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

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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 HSI) in pituitary chromatin. These HSSs 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)† 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, TAF1, 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 HSIV), 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, HSII 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 HSII function in vivo, but the mechanistic basis...
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 GHTPtI preemotatrophe 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 GHTPtI/HSIII cell line was generated by transfection of GHTPtI cells with the HSIII/hGHneo construct. A portion of a surgically removed GH-secreting human pituitary adenoma was utilized. Mouse pituitaries and livers were isolated from the indicated transgenic line. 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 GHTPtI/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 MgCl₂, 1.0 mM CaCl₂, 10 mM sodium butyrate, and 0.1 mM phenylmethylsulfonyl fluoride) at a concentration of 0.3 mg/ml. They were incubated with anti-acetylated lysine residues of histone H3 or H4 were used. Alternatively, 15 µl of specific antibodies were used in a 1:1 mixture. The probes were then Western-blotted and immunostained with antibodies specific to the acetylated lysine residues of histone H3 and H4. DNA and proteins were separately isolated from fixed nuclei and centrifuged at 12,000 × g in a microcentrifuge for 10 min at 4 °C to generate supernatant S1. The nuclei were 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. The antibodies were then Western-blotted and immunostained with antibodies specific to the acetylated lysine residues of histone H3 and H4. DNA and proteins were separately isolated from fixed nuclei and centrifuged at 12,000 × g in a microcentrifuge for 10 min at 4 °C to generate supernatant S1. The nuclei were 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).

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, GHTPtI/HSIII preemotatrophe 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 to ~500 bp (mononucleosomes) to 1 kb, and minimal DNA could be visualized above 2 kb (Fig. 1A).

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 each cell type shown in Fig. 1A were immunoprecipitated with a 1:1 mixture of antibodies specific to acetylated histones H3 and H4. DNA and proteins were separately isolated from the bound chromatin fraction by phenol/chloroform extraction. The bound 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. 1B) and then Western-blotted and immunostained with antibodies spe-
cific to acetylated H3 or H4 (Fig. 1C). 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 bound fraction (Fig. 1C, 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 z-globin and unexpressed hGH (Fig. 1D). 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, the ratios for the inactive hGH promoter was 2.8. In contrast, the ratios for the inactive a-fetoprotein and z-globin genes in the same chromatin samples were 1.1 and 1.2, respectively (Fig. 2B). In four independent experiments using GHFT1/HSII chromatin, the ratios of HSIII between 3.2 and 3.5. Specificity was validated by demonstrating that antibodies to unacetylated H3 failed to enrich for HSIII sequences (Fig. 2B). 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
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. 3A. 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. 3B). The most highly-modified region coincided with HSII (3.4-fold enrichment). Acetylation at the two sites upstream of the LCR (probes p7 and p8) (Fig. 3B) 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 HSII 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 (follitropin 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 extended to establish the chromatin

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/H3III 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 β-globin (hβ-Globin) promoter and the inactive hGH-N promoter (hGHp) in K562 erythroid cells. The human erythroblastemia cell line K562 expresses the human embryonic β-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 β-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 β-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 β-globin gene was significantly greater that that of the hGH promoter (p < 0.001).
HSIII/hGH-neor construct. The HSIII/hGH-neor construct, shown at the bottom, contains a 3.15-kb genomic fragment encompassing HSIII linked to the hGH-N promoter sequences in the integrated transgenic mouse line. The acetylation status of each HS in the integrated transgenic mouse pituitaries was accentuated to 5–10 times normal size, composed predominantly of somatotropes and containing HSI–III and HSIV.

Pooled pituitaries from hGH/P1×GRF adult mice were isolated and analyzed by the ChIP assay (Fig. 4B). 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 parallel to that seen in primary human pituitary chromatin (compare Figs. 3B and 4B). 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 HSII 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 HSII in the hGH/P1×GRF pituitaries was accentuated to 8.8-fold above control levels and was 3.5-fold higher than in the endogenous mouse and human GH. Such mice were 1.5–2 times greater than normal size by 3 months of age, and their pituitaries were more than normal size by 3 months of age, and their pituitaries were significantly higher than that seen in primary human pituitary (compare Figs. 3B and 4B). 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 HSII 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 HSII in the hGH/P1×GRF pituitaries was accentuated to 8.8-fold above control levels and was 3.5-fold higher than in the

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. 4A). 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 HSIV.

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active hGH-N promoter region. The acetylation enrichment of the LCR chromatin domain seen in the pituitaries of hGH/P1 3 GRF mice was specifically absent in the livers of the same animals (Fig. 4C). 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.

**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 array of functional Pit-1-binding sites critical for activation of the hGH transgene expression in the somatotrope cells of the pituitary (36). These observations suggest a model in which CBP/p300 is specifically recruited to HSII through its interaction with Pit-1. The HSII-bound complex containing DNA-binding factors and associated histone acetyltransferase coactivators might then “track” via small steps along the LCR, concomitantly modifying chromatin structure (36). 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.

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