MOLECULAR AND CELLULAR BIOLOGY, Sept. 2006, p. 6535–6546 Vol. 26, No. 17
0270-7306/06/$08.00+0 doi:10.1128/MCB.00267-06

A Single Base Difference between Pit-1 Binding Sites at the *hGH* Promoter and Locus Control Region Specifies Distinct Pit-1 Conformations and Functions

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Received 13 February 2006/Returned for modification 22 March 2006/Accepted 20 June 2006

Activation of the human growth hormone (*hGH-N*) gene in pituitary somatotropes is mediated by a locus control region (LCR). This LCR is composed of DNase I-hypersensitive sites (HS) located −14.5 kb to −32 kb relative to the *hGH-N* promoter. HSI, at −14.5 kb, is the dominant determinant of *hGH-N* expression and is essential for establishment of a 32-kb domain of histone acetylation that encompasses the active *hGH* locus. This activity is conferred by three binding sites for the POU domain transcription factor Pit-1. These Pit-1 elements are sufficient to activate *hGH-N* expression in the mouse pituitary. In contrast, Pit-1 sites at the *hGH-N* promoter are consistently unable to mediate similar activity. In the present study, we demonstrate that the functional difference between the promoter-proximal and the HSI Pit-1 binding sites can be attributed in part to a single base difference. This base affects the conformation of the Pit-1/DNA complex, and reciprocal exchange of the divergent bases between the two sets of Pit-1 elements results in a partial reversal of their transgenic activities. These data support a model in which the Pit-1 binding sites in the *hGH* LCR allosterically program the bound Pit-1 complex for chromatin activating functions.

The transcription factor Pit-1 (POU1F1) is essential for anterior pituitary development and function. In mice, expression of Pit-1 is first observed in the developing anterior pituitary gland on day 13.5 of embryogenesis (35). In the mature pituitary, Pit-1 is expressed in three of the functionally distinct cell types, somatotropes, lactotropes, and thyrotropes, but not in corticotropes, gonadotropes, or follicular-stellate cells (12, 35). Pit-1 is required for differentiation and proliferation of these three anterior pituitary cell lineages, evidenced by the absence of these lineages in the hypoplastic pituitaries of mice (21) and humans (8, 25, 27) homozygous for inactivating *Pit-1* mutations. Pit-1 transactivates the promoters of the cognate peptide hormone-encoding genes in all three cell types: growth hormone (*GH*) in somatotropes, prolactin (*Prl*) in lactotropes, and thyroid-stimulating hormone beta subunit (*TSH*-β) in thyrotropes (reviewed in reference 1). Each of these genes contains multiple functional Pit-1 binding sites in their proximal 5′-flanking sequences. In addition, the *Pit-1* gene itself is autoregulated by multiple promoter-proximal Pit-1 elements, contributing to self maintenance of its own expression (6, 23, 29). These data support a clear and essential role for Pit-1 in pituitary development and corresponding gene expression.

GH promoters from all species studied contain a pair of conserved Pit-1 binding sites within the first 200 bp of 5′-flanking sequence (5, 22). It was therefore remarkable to observe that the promoter of the *hGH-N* gene, containing the conserved pair of Pit-1 binding sites, was not sufficient to activate *hGH-N* transgene expression in the mouse pituitary. An *hGH-N* transgene with as much as 7.5 kb of 5′-flanking sequence was either not expressed or expressed only at very low levels with loss of somatotrope specificity (16). These data suggested that remote regulatory determinants are required to establish a transcriptionally active chromatin domain at the *hGH* locus during pituitary ontogeny. A search for distal regulatory elements revealed a set of four DNase I-hypersensitive sites (HS) located −14.5 to −32 kb relative to the *hGH-N* gene promoter in pituitary chromatin (16). The full set of HS is required for the robust tissue-specific, position-independent, copy number-dependent expression of the genes of the *hGH* cluster in transgenic mice, establishing these determinants as the *hGH* locus control region (LCR) (16, 37). Further analysis of the *hGH* LCR identified the pituitary-specific HSI at −14.5 kb (Fig. 1A) as the major determinant of pituitary *hGH-N* transgene activation in vivo. Linkage of the *hGH-N* gene with its 500-bp proximal promoter to a 404-bp LCR subfragment encompassing HSI demonstrated high-level, position-independent expression in transgenic mouse somatotropes (4, 34). A search for functional *trans*-factor binding sites in this region identified an array of three clustered Pit-1 binding sites that mediate HSI action in vivo (34). Thus, the three Pit-1 binding sites at HSI were proposed to play a central and essential role in *hGH* LCR function.

The long-range activation of the *hGH-N* gene by the LCR appears to be mediated by alterations in core histone acetylation at the *hGH* locus. A pituitary-specific 32-kb domain of histone H3/H4 hyperacetylation encompassing the *hGH* LCR was revealed by chromatin immunoprecipitation (ChIP) assays in transgenic mice carrying the intact *hGH* locus (10, 11). The levels of acetylation display a central peak coincident with HSI and extend 5′ to encompass HSV and 3′ to encompass the *hGH-N* 5′-flanking region (10). This distribution suggests that

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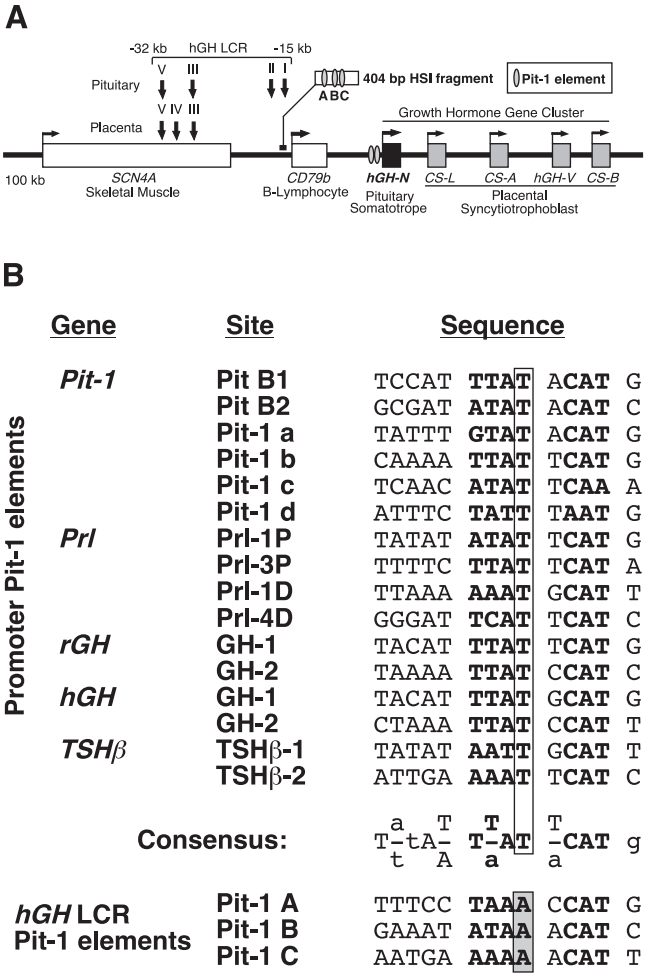

FIG. 1. *hGH* locus contains multiple Pit-1 binding sites. (A) Location of Pit-1 binding sites within the *hGH-N* gene promoter and LCR. The pituitary growth hormone gene (*hGH-N*) (black box), the placentally expressed genes in the cluster (gray boxes), and the unrelated but closely linked *SCN4a* and *CD79b* genes (white boxes) are shown. The transcriptional orientations of each gene are shown (horizontal arrows). The position of each HS in the *hGH* LCR is marked (downward arrow). The 404-bp LCR subfragment encompassing HSI (4, 33) is shown above the map. The positions of the three Pit-1 sites in this active HSI segment are indicated (shaded ovals marked A, B, and C). Each of these three sites contributes to overall HSI activity (33, 34). Two Pit-1 sites are present at the *hGH-N* promoter (shaded ovals). (B) HSI Pit-1 binding site sequences contain an LCR-specific base. An alignment of previously identified high-affinity Pit-1 binding sites from *GH*, prolactin (*Prl*), *Pit-1*, and thyroid-stimulating hormone beta subunit (*TSH*-β) gene promoters is shown (adapted from reference 1). The consensus recognition sequence is indicated below the alignment. Capital letters represent a strong consensus; lowercase letters indicate a weak consensus. The sequences of the three Pit-1 binding sites at HSI of the *hGH* LCR are aligned below the promoter Pit-1 consensus. The T and A bases that distinguish the promoter and HSI groups of Pit-1 sites are isolated in the open and shaded boxes, respectively.

determinants in this region are involved in the recruitment of histone acetyltransferase (HAT) cofactors to the *hGH* locus. Targeted deletion of the Pit-1 binding site array at HSI from an 86-kb *hGH* locus transgene (*hGH/P1*) resulted in a loss of acetylation across the 32-kb LCR domain and a loss of *hGH-N* transgene expression (11). Thus, the recruitment of HAT activity to the *hGH* locus and the corresponding transcriptional activation of the *hGH-N* gene require the Pit-1 elements at HSI. The functional specificity of the HSI Pit-1 binding site array was confirmed by direct comparison to the Pit-1 binding sites at the *hGH-N* promoter. Although the HSI and the promoter Pit-1 sites displayed identical Pit-1 binding activity in vitro and comparable enhancer activities in conventional cell transfection assays, the multimerized array of Pit-1 sites from the *hGH-N* promoter was unable to mediate the consistent *hGH-N* transgene activation that was observed with the HSI elements (33). These data support the model that the Pit-1 binding site array at HSI mediates chromatin modification and long-range activation of the *hGH-N* gene that cannot be conferred by the set of promoter-based Pit-1 binding sites.

The basis for the distinct functions of the Pit-1 binding sites at HSI and at the *hGH-N* promoter remains unclear. However, a special characteristic of Pit-1 is its ability to bind to a range of DNA sequences, resulting in a relatively loose binding site consensus sequence. Pit-1 accommodates these different binding sequences by altering its conformation in order to make contacts between DNA-binding domain amino acids and specific bases (14, 32). This structural flexibility provides a mechanism for allosteric regulation of Pit-1 by its DNA binding site. In support of this model, changes in Pit-1 topology, with selective recruitment of specific cofactors by different Pit-1-DNA complexes, appear to play a role in defining the selective activation of *GH* and *Prl* by Pit-1 in somatotropes and lactotropes, respectively (32). In the present report, we identify a single base that is distinct between promoter-associated Pit-1 sites and the three Pit-1 sites within HSI. We test whether this divergent base dictates the locus-activating function of the *hGH* LCR in vivo. The data presented support this model and complement a growing body of evidence for the allosteric control of transcription factor function by DNA recognition sequences.

MATERIALS AND METHODS

Preparation of recombinant Pit-1. A glutathione *S*-transferase-linked rat *Pit-1* (*rPit-1*) cDNA (gift of Sally Radovick, University of Chicago) was expressed in bacteria and affinity purified as described previously (9).

Methylation interference assay. The sequences of synthetic oligonucleotides used as probes in methylation interference assays are shown in Fig. 2C. To generate double-stranded probes, one strand was 5′ end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase and annealed to the unlabeled complementary strand. Assays were performed as described previously (3) with recombinant rPit-1. Conditions for the binding reactions with methylated probes and the gel separation of Pit-1-bound and unbound probe fractions were as described for the electrophoretic mobility shift assay (EMSA) (below).

Proteolytic clipping bandshift assay (PCBA). The sequences of synthetic oligonucleotides used as PCBA probes are shown in Fig. 2C. Double-stranded oligonucleotides were 5′ end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. Binding reactions (20 μl) each contained 200 ng (8 pmol) of labeled DNA probe in buffer composed of 10 mM HEPES (pH 7.9), 100 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT), and 10% glycerol. Approximately 200 ng (4 pmol) of purified recombinant rPit-1 was added last, and the mixture was incubated for 30 min at room temperature. One microliter each of a protease dilution series was added to the replicate binding reactions to final concentrations of 0.2, 1, 5, 25, and 125 ng/μl. Proteases were purchased from Sigma. Proteolysis reactions were incubated for 2 min at room temperature and stopped with 2 μl of 1-g/liter soybean protease inhibitor (Sigma). Samples were resolved on a 5% nondenaturing polyacrylamide (19:1 acrylamide/bisacrylamide) gel in 2× Tris-glycine buffer, dried, and visualized by autoradiography.

Electrophoretic mobility shift assay (EMSA). For EMSA, double-stranded oligonucleotide probes were 5′ end labeled with [γ-^{32}P]ATP and T4 polynucle-

otide kinase. Binding reactions (20 μl) contained 20 ng of DNA probe in buffer composed of 10 mM HEPES, pH 7.9, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, and 0.3 g/liter bovine serum albumin (BSA). Purified recombinant rPit-1 was added last, and the mixture was incubated for 20 min at room temperature. Samples were resolved on a 5% nondenaturing polyacrylamide (19:1 acrylamide/bisacrylamide) gel in 2× Tris-glycine buffer, dried, and visualized by autoradiography.

Construction of recombinant plasmids. The *3GH1*(*T*/*A*)*-hGH* and *PitABC*(*A*/*T*)*-hGH* plasmid constructs were prepared by replacing the wild-type 3GH1 and PitABC Pit-1 site trimer arrays in the previously described constructs *3GH1-hGH* and *PitABC-hGH* (33) with duplex synthetic oligonucleotides comprised of the 3GH1(T/A) and PitABC(A/T) Pit-1 site trimer arrays containing single base changes in the Pit-1 elements (see Fig. 5A). The modified arrays maintain the same orientation of the Pit-1 binding sites relative to the *hGH-N* gene as the original constructs.

Transient transfection. Presomatotrope GHFT1 cells (20) were transfected by calcium phosphate-DNA precipitation as described previously (17). Cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Ten-centimeter culture dishes at 75% confluence were transfected with 5 μg of supercoiled test plasmid. Five micrograms of pRL-CMV (Promega), which encodes *Renilla* luciferase, was included in the transfection mix to control for transfection efficiency. After 48 h, hGH expression was determined by hGH enzyme-linked immunosorbent assay (Roche Diagnostics) with aliquots of transfected cell culture medium. Luciferase activity in transfected cell lysates was determined with the dual-luciferase reporter system (Promega). hGH levels were normalized to luciferase activities to correct for transfection efficiency. The data were represented as the means of three independent transfections.

Generation of transgenic mouse lines. The *3GH1*(*T*/*A*)*-hGH* and *PitABC*(*A*/*T*)*-hGH* constructs were released from vector sequences of cesium chloride gradient-purified plasmid DNA with BssHII and purified through an Elutip (Schleicher and Schuell). The DNA was diluted to 2 ng/μl in 10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA and was microinjected into the pronucleus of C57Bl/6J × SJL mouse zygotes. Founders in litters yielded from implanted embryos were identified by dot blot analysis of tail DNA using as probe a 1.37-kb SmaI fragment from *hGH-N* (−494 to +876 relative to the initiation site of *hGH-N* transcription). The presence of intact transgenes in the resulting mouse lines was confirmed by Southern blot analysis of genomic DNA prepared from tail biopsies of F_1 animals, digested with HindIII using standard methods and the above *hGH-N* fragment probe. The single HindIII site within the transgene resulted in detection of a ~2.7-kb fragment yielded by digestion of the tandem array of transgene copies. After stripping the *hGH-N* probe from the membrane, a mouse zeta-globin fragment probe was hybridized to control for DNA loading.

RT-PCR assay for *hGH-N* and *mGH* mRNAs. Pituitary RNA was prepared from F_1 animals with Trizol reagent (LifeTechnologies). Levels of *hGH-N* and *mGH* mRNAs were determined by coamplification of *hGH-N* and *mGH* mRNAs by reverse transcription-PCR (RT-PCR) using primers complementary to both sequences as described previously (16). The amplified *mGH* and *hGH-N* cDNAs were distinguished by unique restriction sites, resolved on a 5% nondenaturing polyacrylamide (19:1 acrylamide/bisacrylamide) gel, and visualized by autoradiography.

Immunofluorescent staining. A monoclonal antibody specific to hGH (MAb9) has been described already (4). Monkey anti-rGH, which cross-reacts with mouse GH (mGH) but not hGH, was obtained from the National Hormone and Pituitary Program. Sections (4 to 5 μm) of paraffin-embedded adult pituitaries were dewaxed and rehydrated, followed by microwave antigen retrieval in 10 mM citric acid monohydrate, pH 6.0, and washing in phosphate-buffered saline (PBS). Sections were blocked with protein blocking reagent (Vector Laboratories) and incubated with the primary anti-hGH and anti-mGH antibodies (1:2,000 in PBS containing 10% BSA and 10% Triton X-100) simultaneously at 4°C overnight, followed by washing in PBS. Sections were next incubated with Cy2-conjugated goat anti-mouse immunoglobulin G and Cy3-conjugated goat anti-human immunoglobulin G (Jackson ImmunoResearch) secondary antibodies simultaneously at room temperature for 2 h (1:200 in phosphate-buffered saline containing 10% BSA and 10% Triton X-100), followed by washing in PBS. Slides were mounted in 50 mM Tris, pH 7.5, 80% glycerol and visualized by fluorescence microscopy with the appropriate filters. Images were captured with a color charge-coupled device camera and Photoshop (Adobe) software.

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation (ChIP) assays were performed as previously described (11). The antibody specific for CBP (sc-369) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Normal rabbit serum (Santa Cruz) was used as control for the ChIP assays. The input and bound DNA fractions were amplified by PCR. The sequences of the primers are the following: HSV, 5′-CTTGCCCAGTCCTCACACTT-3′ and 5′-CTGAGGCTTCTGTCCTCCTT-3′; HSI, 5′-CCAAGCCTTTCCCAGTTATAC-3′ and 5′-GATCTTGGCCTAGGCCTCGGA-3′; hGH-N promoter, 5′-CAGGGCTATGGGAGGAAGAGCTT-3′ and 5′-CTTCTCTCCCACTGTTGCCC-3′; mGH promoter, 5′-GTGGGATGTTGCATAACC-3′ and 5′-GGTCGACCCCAAGGCTCC. PCR products were resolved on 1.5% agarose gels, transferred to nylon membranes, and hybridized with ^{32}P-labeled probes. The probes were generated by PCR using the same primers. The signals were quantified by PhosphorImager analysis. A series of dilutions of the chromatin input fraction were used to determine the linear region for the PCR amplification at each site as described previously (11). The signals of bound fractions were normalized to the signals of 0.03% of the input and corrected for background by comparison with the ratio obtained with normal rabbit serum.

RESULTS

The three Pit-1 binding sites at HSI of the *hGH* LCR have a unique consensus sequence. Previously reported mouse transgenic studies have revealed that activation of *hGH-N* transgene expression in pituitary somatotropes is mediated by an array of three Pit-1 binding sites at the pituitary-specific HSI of the *hGH* LCR (34) (Fig. 1A). These studies have further demonstrated that the chromatin-modifying and gene activation functions of these HSI Pit-1 elements are distinct from those of Pit-1 elements within the *hGH-N* promoter (33). To explore the basis for these functional differences, we compared the DNA sequences of the three HSI Pit-1 sites (Pit-1A, Pit-1B, and Pit-1C) with Pit-1 binding sites from the promoters of a set of Pit-1-regulated genes (Fig. 1B) (1). This comparison revealed that the promoter-associated Pit-1 sites uniquely contain an invariant thymine (T) in their Pit-1 element consensus. This T is replaced by an adenine (A) at the corresponding position in each of the three HSI-associated Pit-1 binding sites. Of note, the conserved T of the promoter-associated Pit-1 binding sites, and at some sites also its complementary A, are known from prior crystallographic analyses to make contacts with specific amino acid side chains of bound Pit-1 (14, 32). The presence of the A instead of T at this position in all three HSI Pit-1 sites suggests that this base may contribute to their observed functional specificity in transgenic assays of *hGH* locus activation (33, 34).

Methylation interference assays were performed to determine whether the A specific to the HSI Pit-1 binding sites makes contact with Pit-1. The assays were carried out on DNA fragments encompassing a representative promoter Pit-1 site (rGH1) and on each of the individual HSI Pit-1 elements (Pit-1A, Pit-1B, and Pit-1C) (Fig. 2A and B). The results of mapping both strands are summarized, with critical purines in boldface (Fig. 2C). This analysis demonstrated that methylation of the HSI-specific A decreased Pit-1 binding to all three of the HSI Pit-1 sites: Pit-1A, Pit-1B, and Pit-1C (Fig. 2A). The data also demonstrated that each binding site has a unique pattern of Pit-1–purine contacts. This diversity reflects the unusual level of sequence variation among functional Pit-1 binding sites (1). The extent of DNA contacts was, however, similar among the four binding sites, suggesting that Pit-1 is binding as a dimer to the HSI Pit-1 elements as has been described previously for the promoter GH1 element (13, 32). Of note, methylation of the A in the GH1 element that pairs with the promoter consensus-specific T did not affect Pit-1 binding. This is consistent with the crystallographic data for the Pit-1/GH1 element complex, which showed that this adenine

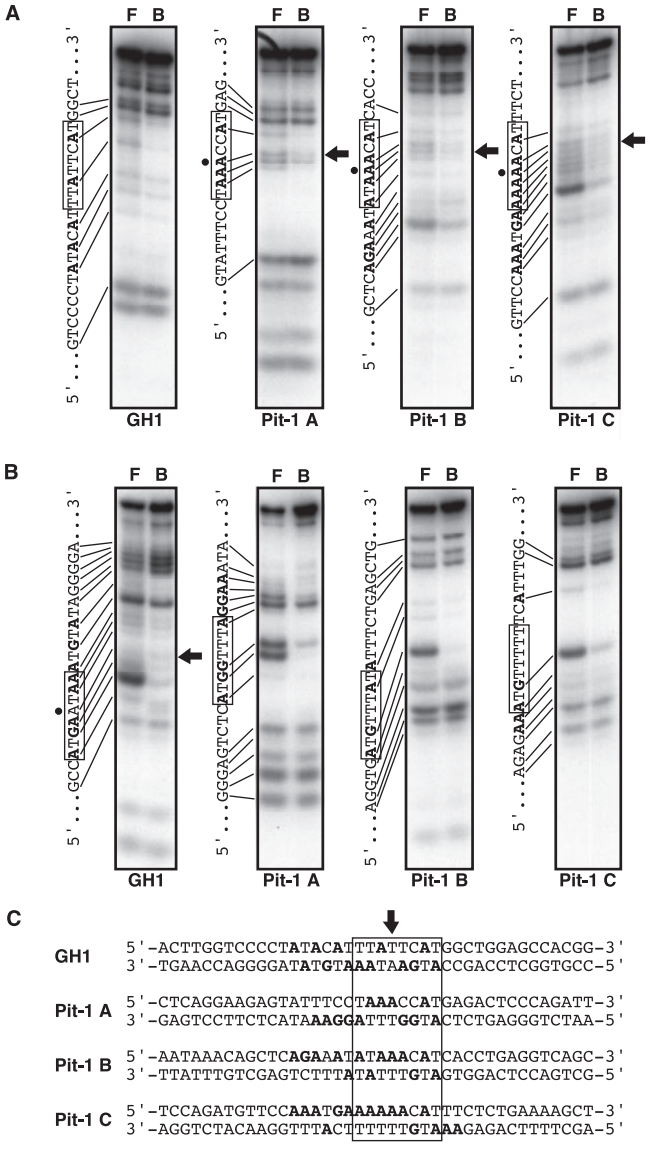

FIG. 2. Methylation interference footprinting of HSI Pit-1 elements identifies contacts between Pit-1 and the HSI-specific adenine. (A) Analysis of the top (sequence consensus) strand. The sequence around the footprinted region is indicated to the left of the autoradiogram for each probe, and the consensus sequences are indicated (boxes). The band generated by cleavage at each methylated purine is indicated (connecting lines). Analyses of the free probe fraction (F) and of probe bound with recombinant Pit-1 (B) are indicated above the autoradiogram. Purines that decreased Pit-1 binding when methylated are indicated to the left (boldface type). The HSI-specific adenines in the Pit-1A, Pit-1B, and Pit-1C probes are indicated (black dot), and the corresponding band in each case is marked (black arrowhead). (B) Analysis of the bottom (sequence consensus complementary) strand. The adenine that base pairs with the promoter-specific T is indicated (black dot), and the corresponding band is marked (black arrowhead). (C) The full double-stranded sequences of each binding site. The Pit-1 consensus is oriented as the top strand and is indicated (box). Purines involved in the Pit-1-induced footprint, based on the experiments depicted in panels A and B, are indicated (boldfaced type), and the divergent base is marked (downward arrow).

does not contact Pit-1 (32). It should be noted that only purines are methylated in this assay, and thus additional protein-base interactions remain indeterminate. These observations suggest that the unique sequences of the *hGH* LCR HSI Pit-1 binding sites may directly affect the conformation of the cognate Pit-1 homodimer complexes.

Protease analysis of Pit-1 complexes indicates that Pit-1 adopts different conformations when bound to *GH* promoter and HSI binding sites. The possibility that Pit-1/DNA complexes formed on promoter-associated and HSI-associated Pit-1 sites differ in their conformations was explored by a proteolytic clipping bandshift assay (PCBA). Limited protease digestion of Pit-1 complexed with ^{32}P-labeled DNA was followed by resolution of digestion intermediates retaining DNA binding by electrophoretic mobility on a native gel. Differences in the pattern and concentrations of digestion products reflect distinct configurations of the protein bound to corresponding DNA binding sites. PCBA has been used successfully in prior studies to distinguish complexes containing different octamer sequence binding (Oct) proteins in B cells (31). It has also been applied to distinguish the conformations of functionally distinct PRMT1 complexes in yeast (38) and to reveal different conformations of the transcription factor AP1 when bound to functionally distinct binding sites in the rat malic enzyme and collagenase-1 gene promoters (2, 36).

PCBA was performed on complexes of Pit-1 bound to the promoter-associated and HSI-associated Pit-1 binding sites using three proteases with distinct cleavage site specificities (Fig. 3). Clipping of the GH1 Pit-1 complex with proteinase K generated six major digestion intermediates (Fig. 3A, lanes 1 to 7). Parallel analysis of the Pit-1 A complex (Fig. 3A, lanes 8 to 14) generated intermediates 1 and 2, but it did so at a lower intensity than that of the GH1 complex (compare lane 11 with lane 4). Intermediate 3 was not produced at all, intermediate 4 was faint (compare lane 12 with lane 5), and products 5 and 6, seen clearly with the GH1 Pit-1 complex, were replaced by smeared products (compare lanes 12 through 14 with lanes 5 through 7). Clipping of the HSI Pit-1B element failed to generate intermediates 1 through 4. Complexes 5 and 6 were replaced by diffuse smears similar to those generated from the HSI Pit-1A complex. Clipping of the HSI Pit-1C complex yielded a pattern of digestion intermediates nearly identical to that shown by the GH1 sequence, with the exception of the strong smears corresponding to complexes 5 and 6 as seen with HSI Pit-1A and Pit-1B complexes. These proteinase K data revealed that each of the studied Pit-1 complexes demonstrates a unique proteolytic clipping pattern. Such variation is consistent with allosteric effects of the divergent binding sites on the bound Pit-1 protein.

Further analysis was carried out using chymotrypsin and pronase E. Chymotrypsin digestion of the GH1 Pit-1 complex yielded seven intermediates (Fig. 3B, lanes 1 to 7). The pattern generated from the three HSI Pit-1 complexes, while similar, had a few major differences (Fig. 3B, lanes 8 to 28). Product 5 was only generated from the Pit-1A complex (compare lane 13 with lanes 6, 20, and 27), and analysis of the Pit-1B and Pit-1C complexes revealed an absence of product 1 (compare lanes 18 and 25 with lanes 4 and 11). Furthermore, product 6 was generated from the GH1 and Pit-1C complexes but not the Pit-1A and Pit-1B complexes (compare lanes 13 and 20 with lanes 6 and 27), and product 8 was clearly more intense in the Pit-1B and Pit-1C reactions. Pronase E clipping also yielded digestion patterns specific to the individual complexes (Fig. 3C). The most notable distinction was the generation of inter-

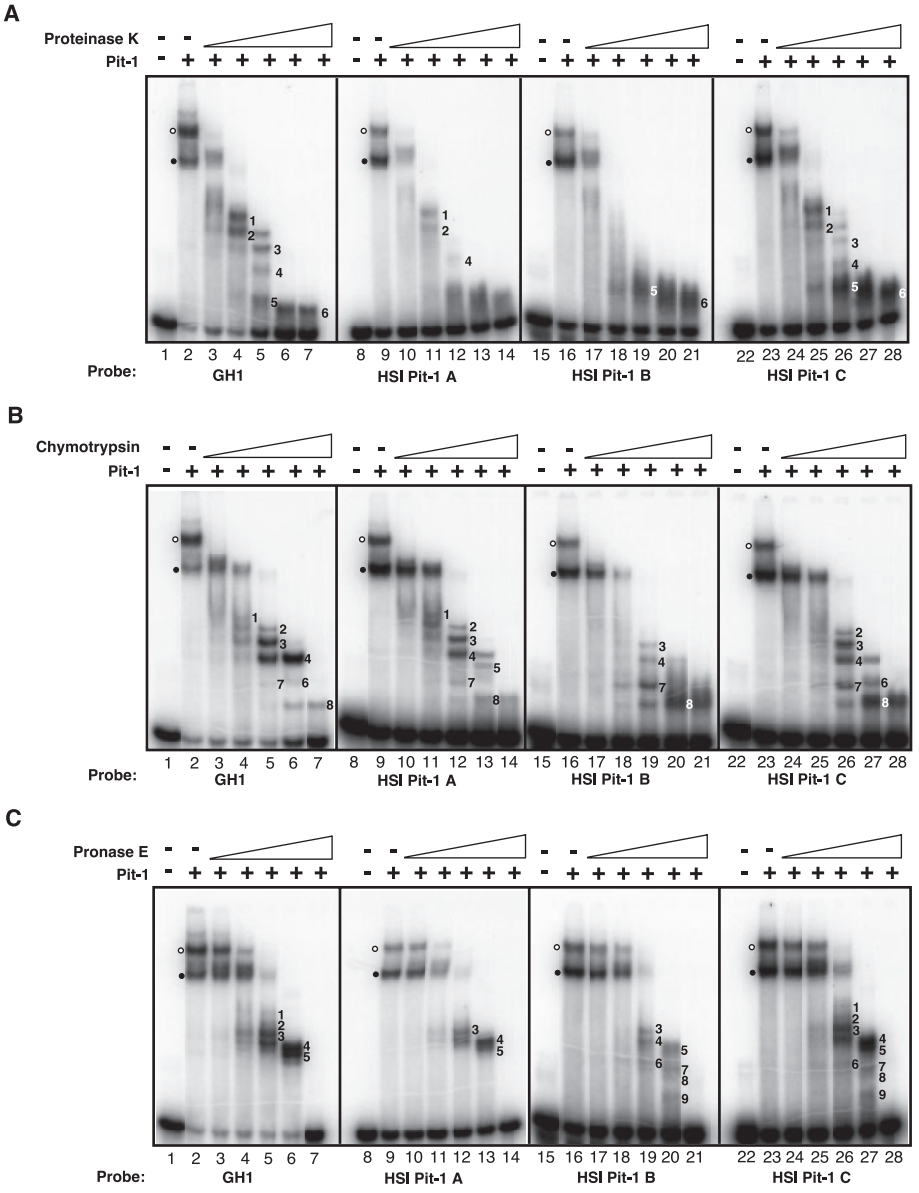

FIG. 3. Pit-1 complex conformations are determined by binding site sequences. The Pit-1 binding site sequences analyzed in this proteolytic clipping bandshift assay (PCBA) are shown in Fig. 2C and are indicated below each respective autoradiogram. The absence or presence of protease (absence, minus sign; presence, wedge) and Pit-1 (absence, minus sign; presence, plus sign) is indicated above each autoradiogram. The band indicated by a black dot in the first Pit-1-containing lane in each autoradiogram is the Pit-1 dimer/probe complex. The lower mobility species indicated (white dots) likely reflect an interaction between two Pit-1 dimer/probe complexes through the Pit-1 glutathione *S*-transferase tags (15, 24). The related identities of these two complexes was confirmed by methylation interference footprinting of both species, which showed identical Pit-1 footprints (data not shown). This interaction is lost at the lowest protease concentrations due to the cleavage of the exposed glutathione *S*-transferase moiety by the protease. The major DNA-bound proteolysis products are numbered to the right of the corresponding bands from lowest to highest mobility. Note that each binding site generates unique patterns of proteolytically clipped complexes. (A) PCBA with proteinase K; (B) PCBA with chymotrypsin; (C) PCBA with pronase E.

mediate products 6 to 9 from the Pit-1B and Pit-1C complexes but not from GH1 or Pit-1A complexes (compare lanes 19 through 20 and lanes 26 through 27 with lanes 5 through 6 and lanes 12 through 13). In addition, the faint product 1 was only observed with the GH1 and Pit-1C complexes (compare lanes 5 and 26 with lanes 12 and 19). Thus, each of these two additional proteolytic clipping studies confirmed the effects of distinct Pit-1 binding site sequences on Pit-1 protein conformation.

A thymine-to-adenine mutation within the promoter Pit-1 element alters the conformation of bound Pit-1. The impact of the T/A difference between the promoter-associated and HSI-associated Pit-1 binding sites was directly addressed by introducing a single-base T-to-A alteration in the GH1 sequence. PCBA mapping was carried out to determine whether this substitution had an impact on the conformation of the Pit-1 complex. The analysis revealed that the T-to-A transversion within the promoter GH1 Pit-1 element altered the chymo-

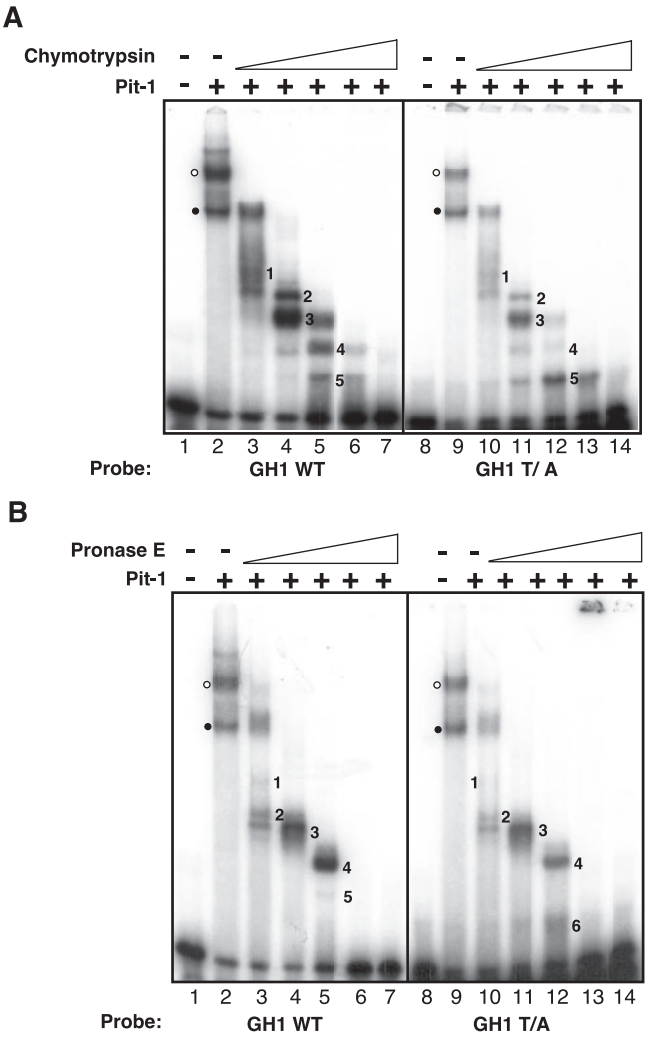

FIG. 4. Transversion of the conserved thymine to adenine alters the GH1 element Pit-1 PCBA profile. (A) Chymotrypsin digestion profile. A comparison of the wild-type promoter Pit-1 binding site (GH1 WT) and the thymine-to-adenine mutant (GH1 T/A) in the PCBA assay after chymotrypsin digestion is shown. The figure is labeled as described in the legend to Fig. 3. (B) Pronase E digestion profile.

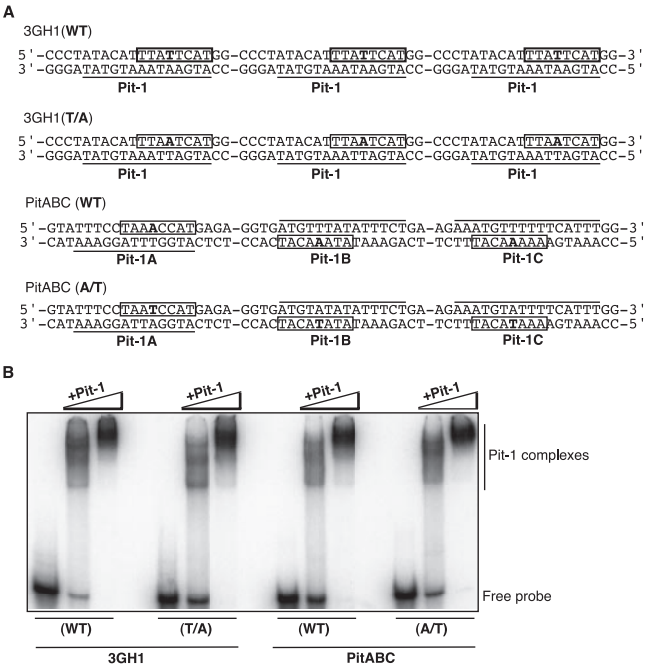

FIG. 5. Wild-type and T/A transversion trimer arrays of the *GH* promoter and HSI Pit-1 sites bind Pit-1 with the same affinity. (A) Structures of the wild-type *3GH1* [*3GH1*(*WT*)], mutant *3GH1*(*T*/*A*), wild-type *PitABC* [*PitABC*(*WT*)], and mutant *PitABC*(*A*/*T*) Pit-1 element trimer arrays. The sequences of both strands are shown, and the Pit-1 recognition consensus sequence is indicated (boxes). The HSI Pit-1 elements are arranged in their natural relative orientations. The full Pit-1 footprinted regions are shown on the consensus-complementary strand (underlined or overlined). (B) EMSA analysis of Pit-1 binding. Double-stranded *3GH1*(*WT*), *3GH1*(*T*/*A*), *PitABC*(*WT*), and *PitABC*(*A*/*T*) trimer arrays were end labeled and used as probes (labeled below the autoradiogram) in an EMSA after incubation with increasing concentrations of purified recombinant Pit-1 (GST–Pit-1) (wedges). The positions of free probe and Pit-1/DNA complexes are labeled to the right.

trypsin digestion pattern of the new Pit-1-DNA complex compared to that of the original complex (Fig. 4A). Most notably, the relative concentration of intermediate 5 was markedly increased in the mutant complex compared to that of the wild-type complex (compare lanes 11 to 13 with lanes 4 to 6). The T-to-A alteration also affected the pronase E digestion profile of the GH1 element–Pit-1 complex (Fig. 4B). Intermediate product 5 produced in the wild-type GH1 complex (lane 5) was absent from the digestion profile of the mutant GH1 complex (lane 12). Digestion product 6 was observed only in the mutant complex (compare lane 12 to lane 5). The assays were repeated to evaluate the reproducibility of the results, which were consistent between experiments. These data suggest that the T-to-A mutation in the promoter-associated Pit-1 site alters the conformation of the Pit-1 complex, as reflected by the changes in protease accessibility in the bound Pit-1.

Reciprocal A/T exchange between promoter-associated and HSI-associated Pit-1 sites does not affect in vitro protein binding affinity or gene expression in transiently transfected cells. In prior in vivo studies, an array of three linked copies of the promoter GH1 binding sequence was compared to the HSI Pit-1A, Pit-1B, and Pit-1C binding site trimer array (33). Using these trimerized binding site constructs as a starting point, a reciprocal exchange was carried out between T and A; the conserved T in each GH1 site was replaced by an A to generate 3GH1(T/A), and the conserved A in each HSI Pit-1 binding site was replaced with a T to generate PitABC(A/T) (Fig. 5A). EMSA of these mutated Pit-1 arrays failed to reveal any alteration in Pit-1 binding affinity (Fig. 5B); the pattern and intensity of Pit-1 complexes with the mutant trimers was comparable to that of the wild-type sequences, showing the same increase in Pit-1 complex formation with increasing amounts of Pit-1.

The function of the substituted Pit-1 trimer arrays was next assessed in a conventional cell transfection study. The 3GH1(T/A) and PitABC(A/T) arrays were linked to the *hGH-N* gene with its contiguous 500 bp of 5′-flanking promoter sequences [constructs *3GH1*(*T*/*A*)-*hGH* and *PitABC*(*A*/*T*)-*hGH*;

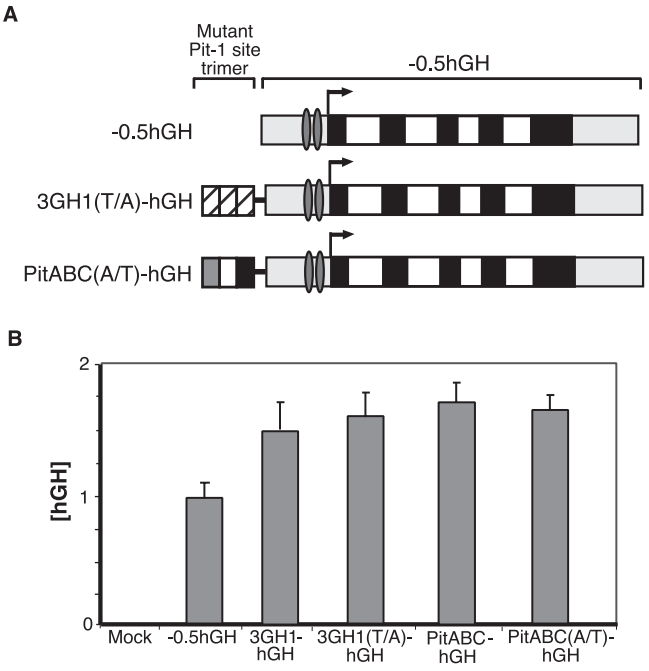

FIG. 6. Single nucleotide transversions within the *hGH-N* promoter and HSI Pit-1 element trimers have no appreciable impact on the expression of a linked *hGH-N* gene in transiently transfected GHFT1 pituitary cells. (A) Schematic diagrams of the mutant *GH* promoter [*3GH1*(*T*/*A*)-*hGH*] and HSI [*PitABC*(*A*/*T*)-*hGH*] synthetic Pit-1 element trimer transgene constructs. The Pit-1 element trimers are represented (shaded or hatched boxes), and the two native *hGH-N* promoter-proximal Pit-1 sites are shown (ovals). The *−0.5hGH* fragment contains the five exons and four introns of the *hGH-N* gene with 0.5 kb of 5′-flanking sequence. (B) *hGH-N* expression levels after transient GHFT1 cell transfections are unaffected by T/A exchange within the Pit-1 trimer arrays. The levels of GH protein expression, corrected for transfection efficiency, represent the means (±standard deviations; error bars) of three independent transfections, normalized to the level obtained with the *−0.5hGH* construct, which was set at 1. The identity of each transfected construct is indicated below the graph. Results from the *3GH1-hGH* and *PitABC-hGH* constructs lacking the T/A substitutions are included for comparison.

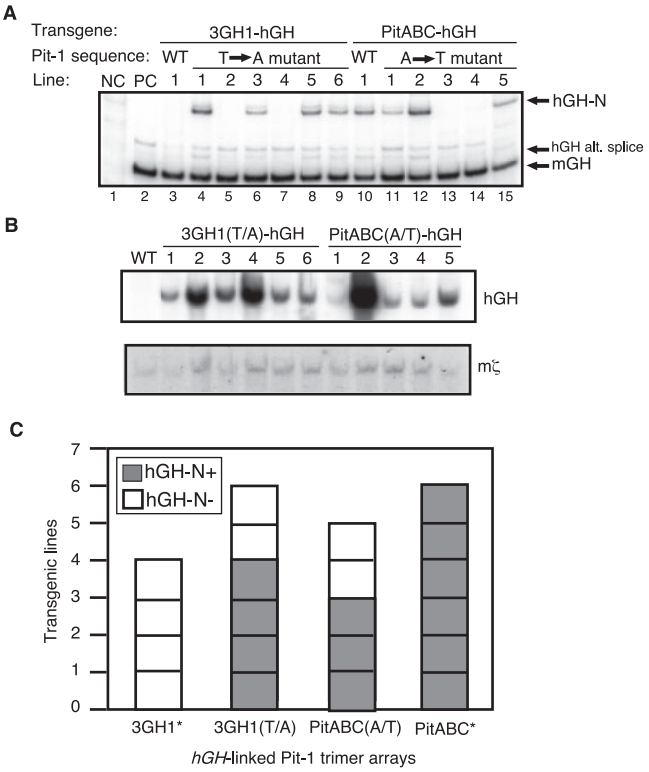

FIG. 7. Single nucleotide transversions within the GH promoter and HSI Pit-1 element trimers partially interconvert their activities on *hGH-N* transgene expression. (A) *hGH-N* transgene expression of the mutant constructs. Pituitaries were isolated from F_1 transgenic mice from each of six *3GH1*(*T*/*A*)-*hGH* and five *PitABC*(*A*/*T*)-*hGH* lines. RT-PCR detecting *hGH-N* and *mGH* RNAs was performed with the pituitary RNA. The positions of the three coamplified *GH* RT-PCR products are labeled (to the right of the autoradiograph). Each lane corresponds to an independent mouse line with a unique transgene integration site. The specific transgene carried by each line, the specific Pit-1 element trimer (wild type [WT] or mutant, GH1 or PitABC), and the line designation (numbers) are indicated above the autoradiograph. Wild-type mouse brain RNA served as a negative control (NC), wild-type mouse pituitary RNA as a positive control (PC). (B) Southern blot hybridization for *hGH-N* and *mζ-globin* confirm the presence of the *hGH-N* transgenes compared with an endogenous control. The *hGH-N* hybridization signal is proportional to the *hGH-N* transgene copy number. (C) The frequency of *hGH-N* transgene activation in the mouse pituitary is responsive to the sequence of the linked Pit-1 trimer array. The bar in each case represents the total number of lines generated with each indicated transgene, and the shaded region indicates the number of those lines that express the *hGH-N* transgene in the pituitary. The 3GH1 and PitABC results indicated by an asterisk (extreme right and left bars, respectively) have been previously reported (33) and are displayed for the sake of comparison to the summary of the analysis of the *3GH1*(*T*/*A*) and *PitABC*(*A*/*T*) transgenes (A). alt., alternative.

Fig. 6A]. Each of these recombinant genes was analyzed for *hGH-N* expression in transfected GHFT1 presomatotrope cells, which contain substantial levels of Pit-1 (20; our unpublished data). The two substituted Pit-1 arrays conferred an equivalent and modest enhancement of expression from the linked *−0.5hGH* gene. These effects were identical to the activities of the wild-type 3GH1 and PitABC arrays in this assay (Fig. 6B). Thus, the single A/T base substitutions within the *GH* promoter and LCR Pit-1 element arrays have no effect on their in vitro binding properties as assessed by EMSA, nor do they impact on the enhanced expression of a linked *hGH-N* gene as assessed in transiently transfected cells.

Reciprocal A/T exchange between promoter-associated and HSI-associated Pit-1 arrays alters *hGH-N* transgene activation in the mouse pituitary. The impact of the single base substitutions was tested in the context of a mouse transgenic model. The *3GH1*(*T*/*A*)-*hGH* and *PitABC*(*A*/*T*)-*hGH* transgenes (Fig. 6A) were individually injected into fertilized mouse oocytes, and multiple transgenic lines were established. The pituitary level of *hGH-N* mRNA in F_1 mice from each line was directly compared to the endogenous *mGH* mRNA levels by a validated RT-PCR coamplification assay (33) (Fig. 7A). Analyses of pituitary RNA from the previously reported lines carrying the wild-type versions of the Pit-1 arrays linked to the *hGH-N* gene were included as negative and positive expression controls, respectively (compare lane 3 with lane 10) (33). In contrast to the consistent lack of expression of the wild-type *3GH1-hGH* transgene (*hGH-N* expression in 0 of 4 transgenic lines [33]), the substitution of A for T [*3GH1*(*T*/*A*)-*hGH* trans-

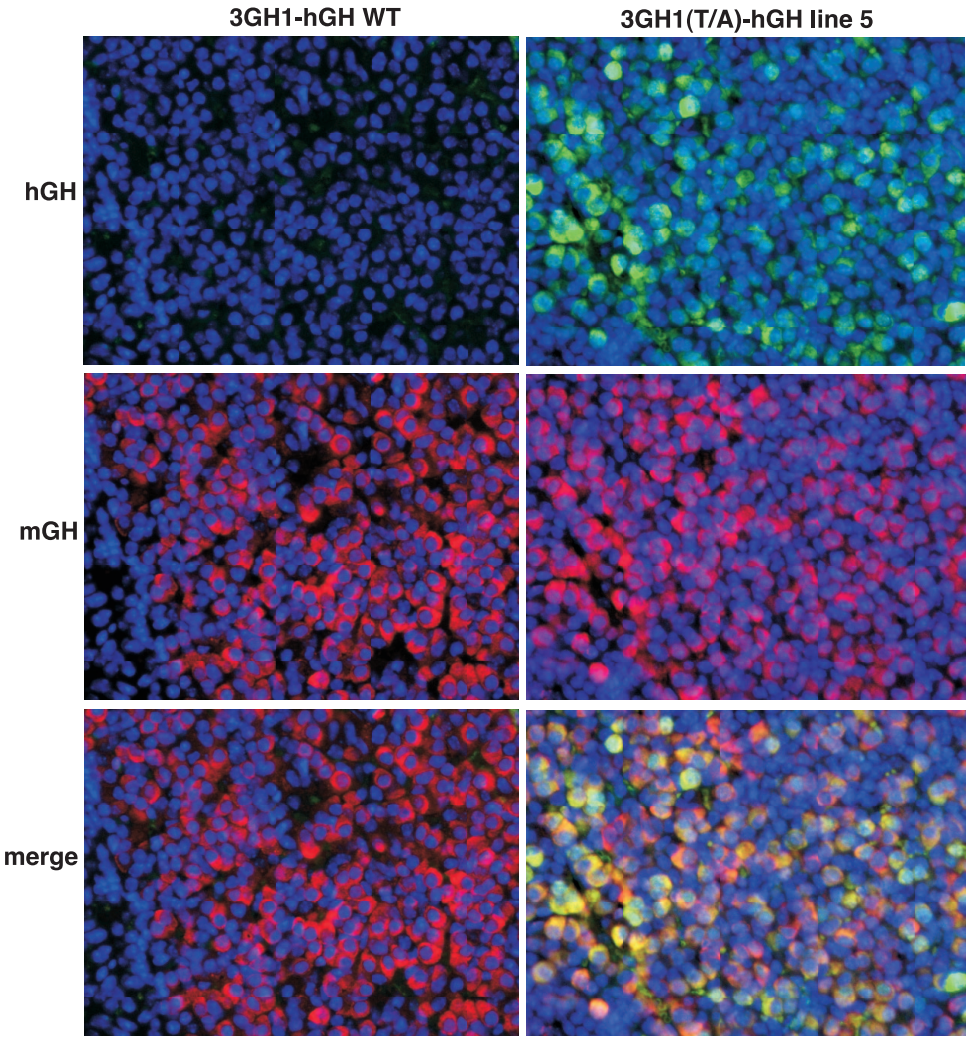

FIG. 8. Pituitary hGH protein in a *3GH1*(*T*/*A*)-*hGH* mouse line colocalizes with endogenous mGH. Pituitary sections from an adult mouse carrying the wild-type (WT) *3GH1-hGH* transgene (line 1) and from a mouse carrying the mutant *3GH1*(*T*/*A*)-*hGH* transgene (line 5) were prepared. After incubations with the relevant antibodies, hGH (green) and mGH (red) immunofluorescence was assessed. As predicted on the basis of prior studies (33), wild-type *3GH1-hGH* failed to express hGH protein. In contrast, the individual and merged images revealed robust expression of hGH from the *3GH1*(*T*/*A*)-*hGH* transgenic line with colocalization of the hGH and mGH signals (yellow-orange). Sections were counterstained with 4′,6-diamidino-2-phenylindole to indicate nuclei (blue). Sections were observed at ×400 magnification.

gene] resulted in activation of the linked *hGH-N* in four of six lines (lanes 4 to 9). The transcript levels in the pituitaries of these mice were comparable to those observed with the wild-type *PitABC-hGH* line (lane 10). The reciprocal transversion was also assessed. In comparison to the consistent position-independent activation of the *hGH-N* gene by a linked HSI-derived PitABC trimer (transgene expression in six of six lines [33]), the mutated PitABC sequence bearing the A-to-T mutations [*PitABC*(*A*/*T*)-*hGH* transgene] failed to activate *hGH-N* transgene expression in two of five lines (lanes 11 to 15). As shown in Fig. 7B, the range of transgene copy number was comparable between the *3GH1*(*T*/*A*)-*hGH* and *PitABC*(*A*/*T*)-*hGH* mouse lines. The observation that HSI-mediated *hGH-N* transgene activation, while position independent, is not copy number dependent has been previously documented (33, 34). These transgenic mouse expression data, summarized in Fig. 7C, demonstrate that the T/A divergence between the promoter-derived and the HSI-derived Pit-1 sites impacts gene activation specifically in vivo. The observation that the single T/A base exchange does not completely interconvert the transgenic activities of these Pit-1 elements is addressed in Discussion.

Previous studies have demonstrated that activation of the *hGH-N* gene by its LCR and more specifically by the isolated PitABC trimer is nonvariegated and somatotrope cell specific (33). We determined whether the promoter Pit-1 elements containing the A-to-T mutation are capable of establishing this pattern of *hGH-N* expression. A pituitary from an adult *3GH1*(*T*/*A*)-*hGH* mouse (line 5) was studied by immunofluorescence. Human GH protein was observed in all cells that were positive for mGH, and *hGH-N* transgene expression was restricted to these somatotropes (Fig. 8). Thus, the pituitary *hGH-N* expression pattern conferred by the *3GH1*(*T*/*A*)-*GH* transgene is identical to that previously observed with the *PitABC-GH* transgene. In clear contrast, analysis of the pitu-

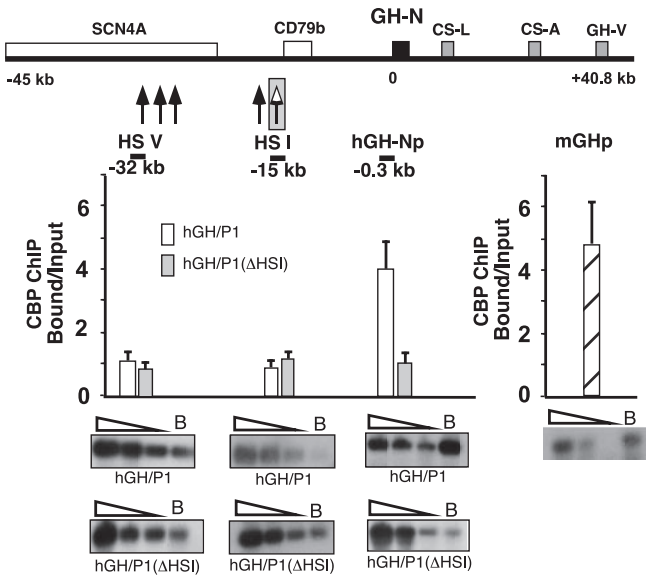

FIG. 9. CBP is selectively recruited to the promoters of *hGH-N* and *mGH* but not to HSI. The diagram at the top of the figure represents the *hGHP1* transgene. The companion *hGH/P1ΔHSI* line used in the analysis has a 99-bp deletion that removes critical Pit-1 binding sites from HSI. This inactivation of HSI is represented by the shaded box over HSI. The location of each of the three amplicons used in the ChIP analysis of the *hGH* locus (HSV, HSI, and hGH-Np) is indicated below the *hGH* locus transgene map. A separate *mGH* promoter amplicon was used to detect the *mGH* promoter sequences (not shown on the map). The autoradiograms at the bottom of the figure are representative Southern hybridizations of the PCR-amplified fragments from the CBP ChIP. The wedges indicate serial dilutions of each of the indicated input DNA samples. Pituitary chromatin was isolated from *hGH/P1* and *hGH/P1*(Δ*HSI*) transgenic mouse lines (indicated below the autoradiograms), pooled from five pituitaries per assay. Signals obtained from the CBP antibody-bound fraction (B) were normalized to 0.03% of input. The bound/input ratios were corrected for the background ratio obtained with normal serum, which was set at 1. The normalized values are plotted on the histogram; the levels represent the means (± standard deviations) of four independent experiments (performed with two different mouse lines in duplicate). The bound/input ratio of the endogenous *mGH* promoter (mGHp) is also shown.

itary from a representative *3GH1-hGH* wild-type line shows a total absence of *hGH* transgene expression, confirming the lack of activity of the wild-type promoter-derived Pit-1 sites in the transgenic setting.

CBP is recruited to the *hGH-N* and *mGH* promoters but not to HSI in the transgenic mouse pituitary. Our previous results indicated that Pit-1 occupancy at the *hGH-N* promoter is dependent on intact Pit-1 sites at HSI (11). Furthermore, the *hGH-N* promoter is not sufficient to activate *hGH-N* expression in somatotropes and needs HSI in *cis* to acquire this activity (16, 34). These observations suggest that the two sets of Pit-1 binding sites, one at HSI and the other at the *hGH* promoter, play distinct roles in *hGH-N* activation. These distinct functions may reflect the recruitment of different sets of coactivator proteins. The histone acetyltransferase CBP has been previously shown in cell-based assays to interact with Pit-1 at the *hGH-N* promoter, where it functions as a transcriptional coactivator (7). Thus, our model would predict that CBP recruitment occurs exclusively at the promoter Pit-1 complexes and that a distinct coactivator(s) is recruited to the Pit-1 complex at HSI.

The model showing that different coactivators are recruited to the Pit-1 complexes at the *hGH* promoter and HSI was tested by chromatin immunoprecipitation (ChIP) assays for CBP. The initial analysis was done on pituitary chromatin isolated from mice carrying an 86-kb human genomic transgene that encompasses the entire *hGH* LCR and *hGH-N* gene (*hGH/P1* transgene [37]) (Fig. 9, top). Accurate expression of this *hGH/P1* transgene has been previously validated (37). ChIP assays in two mouse lines carrying this transgene (lines 811D and 809F [37]) revealed robust CBP occupancy at the *hGH-N* promoter and at the endogenous *mGH* promoter (Fig. 9). In marked contrast, there was no evidence for CBP occupancy at HSI (Fig. 9). A second set of CBP ChIP assays was performed on pituitary chromatin from mice carrying the *hGH/P1* transgene specifically lacking two essential Pit-1 binding sites at HSI [*hGH/P1*(Δ*HSI*) transgene; lines 960G and 969E] (11). This Pit-1 binding site deletion has been previously shown to result in loss of HSI formation, loss of histone acetylation throughout the LCR, loss of Pit-1 occupancy at the *hGH-N* promoter, and a dramatic repression of *hGH-N* expression (11). ChIP analysis revealed that HSI inactivation markedly repressed CBP occupancy at the *hGH-N* promoter. Because this loss of CBP occupancy was concomitant with the loss of *hGH-N* promoter Pit-1 occupancy (11), we conclude that CBP is selectively recruited to the *hGH-N* promoter Pit-1 complexes. In contrast, HSI-mediated LCR function is dependent on a distinct and as-yet unidentified Pit-1 coactivator complex (see Discussion).

DISCUSSION

The Pit-1 binding sites at HSI are the primary determinants of *hGH-N* gene activation by the *hGH* LCR (33, 34). These three discrete Pit-1 elements are necessary (4, 11, 16, 34) and sufficient (33) for the high-level, position-independent, and somatotrope-specific expression of a linked *hGH-N* transgene in mice. HSI is located at the central peak of a 32-kb domain of histone H3/H4 hyperacetylation in pituitary somatotropes (10). In the context of a contiguous 86-kb transgene encompassing the intact *hGH* locus, deletion of the HSI Pit-1 elements results in the loss of the hyperacetylated domain and *hGH-N* expression. This is accompanied by a reduction in Pit-1 binding at the *hGH-N* promoter sites 15 kb downstream (11). The coincidence of these effects suggests that Pit-1 complexes at HSI are responsible for the recruitment of histone acetyltransferase (HAT) activity to the *hGH* locus and that this recruitment is essential for *hGH-N* gene activation. These observations also indicate that the HSI Pit-1 complexes are functionally distinct from the promoter Pit-1 complexes.

The *hGH-N* activation mechanism is specifically dependent upon the array of Pit-1 complexes at HSI. Three copies of the promoter GH1 Pit-1 recognition sequence are not able to recapitulate the consistent activation of an *hGH-N* transgene in pituitary somatotropes that is mediated by a synthetic array of the three HSI Pit-1 elements. This distinction in in vivo activity of HSI and GH promoter sequences was observed despite identical Pit-1 binding affinities and enhancer activities in transiently transfected cells (33). The activity of isolated HSI

Pit-1 binding sites in these constructs strongly suggested that the observed selectivity relates to specific conformations of the Pit-1 complexes adopted upon binding to the unique recognition sequences at HSI.

Here we show that the nucleotide sequences of the HSI Pit-1 binding sites differ from the *GH* promoter Pit-1 sequences. Most notably, an adenine common to all three HSI elements contrasts with a thymine conserved at the corresponding position in the promoter Pit-1 elements (Fig. 1B). The methylation interference studies indicated that DNA contacts differed among the promoter and HSI Pit-1 complexes. Of interest, the adenine unique to the HSI Pit-1 elements is involved in the interaction with Pit-1 (Fig. 2). Results from the PCBA indicated that these recognition sequence differences can alter the conformation of the Pit-1/DNA complex. Thus, the functional distinction between the promoter and HSI Pit-1 elements may be related to critical differences in Pit-1 complex topology (Fig. 3). In support of this model, reciprocal exchange of the *GH* promoter-specific thymine and HSI-specific adenine interconverts their activities in a transgenic mouse model (Fig. 6, 7, and 8). This functional interchange is accompanied by a change in the conformation of the Pit-1/DNA complex in vitro (Fig. 4). Together these observations support a model in which the sequence of the Pit-1 binding site mediates allosteric specification of Pit-1 complex activity. The adenine-containing Pit-1 complex conformation appears to significantly contribute to the unique activities of the Pit-1 elements at HSI.

It should be noted that the proteolysis patterns obtained by PCBA were not identical among the HSI Pit-1 complexes. This lack of uniformity most likely reflects differences in the sequences of these sites beyond the conserved adenine. Such differences in overall Pit-1 conformation of the three HSI-derived site complexes is consistent with their differences in relative potency assessed in transgenic models (33, 34). The involvement of additional bases in the specificity of HSI Pit-1 complex activities is further suggested by the transgenic analyses. The HSI-specific adenine is not fully sufficient for characteristic HSI activity, as two of the six *3GH1*(*T*/*A*)-*hGH* lines did not express the transgene. The reciprocal observation that the activity of the HSI Pit-1 element trimer is not completely abrogated by the A-to-T alteration also suggests the involvement of additional base pairs in the specificity mechanism. However, no additional sequences within the aligned binding sites perfectly segregate between the HSI and promoter Pit-1 elements, masking any obvious candidates. We conclude that the ability to alter the activity of Pit-1 elements in a transgenic system by a single base substitution, without affecting Pit-1 affinity, supports an allosteric model for the specificity of the *hGH* LCR HSI Pit-1 complexes.

The ability of Pit-1 to be allosterically controlled by DNA sequence is supported by crystallographic analyses of POU domain/DNA complexes (14, 18, 32) and can be attributed to the unique structural nature of the POU class of proteins. Pit-1 is a member of a family of transcription factors defined by a region of homology, the POU domain (for "Pit-1, Oct-1/2, Unc-86"). The POU domain is a bipartite DNA binding domain comprised of an amino-terminal 75-amino-acid POU-specific domain (POU_S) tethered by a disordered linker of variable length and composition to a carboxy-terminal 60-amino-acid homeodomain (POU_H) (reviewed in reference 30). The POU_S domain consists of a cluster of four alpha helices. The central two helices, α2 and α3, form a helix-turn-helix DNA binding domain similar to the DNA binding domains of the phage 434 and λ repressors. The POU_H domain is comprised of three alpha helices; the carboxy-terminal helices α2 and α3 form an helix-turn-helix motif (14). Crystallographic studies of the Pit-1 and Oct-1 POU domains bound to DNA have revealed that the flexibility of the disordered linker region allows the subdomains to bind DNA in multiple orientations. In addition, the alpha-helix side chains of the POU domain are involved in DNA binding through a diversity of potential interactions with multiple bases and with the phosphate backbone. Thus, Pit-1 can bind to a range of DNA sequences with variable conformations dictated by the underlying nucleotide sequence (14, 18). An additional layer of complexity results from the fact that Pit-1 typically binds to DNA as a cooperative dimer (13). The nature of the interaction of one Pit-1 subunit with the POU domain of its dimerizing partner is also facilitated by the loose binding specificity and linker flexibility and is dictated by the structure of the DNA binding site (14). The resulting differences in subunit interactions allow for further structural distinctions between Pit-1 dimer complexes assembled at distinct binding sites.

The contacts between amino acids in the POU domain and DNA bases revealed by structural analysis of Pit-1 POU domain/DNA complexes support the specificity of the HSI Pit-1 sites. In complexes containing either a synthetic promoter-type Pit-1 element (13) or the natural *GH* promoter GH1 or *Prl* promoter Prl-1P Pit-1 elements (32), specificity for the conserved thymine in the DNA sequence (Fig. 1B) is conferred by a major groove hydrogen bond linking this base to the threonine at position 45 (Thr-45) of the POU_S α3 helix. Specificity for this base is further conferred by van der Waals contacts between its methyl group and the Cβ methylene groups of Thr-45 and Ser-43 of the POU_S domain (14). The thymine at this position in the Prl-1P element (32), and its paired adenine in the synthetic Pit-1 element (14), can also contribute to the conformation of the POU domain dimer by forming a minor groove hydrogen bond with Arg-5 of the POU_H α1 helix in the partner POU domain monomer. Thus, one would predict that the presence of an adenine in place of a thymine in this position, as is seen in the HSI Pit-1 elements (Fig. 1B), would result in distinct contacts with both POU domains of the Pit-1 dimer and the potential for a concomitant adjustment in the topology of the complex.

The unique flexibility of the POU domain-DNA interactions is postulated to modulate and specify interactions of bound POU domain proteins with cofactors (14, 26, 30). In this manner, a POU domain protein can display distinct context-specific biological activities when bound to different DNA elements (14). Direct evidence in support of this model has been gathering in recent years. A study of two different Oct-1 binding sites revealed that both binding sites have equal affinities for Oct-1, but only one of the complexes can recruit the B-cell coactivator OBF-1 (OCA-B, Bob-1) (39). This specificity reflects an allosteric induction of the required OBF-1 interaction surface on the Oct-1 dimer by the underlying DNA element (28). Allosteric specification has also been observed with Pit-1. In this context, an additional two base pairs between the POU_S and POU_H interaction sites in the prolactin gene Prl-1P Pit-1

element prevents the recruitment of the nuclear receptor corepressor N-CoR to the complex, unlike the *GH* gene GH1 Pit-1 element. In contrast, the more compact Pit-1 dimer complex conferred by the GH-1 element is able to interact with N-CoR (32). This paradigm for the allosteric control of cofactor recruitment by nucleotide sequence has recently extended beyond the POU domain proteins. Like Pit-1, the dimeric transcription factor NF-κB recognizes a particularly loose consensus sequence. Investigation of the mechanistic basis for a functional distinction among different NF-κB binding sites revealed that a single nucleotide difference is able to determine which cofactor is recruited by the NF-κB dimer (19).

The results of our studies, along with a body of precedents in the literature, lead us to conclude that the HSI Pit-1 complexes recruit cofactors critical to LCR functions and that the nature of these cofactors is specified by a DNA-mediated allosteric mechanism. Studies by others have documented an interaction between Pit-1 and the HAT coactivator CBP (7, 40). Thus, CBP was an attractive candidate for the HAT recruited by HSI and was responsible for the acetylation of the *hGH* locus in the pituitary. ChIP assays revealed that CBP is in fact recruited to the *hGH-N* promoter but appears not to be targeted to HSI. Interestingly, the recruitment of CBP to the *hGH-N* promoter is itself dependent upon HSI action (Fig. 9) and parallels the HSI-dependent Pit-1 occupancy at the *hGH-N* promoter (11). This suggests that CBP is recruited to the *hGH-N* promoter by the promoter Pit-1 complexes, the assembly of which is dependent upon global *hGH* locus histone acetylation. These data support our hypothesis that the distinct Pit-1 element sequences at HSI and the *hGH-N* promoter result in differential cofactor recruitment. ChIP analysis with antibodies to several other HATs (p300, TAF-250, PCAF, and GCN5) failed to detect the HSI region (data not shown), indicating that they are unlikely to be involved in Pit-1-mediated *hGH* locus acetylation. Thus, the HAT activity(ies) recruited by HSI involved in the initial LCR action at the *hGH* locus remains unknown. Future studies will address the characterization of such pioneer HAT cofactor complexes. The eventual goal is to correlate high-resolution structural analysis of the Pit-1 complex and cofactor interactions at HSI and at the *hGH-N* promoter and to define the order and timing of their respective functions in pituitary organogenesis and *hGH* gene activation.

ACKNOWLEDGMENTS

We thank the University of Pennsylvania Transgenic and Chimeric Core for help in generating transgenic mice, the University of Pennsylvania Morphology Core for help in preparing mouse pituitary sections, and Arthur Gutierrez-Hartmann, University of Colorado, for helpful suggestions.

The work was supported by NIH K01 DK62164 (B.M.S.), NIH K01 DK064011 (Y.H.), and R01 HD25147 (N.E.C. and S.A.L.).

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