THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 18, Issue of May 5, pp. 13827–13834, 2000

Printed in U.S.A.

Targeted Recruitment of Histone Acetyltransferase Activity to a Locus Control Region*

Received for publication, October 19, 1999, and in revised form, February 7, 2000

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Locus control regions (LCRs) are capable of activating target genes over substantial distances and establishing autonomously regulated chromatin domains. The basis for this action is poorly defined. Human growth hormone gene (*hGH-N*) expression is activated by an LCR marked by a series of DNase I-hypersensitive sites (HSI–III and HSV) in pituitary chromatin. These HSs are located between −15 and −32 kilobases (kb) relative to the *hGH* transcription start site. To establish a mechanistic basis for *hGH* LCR function, we carried out acetylation mapping of core histones H3 and H4 in chromatin encompassing the *hGH* cluster. These studies revealed that the entire LCR was selectively enriched for acetylation in chromatin isolated from a human pituitary somatotrope adenoma and in pituitaries of mice transgenic for the *hGH* locus, but not in hepatic or erythroid cells. Quantification of histone modification in the pituitary revealed a dramatic peak at HSI/II, the major pituitary-specific *hGH* LCR determinant (−15 kb), with gradually decreasing levels of modification extending from this site in both 5′- and 3′-directions. The 5′-border of the acetylated domain coincided with the 5′ most *hGH* LCR element, HSV (−34 kb); and the 3′-border included the expressed *hGH-N* gene, but did not extend farther 3′ into the placenta-specific region of the gene cluster. These data support a model of LCR function involving targeted recruitment and subsequent spreading of histone acetyltransferase activity to encompass and activate a remote target gene.

The majority of DNA in the eukaryotic nucleus is packaged into a compact chromatin conformation. For a gene to be expressed, this chromatin structure must be disrupted to accommodate transcription factor binding and RNA polymerase assembly and passage (1, 2). A specific set of distal regulatory elements termed locus control regions (LCRs)[1] are postulated to function in promoting changes in chromatin structure conducive to gene activation. Critical determinants that constitute an LCR co-map to one or more DNase I-hypersensitive sites (HSs) flanking LCR-dependent genes in the chromatin of expressing cells (3). The functions of such LCR determinants are operationally defined by their ability to establish autonomously functioning transgene chromatin domains that are independent of the site of integration within the host genome (4, 5). The biochemical mechanism(s) by which LCRs activate their target genes has not been determined.

Histone-modifying enzymes are directly involved in modulating chromatin structures relevant to gene transcription (6–11). The acetylation of nucleosomal histones at the promoters of certain genes promotes chromatin disruption (12–15) and facilitates transcriptional activation (16–18). Histone deacetylase activity has the opposite effect, resulting in gene silencing (19, 20). The linkage between histone acetylation and transcriptional activation was strongly supported by the discovery that a number of transcriptional coactivators (GCN5, CBP/p300, SRC1, $TAF_{11}250$, and PCAF) possess histone acetyltransferase activity (7, 21–25), whereas a number of transcriptional repressors (Rpd3, HDAC1, and HDAC2) associate with histone deacetylases (26–31). Collectively, these data provide strong evidence that acetylases and deacetylases activate or repress gene expression by being recruited to specific promoters and/or proximal enhancer elements. The role of such histone acetylation and deacetylation in LCR function remains to be explored.

The human growth hormone gene (*hGH*) cluster comprises five closely linked genes: 5′-*hGH-N*/*hCS-L*/*hCS-A*/*hGH-V*/*hCS-B*-3′. Expression of *hGH-N* is limited to the somatotrope and somatolactotrope cells of the anterior pituitary, whereas expression of the remaining four genes is restricted to the syncytiotrophoblast layer of the placental villi (32, 33). A set of tissue-specific DNase I-hypersensitive sites located between −15 and −32 kb upstream of the *hGH* gene have been identified and shown to be required for appropriate tissue-specific expression of the *hGH* gene cluster in transgenic mice. Pituitary chromatin contains a subset of four HSs (HSI–III and HSV), and chromatin isolated from placental syncytiotrophoblasts contains a partially overlapping set of three HSs (HSIII–V). The full set of HSs renders expression of *hGH-N* transgenes reproducibly copy number-dependent and site of integration-independent in the mouse pituitary (34). HSI and HSII, which are closely linked and thus considered as a single determinant, are located 15 kb 5′ to the *hGH* gene and are unique to the pituitary (34). They are fully sufficient to confer high level, developmentally appropriate, somatotrope-specific and position-independent expression on a linked *hGH-N* transgene (35). As such, HSI/II constitutes the major element of the *hGH* LCR in the pituitary. Critical *cis*-acting determinants that bind the pituitary-specific POU homeodomain *trans*-factor Pit-1 have recently been identified within HSI/II (36). These sites are necessary for HSI/II function *in vivo*, but the mechanistic basis

* This work was supported by National Institutes of Health Grant HD25147 (to N. E. C. and S. A. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: LCRs, locus control regions; HS, DNase I-hypersensitive site; CBP, cAMP-responsive element-binding protein-binding protein; hGH, human growth hormone; GH, growth hormone; kb, kilobase(s); bp, base pair(s); GRF, growth hormone-releasing factor; ChIP, chromatin immunoprecipitation; ACTH, adrenocorticotropic hormone.

for their action is not defined.

A central question raised by studies of LCR function concerns the mechanism by which LCRs selectively alter chromatin structures and establish transcriptionally productive chromatin environments in specific tissues or cell types. Given the effects of histone modification on modulating chromatin structure, we have investigated the potential association of histone acetylation with LCR function. The data support a role for LCR-mediated histone acetylation in the establishment of a pituitary-specific, transcriptionally active chromatin environment required for *hGH-N* gene activation.

EXPERIMENTAL PROCEDURES

Tissue Culture and Primary Cells—Mouse GHFT1 presomatotrope cells (37) and human K562 erythroid cells were maintained in Dulbecco's modified Eagle's medium and RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. The GHFT1/HSIII cell line was generated by transfection of GHFT1 cells with the *HSIII/hGHneo*^{r} construct.[2] A portion of a surgically removed GH-secreting human pituitary adenoma was utilized. Mouse pituitaries and livers were isolated from the indicated transgenic lines. Pituitary and liver samples were dissociated in cell dissociation buffer (Life Technologies, Inc.). Nuclei were isolated from the dissociated pituitary and liver cells and cultured GHFT1/HSIII and K562 cells by hypotonic lysis in the presence of mild detergent as described previously (38).

Transgenic Mice—Mice carrying the *hGH/P1* transgene were generated by microinjection of a linearized P1 plasmid carrying the *hGH/P1* clone (encompassing the entire *hGH* LCR and the first four genes of the cluster) into fertilized mouse oocytes to establish founder lines (39). Frozen embryos carrying the human growth hormone-releasing factor (*GRF*) transgene were a kind gift from R. Brinster (University of Pennsylvania). To generate *hGH/P1*×*GRF* compound transgenic mice, an *hGH/P1* transgenic line was crossed to the *GRF* line. Doubly positive transgenic mice were identified by dot blot analysis of tail DNA using the probes described below.

Preparation of Unfixed Chromatin—Nuclei were resuspended in 1.0 ml of digestion buffer (50 mM NaCl, 20 mM Tris-HCl (pH 7.5), 3.0 mM $MgCl_2$, 1.0 mM $CaCl_2$, 10 mM sodium butyrate, and 0.1 mM phenylmethylsulfonyl fluoride) at a concentration of 0.3 mg/ml. They were digested with 25 units of micrococcal nuclease (Amersham Pharmacia Biotech) for 6 min at 37 °C. The digestion was terminated by the addition of EDTA to 0.5 mM, and the sample was centrifuged at 12,000 × *g* in a microcentrifuge for 10 min at 4 °C to generate supernatant S1. The pellet was resuspended in 300 μl of low salt lysis buffer (10 mM Tris-HCl (pH 7.5), 10 mM sodium butyrate, 0.25 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride), incubated on ice for 2 min, and then centrifuged as before. The resulting supernatant, S2, was combined with S1. The soluble chromatin was concentrated using Microcon centrifugal filters (Amicon, Inc., Bedford, MA).

Immunoprecipitation of Unfixed Chromatin—The chromatin immunoprecipitation (ChIP) procedure was carried out according to previously reported methods (40, 41) with minor modifications. Antisera specific to the acetylated lysine residues of histone H3 or H4 were diluted 1:100 and used in a 1:1 mixture. Alternatively, 15 μl of antiserum to unacetylated histone H3 was used. Each antiserum was kindly provided by C. D. Allis (University of Virginia). Immunoprecipitations contained 250-μg aliquots of chromatin DNA in a total volume of 500 μl. Protein A-Sepharose (Amersham Pharmacia Biotech) precipitates were generated and washed, and DNA and proteins were harvested from pellets and supernatants according to published protocols (40). Input, unbound, and bound DNA samples were each analyzed by electrophoresis on 1% agarose gels stained with ethidium bromide prior to use to ensure the quality of oligonucleosome preparations. Southern hybridization of micrococcal nuclease-digested input DNA using sheared ^{32}P-labeled total genomic DNA as a probe demonstrated that the majority of DNA ranged in size from ~0.16 to 1 kb.

Proteins from bound and unbound fractions were obtained from the first phenol/chloroform phase to which 8 μg of bovine serum albumin was added as carrier. HCl was then added to 0.1 M, followed by precipitation with 12 volumes of acetone as described previously (40, 41). Proteins were analyzed by electrophoresis on 15% SDS-polyacrylamide gels and then transferred to nitrocellulose filters and separately incubated with anti-acetylated H4 and anti-acetylated H3 (both at 1:1000 dilution) and anti-unacetylated H3 (1:250 dilution) antibodies. The blots were developed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG (used at 1:3000 dilution; Roche Molecular Biochemicals), and immune complexes were visualized by the Lumi-Light Western blotting substrate system (Roche Molecular Biochemicals).

Slot Blots and PhosphorImager Quantification—Equal masses of DNA (1.0 μg) from input (unfractionated chromatin), antibody-bound, and unbound fractions were loaded onto Zetabind membranes (Cuno, Inc., Meriden, CT) using a slot-blot manifold. The blots were incubated overnight at 65 °C with hybridization solution containing 1–2 × 10^6 cpm/ml random primer-labeled probe. Subsequent washes were at 60 °C in 0.1% SDS and 0.5× SSC. Signals were quantified by PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA) using ImageQuant software, and the ratios between bound and unbound DNA fractions were calculated for each probe used. To correct for potentially unequal slot-blot loadings, each blot was rehybridized with random primer-labeled sheared genomic DNA (see below). All ratios are reported after normalization to this loading control.

Preparation of DNA Probes—The majority of DNA fragments used as hybridization probes were generated by polymerase chain reaction using AmpliTaq DNA polymerase (Perkin-Elmer). The template for the polymerase chain reaction was a P1 clone encompassing the *hGH* LCR and the first four of the five clustered *hGH* genes (39). The primer sets are as shown in Table I. Probe p9 was a subcloned 263-bp *Eco*RI fragment of a repeated element ("P-element") (42) located 2 kb 5′ to *hCS-L*, *hCS-A*, *hGH-V*, and *hCS-B*. Probe p10 was an amplified fragment of a repeated element ("enhancer element") (43) located 2 kb 3′ to *hCS-L*, *hCS-A*, and *hCS-B*. Due to the high sequence homology between each of the *hGH* promoters, primers specific for the *hGH-N* promoter were designed to amplify a 200-bp region −0.8 kb to −1 kb upstream of the *hGH-N* transcription start site. The mouse ζ-globin probe was a 1.3-kb *Bam*HI fragment encompassing the mouse ζ-globin coding region. The human ζ-globin probe was a 1.8-kb *Bgl*II fragment encompassing the human ζ-globin coding region. The probe used as a loading control was generated by random primer labeling of sonicated mouse or human total genomic DNA. Transgenic genotypes were determined by tail blots using two probes: the *GRF* probe was a 1.7-kb *Bgl*II/*Hin*dIII fragment encompassing the human *GRF* coding region, and the *hGH* probe was a 500-bp *Eco*RI/*Bam*HI fragment encompassing the *hGH-N* promoter.

RESULTS

Specificity of the Chromatin Immunoprecipitation Procedure—A set of studies were designed to establish the specificity and accuracy of the ChIP approach used in this report. Salt-soluble, unfixed oligonucleosome preparations from each cell or tissue type studied (K562 erythroid cells, GHFT1/HSIII presomatotrope cells, human pituitary tumor tissue, and P1/GRF transgenic mouse pituitary and liver tissue) were generated by micrococcal nuclease digestion of purified nuclei. To ensure the quality of the chromatin preparations and to determine the size distribution of the resulting oligonucleosomes for each sample, the DNA from the digested chromatin preparation was analyzed by gel electrophoresis. In each case, this analysis revealed a typical oligonucleosome ladder; the majority of DNA ranged from ~160 bp (mononucleosomes) to 1 kb, and minimal DNA could be visualized above 2 kb (Fig. 1*A*).

To establish the specificity of the antibodies for acetylated H3 and H4 under the experimental ChIP conditions used for our studies, soluble oligonucleosomes prepared from the K562 erythroleukemia cell line (Fig. 1*A*) were immunoprecipitated with a 1:1 mixture of antibodies specific to acetylated histones H3 and H4. DNA and proteins were separately isolated from the total input chromatin, the unacetylated (unbound) chromatin, and the acetylated (bound) chromatin. DNA from each fraction was applied via a slot-blot manifold to nylon membranes for hybridization analysis (see below). In parallel, equal amounts of core histones isolated from each fraction were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 1*B*) and then Western-blotted and immunostained with antibodies spe-

[2] I. Bennani-Baiti, A. Abu-Daya, N. E. Cooke, and S. A. Liebhaber, unpublished data.

TABLE I
Amplification primer sets

Probe	Size	Primer set
	bp	
HSI/II	400	5′-GGAATTCCCAAGCCTTTCCCAGTTATAC-3′ 5′-GGAATTCGATCTTGGCCTAGGCCTCGGA-3′
HSIII	363	5′-GCGAATTCGAGGAGAGACTAGAGAAGCACCCAG-3′ 5′-AAGGATCCACTCATAACCCACCCATAAACACC-3′
HSIV	460	5′-TGCCTCTACGTGGACATCTC-3′ 5′-TATCAGCAGAGAGTGCACAA-3′
HSV	500	5′-CGAGTGGACCACCTTAACTT-3′ 5′-TAGAGGATAAGTGTGAGGAC-3′
p1	340	5′-GATTACAAGCGCCCACTACC-3′ 5′-GGGAGAGAATAAGCCAGGAGGTG-3′
p2	405	5′-TGCTCAGACCAGCCTATGCA-3′ 5′-TCAACAGGAAGTGGAGCACA-3′
p3	220	5′-GCTCCCCCAGAAGACTGAGAG-3′ 5′-GGAGGAAAACGTGRAACTGC-3′
p4	470	5′-GCTGTATTCTTCCAGACAAG-3′ 5′-GAGCTAAGCTATGAGGATGC-3′
p5	320	5′-GCCTCAAAACCTGATTGG-3′ 5′-GGAGATCTCTGAGGCTGG-3′
p6	260	5′-CCTGGGTGGCGTAGAGATG-3′ 5′-GACCCACGTTGTCGTAGTTG-3′
p7	416	5′-GCTCATCAAGATCATTGGCA-3′ 5′-AGTAAATTGAGAACTACGGG-3′
p8	240	5′-TGCCAGGAGGTGGGAGTT-3′ 5′-TGGCCCTGGGCTTTTGTGTAC-3′
p10	240	5′-GTCTACATTTCAGCTCATCA-3′ 5′-CCTTGTTTACATGTTAGAATC-3′
hGH-N-specific probe	200	5′-GCCTCGCCACCCCTGT-3′ 5′-CTTCCATGTTCCTCC-3′

cific to acetylated H3 or H4 (Fig. 1*C*). These results demonstrated that the immunoprecipitates were enriched for acetylated histones H3 and H4 compared with the unbound fractions. The specificity of the immunoprecipitation was further validated by demonstrating a reciprocal enrichment of unacetylated histone H3 in the unbound fraction (Fig. 1*C*, *lower panel*). As expected, prolonged exposure of the blot revealed a small amount of residual unacetylated H3 in the bound fraction as well (not shown). These protein studies demonstrated that this ChIP procedure enriched chromatin fractions for acetylated histones H3 and H4.

K562 DNA isolated by the ChIP procedure was analyzed by hybridization using probes corresponding to promoter sequences of actively transcribed ζ-globin and unexpressed *hGH* (Fig. 1*D*). Equal amounts of intact DNA extracted from input, unacetylated (unbound), and acetylated (bound) chromatin were applied to membranes via a slot-blot manifold and sequentially probed for specific DNA sequence content. Hybridization signal intensities in each of these three fractions were normalized for minor differences in DNA loading by directly quantifying the DNA in each slot using a labeled total genomic DNA probe. As expected from previous acetylation studies of active human globin genes (44), the chromatin encompassing the expressed ζ-globin promoter was enriched for acetylation (bound *versus* unbound ratio of 3.7). The acetylation of the chromatin encompassing the silent *hGH* promoter was at background levels (ratio of 1.0). The difference between these two ratios was highly significant ($p < 0.001$). In five independent ChIP experiments using K562 chromatin, the ratios of the ζ-globin promoter ranged between 3.5 and 3.8. The specificity of the immunoprecipitation was further validated by demonstrating that K562 nuclear chromatin immunoprecipitated with antibodies to unacetylated histone H3 was not enriched for ζ-globin promoter sequences. These DNA hybridization studies demonstrated the specificity and reproducibility of the ChIP procedure that was used in the studies that follow.

HSIII of the hGH LCR Is Acetylated when Integrated into the Chromatin of a Pituitary Presomatotrope Cell Line—The association of HS formation with localized histone acetylation was initially tested in a cell culture setting. HSIII of the *hGH* LCR (Fig. 2*A*) was used for these studies because the DNase I-hypersensitive structure of this chromatin element could be reproduced in stably transfected pituitary cells.[2] A 3150-kb fragment encompassing HSIII was linked to 500 bp of the *hGH-N* promoter region driving a neomycin resistance (neo^r) cassette (*HSIII/hGHneo*^{r}) (Fig. 2*B*, *bottom*). Following stable transfection into GHFT1 presomatotrope cells, a neo^r cell line (GHFT1/HSIII) was obtained. A DNase I-hypersensitive site formed in the integrated 3150-bp HSIII segment at a position corresponding precisely to that of native HSIII in primary pituitary and placental tissue (data not shown). Cell nuclei isolated from these neo^r cells were digested with micrococcal nuclease, and the resulting soluble oligonucleosomes were immunoprecipitated with a 1:1 mixture of antibodies specific to acetylated histones H3 and H4 using the ChIP procedure (40, 41). Equal amounts of intact DNA extracted from the input, unbound, and bound chromatin fractions were sequentially probed for specific DNA sequence content as described above. The normalized ratio of HSIII sequences in the bound *versus* unbound chromatin fractions was 3.3, and that for the *hGH-N* promoter was 2.8. In contrast, the ratios for the inactive α-fetoprotein and ζ-globin genes in the same chromatin samples were 1.1 and 1.2, respectively (Fig. 2*B*). In four independent experiments using GHFT1/HSIII chromatin, the ratios of HSIII ranged between 3.2 and 3.5. Specificity was validated by demonstrating that antibodies to unacetylated H3 failed to enrich for HSIII sequences (Fig. 2*B*). These data further established the specificity and reproducibility of this ChIP procedure and demonstrated the enrichment of HSIII in the acetylated chromatin fraction of a stably transfected, pituitary-derived cell line.

Each HS of the hGH LCR Is Enriched in the Acetylated Chromatin Fraction of a GH-secreting Human Pituitary Adenoma—Analysis of *hGH* LCR chromatin acetylation was next expanded to an *in vivo* setting in which the entire *hGH* cluster and adjacent sequences could be studied in their native setting. The chromatin of a primary GH-secreting human pituitary

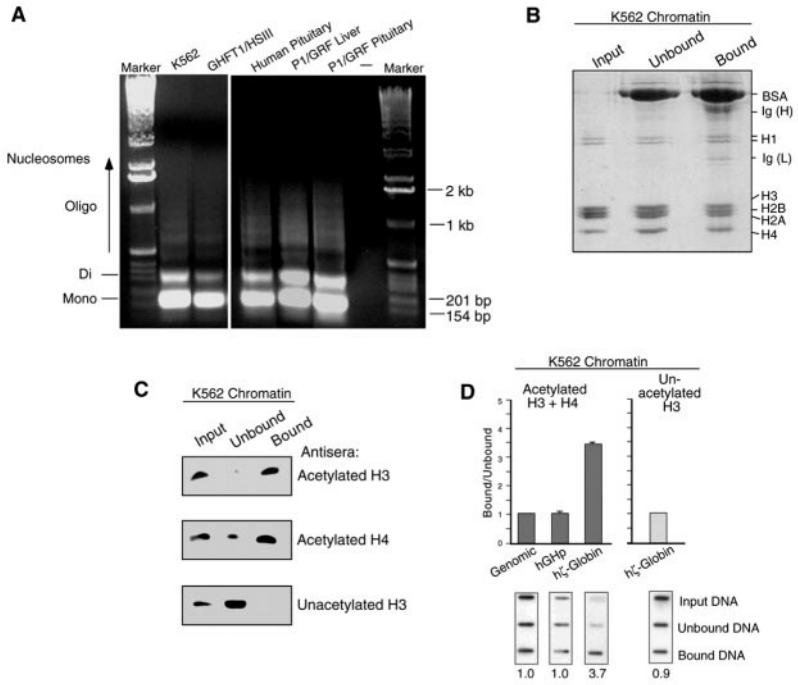

FIG. 1. **Analysis of proteins and DNA by the ChIP procedure.** *A*, size distribution of oligonucleosomes used for ChIP analysis. Nuclei were isolated from the following cell and tissue types: K562 erythroid cells, GHFT1/HSIII presomatotrope cells, GH-secreting human pituitary tumor, P1/GRF transgenic mouse liver, and P1/GRF transgenic mouse pituitary tissue. The nuclei were subjected to micrococcal nuclease digestion to yield a soluble oligonucleosome fraction. DNA from each preparation was resolved on a 1% agarose gel and stained with ethidium bromide. Size markers are shown for each of the two panels (derived from two independently run gels), and the markers on the *right panel* are labeled according to size. The predicted positions of the mono-, di-, and oligonucleosomes (as inferred from the molecular size markers) are indicated on the *left panel*. *B*, SDS-polyacrylamide gel electrophoresis of proteins isolated after the ChIP procedure using oligonucleosomes obtained from K562 cells. Proteins were isolated from input, unbound, and bound fractions following immunoprecipitation with a 1:1 mixture of antibodies specific to acetylated histones H3 and H4. Core histones from each fraction were resolved by 15% SDS-polyacrylamide gel electrophoresis and stained with Coomassie Blue. The positions of each of the histone proteins as well as the immunoglobulin subunits (heavy (*H*) and light (*L*)) and the bovine serum albumin (*BSA*; present as carrier in the precipitation procedure) are indicated to the right. *C*, Western analysis of core histones isolated by the ChIP procedure. Equal amounts of core histones isolated from input, unbound, and bound fractions (as estimated from *B* above) were resolved by 15% SDS-polyacrylamide gel electrophoresis, Western-blotted, and immunostained with antibodies to acetylated H3, acetylated H4, or unacetylated H3. The identity of each of the antiserum used is indicated to the right of the respective panels. *D*, acetylation status of the chromatin encompassing the active human ζ-globin (*hζ-Globin*) promoter and the inactive *hGH-N* promoter (*hGHp*) in K562 erythroid cells. The human erythroleukemia cell line K562 expresses the human embryonic ζ-globin gene. Nuclear chromatin from these cells was subjected to ChIP using a 1:1 mixture of antisera against acetylated histones H3 and H4. Equal amounts of DNA purified from starting chromatin (*Input DNA*), unacetylated (*Unbound DNA*), and acetylated antibody-bound (*Bound DNA*) fractions were slot-blotted onto nylon membranes and then hybridized sequentially with probes to total genomic DNA, inactive *hGH-N* promoter, and active human ζ-globin promoter sequences. The normalized ratios (bound/unbound) are indicated below each hybridization panel (autoradiograph) and are summarized in the histogram. As a specificity control, antiserum specific to unacetylated histone H3 was used in the ChIP procedure and hybridized with the human ζ-globin promoter. The mean value from at least three determinations from individual ChIP assays is shown; *error bars* indicate S.E. All ratios were normalized to the ratio obtained using a probe for total genomic DNA as a loading control. The ratio of acetylation of chromatin encompassing the human ζ-globin gene was significantly greater that that of the *hGH* promoter ($p < 0.001$).

adenoma was analyzed using a set of probes corresponding to the *hGH-N* promoter, each of the *hGH* LCR HSs, and two sites located 5′ to the LCR. Tissue specificity of the acetylation map was established by comparing these results with those obtained from analysis of chromatin isolated from the human K562 erythroid cell line. The positions of the probes in relation to the *hGH* cluster, the closely linked *CD79b* (the B lymphocyte-specific immunoglobulin receptor subunit gene encoding Igβ) (45), and *SCN4A* (the striated muscle-specific sodium channel gene) (46) are shown in Fig. 3*A*. ChIP assays of the human pituitary adenoma chromatin revealed that the segments encompassing each of the five HSs as well as the adjacent *hGH-N* promoter were all enriched for acetylation (all ratios = 2.0) (Fig. 3*B*). The most highly modified region coincided with HSI/II (3.4-fold enrichment). Acetylation at the two sites upstream of the LCR (probes p7 and p8) (Fig. 3*B*) was insignificant (ratios of 1.4). There was virtually no acetylation enrichment at any of these sites in erythroid (K562) cell chromatin. These data demonstrated tissue-specific enrichment of acetylation at all HSs in the chromatin of this human pituitary cell line enriched for somatotropes. Furthermore, they identified a 5′-boundary to the LCR modification in the pituitary just upstream of HSV and suggested that HSI/II was the most highly modified among the HSs. Of additional note was the acetylation of HSIV. Because HSIV does not form in the pituitary, its modification in this tissue suggested that acetylation of the *hGH* locus in pituitary chromatin might not be limited to the immediate locale of the active HSs, but might instead be generally distributed throughout the LCR domain.

Histone Acetylation of the hGH LCR Is Recapitulated and Enhanced in Somatotrope-enriched Transgenic Mouse Pituitaries—The pituitary is made up of six differentiated hormone-secreting cell types: the somatotropes (secreting GH), somatolactotropes (GH and prolactin), lactotropes (prolactin), thyrotropes (thyrotropin-releasing hormone), gonadotropes (luteinizing hormone and follicle-stimulating hormone), and corticotropes (ACTH). The representation of somatotropes in the surgical human pituitary tumor sample was difficult to quantify, and the scarcity of the material limited the number of chromatin regions that could be experimentally assessed. Therefore, a surrogate mouse model with pituitary somatotrope hyperplasia was established to extend the chromatin

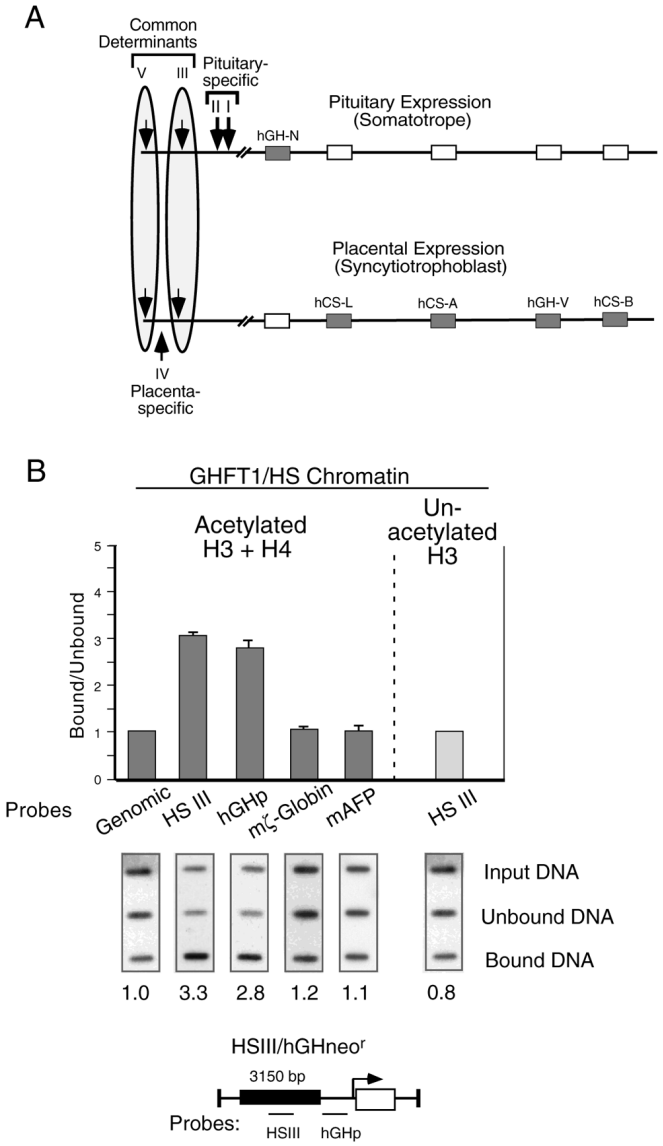

FIG. 2. **HSIII of the *hGH* LCR is hyperacetylated in stably transfected GHFT1 cells.** *A*, diagram of the *hGH* gene cluster and adjacent LCR. The *black boxes* are expressed genes, and the *white boxes* are silent genes. The *top line* represents the status of the cluster in pituitary somatotropes, and the *bottom line* represents the status of the cluster in placental syncytiotrophoblasts. HSs common to both tissues are indicated by the *elongated shaded ovals* (HSIII and HSV); HSs specific to the pituitary (HSI/II) or placenta (HSIV) are indicated separately. *B*, acetylation status of the *hGH* promoter (*hGHp*) and HSIII in GHFT1 presomatotrope cells stably transfected with the *HSIII/hGHneor* construct. The *HSIII/hGHneor* construct, shown at the bottom, contains a 3.15-kb genomic fragment encompassing HSIII linked to the *hGH-N* promoter and *neor* coding region. After transfection of GHFT1 presomatotrope cells with this neor vector, cells were selected; nuclei were isolated; and soluble chromatin was immunoprecipitated with antisera against acetylated H3 and H4 or, as a specificity control, antiserum recognizing unacetylated H3. Equal amounts of DNA purified from input chromatin, acetylated antibody-bound, and unacetylated unbound fractions were slot-blotted as described for Fig. 1*D* and hybridized sequentially with probes to total genomic DNA, HSIII, and *hGH-N* promoter sequences in the integrated *HSIII/hGHneor* construct and the endogenous mouse ζ-globin (*mζ-Globin*) and mouse α-fetoprotein (*mAFP*) loci. All ratios were normalized to the ratio obtained using a probe for total genomic DNA as a loading control. The ratios of signals in the bound *versus* unbound chromatin are shown below each slot-blot panel and are summarized in the histogram. The mean value from at least three individual ChIP assays is shown; *error bars* indicate S.E. The ratios of HSIII and the hGH promoter were not significantly different ($p > 0.1$), and both were significantly greater than ratios detected with the other probes ($p < 0.001$).

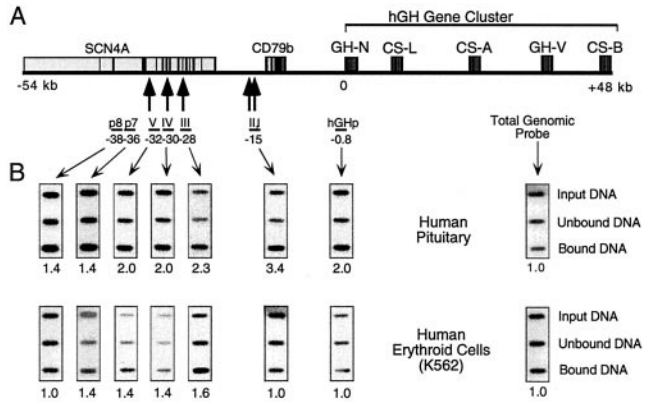

FIG. 3. **Each HS of the *hGH* LCR is enriched in the acetylated chromatin fraction isolated from a GH-secreting human pituitary adenoma.** *A*, schematic representation of the *hGH* LCR and *hGH* gene cluster contiguous with the two linked upstream genes, *CD79b* and *SCN4A*. Each of the genes is shown as a *shaded box*, and the *dark vertical lines* indicate exons. Probes used for acetylation mapping are *underlined*, and their coordinates relative to the *hGH-N* transcription start site are indicated below. *B*, acetylation status of each HS in human pituitary adenoma and K562 erythroid chromatin. Soluble chromatin samples isolated from each of the indicated sources were immunoprecipitated with anti-acetylated H3 and H4 antibodies. Equal amounts of DNA purified from input, antibody-bound, and unbound fractions were slot-blotted onto nylon membranes and then sequentially hybridized with the indicated probes (*underlined*). Ratios (bound/unbound) are shown beneath each blot. All ratios were normalized to the ratio obtained using a probe for total human genomic DNA as a loading control (shown to the right).

analysis. A transgenic mouse line was generated carrying an extensive human genomic DNA insert isolated from a P1 bacteriophage library that encompassed the entire *hGH* LCR and first four genes of the cluster ("P1 clone") (Fig. 4*A*). Lines containing this *hGH/P1* transgene expressed the *hGH-N* gene in a physiologically appropriate manner in the pituitary (39). A second mouse line was obtained that was transgenic for an overexpressing human *GRF* transgene. This *GRF* overexpression stimulates hyperproliferation of pituitary somatotropes (47). Crossing the *hGH/P1* and *GRF* lines generated compound transgenic mice (*hGH/P1×GRF*) expressing high levels of mouse and human GH. Such mice were 1.5–2 times greater than normal size by 3 months of age, and their pituitaries were 5–10 times normal size, composed predominantly of somatotropes and containing HSI–III and HSV.[3]

Pooled pituitaries from *hGH/P1×GRF* adult mice were isolated and analyzed by the ChIP assay (Fig. 4*B*). Livers of the same *hGH/P1×GRF* mice served as a source of chromatin from a non-expressing tissue. The *hGH* LCR in *hGH/P1×GRF* chromatin displayed a pattern of acetylation enrichment paralleling that seen in primary human pituitary chromatin (compare Figs. 3*B* and 4*B*). As in the primary human pituitary, each of the HSs and the adjacent *hGH-N* promoter were enriched in the acetylated chromatin fraction, whereas regions immediately 5′ to HSV were unacetylated. However, when compared with the primary human pituitary sample, the overall level of modification was significantly greater in the *hGH/P1×GRF* transgenic mouse pituitaries. This difference was particularly marked toward the central region of the LCR, with the enrichment of HSIV, HSIII, and HSI/II in the acetylated chromatin fraction being 2–2.5-fold higher in this tissue than that seen in the human pituitary adenoma. Thus, the acetylation peak at HSI/II in the *hGH/P1×GRF* pituitaries was accentuated to 8.8-fold above control levels and was 3.5-fold higher than in the

[3] F. Elefant, A. Zhang, N. E. Cooke, and S. A. Liebhaber, unpublished data.

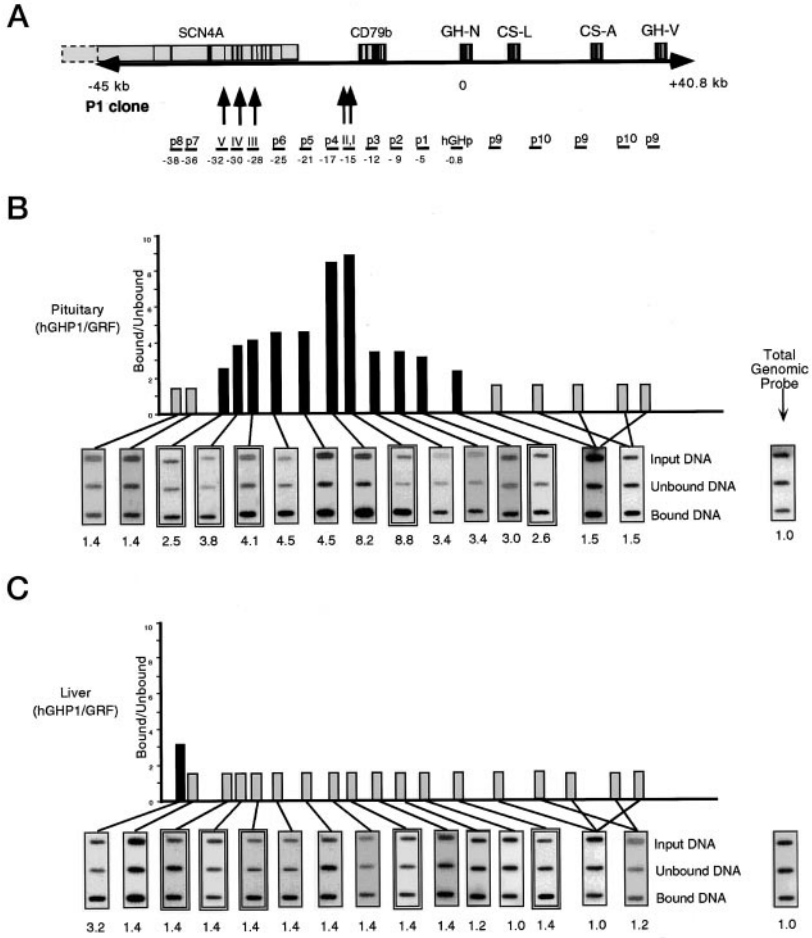

FIG. 4. **The chromatin domain encompassing the *hGH* locus transgene in the mouse pituitary recapitulates a peak of acetylation at HSI/II; the modified domain encompasses all HSs and intervening regions and extends to the *hGH-N* promoter.** *A*, shown is a schematic representation of the *hGH/P1* transgene. The extent of the transgene (P1 clone) relative to the positions of the *hGH* locus is defined by the *double-headed arrow* (coordinates −45 to +40.8). The positions of the HSs and the probes used in the study (*underlined*) are shown below the diagram. *B* and *C*, autoradiographs using hybridization probes corresponding to the HSs and the *hGH-N* promoter in *hGH/P1×GRF* transgenic mouse pituitary **(*B*)** and liver chromatin **(*C*)** are *double-boxed*. Autoradiographs using hybridization probes corresponding to sites lying upstream of the LCR (p7 and p8), between the HSs (p6, p5, and p4), between the HSs and the *hGH-N* promoter (p3, p2, and p1), or 3′ to *hGH-N* (p9 and p10) are *single-boxed*. Soluble chromatin samples isolated from *hGH/P1×GRF* compound transgenic mouse pituitaries (enriched for somatotropes) and liver tissue from the same animals were immunoprecipitated with anti-acetylated H3 and H4 antibodies and analyzed as described for Fig. 2. All ratios were normalized to the ratio obtained using a probe for total genomic mouse DNA as a loading control (shown on the right). The ratios of signals in bound *versus* unbound chromatin were determined as described for Fig. 1 and are shown below each autoradiograph. The ratios are summarized in the histograms. *Black bars* (ratios > 2.0) are considered to be enriched for acetylation, and *shaded bars* (ratios < 2.0) are considered to represent background.

active *hGH-N* promoter region. The acetylation enrichment of the LCR chromatin domain seen in the pituitaries of *hGH/P1×GRF* mice was specifically absent in the livers of the same animals (Fig. 4*C*). These data demonstrated a localized peak of modification of HSI/II and supported the conclusion that the acetylation enrichment of the *hGH* LCR was somatotrope cell-specific, thus confirming and extending the observations obtained using primary human pituitary tissue.

Acetylation of the hGH LCR Chromatin Domain Extends beyond the HSs and Reveals Defined 5′- and 3′-Borders—Additional probes were generated to determine the extent of histone acetylation in pituitary chromatin segments between and surrounding the HSs (Fig. 4*A*). Probes located between the *hGH-N* gene and HSI/II (probes p1, p2, and p3, located at −5, −9, and −12 kb, respectively) and between HSI/II and HSIII (probes p4, p5, and p6, located at −17, −21, and −25 kb, respectively) were used in the ChIP assay. These data demonstrated that all regions tested between HSV and the *hGH-N* promoter were highly enriched in the acetylated chromatin fraction (Fig. 4*B*, *black bars*). The levels of acetylation at each of the sites flanking the HSI/II peak decreased in a graded fashion, culminating at discrete 5′- and 3′-borders. The 5′-border was deemed to be coincident with HSV because the upstream probes p7 and p8 were unacetylated both in the human pituitary and in the *hGH/P1×GRF* transgenic pituitary (Figs. 3*B* and 4*B*). The 3′-border of the acetylated domain was sought by mapping internal regions of the *hGH* transgene cluster using probes that recognize two elements flanking *hCS-L* (as well as *hCS-A*) and *hGH-V* (probes p9 and p10). These two elements, generated by localized duplication events during the evolution of the *hGH* cluster (48) and not represented elsewhere in the genome (data not shown), were both unacetylated (Fig. 4*B*). Thus, the acetylation present in the LCR encompassed pituitary-expressed *hGH-N*, but did not extend farther 3′ into the cluster. These data suggested that the acetylation activity, once recruited to the LCR, modified a 32-kb domain extending from HSV through the activated *hGH-N* locus.

DISCUSSION

Histone acetyltransferases modulate chromatin structure by acetylation of specific basic lysine residues present on the N-

terminal tails of histones. This modification neutralizes the positively charged lysines, thereby weakening interactions between neighboring nucleosomes. This effect promotes the destabilization of higher order chromatin structure, thus facilitating the transcription process (49–51). *In vitro* studies suggest that acetylation of histones increases the accessibility of transcription factors for nucleosomal DNA (52) and facilitates recruitment of SWI/SNF-like chromatin-remodeling factors (53). The work presented here suggests that chromatin acetylation may be a critical step in LCR function in addition to its more established correlation with enhancer activation (54, 55).

Previous studies in yeast strongly support a mechanism by which highly localized, promoter-targeted histone-modifying activities lead to selective effects on transcriptional regulation (Refs. 17, 20, and 31; reviewed in Ref. 2). More recently, Parekh and Maniatis (56) demonstrated that promoter-localized histone acetylation is also required for metazoan gene activation. This work demonstrated that transcriptional activation of the virally induced human interferon-β gene is associated with promoter-localized hyperacetylated histones H3 and H4. Our data would suggest that LCR elements are similarly recruiting histone acetylation activity in a targeted manner. However, in clear contrast to the highly restricted promoter-localized histone acetylation observed for inducible genes, acetylase activity, once targeted by the *hGH* LCR, appeared to extend throughout the extensive LCR chromatin domain and encompass the *hGH-N* promoter. The capacity for acetylation spreading was initially suggested by the extensive acetylation of an episomal Epstein-Barr virus-derived plasmid containing the *IgH* LCR (57). The acetylase activity may be targeted by a single component of the LCR such as HSI/II or by several of the LCR HS elements at differing levels. In either case, the general modification between these elements suggests a subsequent spreading mechanism.

The observation that the entire domain of the *hGH* LCR is hyperacetylated needs to be compared with prior observations of acetylation of the domain spanning the chicken β-globin gene cluster and its 5′-LCR (58). The pattern of modification for the *hGH* LCR demonstrates a distinct central peak corresponding to the major pituitary HS. Its extension into the *hGH* cluster is limited to the 5′ most (expressed) *hGH-N* gene (Figs. 3 and 4). This pattern suggests a mechanism involving targeted recruitment and spreading of a histone acetyltransferase activity by the LCR. In contrast, the chicken β-globin LCR modification is uniform throughout the domain and encompasses the entire cluster. This lack of localized peaks suggests that, in this system, the modification may not be reflecting specific targeting to HSs. A strict comparison between these two systems is, however, complicated by the fact that the differences may partially reflect the use of antibodies recognizing the *N*-acetyllysine on all histones in the former study (58) rather than the specific acetyllysine H3 and H4 antibodies used in the present study. Thus, generalizations on the basis of comparisons between these two systems may be premature at this point.

How might histone acetyltransferase activity be recruited to the HSI/II LCR element specifically in pituitary chromatin? Recent studies have demonstrated that certain transcriptional cofactors such as CBP/p300 and PCAF possess histone acetyltransferase activities (22, 24), acetylate histones H3 and H4 *in vitro* (59), and associate with numerous tissue-specific transcription factors (60–64). In this regard, Pit-1, a pituitary-specific DNA-binding factor shown to be central to the regulation of somatotrope-restricted *hGH* expression, was recently shown to associate with CBP in the pituitary cell (65). Additionally, we have recently demonstrated that HSI/II contains an array of functional Pit-1-binding sites critical for activation of *hGH* transgene expression in the somatotrope cells of the pituitary (36). These observations suggest a model in which CBP/p300 is specifically recruited to HSI/II through its interaction with Pit-1. The HSI/II-bound complex containing DNA-binding factors and associated histone acetyltransferase coactivators might then "track" via small steps along the LCR, concomitantly modifying chromatin structure (66). Alternatively, further acetylation of the domain might reflect recruitment of histone acetyltransferase coactivators by each of the pituitary-specific HSs that subsequently spread the modification throughout the LCR from these multiple target sites. Subsequent studies can now be designed to further refine this model.

Acknowledgments—We gratefully acknowledge Yuhua Su for the *hGH/P1* transgenic mouse line, Idriss Bennani-Baiti and Anita Abu-Daya for establishing and DNase I-mapping the GHFT1/HSIII cell culture line, Brian M. Shewchuk and Yugong Ho for critically reading the manuscript, and Jessie Harper for excellent secretarial support.

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