an extensive region of conserved sequence identity associated with each placental gene. The repressive action of the P-element demonstrated in these cell transfection studies has not been addressed in a developmentally dynamic setting, and the possibility that the placental genes in the hGH cluster are regulated by a positively acting determinant has not been thoroughly tested. In the present study, we sought to expand our understanding of hGH LCR function by investigating the mechanistic basis for placenta-specific *hCS* activation. The results suggest a model in which specific positive regulatory elements can target distinct patterns of chromatin modification at the hGH locus in the pituitary and placenta. Such distinct patterns of chromatin modification may reflect the ability of the hGH LCR to activate genes within its multigene cluster via two selective and mutually exclusive pathways.

Results

HSIII and HSV were selectively enriched for acetylated histones in placental chromatin

The genes of the hGH cluster are expressed at high levels in two distinct tissues; *hCS-A* and *hCS-B* in placental syncytiotrophoblasts and *hGH-N* in pituitary somatotropes. The remaining two genes, *hGH-V* and *hCS-L*, are also placenta specific, although they are expressed at very low levels. Expression of the genes of the hGH cluster in both tissues is LCR dependent (Jones *et al.*, 1995; Su *et al.*, 2000). Initial studies were designed to determine whether the acetylation pattern of the *hGH* LCR in the syncytiotrophoblasts paralleled that previously determined in the pituitary (Elefant *et al.*, 2000). Syncytiotrophoblast nuclei were selectively released from the villi of a normal human term placenta (see Materials and methods) and subjected to DNase I mapping and chromatin immunoprecipitation (ChIP). The DNase I analysis of the LCR region confirmed the placenta-specific pattern of HS (HSIII, HSIV and HSV) (Figure 1A). The same nuclear preparation was then analyzed by ChIP; solubilized placental nucleosomes were immunoprecipitated with a mixture of antibodies recognizing acetyl-lysine residues on histones H3 and H4

(Figure 1C). Levels of acetylation at specific sites were determined by hybridization with a series of unique sequence probes. Hybridization signal intensities in each of the three ChIP fractions (input, unbound and bound) were normalized for DNA loading by rehybridizing the membrane with a total genomic DNA probe. The normalized ratios of histone acetylation enrichment (bound/unbound) were determined.

The initial ChIP studies monitored levels of histone acetylation at specific sites 5' to the hGH gene cluster. These sites included the hGH-N promoter, each of the hGH LCR HS, and regions between and surrounding the HS (Figure 1B). The positions of each of these probes could be exactly defined in relation to the hGH cluster and to the closely linked B-lymphocyte-specific *CD79b* (Bennani-Baiti *et al.*, 1998b) and striated muscle-specific *SCN4A* genes (Bennani-Baiti *et al.*, 1995) based on the sequence of this entire contiguous region (DDBJ/EMBL/GenBank accession No. AC005803). This analysis revealed a pattern of acetylation in placental chromatin that was clearly distinct from the extensive pattern of acetylation of the LCR previously determined in the pituitary. The pituitary ChIP data are summarized in Figure 1C as white bars for reference (data taken from Elefant *et al.*, 2000). Acetylation in the placental chromatin was limited to HSIII and HSV (bound:unbound DNA ratios of 4.0 and 2.8, respectively). The acetylation levels at these two sites were essentially identical to those observed in the pituitary (compare black and white bars; Figure 1C). Surprisingly, acetylation at the placenta-specific HSIV was not significantly enriched over that of total genomic DNA (bound:unbound DNA <2.0; the background level was observed in non-expressing tissues; Elefant *et al.*, 2000). Thus the patterns of hGH LCR chromatin acetylation in pituitary and placenta shared the modification of HSIII and HSV, but were otherwise distinct.

The P-element failed to repress expression of hGH-N in the transgenic mouse pituitary

The lack of a placenta-specific site of histone acetylation in the 5'-flanking domain of the hGH gene cluster suggested that one or more element(s) relevant to activation of the *hCS* might exist elsewhere. A candidate for such a placental restriction determinant was the conserved P-element. The P-element is a 263 bp fragment positioned 2 kb 5' to each of the placentally expressed genes in the hGH cluster (see Figure 4A) (Chen *et al.*, 1989). A previous study, based upon cell transfection assays, concluded that the P-element controlled the placental restriction of the *hCS* genes by repressing their expression in the pituitary. In these studies, the repressive effect of the P-element did not appear to be *hCS* specific because it was equally effective in repressing a juxtaposed *hGH-N* promoter (Nachtigal *et al.*, 1993). To determine whether the P-element could repress *hGH-N* expression *in vivo*, as it does in transfected pituitary cells, a transgene was constructed in which *hGH-N* linked to HSI,II was flanked by P-elements (*HSI,II/PhGHP*; Figure 2A). We have demonstrated previously that linking HSI,II to *hGH-N* (*HSI,II/hGH* transgene) results in consistent high-level expression in somatotrope cells of the anterior pituitary, causing a consistent phenotype of gigantism in

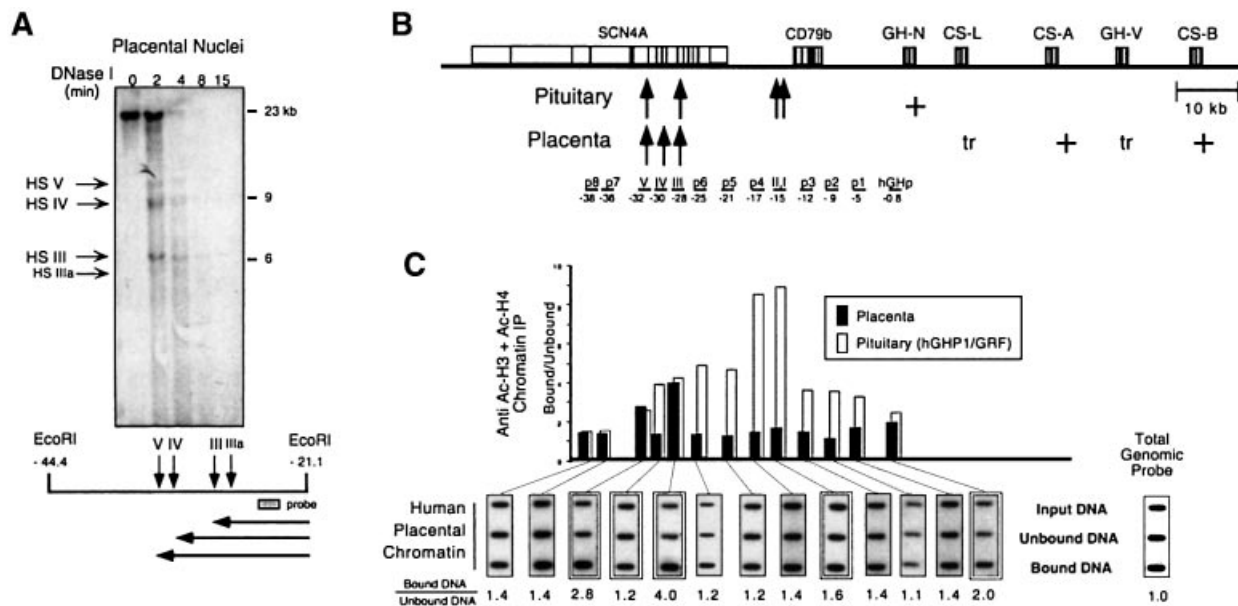


Fig. 1. HSIII and HSV but not HSIV are enriched for acetylated histones in placental chromatin. **(A)** Placental chromatin contains three prominent DNase I hypersensitive sites, HSIII, HSIV and HSV, located between 28 and 32 kb 5' to the hGH multigene cluster. Nuclei selectively isolated from the hCS-expressing syncytiotrophoblasts of a normal human term placenta were digested with DNase I for increasing periods of time (indicated above respective lanes in minutes). DNA was isolated at each time point, digested to completion with *EcoRI* and analyzed by Southern blotting using the ³²P-labeled probe (represented diagrammatically in the map below the autoradiograph). The identity of the HS that resulted in the generation of each of the sub-bands is indicated to the left of the autoradiograph and the size markers are shown to the right. The diagram below the Southern blot illustrates the position of each DNase I HS site (vertical arrows) relative to the *EcoRI*-defined 3' terminus. The horizontal lines represent the lengths of each corresponding sub-band. The coordinates represent the number of kilobases 5' to the transcription start site of *hGH-N*. Three major HS (HSIII, HSIV and HSV) and a minor HS (IIIa) were detected. **(B)** Diagram of the hGH cluster and its LCR. A schematic representation of the hGH multigene cluster and its associated LCR are shown. The positions of the closely linked B-lymphocyte-specific *CD79b* gene (Bennani-Baiti *et al.*, 1998b) and the striated muscle-specific *SCN4A* gene (Bennani-Baiti *et al.*, 1995) are also indicated. The shaded rectangles represent each of the genes (labeled) and the vertical lines within each rectangle indicate their respective exons. The presence of HS in placenta or pituitary are indicated (arrows). The expression patterns of each gene are indicated as strong (+) or trace (tr). The probes used to map chromatin acetylation levels across this region are labeled and underlined below the diagram, and their coordinates relative to the transcription start site of *hGH-N* are indicated. Three probes correspond to segments between *hGH-N* and HSI,II (p1, p2 and p3 located at -5, -9 and -12 kb, respectively); three to segments between HSI,II and HSIII (p4, p5 and p6 located at -17, -21 and -25 kb, respectively); and two to segments 5' of the LCR (p7 and p8 located at -36 and -38 kb, respectively). **(C)** Acetylation of the hGH LCR in human placental chromatin is limited to segments encompassing HSIII and HSV. Chromatin from nuclei selectively released from placental syncytiotrophoblasts (the chromatin preparation analyzed in Figure 1A) was subjected to ChIP analysis. Soluble nuclear chromatin was immunoprecipitated with a mixture of antisera specific to acetylated histones H3 and H4. Equal amounts of DNA purified from starting chromatin (Input DNA), unacetylated chromatin (Unbound DNA) and acetylated antibody-bound chromatin (Bound DNA) were applied to nylon membranes via a slot-blot manifold. The membranes were then sequentially hybridized with the ³²P-labeled probes underlined in (B). The autoradiographs generated using hybridization probes corresponding to each HS of the hGH LCR (including the pituitary-specific HSI,II) and by the *hGH-N* promoter are doubly boxed. Ratios of hybridization signal intensities in the bound and unbound chromatin fractions were normalized to the corresponding ratios obtained using a loading control probe (Total Genomic Probe) and are indicated below each autoradiograph. The normalized ratios are summarized in the histogram. Each bar in the histogram is centered below its respective probe. Black histogram bars represent placental chromatin. This figure shows the ChIP analysis from one representative experiment using chromatin isolated from a single placental preparation shown in (A). All chromatin immunoprecipitations and slot-blot analyses reported were repeated twice with consistent results. White histogram bars represent previously published data obtained from analyses of transgenic mouse lines carrying the entire hGH LCR and linked hGH gene cluster on a P1 transgene (*hGH/P1*) (Elefant *et al.*, 2000). These data are included to facilitate direct comparison with the placental study.

host mice (Jones *et al.*, 1995). A repressive effect of the P-element on transgene expression would be predicted to repress this phenotype of gigantism. Six transgenic mouse lines were generated carrying *HSI,II/PhGHP* and five lines were generated in a parallel set of injections carrying the control *HSI,II/hGH*. Transgene expression was initially monitored by growth curves of the male founders for each of the lines (Figure 2B). In comparison with the growth rate of wild-type littermates, each of the *HSI,II/PhGHP* lines demonstrated clear-cut gigantism. This phenotype was indistinguishable from that of mice carrying the corresponding transgene lacking the P-element. Three founders died secondary to complications of this gigantism (Brem *et al.*, 1989) during the weighing period (Figure 2B). Quantitative analysis of pituitary hGH-N mRNA levels in

three founders from each of the two transgenic groups (three *HSI,II/PhGHP* lines and three *HSI,II/hGH* lines) revealed equivalent high levels of transgene expression (Figure 2C and D). These data suggested that the P-element was unable to repress *hGH-N* expression in the transgenic mouse pituitary.

The P-element stimulated linked transgene expression in the mouse placenta

The lack of a repressive effect of the P-element on gene expression in the pituitary led us to consider that the placental restriction might reflect a positive effect in the placenta. Because the transgenic mouse accurately models placental as well as pituitary expression from the hGH gene cluster (Su *et al.*, 2000), the possibility that the

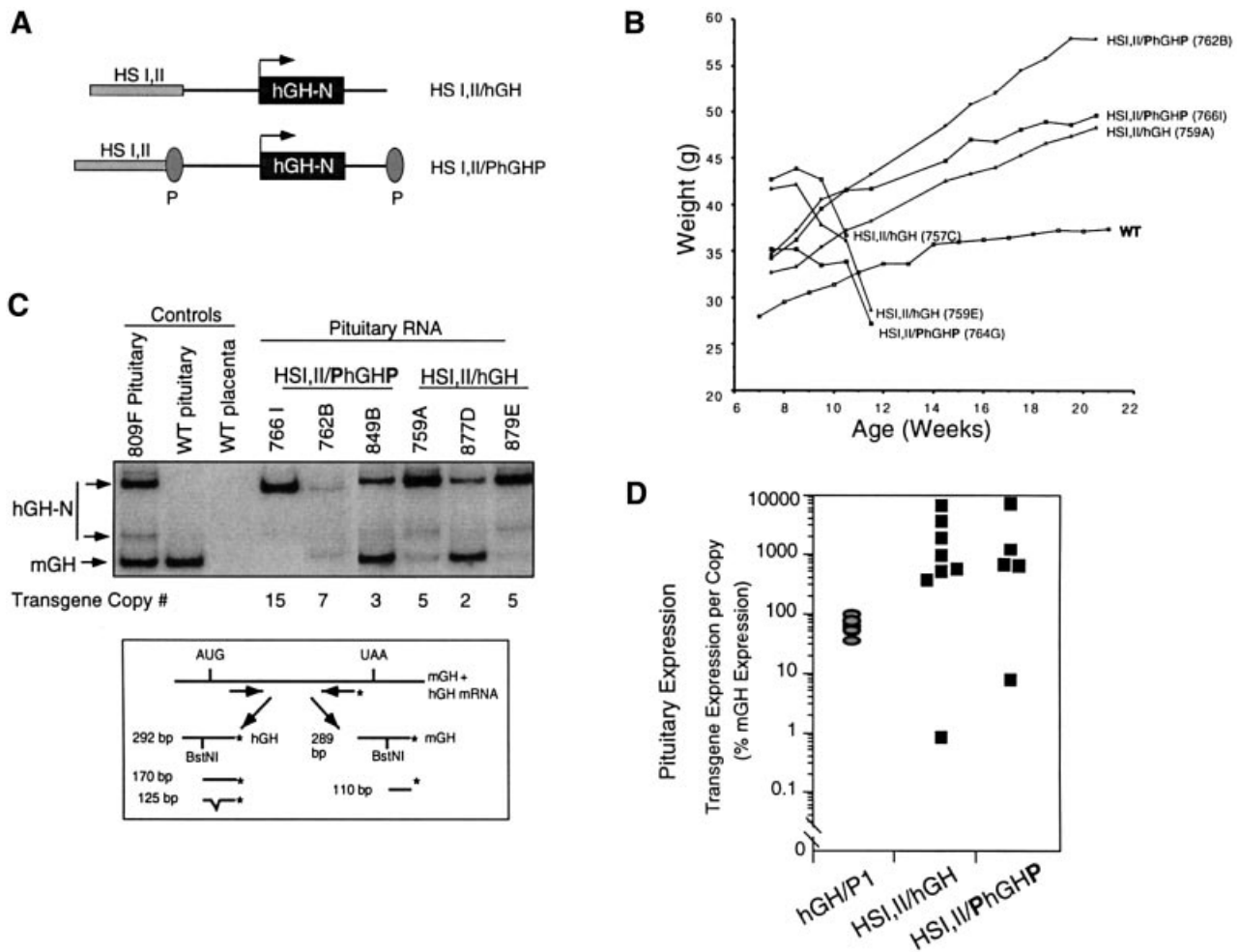


Fig. 2. The P-element fails to repress expression of *hGH-N* in the transgenic pituitary. (A) *HSI,III/hGH* and *HSI,III/PhGHP* transgene constructs. The P-element, previously demonstrated to silence expression of linked genes in transfected pituitary-derived cell lines (Nachtigal *et al.*, 1993), was inserted on either side of *hGH-N* in the context of the pituitary-specific *HSI,III/hGH* transgene to generate the *HSI,III/PhGHP* transgene. (B) Growth curves of mice containing the *HSI,III/hGH* or the derivative *HSI,III/PhGHP* transgenes. Body weights (ordinate) of each male founder transgenic for either transgene were monitored for 22 weeks (abscissa). Each growth curve is labeled with the respective transgene construct and identifying line number. The growth curve for a control, non-transgenic mouse is shown for comparison (WT). The three founders exhibiting the most extreme initial weights experienced rapid pre-morbid decreases in their body weights and died during the study period. (C) *hGH-N* mRNA levels in the pituitaries of *HSI,III/hGH* or *HSI,III/PhGHP* transgenic mice. A diagram depicting the assay is shown below the autoradiograph. *hGH-N* and *mGH* mRNAs were co-amplified from pituitary RNA samples isolated from each of the indicated lines. The 32 P-end-labeled (asterisk) PCR product was digested with *Bst*NI to differentiate between the co-amplified *mGH* and *hGH* mRNA species (see Materials and methods for details). The identity of each band is indicated by the corresponding labeled arrows to the left of the gel. Analyses of *mGH* and *hGH* mRNA content in the pituitaries of three *HSI,III/hGH* and three *HSI,III/PhGHP* transgenic mice are shown. Three of the lines (849B, 877D and 879E) were generated subsequent to the growth curve experiment to compensate for founder deaths. Controls included mRNA from the pituitary of the mouse line expressing the *hGH-N* transgene (line 809F; Jones *et al.*, 1995), mRNA isolated from a wild-type (WT) mouse pituitary (showing only the 110 bp *mGH* cDNA band) and mRNA from wild-type mouse placenta (showing no *hGH-N* or *mGH* expression). (D) Equivalent expression of *HSI,III/hGH* and *HSI,III/PhGHP* transgenes in the mouse pituitary. Band intensities in (C) were quantified by PhosphorImager (Molecular Dynamics) analyses and the levels of *hGH-N* mRNA for each line were normalized to both *mGH* mRNA and transgene copy numbers ($\text{hGH-N mRNA/transgene copy/mGH RNA} = \% \text{ mGH expression}$). This value represents the level of expression from a single transgene copy as a percentage of the expression of a single endogenous *mGH* gene (ordinate; logarithmic scale). These results are compared to previously documented, copy-number-dependent *hGH-N* expression in the pituitaries of mice carrying the *hGHP1* transgene encompassing the entire *hGH* multigene cluster and LCR (gray ovals) (Su *et al.*, 2000). Each data point represents expression from a single transgenic line with a unique transgene insertion site.

P-element serves a direct stimulatory role in placental expression could be tested in this system. The six transgenic founders surviving the morbidity of chronic *hGH* oversecretion (Figure 2B plus the female founders, not shown) were bred to generate transgenic embryos. Placental RNA was isolated on embryonic day (e) 18.5 from each of the embryos identified as transgenic. The *hGH-N* mRNA content in the placenta was analyzed by RT-PCR. Three *HSI,III/hGH* lines (759A, 877D and 879E) and three *HSI,III/PhGHP* lines (766I, 762B and 849B) were

studied. *hGH-N* mRNA was detected in the placentas of two of the three *HSI,III/PhGHP* lines and in none of the three *HSI,III/hGH* lines (Figure 3). Expression of these transgenes in other tissues was limited to trace levels of ectopic expression in brain, heart or liver that varied from line to line, and was unrelated to the presence or absence of the P-element (data not shown). These data suggested that the P-element was driving the expression of *hGH-N* in the placenta and thus might be involved in the normal activation of the *hCS* genes.

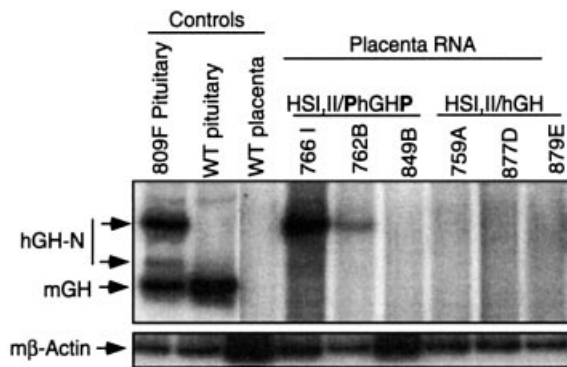


Fig. 3. The P-element activates hGH-N transgene expression in the mouse placenta. mRNA was isolated from the placentas of transgenic embryos derived from each of the six surviving transgenic lines and was analyzed for the presence of hGH-N mRNA by RT-PCR as described in Figure 2C. hGH-N expression was detected in two of three *HSI,II/PhGHP* line placentas (766I and 762B) and in none of three *HSI,II/hGH* line placentas. RT-PCR for mouse β -actin RNA served as control for RNA quality.

The P-element was associated with a focused peak of histone acetylation in placental chromatin

Histone acetylation is associated with positive transcriptional activation in a number of studied systems and tends to be maximal at sites of the corresponding *cis*-acting regulatory element (see Discussion). HSI,II, the major somatotrope-specific positive regulatory element of the hGH LCR, is acetylated in primary human pituitary chromatin and in the pituitaries of transgenic mice carrying the hGH gene cluster linked to and contiguous with the LCR (Elephant *et al.*, 2000). To determine whether acetylation was also involved in gene activation by the conserved P-element, histone modification was assessed at this site in human placental syncytiotrophoblast chromatin. Acetylation enrichment was also assessed at a region 2 kb 3' to *hCS-L*, *hCS-A* and *hCS-B* genes. These regions each contain a conserved element shown to enhance *hCS* expression in transfected, cultured placental cell lines and, on the basis of these studies, have been referred to as the 'hCS enhancers' (Walker *et al.*, 1990) (Figure 4A). ChIP analysis revealed a peak of histone acetylation at the P-elements that was 17-fold above total genomic control levels. In marked contrast, the 3' hCS enhancer elements were not enriched for acetylation, and neither were the P-elements and enhancer elements in primary human pituitary chromatin (Figure 4B). Therefore, the chromatin encompassing the P-element was highly and specifically acetylated in the placenta.

Extension of P-element chromatin acetylation in placental cells was examined next. We had previously demonstrated that the high level of acetylation at HSI,II in pituitary chromatin extends at lower, but still substantial levels bidirectionally for ~32 kb (see Figure 1C, gray bars). To determine whether acetylation within the hGH gene cluster in the placental chromatin was similarly distributed, regions flanking the P-element were probed in the ChIP assay. Probes located -1.95 and -2.4 kb upstream of the P-element (p12 and p13, respectively) revealed essentially no acetylation above background, and the two probes most closely bracketing the P-elements (p11 at -0.5 kb upstream and Pr at 1.5 kb downstream of the

P-element) revealed only low levels of acetylation (Figure 4B). Thus, placental syncytiotrophoblast chromatin acetylation was highly localized to the P-element.

Discussion

Previous studies of the hGH multigene cluster have demonstrated that pituitary-restricted *hGH-N* expression as well as placenta-restricted *hCS-L*, *hCS-A*, *hGH-V* and *hCS-B* expression are dependent upon the hGH LCR. Gene expression in these two tissues is paralleled by the establishment of overlapping sets of tissue-specific DNase I HS in the LCR (Jones *et al.*, 1995; Su *et al.*, 2000). The findings of previous studies, when combined with the present data, suggest that the hGH LCR functions along distinct pathways of chromatin activation to effect mutually exclusive patterns of expression in pituitary and placenta. LCR-mediated *hGH-N* activation in the pituitary correlates with histone acetylation of a 32 kb chromatin domain encompassing the entire LCR. This pattern of chromatin modification, which is found in human pituitaries and is reliably reproduced in transgenic models (Elephant *et al.*, 2000), demonstrates a distinct central peak at the pituitary-specific HSI,II (see Figure 1C, white bars). HSI,II has been demonstrated in transgenic studies to comprise the major positive regulatory element in the LCR for somatotrope expression of *hGH-N* (Jones *et al.*, 1995; Bennani-Baiti *et al.*, 1998a; Shewchuk *et al.*, 1999). These data suggest that histone acetylation, targeted primarily to HSI,II, contributes to pituitary-specific changes in chromatin structure that direct and restrict *hGH-N* expression in this tissue.

The data in the present study demonstrated that the pattern of histone acetylation in placental chromatin was distinct from that in the pituitary. Overlap in the acetylation patterns at the hGH locus in pituitary and placental chromatin was limited to HSIII and HSV; these two sites form in both tissues and were modified to the same extent. The conservation of chromatin structure at these two sites (Figure 1C) implied that they mediate a function(s) shared in pituitary and placenta. The positioning of HSV at the 5' border of the acetylated LCR domain in pituitary chromatin (Elephant *et al.*, 2000) suggested that it may have an insulator role. The 5' and 3' borders of the chicken β -globin gene cluster are similarly bounded by LCR elements that are highly acetylated (Hebbes *et al.*, 1994). These elements have been shown to exert local insulator functions and to contain *cis*-acting elements shared among insulators in unrelated gene systems (Chung *et al.*, 1993; Pikaart *et al.*, 1998; Prioleau *et al.*, 1999; Saitoh *et al.*, 2000). An insulator function for HSV, and possibly HSIII, in the hGH LCR is directly supported by previous studies demonstrating that a fragment containing HSIII and HSV can shield a weakly expressed hGH transgene from position effects in transgenic mice (Jones *et al.*, 1995). Thus, the distal LCR region encompassing HSIII and HSV may serve as the boundary that marks the 5' end of the active hGH locus chromatin domain in both the pituitary and placenta. Additional tests of this function are currently under way.

The initial DNase I mapping of the hGH LCR suggested that HSI,II would be of particular importance to pituitary expression and HSIV in placental expression. The

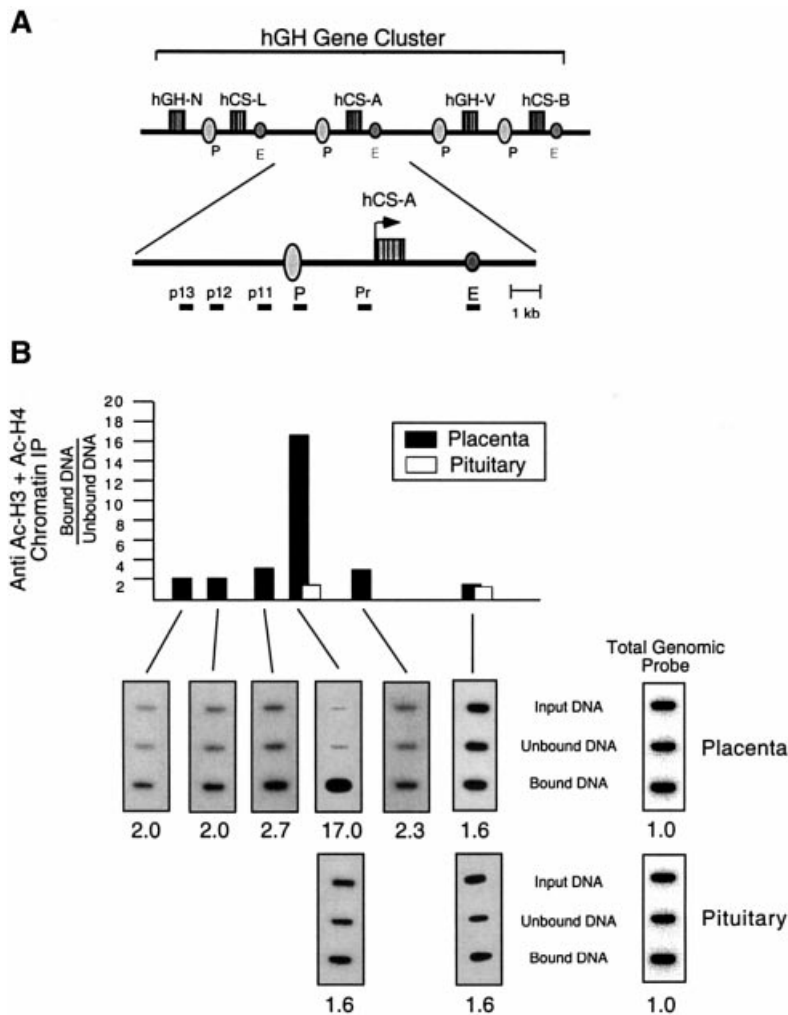


Fig. 4. The P-element displays a localized peak of hyperacetylation in placental chromatin. (A) Diagram of the 48 kb hGH multigene cluster with an enlargement of the hCS-A gene region. An expanded schematic of the hCS-A gene and flanking regions is provided to show the precise spacing of the probes used in this study. The conserved P-elements (P) are located 2 kb 5' to each of the placentially expressed genes. A set of conserved enhancer elements (E) (Jiang and Eberhardt, 1997) are located 2 kb downstream from *hCS-L*, *hCS-A* and *hCS-B*. Due to the high level of sequence identity (92–98%) in the immediate flanking and intervening regions among these genes, we were unable to generate probes specific for each placental locus (see Materials and methods). Therefore, only p13 is specific for the hCS-A locus; each of the remaining probes hybridize to conserved regions adjacent to each of the placentially expressed genes (see Materials and methods). None of these probes, however, are represented elsewhere in the genome (confirmed by Southern blotting; data not shown). (B) Representative ChIP analyses of placental and pituitary chromatin at the hCS-A locus. Soluble nuclear chromatin prepared from each of the indicated sources was immunoprecipitated with anti-acetylated histone H3 and H4 antibodies, and analyzed as in Figure 2. All ratios were normalized to the ratio obtained using a probe for total genomic human DNA as a loading control (shown to the right). The normalized ratios detected in the bound versus unbound chromatin fractions are shown below each respective autoradiograph and are summarized in the histogram. The black histogram bars represent human placental chromatin. White histogram bars represent analysis of human pituitary chromatin at the P-element and enhancer regions.

subsequent studies, summarized above, have confirmed that HSI,II is the main positive regulatory element within the hGH LCR (Jones *et al.*, 1995; Bennani-Baiti *et al.*, 1998a; Shewchuk *et al.*, 1999). HSIV, being unique to the placenta, might have a comparable function in that tissue. However, its role remains less well defined. Although it forms in placental chromatin (Figure 1A), the region encompassing HSIV is not acetylated (Figure 1C). The specific lack of HSIV acetylation in the placenta implies that any effect that it might be exerting on gene expression in this tissue is mediated by a mechanism independent of histone modification. These results suggested that one or more regulatory element(s) in addition to the 5'-flanking LCR might be involved in the selective expression of the subset of the hGH gene cluster in the placenta. The

P-element may fill this role. Tissue culture-based studies had suggested that the P-elements, located 2 kb upstream of each placentially expressed gene in the hGH cluster, selectively repress the placentially expressed genes in the pituitary (Nachtigal *et al.*, 1993). Our *in vivo* test of the P-element using transgenic mice failed to support this repressive model (Figure 2). Instead, the *in vivo* expression studies (Figure 3) and dramatic acetylation of the P-element in placental chromatin (Figure 4) support a model in which the placental activation of *hCS* reflects action by the P-element.

The mechanism of P-element function is not known. The P-elements upstream of *hCS-L*, *hGH-V*, *hCS-A* and *hCS-B* are each directly preceded by a 30- to 50-nucleotide-long polypurine tract (A_nG) (Chen *et al.*,

1989). Such tracts have been shown to form unusual helical structures that influence the binding of specific *trans*-acting factors and exclude nucleosomes (Gross and Garrard, 1988). Such a role for the P-element in chromatin modulation may explain its lack of stimulatory activity in transient transfection assays, because such assays, based on expression of loosely packaged episomes, may not accurately recapitulate chromatin-based mechanisms of gene activation (Jeong and Stein, 1994). Similarly, the lack of acetylation at the chromatin segment encompassing the *hCS* 3' enhancer elements in primary syncytiotrophoblast nuclei was unexpected. These enhancers demonstrate a clear stimulatory action on *hCS* expression in cell transfection studies (Walker *et al.*, 1990; Jiang and Eberhardt, 1995). These data run counter to the general correlation between enhancer action and histone acetylation (Sheridan *et al.*, 1997; Krumm *et al.*, 1998; Forrester *et al.*, 1999; Forsberg *et al.*, 1999; McMurry and Krangel, 2000). Because the *hCS* enhancer has only been defined in the context of cell transfection studies, a firm resolution of its effects *in vivo* and in the context of chromatin must await further analysis in appropriate systems.

The acetylation of the P-element was spatially localized, substantial, and restricted to placental chromatin (Figure 4). This suggested specific targeting of histone acetyl transferase (HAT)-containing co-activators to this site. Similarly, targeted and localized acetylation has been found at a limited number of studied genes and has been demonstrated to be tightly linked to transcriptional activation (Kuo *et al.*, 1998; Chen *et al.*, 1999; Parekh and Maniatis, 1999; Schubeler *et al.*, 2000; Vignali *et al.*, 2000). In support of a stimulatory role for the P-element in placental *hCS* activation, two of three *HSI,II/PhGHP* transgenic lines expressed *hGH* mRNA in the mouse placenta whereas no *hCS* expression was detected in any of the three *HSI,II/hGH* lines tested (Figure 3). The line-to-line variability in the levels of *HSI,II/PhGHP* transgene expression in the placenta (Figure 3; lines with high, low and no appreciable expression) suggested site-of-integration position effects. This position dependence was consistent with our previous data demonstrating a similar lack of copy-number dependence for the *HSI,II/hGH* transgene in the pituitary (Jones *et al.*, 1995) (Figure 2D). In both cases, the lack of copy-number-dependent expression may reflect the absence of LCR component(s) such as HSV and HSIII that serve to insulate the transgene from surrounding influences at the insertion site (see above).

Of note, the present ChIP data were generated with a mix of anti-H3 and anti-H4 antibodies. Using antibodies specific for each of the histones in higher eukaryotic systems may (Schubeler *et al.*, 2000) or may not (Parekh and Maniatis, 1999) reveal selectivity in the distribution of acetylation. In the present case, the observation of a dramatic peak of acetylation at the P-element and a lack of acetylation in the domain upstream of the *hGH* cluster (between the *hGH* gene and HSIII) clearly distinguish the placental from pituitary patterns of chromatin modification. This distinction would not be altered by more defined studies. The present data suggests that the stimulatory effect attributed to the P-element could be mediated by the recruitment to that site of a tissue-specific and highly restricted HAT activity. Distinctions between the acetylation of separate classes of histones, and among the various

target lysines on each particular histone, may be of interest in future studies as the HAT complexes responsible for the modifications in the placenta and pituitary are identified.

The distinct differences in the patterns of histone acetylation at the *hGH* LCR in placenta and pituitary appear to reflect distinct mechanisms by which HAT activities are involved in the regulation of gene transcription over long distances. The mechanism mediated by *HSI,II* in the pituitary may reflect extended propagation of histone acetylation with resultant creation and/or maintenance of an activated chromatin domain (Hebbes *et al.*, 1994; Madisen *et al.*, 1998; Elefant *et al.*, 2000; Schubeler *et al.*, 2000). In contrast, the P-element appears to exert a more localized effect on chromatin structure (Kuo *et al.*, 1998; Struhl, 1998; Parekh and Maniatis, 1999; Schubeler *et al.*, 2000). The recent identification of two distinct HAT-containing complexes that mediate either restricted or extended modification of chromatin templates *in vitro* supports the two modes of chromatin modification observed in the expression of the *GH* locus (Vignali *et al.*, 2000). Thus the distinct patterns of acetylation observed within the *hGH* locus in pituitary and placental chromatin suggest a model in which specific positive regulatory elements can in a tissue-specific manner target distinct HATs to a single LCR and multigene locus. It will be of considerable interest to identify the specific HATs involved in the extensive versus localized pattern of hyperacetylation in pituitary and placental chromatin, in order to test this model *in vivo*.

Materials and methods

Transgene construction

To create *HSI,II/hGH*, a 1.6 kb *Bgl*II restriction fragment containing *HSI,II* was isolated and inserted in the native orientation upstream of *hGH-N* (2.6 kb *Eco*RI fragment R2 of *hGH-N*; Chen *et al.*, 1989) at the *Bam*HI site of *pBSIIKS-hGH-N* (*pBSIIKS*; Stratagene). This *hGH-N* gene fragment contained 494 bp of 5'- and 530 bp of 3'-flanking sequences. To create *HSI,II/PhGHP*, *Eco*RI linkers were added to a Klenow-generated and blunt-ended 263 bp *Bam*HI and *Hinf*I restriction fragment encompassing the P-element sequence (Nachtigal *et al.*, 1993). This end-modified P-element was inserted in its native orientation both 5' and 3' of *hGH-N* in the *HSI,II/hGH* plasmid.

Generation and analysis of transgenic mice

Transgene inserts released from vector sequences by *Bss*HII digestion were resolved on agarose gels, isolated using a Qiaex II gel extraction kit, purified through an Elutip (Schleicher and Schuell) and diluted to 2 ng/ μ l in 10 mM Tris-HCl pH 7.6, 0.1 mM EDTA. This DNA solution was then micro-injected into fertilized C57BL/6J \times SJL mouse embryos. Transgenic founders were detected by dot-blot analysis of tail DNA using a 1.37 kb *Sma*I fragment derived from *hGH-N* (coordinates -494 to +876 relative to the start of *hGH-N* transcription) as probe. The integrity of the transgenes and their copy numbers were determined by Southern blot analyses of *Bgl*II-digested tail DNA using the *hGH* genomic probe. Only founders carrying intact transgenes (as detected by a 4.8 kb hybridizing band for *HSI,II/PhGHP* lines and a 4.2 kb hybridizing band for *HSI,II/hGH* lines) were used for subsequent studies. Transgene copy number was established by comparing the signal intensity of the repeated transgene hybridization band with that of the hybridizing band corresponding to the single-copy junction fragment.

Detection of *hGH* transcripts in transgenic pituitary and placental RNA by RT-PCR

Pituitary and placental RNAs were prepared from tissues isolated from mice immediately following decapitation as described (Jones *et al.*, 1995). *hGH-N* expression in the pituitary and placenta was detected by RT-PCR using individual organs as previously described (Jones *et al.*, 1995; Su *et al.*, 2000). Briefly, reverse transcription using AMV RT

Table I. Oligonucleotide primers used in mRNA detection or as probes for ChIP analyses

Probe	Size	Primer set
<i>mhGH</i>	290 bp	(5'-GCCTGCTCTGCCTGC-3') (5'-GACTGGATGAGCAGCAG-3')
p11	180 bp	(5'-GAATCCCAAGTCTAATGC-3') (5'-CCTTTATAAGGGGTTACC-3')
p12	194 bp	(5'-CCTGGACATATCATATGGC-3') (5'-CAAGGAACCTCAGCCACAG-3')
p13	175 bp	(5'-GAGCTCTGATTCTCTAGCCC-3') (5'-CATGGTTCTCACTGCCC-3')

(Promega, Madison, WI) was performed using 0.2–0.5 µg of total RNA. Subsequent PCR was carried out using primers corresponding to perfectly conserved regions between mouse (m) *GH* and *hGH* (primers in Table I). Following 30 cycles of PCR, the 3'-end-labeled cDNA products were digested with *Bst*NI to yield fragments specific to hGH-N (170 and 125 bp) and mGH (110 bp) mRNAs. To compare the levels of hGH mRNA relative to endogenous mGH mRNA in the pituitary, the intensities of the mGH- and hGH-specific bands were quantified by PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA) analysis using ImageQuant software. The ratio was then divided by transgene copy number (see above) to establish the transgene expression per copy.

Isolation of nuclei from placenta

Intact nuclei were selectively isolated from the syncytiotrophoblast cells of human term placentas as described previously (Jones *et al.*, 1995) with the following minor modifications. Villous fragments were excised from placenta, resuspended in cold phosphate-buffered saline and finely minced. Fragments that passed through a 10-gauge screen were pelleted, resuspended in 150 mM NH₄Cl containing 0.5 g of NH₄HCO₂ per 10 g of tissue, and incubated at 4°C. After 45 min, the majority of syncytiotrophoblast cells had undergone osmotic lysis due to their high levels of carbonic anhydrase. The preparation was pelleted at 1000 g to remove unlysed cells and residual NH₄HCO₂. The nuclear pellets were resuspended in 45 ml cold RB [0.1 M NaCl, 50 mM Tris-HCl pH 8.0, 3 mM MgCl₂, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM sodium butyrate] and the preparation was passed through a cheesecloth to separate the syncytiotrophoblast nuclei from tissue debris. Preparations were examined under the microscope to confirm the presence of intact nuclei.

DNase I HS mapping

Nuclei were washed and resuspended in RB buffer and digested with 1.0 µg/ml of DNase I (Gibco-BRL, Grand Island, NY) at 37°C for increasing amounts of time. Reactions were terminated by the addition of Na₂EDTA to 50 mM and the nuclei were lysed by overnight incubation at 37°C in 0.8 M NaCl, 50 mM EDTA, 0.5% SDS, 200 µg/ml of proteinase K. The samples were then phenol-chloroform extracted, ethanol precipitated, and digested with *Eco*RI prior to Southern blotting. DNA samples were resolved on 0.8% agarose gels, transferred to Zetabind nylon membranes (Cuno Inc., Meriden, CT), and pre-hybridized at 65°C overnight as previously described (Su *et al.*, 2000). The membranes were subsequently incubated overnight at 65°C with hybridization solution containing 1–2 × 10⁶ c.p.m./ml of random primer-labeled probe. Subsequent washes were at 60°C in 0.1% SDS and 0.1 × SSC, followed by autoradiography.

Immunoprecipitation of unfixed chromatin

Preparation of unfixed chromatin and the chromatin immunoprecipitation (ChIP) procedure were both carried out as previously described (Hebbes *et al.*, 1994; O'Neill and Turner, 1995, 1996; Crane-Robinson *et al.*, 1997) with certain modifications (Elefant *et al.*, 2000). Briefly, 0.3 mg nuclei were digested with 25 U of micrococcal nuclease at 37°C for 6 min in 1 ml of digestion buffer containing 50 mM NaCl, 20 mM Tris-HCl pH 7.5, 3.0 mM MgCl₂, 1.0 mM CaCl₂, 10 mM sodium butyrate, 0.1 mM PMSF. The digestion was terminated by the addition of Na₂EDTA to 0.5 mM and the salt-soluble chromatin was isolated as described (Hebbes *et al.*, 1994). Soluble chromatin was concentrated using a Microcon centrifugal filter (Amicon Inc., Bedford, PA) and 250 µg of this chromatin (input) was incubated with 10 µl each of antisera specific for the acetylated forms of histones H3 or H4 (Upstate Biotech., Lake Placid, NY) in a total volume of 500 µl. Protein A-Sepharose (Amersham Pharmacia Biotech) precipitates were generated, washed, and DNA was purified from the pellets (bound) and supernatants (unbound) as previously described (O'Neill and Turner, 1996). DNA samples from

the input, bound and unbound fractions were analyzed by electrophoresis on 1% agarose gels to determine the size distribution of the resulting oligonucleosomes. The majority of DNA ranged from 160 bp (mononucleosomes) to 1 kb; only trace DNA could be visualized above 2 kb.

Equal masses of DNA (1.0 µg) from input, unbound and antibody-bound DNAs were loaded onto Zetabind nylon membranes using a slot-blot manifold. The blots were incubated overnight at 65°C with hybridization solution containing 1–2 × 10⁶ c.p.m./ml random primer-labeled probe. Subsequent washes were at 60°C in 0.1% SDS and 0.5 × SSC. Signals were quantified by PhosphorImager analysis. The ratios between bound and unbound DNA fractions were calculated for each probe used. All ratios were normalized for total DNA loading onto the slot blot by rehybridizing the membrane with ³²P-labeled total genomic DNA (Elefant *et al.*, 2000). All ratios were verified in a minimum of two separate experiments.

Hybridization probe preparation

The majority of hybridization probes were generated by PCR using AmpliTaq DNA polymerase (Perkin-Elmer). A P1 clone encompassing the hGH LCR and the first four genes of the hGH cluster (Su *et al.*, 2000) served as the template. The primer sets used for generating probes p1–p8 and probes corresponding to HSI,II, HSIII, HSIV and HSV (Figure 1) were as previously described (Elefant *et al.*, 2000). The sequences of the additional primer sets used for the present studies and the predicted sizes of the generated probes in bp are summarized in Table I. The P-element probe consisted of a subcloned 263 bp *Eco*RI fragment (a kind gift from Dr Peter Cattini, University of Manitoba, Canada) (Nachtigal *et al.*, 1993). The *hCS* enhancer probe was a 240-bp amplified fragment of the repeated enhancer element sequence (Jiang and Eberhardt, 1997). Probe p13 is specific for the *hCS*-A upstream region; the p12, p11, P-element and E probes hybridize to conserved elements adjacent to each of the placental genes. The hGH promoter probe was a 500 bp *Bam*HI fragment encompassing the hGH-N gene promoter region. This probe cross hybridized with the *hCS* and hGH-V promoters due to their high degree of sequence identity. A genomic DNA loading control probe was generated by random primer labeling of sonicated human total genomic DNA.

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