Pit-1 Binding Sites at the Somatotrope-specific DNase I Hypersensitive Sites I, II of the Human Growth Hormone Locus Control Region Are Essential for in Vivo hGH-N Gene Activation*

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The human growth hormone gene cluster is composed of five closely related genes. The 5′-most gene in the cluster, hGH-N, is expressed exclusively in somatotropes and lactosomatotropes of the anterior pituitary. Although the hGH-N promoter contains functional binding sites for multiple transcription factors, including Sp1, Zn-15, and Pit-1, predictable and developmentally appropriate expression of hGH-N transgenes in the mouse pituitary requires the presence of a previously characterized locus control region (LCR) composed of multiple chromatin DNase I hypersensitive sites (HS). LCR determinant(s) necessary for hGH-N transgene activation are largely conferred by two closely spaced HS (HS II) located 14.5 kilobase pairs upstream of the hGH-N gene. The region sufficient to mediate this activity has recently been sublocalized to a 404-base pair segment of HS II (F14 segment). In the present study, we identified multiple binding sites for the pituitary POU domain transcription factor Pit-1 within this segment. Using a transgenic founder assay, these sites were shown to be required for high level, position-independent, and somatotrope-specific expression of a linked hGH-N transgene. Because the Pit-1 sites in the hGH-N gene promoter are insufficient for such gene activation in vivo, these data suggested a unique chromatin-mediated developmental role for Pit-1 in the hGH LCR.

Tissue-specific regulation of eukaryotic genes is mediated by the combined action of ubiquitous and tissue-restricted transcription factors. These factors are brought into functional concert by binding to multiple classes of gene regulatory determinants, leading to the appropriate induction of transcription (reviewed in Ref. 1). One class of regulatory elements appears to function primarily at the level of chromatin structure. The role of these elements is to establish transcriptionally competent domains that are available for interactions with the appropriate trans-factors. These chromatin elements are most reliably detected and mapped based on their ability to support expression of linked transgenes independent of the site of integration in the host genome (2). When detected in the mammalian genome, these elements are termed locus control regions (LCRs); reviewed in Ref. 3. Components of locus control regions, which may be situated at significant distances from their target genes, can be identified by their ability to establish DNase I hypersensitive sites (HS) in the chromatin of expressing tissues. The mechanism for LCR function is inferred to be biphasic, involving the initial alteration of chromatin structure and subsequent transcriptional activation (reviewed in Ref. 4).

The human growth hormone gene, hGH-N, is the 5′-most member of a five-gene cluster on chromosome 17. These genes share greater than 95% sequence identity and encode structurally similar proteins (5) (Fig. 1). Despite this structural similarity, the hGH-N gene is expressed exclusively in the somatotropes and lactosomatotropes of the anterior pituitary, while the remaining genes of the cluster (hCS-L, hCS-A, hGH-V, and hCS-B) are expressed exclusively in the syncytiotrophoblasts of the placental villi (6–8). This tissue specificity does not appear to be attributable to proximal transcriptional control elements per se, as evidenced by the similar expression of hGH-N and the placenta-specific genes in transplanted pituitary cells (9). Furthermore, retention of as much as 7.5 kb of 5′-flanking sequence in cis to the hGH-N gene is insufficient to recapitulate appropriate levels and specificity of expression in transgenic mice (10). These data suggest the participation of distal regulatory element(s) in the developmental activation of hGH-N gene expression.

A set of DNase I HS located between 14.5 and 30 kb upstream of the hGH gene cluster has been identified exclusively in pituitary and placental chromatin (HS I–V; Fig. 1). The tissue-specific nature of these sites implies that they play a role in regulation of the hGH gene cluster. Human GH-N transgenes with extensive 5′-flanking regions encompassing these remote HS are expressed at high levels and in a position-independent and copy number-dependent manner in the mouse pituitary. These data indicate that these DNase I HS constitute an LCR for the hGH gene cluster and that the transgenic mouse is an appropriate model system in which to study its function (10). The somatotrope-specific determinants of the LCR map to the region marked by two closely linked HS approximately 14.5 kb 5′ to the hGH-N gene (HS II; Fig. 1) (10). HS II activity was initially isolated on a 1.6-kb fragment and was subsequently sublocalized to a 404-bp segment (the F14 segment) (11). This F14 segment is sufficient to confer high level, somatotrope-specific, and position-independent expression of a linked hGH-N gene in transgenic mouse pituitary.

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1 The abbreviations used are: LCR, locus control region; EMSA, electrophoretic mobility shift assay; hGH, human growth hormone; mGH, murine growth hormone; HS, hypersensitive site(s); PCR, polymerase chain reaction; bp, base pair(s); kb, kilobase pair(s); mAb, monoclonal antibody.
Furthermore, the F14 segment mediates appropriately timed induction of the linked hGH-N transgene during mouse embryonic development, paralleling induction of the endogenous mGH gene (11). In the present study, we identified multiple binding sites for the Pou domain transcription factor Pit-1 within the F14 segment of the HS I,II region and have established that these sites are essential components of LCR-mediated gene activation in the pituitary in vivo.

**EXPERIMENTAL PROCEDURES**

Preparation of DNA Probes for Binding Studies—The previously described F14 segment contains the 3′-terminal 404 bp of the 1602-bp HS I,II sequence (GenBank™ accession no. AF039413). DNA fragments used as probes in electrophoretic mobility shift assays (EMSAs) and DNase I footprinting assays were generated by PCR using a plasmid (pHSI, II-11) containing the 1602-bp HS I,II region as a template. PCRs consisted of 10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 0.2 mM each dNTP, 0.5 μg each primer, 10 ng of template DNA, and 2.5 units of AmpliTag DNA polymerase (Perkin-Elmer) in a total of 100 μl. Primers for fragments F14.1, F14.2, F14.3, F14.4, and F14.5 were designed to add EcoRI sites to both ends of the resulting PCR product to allow 3′-end labeling (Table I). The end points of these fragments relative to the 1602-bp HS I,II region are as follows: F14.1, 1164–1225; F14.2, 1275–1359; F14.3, 1356–1455; F14.4, 1435–1524; F14.5, 1520–1602. The F14 region DNase I footprinting probe containing nucleotides 1164–1125 was amplified with primers designed to add an EcoRI site at one end of the amplicon to allow the sense strand relative to hGH-N to be 3′-end-labeled. The 3′-ends of probes digested with EcoRI were radioiodolated using [α-32P]dATP and Klenow DNA polymerase. Fragments F14.1.3, F14.2, F14.3.3, and F14.3.4 were generated by annealing complementary synthetic oligonucleotides and 5′-end labeling with [32P]dATP and T4 polynucleotide kinase. All radioiodolated probes and unlabeled competitor fragments were purified on 5% polyacrylamide. Wild type and mutant duplex Pit-1 oligonucleotides were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

**Tissue Culture—**GH3 (12), GHFT1 (13), and NIH3T3 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin.

Preparation of Crude Nuclear Extracts—Nuclear extracts of NIH/3T3, GHFT1, GH3, and primary mouse pituitary cells were prepared as described (14). Pituitaries excised from multiple CD-1 mice were placed in ice-cold phosphate-buffered saline and collected by centrifugation at 1000 × g for 10 min. After removing the supernatant, the pituitaries were incubated in dissociation buffer (Life Technologies, Inc.) for 1 min at room temperature and then disaggregated by repeated pipetting, and the larger material was allowed to sediment. The buffer and dissociated cells were removed to a tube containing 40 ml of cold Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. This process was repeated until the pituitary tissue was completely dissociated.

EMSAs—Protein-DNA complexes were detected in vitro on the basis of changes in the electrophoretic mobility of a radioiodolated DNA probe (15). Binding reactions (25 μl) contained 5 ng of an end-labeled, double-stranded DNA fragment in buffer composed of 10 mM HEPES (pH 7.9), 1 mM EDTA, 1 mM dithiothreitol, 32 mM KCl, 10% glycerol, 0.08 μl/giter poly[d(dC)] and 0.3 μl/giter bovine serum albumin. Nuclear extract (4 μg) was added last, and the mixture was incubated for 20 min at room temperature. Samples were resolved on a 5% nondenaturing polyacryl
The human growth hormone gene cluster. The growth hormone gene (hGH-N) is shown as a black box. The growth hormone variant (hGH-V) gene and the three chorionic somatomammotropin (hCS) genes are shown as dark gray boxes. The unrelated but closely linked SCN4a and CD79b genes are shown as light gray boxes (51, 52). An arrow above each gene shows its transcriptional orientation. The position of HS in pituitary and placental chromatin are marked by the down arrow pointing arrows.

Immunohistochemical Staining—A highly specific anti-hGH monoclonal antibody has been previously described (mAb 9) (11). Anti-rat growth hormone, which cross-reacts with mGH, was obtained from the National Hormone and Pituitary Program. Sections of paraffin-embedded mouse embryos were stained with hematoxylin and eosin. Anti-rat growth hormone was used at a 1:2500 dilution, and mAb 9 was used at 1:1500; incubations were for 24 h at room temperature. The specificity of immunostaining was documented by nonimmune sera and by competition with purified hGH (Oako). All reactions were visualized with streptavidin-biotin-peroxidase (Autoprobe III detection system; Biomeda) and were revealed with the chromagen 3,3’-diaminobenzidine (11). Co-localization of hGH and mGH was determined by staining adjacent sections. Scoring of embryos was performed by an individual who was blind to transgene presence or identity. Because of the non-quantitative nature of the immunohistochemistry assay and the fact that HS I,II alone does not confer copy number dependence (10), expression level per transgene copy was not addressed. Copy number was determined to control for the range of copy numbers carried by each construct were compared. It should be noted that although some of the hGH-positive embryos carried a relatively high transgene copy number, this did not bias the staining assay or expression data because F14.3 spontaneous embryos with comparable copy numbers were all negative for hGH-N expression.

RESULTS

Somatotrope-specific Nuclear Proteins Bind Specifically to Sequences within the 404-bp F14 Segment of HS I,II—Formation of HS I,II of the hGH LCR is specific to nuclear chromatin from pituitary cells (Fig. 1) (10). The determinants of HS I,II involved in the somatotrope-specific activation of the linked hGH-N transgene have been localized to a 404-bp subsegment, F14 (nucleotides 1198–1602 in GenBank™ accession no. AF039413) (11). Computer-assisted sequence analysis of this region identified multiple potential trans-factor binding sites, some of which are recognized by ubiquitous transcription factors and nuclear hormone receptors that could possibly contribute to its function. However, no motifs were identified that fit the pituitary-specific profile of F14 activity. To determine whether F14 could interact with one or more pituitary-specific factors, EMSAs were performed using nuclear extract from the mouse fibroblast cell line NIH3T3 and the mouse presomatotrope cell line GHFT1, as well as from the growth hormone-expressing rat somatotrope cell line GH3 and primary mouse pituitary cells. As an initial control to confirm the phenotype of these four extracts, each was incubated with a probe containing a binding site for the pituitary-enriched POU domain transcription factor Pit-1 (Fig. 2A). Consistent with the ordered phenotype of each of the cells, strong binding to the Pit-1 probe was observed with extracts from the GHFT1, GH3, and mouse pituitary nuclei but not with NIH3T3 nuclear extracts (Fig. 2A). The Pit-1 complexes formed by the three pituitary cell lines consisted of a strong doublet. An additional minor Pit-1 complex of higher mobility was formed by the two extracts originating from the mature somatotrope cells (GH3 and primary pituitary; Fig. 2A). The presence of multiple Pit-1 complexes was attributed to the presence of multiple isoforms of Pit-1 (reviewed in Ref. 20) and was not further explored.

To screen the F14 segment of HS I,II for protein binding, the 404-bp segment was divided into five partially overlapping probes: F14.1, F14.2, F14.3, F14.4, and F14.5 (Fig. 2B). Each of these probes was initially screened by incubation with GHFT1 and fibroblast (NIH/3T3) extracts. Whereas all five subfragments formed a variety of weak complexes that were largely observed in both the fibroblast and the GHFT1 extracts, a distinct and strong GHFT1-specific doublet complex was assembled on the F14.3 probe (Fig. 2B; lane 9). Incubation of this F14.3 probe with the full set of nuclear extracts demonstrated formation of the strong doublet by proteins from each of the somatotrope-related cell lines (Fig. 2C, lanes 3–5). The same doublet was also assembled on the F14.1 probe, although this complex was much less intense (Fig. 2B, lane 3). The doublet complex could be self-competed by the addition of a 10- and 50-fold molar excess of unlabelled F14.3 and F14.1 segments, indicating that the corresponding interactions were sequence-specific (Fig. 2C, lanes 6–15). The F14.1 fragment was a weaker competitor than F14.3, consistent with the weaker intensity of these complexes on an F14.1 fragment probe (Fig. 2B; compare lane 3 with lane 9). Thus, the F14 segment of the HS I,II LCR determinant bound to one or more pituitary-specific nuclear proteins, and these binding sites were localized to two subdomains, F14.1 and F14.3.

F14.1 and F14.3 Probes Form Complexes with the Pituitary-enriched POU Domain Transcription Factor Pit-1—The complexes that formed on the F14.1 and F14.3 probes were not to be strikingly similar in gel migration to Pit-1 complexes (compare Fig. 2C, lanes 3–5, with 2A). In addition, the Pit-1, F14.1, and F14.3 probes all formed the intense doublet complex specifically with the nuclear extracts from the three pituitary cell types (double arrows in Fig. 2, A–C). This comparison suggested that the complexes forming on F14.1 and F14.3 contained Pit-1. This hypothesis was confirmed by supershift reactions using polyclonal and monoclonal Pit-1 antibodies. The intensity of the pituitary-specific complexes formed by all three pituitary cell nuclear extracts was diminished by the presence of the antibody, with the concomitant appearance of multiple supershifted complexes (Fig. 2D and data not shown). The complexes formed by the NIH3T3 nuclear extract were unaffected by the Pit-1 antibody (data not shown). As an additional test for the presence of Pit-1 in these complexes, the control oligonucleotide containing a known Pit-1 binding site was used as a cold competitor. The somatotrope-specific complexes were specifically competed by a 30-fold molar excess of the Pit-1 oligonucleotide (Fig. 2D, lane 4). The high mobility Pit-1 complex specific for GH3 and mouse pituitary nuclear extracts was efficiently competed but inefficiently supershifted (Fig. 2D, compare lanes 3 and 4), possibly indicating a minor Pit-1 isoform with a different topology than that of the major 30–33-kDa isoform immunogen. The same antibody supershift and competition experiments were performed with the labeled F14.1 subfragment probe, and the outcome was the same (Fig. 2D, lanes 5–8). Therefore, the full set of EMSA studies indicated that the pituitary-specific complexes formed on the F14.3 and F14.1 probes were due to Pit-1 binding.

Pit-1 Binds Two Discrete Sites within the F14.3 Subfragment and One Site within the F14.1 Subfragment of HS I,II—The EMSA studies detailed above did not define whether Pit-1 was binding to a single site or to multiple sites within the target F14 probes. Because of the relatively loose consensus Pit-1 recognition motif (21), it was difficult to predict the exact site(s) of Pit-1 binding. To directly sublocalize the Pit-1 binding site(s), F14.3 was divided into four partially overlapping sub-
The F14 subfragment of the hGH LCR HS I,II formed pituitary-specific complexes that contained Pit-1. A confirmation of Pit-1 in nuclear extract preparations of lactosomatotrope lineage cell lines. The source of nuclear extract is shown above each lane. The Pit-1 complexes are indicated by arrows. Free refers to uncomplexed probe. B, scanning the F14 fragment for somatotrope-specific nuclear protein binding. The probe used in each reaction is shown below the corresponding lanes and is indicated on the map of the 404-bp HS I,II F14 shown below the gel. The extent of each probe is shown by a horizontal line with the positions of their end points relative to the ends of the 404-bp F14 fragment indicated. The arrows show the major somatotrope-specific complexes. The high background with the F14.1 probe may be due to an extensive palindromic sequence. C, specificity of complexes assembled on the F14.3 subfragment. Major and minor pituitary-specific complexes are shown (double arrow and single arrow, respectively). Cold competitor fragments included in binding reactions are indicated above the lanes. D, supershift with a polyclonal Pit-1 antibody (pAb) and competition with a 30-fold molar excess of an unlabeled Pit-1 element show that the pituitary-specific complexes that formed on F14.3 and F14.1 in an EMSA contained Pit-1. The pituitary-specific complexes (bracket) and supershifted complexes (arrows) are indicated on the left.

The Pit-1 Binding Sites within the F14 Fragment of HS I,II Are Essential for Consistent Activation of the hGH-N Transgene in Somatotropes of the Embryonic Mouse Pituitary—Determinants sufficient to confer high level, position-independent, somatotrope-specific expression of a linked hGH-N gene in transgenic mice have previously been resolved to the F14 segment of HS I,II (11). As described above, the pituitary-specific complexes formed on this fragment corresponded to three Pit-1 binding sites. The functional contribution of these Pit-1 sites to LCR function was next tested. We focused on the Pit-1 sites within the F14.3 sequence because they appeared by EMSA to demonstrate the highest binding affinity (Fig. 2B, compare lanes 3 and 9; Fig. 2D, compare lanes 2 and 6). The entire F14.3 region was deleted from F14 to test the functional role of these two Pit-1 sites in F14.3 (Fig. 4). Several additional footprints were also seen, as predicted by the presence of non-Pit-1 complexes on the EMSA (Fig. 2C’ and data not shown). Thus, the EMSA and footprint analysis were internally consistent in demonstrating an array of three Pit-1 sites in the F14 segment of HS I,II (Fig. 4, bottom).

The positions of the three Pit-1 sites within the F14 fragment were confirmed by DNase I foot printing. Regions of the F14 fragment protected from DNase I digestion by GH3 nuclear extract corresponded to the single Pit-1 site in F14.1 and the two Pit-1 sites in F14.3 (Fig. 4). Several additional footprints were also seen, as predicted by the presence of non-Pit-1 complexes on the EMSA (Fig. 2C’ and data not shown). Thus, the EMSA and footprint analysis were internally consistent in demonstrating an array of three Pit-1 sites in the F14 segment of HS I,II (Fig. 4, bottom).
gene in native orientation. This hGH-N gene contained its own contiguous promoter within 0.5 kb of 5'-flanking sequence encompassing two functional Pit-1 sites (22) (Fig. 5A). Each construct was injected into fertilized oocytes, and reimplanted embryos were harvested at embryonic day 18.5. The transgenic status of each embryo was established by dot-blot analysis of placental DNA. Sagittally sectioned anterior segments of each embryo were fixed. Pituitaries were identified by light microscopy, and the presence of hGH and mGH staining was scored by immunostaining with antibodies specific for hGH or mGH. The immunohistochemical analysis was carried out without prior knowledge of the presence or identity of the corresponding transgene. The results of the immunostains, which could not be rigorously quantified due to the nature of the assay, were grouped into three categories based on the relative intensities of hGH and mGH signals: 1) hGH expression in somatotropes at levels stronger than endogenous mGH; 2) hGH expression at levels equivalent to the endogenous mGH; and 3) no detectable hGH expression, scored as +++, +, and negative, respectively (Fig. 6). In all cases where the hGH-N transgene was expressed, its expression was limited to, and coincident with, that of the endogenous mGH-positive cells. This colocalization of hGH and mGH expression was consistent with the previously documented somatotrope-restricted activity of the HS I,II region (11). All 24 control nontransgenic littermate embryos were negative for hGH. In contrast, all seven embryos carrying the intact F14 fragment linked to hGH-N (F14-GH) were positive for hGH, with the staining intensity greater than that for mGH in three of these (Fig. 7). The high specificity of this assay and the consistent expression of the F14-linked hGH-N transgene were in full agreement with our prior studies (11). In marked contrast to the F14 results, none of the nine transgenic embryos carrying the F14.3-GH mutation were positive for hGH-N expression. Taken together, these data clearly demonstrated that the central region of the F14 fragment, encompassing the two high affinity Pit-1 binding sites, was essential for site-of-integration-independent activation of hGH-N transgene expression in the embryonic pituitary.

The F14.3 region, shown to be essential for the function of the F14 segment of HS I,II (see above), was next tested for functional sufficiency by linking the isolated F14.3 subfragment to the hGH-N gene (F14.3-GH). The F14.3 fragment activated hGH-N expression in three of six transgenic embryos. Only one of these embryos showed higher hGH than mGH staining intensity. Thus, the F14.3 subregion contained determinant(s) that were necessary but not fully sufficient for F14-
mediated activation of hGH-N in a site-of-integration-independent fashion.

The functional contributions of the individual F14.3 Pit-1 binding sites were next tested. Point mutations were introduced at each of the two Pit-1 sites independently (F14mut5’ Pit-1-GH or F14mut3’ Pit-1-GH) and in tandem (F14mut5’,3’ Pit-1-GH). These mutations were carried out in the context of the full F14 fragment (Fig. 5A). The mutations introduced were transversions within the conserved core binding sequence determined by alignment with a compendium of known Pit-1 sites (21). The base substitutions introduced at the Pit-1 sites (Fig. 5B) were demonstrated to destroy Pit-1 binding as assessed by EMSA (data not shown). The effects of the two Pit-1 binding site mutations were remarkably similar; the 5’ and 3’ mutations each decreased the F14 activation frequency from 100% to approximately 50% (five of 10 and three of five, respectively; Fig. 7). This result suggested an additive contribution of the two Pit-1 sites to overall F14 activity.

The contribution of both central Pit-1 sites to F14 function was next tested by mutating the sites in tandem. Combined mutation of both Pit-1 sites essentially abrogated the function of F14; five of six transgenic embryos did not express the hGH-N transgene, and the single positive embryo carried the highest transgene copy number for this set (~20 copies). These data confirm that the two Pit-1 sites within the F14.3 subfragment are critical determinants of HS I,II LCR activity.

DISCUSSION

We have previously described a distal regulatory region located between 15 and 30 kb upstream of the hGH-N gene. This region was identified by mapping DNase I HS specific to the nuclear chromatin of pituitary and/or placental cells (Fig. 1). High level, copy number-dependent, and position-independent expression of hGH-N transgenes in the mouse pituitary could be consistently established by linkage to the full set of these HS. These data suggested that these HS composed an LCR for the hGH-N gene. Of the five HS identified in pituitary and placenta, the HS I,II doublet, located 14.5 kb 5’ to the hGH-N gene, was the only LCR component specific to pituitary chromatin. Transgenic analyses demonstrated that a 1.6-kb restriction fragment encompassing HS I,II was sufficient for establishing high level, somatotrope-specific, position-independent, and appropriately timed expression of the linked hGH-N gene (10). This activity was not copy number-dependent, however, in the absence of the remainder of the LCR. The activity was subsequently localized to the 404-bp F14 subfragment by functional mapping in developing transgenic embryos (11). This F14 segment of HS I,II appeared to encompass the major somatotrope-specific activation determinants of the hGH LCR.

In the present study, we have sought to identify and functionally test specific factor binding events that underlie F14-mediated hGH-N gene activation. Whereas computer-assisted scanning of the DNA sequence of this region failed to uncover pituitary-specific binding sites, in vitro binding assays demonstrated somatotrope-specific binding activity (Fig. 2D). This activity was due to binding of the pituitary POU domain transcription factor Pit-1 (Fig. 2D). Binding assays with a panel of probes scanning the F14 region further sublocalized two Pit-1 binding sites within the central F14 subfragment, F14.3 (Fig. 3), and a third Pit-1 site at the 5’-terminus of F14 in subfragment F14.1 (Figs. 2, B and C, and 3B). Deletion of the
F14.3 subsegment, with its two central Pit-1 sites, eliminated all somatotrope activation by the F14 segment in the transgenic founder assay. A similar loss of activity was observed when the two central (F14.3) Pit-1 sites were simultaneously mutated in the context of the F14 fragment. These data demonstrated that the two Pit-1 binding sites within the F14.3 subfragment were essential for F14-dependent activation of the hGH-N transgene during pituitary development (Fig. 7).

Although the F14.3 segment was critical for the F14-mediated activation of the hGH-N transgene, it was not fully sufficient for this function. Direct ligation of the F14.3 segment resulted in hGH-N expression in only 50% of transgenic embryos. This suggested that the full levels of activation mediated by the intact F14 fragment were based on F14.3 determinants in conjunction with one or more additional element(s) outside the F14.3 segment. A candidate for the additional determinant is the single F14.1 3' Pit-1 site. Thus, the data suggested that a single Pit-1 site was insufficient to confer consistent hGH-N transgene expression (F14Δ.3-GH and F14mut5'-5'Pit-1-GH), two Pit-1 sites could activate expression at 50% of random insertion loci (F14.3-GH, F14mut5'-5'Pit-1-GH, and F14mut3'-5'Pit-1-GH), and full position independence required the concerted action of all three Pit-1 sites (F14-GH). Whereas this model focuses on the defined role of Pit-1, it is recognized that additional factors are likely to be required for full F14 activity. This conjecture is supported by the observation of complexes identified by EMSA that do not involve Pit-1 as well as a corresponding set of undefined DNase I footprints in this region (Figs. 2–4). Further studies will be necessary to test the contribution of the F14.1 Pit-1 site to LCR function and to

**Fig. 6.** Representative immunohistochemistry assay results for embryonic day 18.5 hGH expression. Pituitary sections from embryos representing the three classes of hGH-N transgene expression are shown; levels greater than endogenous mGH (hGH ++), levels equivalent to mGH (hGH +), and no hGH expression (hGH negative). The primary antibody used (anti-hGH or anti-mGH) is indicated at the top. Cells positive for the antigen in each pituitary are indicated by a brown cytoplasmic stain. Orientation of the pituitary section is indicated by the arrows as follows: anterior (A), posterior (P), dorsal (D), and ventral (V). The floor of the sella turcica is indicated (S).
identify additional factors acting at HS I,II.

A central question raised by our results is how a set of Pit-1 elements in the LCR could mediate position-independent expression of the hGH-N transgene, while a similar set of Pit-1 sites in the hGH-N promoter are insufficient for such activation. The promoter-proximal Pit-1 sites have been intensively studied by several groups. These studies have demonstrated that these Pit-1 sites are critical for hGH-N (as well as Prl) promoter action both in cell-free transcription systems (23, 24) and following transient transfections into tissue culture cells (25, 26). The postulated mechanism for this activity, as has been proposed for many transcription factors (reviewed in Refs. 1 and 27), is the facilitated recruitment of the RNA polymerase II holocomplex (24, 26). Although the HS I,II Pit-1 element sequences are congruent with those of known functional Pit-1 sites (alignment available upon request) and bind Pit-1 similarly to the hGH-N promoter by EMSA (data not shown), the Pit-1 sites in the HS I,II region appear to have a functional profile distinct from those in the hGH-N promoter. Whereas HS I,II has a highly robust action on the hGH-N gene in vivo (in the transgenic model), it is remarkably weak in its ability to enhance transcription when linked either to the hGH-N promoter or to a viral promoter in both transient and stable cell transfection studies (11). Its activity in transgenic mice also displays an orientation dependence (10). Thus it cannot be classified as a classical enhancer by conventional operational parameters. Reciprocally, the Pit-1 sites in the hGH-N promoter are highly active in cell transfection studies but are virtually inactive in the transgenic setting (10, 11, 28, 29). The ability of Pit-1 to mediate distinct regulatory pathways when situated at either of these two sites may relate to the range of differential Pit-1 activities that have been observed, apparently specified by binding site sequences themselves (21). Different Pit-1 binding sites are able to specify the ability of DNA-bound Pit-1 to synergize with specific subsets of locally bound proteins (30–32). This flexibility in both DNA binding and interaction with other proteins appears to be a virtue of the POU domain itself (33). This unique structure allows Pit-1 to bind DNA in multiple configurations, in theory presenting different surfaces that specify interactions with other regulatory proteins. This model would predict that the Pit-1 sites at HS I,II may function via a pathway unique from those in the hGH-N proximal promoter, requiring some aspect of the full developmental cascade present in the transgenic model but absent in the developmentally static cell lines.

The presence of sites for the same tissue-specific trans-acting factors at both the proximal promoter/enhancer and at the LCR is not unique to Pit-1 and the hGH gene locus. For example, each of the HS of the β-globin LCR contains multiple GATA-1, AP-1, NF-E2, and CACC binding motifs in varying arrangements (34). These elements are variably required to confer position-independent expression of linked β-like globin genes in transgenic mice (35–37). Although the β-like globin gene proximal sequences also contain arrays of the same elements, they are insufficient for appropriate expression, as evidenced by naturally occurring deletions of the LCR (38, 39) and in transgenic experiments with isolated genes (40–43). Thus, the binding of common factors to distal regulatory elements and target promoters where they act differently may be a common mechanism for specifying functional cooperation between these regions.

Our data suggested that LCRs regulating different genes may function by distinct mechanisms. Two aspects of the presently described hGH LCR are of particular note in this regard. First, unlike the intensively studied β-LCR, the major component of the hGH LCR, HS I,II, is not functional in stably transfected cells, a system that typically reveals chromatin-dependent activities. This characteristic suggested a novel mechanistic requirement for some aspect of the developmental process for HS I,II activity that is provided only by a transgenic model. Second, the observation that only half of embryos transgenic for constructs with two of the three identified Pit-1 binding sites were competent to overcome position effects differed from predictions based upon stochastic models of LCR action in other systems (44–46). Such a model would predict that progressive mutation of functional HS I,II elements would result in a decrease in the number of somatotrope cells expressing hGH-N in all founder pituitaries (position-effect variegation; Refs. 44, 47, and 48). Instead, we observed a decrease in the number of productive founders and no evidence for a decrease in the proportion of hGH-positive somatotropes in expressing pituitaries. It thus appeared that the array of Pit-1 sites in the HS I,II region function in an additive fashion and that at least two sites must be available to achieve a critical threshold for
establishing a transcriptionally competent chromatin domain for hGH-N expression.

How might the Pit-1 sites within HS I,II function to establish such a transcriptionally competent chromatin domain? Recently, Pit-1 has been shown to interact with coactivator complexes containing CBP and pCAF. Furthermore, the histone acetyltransferase domains of these proteins are essential for cAMP and growth factor-mediated Pit-1 function (49). The acetylation of core histones at genomic loci has been implicated as a critical step in the process of remodeling chromatin into an "active" conformation (reviewed in Ref. 50). Thus, one could hypothesize that Pit-1 bound at HS I,II might function via recruitment of histone acetyltransferase activities to this region. This recruitment would require a critical threshold of Pit-1 molecules for full function, leading to local nucleosome remodeling necessary for subsequent access by factors required for transcriptional initiation of the hGH-N gene. Consistent with this hypothesis, we show in a separate report a dramatic peak of pituitary-specific histone hyperacetylation centered at HS I,II that encompasses the entire LCR and extends to the proximal promoter region of hGH-N.2 Taken together, these findings suggest a model for LCR function in which Pit-1 marks an entry point for histone acetyltransferase-containing complexes at the hGH locus, which can then extend the acetylated domain bidirectionally to include the upstream HS III and HS V of the LCR and the downstream hGH-N gene promoter. The exclusive activity of HS I,II in a developmental (transgenic) system may indicate a function for Pit-1 in this context at a critical point in pituitary ontogeny.

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