An Autoregulatory Pathway Establishes the Definitive Chromatin Conformation at the *Pit-1* Locus

Yugong Ho,^a Nancy E. Cooke,^{a,b} Stephen A. Liebhaber^{a,b}

Departments of Genetics^a and Medicine,^b Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania, USA

The transcription factor Pit-1 (POU1-F1) plays a dominant role in cell lineage expansion and differentiation in the anterior pituitary. Prior studies of the mouse *Pit-1* (*mPit-1*) gene revealed that this master regulatory locus is activated at embryonic day 13.5 (E13.5) by an early enhancer (EE), whereas its subsequent expression throughout adult life is maintained by a more distal definitive enhancer (DE). Here, we demonstrate that the sequential actions of these two enhancers are linked to corresponding shifts in their proximities to the *Pit-1* promoter. We further demonstrate that the looping of the definitive enhancer to the *mPit-1* promoter is critically dependent on a self-sustaining autoregulatory mechanism mediated by the Pit-1 protein. These Pit-1-dependent actions are accompanied by localized recruitment of CBP and enrichment for H3K27 acetylation within the *Pit-1* locus. These data support a model in which the sequential actions of two developmentally activated enhancers are linked to a corresponding shift in higher-order chromatin structures. This shift establishes an autoregulatory circuit that maintains durable expression of Pit-1 throughout adult life.

Transcriptional controls and developmental programs within the mammalian genome are tightly linked to the higher-order chromatin structure and organization (1, 2). Sublocalizations of enhancers and boundary elements within the nucleus can impact the levels and coordination of the corresponding transcriptional activities (3, 4). Of particular note is the observation that transcriptional enhancers are frequently located at substantial distances from their target promoters when mapped in the linear genome (5). While the evolutionary advantage of such extensive separations of enhancers and promoters remains to be determined, mechanistic models of long-range enhancer actions are supported in a number of model systems. These mechanisms include polymerase tracking, linear spreading of the chromatin structure, and looping between enhancers and their target promoters (6). Studies in a limited number of model systems suggest that in some cases, enhancers can activate target promoters by combining noncontact (tracking and spreading) and contact (looping) mechanisms (7–9). The use of chromatin conformation capture (3C) technologies has substantiated the existence of long-range looping between enhancers and promoters at numerous loci (10, 11). How these long-range interactions contribute to pathways of developmental control and the degree to which these higher-order conformations are reconfigured during developmental progression remain to be determined.

Development pathways require tight controls over the spatial and temporal expression of specific transcription factors (TFs). These shifts in TF expression direct developmental programs and lineage definitions by recruiting subsets of coactivators, corepressors, and RNA polymerase II (Pol II) holo complexes to target promoters (12). In some cases, the expression of a transcription factor is constrained to a narrow developmental window, while in other cases, it must be durably maintained in an “on” state throughout adult life. The mechanisms underlying transient activation versus stable maintenance of gene expression are likely to differ. Certain of these controls may involve long-range communications and, as such, may depend on realignments of higher-order chromatin configurations to accommodate transitions in gene regulation (13).

The anterior pituitary gland represents a robust model for mammalian development (14). Pituitary development reflects a well-defined cascade of transcription factors and signaling molecules that drive regional expansion and functional differentiation of five distinct hormone-producing cell lineages: somatotropes, lactotropes, thyrotropes, corticotropes, and gonadotropes (14, 15). Pit-1, a pituitary-specific POU homeodomain transcription factor, plays a dominant and essential role in this process (14). Ectopic expression of Pit-1 can activate key genes in this pathway (i.e., *GH*) (16), whereas loss of Pit-1 expression results in a combined pituitary hormone deficiency (CPHD) syndrome reflecting the absence of lactotropes, somatotropes, and a subset of thyrotropes (14). Thus, Pit-1 serves as a master regulator of lineage commitment and expansion in the anterior pituitary. Understanding the controls over *Pit-1* gene regulation is therefore central to the understanding of mammalian development.

Pit-1 transcription in the mouse is activated at embryonic day 13.5 (E13.5). This activation is under the control of an early enhancer (EE) located 5.8 kb 5' to the *Pit-1* promoter (17). EE activity has been reported to require binding of the homeodomain/zinc finger transcription factor Atbf1 (17). Transgenic studies demonstrated that the EE is required for the proper temporal expression of the reporter gene (17). Remarkably, while EE function is critical to the initial activation of *Pit-1*, it is unable to sustain the subsequent expression of *Pit-1* beyond E16.5 (18). Instead, the maintenance of robust *Pit-1* transcription into adult life depends

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Address correspondence to Yugong Ho, yho@mail.med.upenn.edu.

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on a distinct definitive enhancer (DE) located 10.2 kb 5' to the *Pit-1* promoter (18, 19). The DE itself contains an array of Pit-1 binding sites. Based on these observations, it has been postulated that durable maintenance of Pit-1 expression in the adult may be under a self-sustaining autoregulatory control circuit (19, 20). Testing this model, defining the corresponding mechanism(s) of the shift from early to late enhancer action, and testing the corresponding mechanistic model(s) are of central importance to understanding the development of the anterior pituitary.

Here, we explore the structural changes at the *Pit-1* chromatin locus that correspond to, and are likely involved in, switching between the two developmentally controlled *Pit-1* enhancers. The data show that the initial activation of *Pit-1* reflects close physical proximity of the EE to the *Pit-1* promoter, whereas the subsequent maintenance phase of *Pit-1* expression reflects a shift in the locus configuration that positions the DE close to the *Pit-1* promoter. Importantly, the data also demonstrate that the definitive maintenance configuration of the *Pit-1* chromatin locus is dependent on the actions of Pit-1 itself, is accompanied by the recruitment of the histone acetyltransferase (HAT) coactivator CBP to the definitive-enhancer region, and results in the establishment of an expansive domain of acetylated histones. These studies demonstrate a dynamic alteration of the chromatin conformation at the *Pit-1* locus that is mediated in part by an autoregulatory control circuit. This pathway establishes a durable activating chromatin configuration at the *Pit-1* locus in the adult pituitary.

MATERIALS AND METHODS

Cell and transgenic mouse lines. The murine pituitary cell lines GHFT1 (21) and Pit-1/Triple (22) were cultured in Dulbecco's modified eagle medium (DMEM) (Life Technologies) plus 10% fetal bovine serum (FBS) with 1× antibiotic-antimycotic solution (Invitrogen).

Somatotrope-enriched pituitaries were isolated from 8- to 12-week-old mice carrying the human *GRF* (*hGRF*) transgene (23). All mouse studies were reviewed and approved by the University of Pennsylvania Laboratory Animal Use and Care Committee.

3C assay. The 3C procedure was performed on cross-linked pituitary chromatin as described previously (8, 24). Five pituitary glands were pooled to isolate nuclei for each 3C analysis. The GHFT1 cells and Pit-1/Triple cells were fixed with 1% formaldehyde at room temperature for 10 min. Fixation was stopped by adding glycine to a final concentration of 0.125 M, and the cells were washed twice with 20 ml of cold phosphate-buffered saline (PBS) containing protease inhibitors and processed as previously described (8). The frequencies of ligation between the *Pit-1* promoter fragment and the other BglII fragments at the *Pit-1* locus were assessed by semiquantitative PCR. The PCR products were separated on 1.5% agarose gels, stained with SYBR Gold, scanned using a Typhoon phosphorimager system (GE Healthcare), and quantified using the ImageQuant TL program (GE Healthcare). Ligation frequencies were normalized to a parallel ligation of BglII-digested mouse *Pit-1* bacterial artificial chromosome (BAC) clone DNA to establish a relative ligation value. For cells treated with mouse Pit-1 (mPit-1)-specific short hairpin RNA (shRNA) and the nontarget control, this ratio was further normalized to the 3C analysis at a mouse basal transcription factor locus (*Ercc3*) (8) using a pair of identically oriented primers located within two adjacent BglII fragments of the *Ercc3* locus. This was necessary in order to compare the ligation efficiencies between chromatin samples of cells expressing the targeting and control shRNAs. The ligation efficiency was calculated as $(\text{signal}_{\text{pituitary}} / \text{signal}_{\text{ran-dom control}}) / \text{signal}_{\text{ercc3 ligation}}$. The primer sequences used in the 3C assays are available upon request.

ChIP assays. Chromatin immunoprecipitation (ChIP) assays were performed as described previously (24, 25) with minor modifications.

Twenty micrograms of pituitary chromatin was used for each ChIP assay. Antibodies specific for the acetylated histone H3K27 (ab4729) and Pol II phosphorylated at Ser-5 of the C-terminal domain (CTD) (ab5131) were purchased from Abcam (Cambridge, MA). The antibodies for CBP (sc369X), p300 (sc585X), and Pit-1 (sc16288X) were purchased from Santa Cruz (Santa Cruz, CA). Normal rabbit IgG (12-370; Millipore, Billerica, MA) and normal goat IgG (sc2028; Santa Cruz) were used as controls for the ChIP assays. The input and bound DNAs were amplified by PCR, and the amplification products were quantified as described previously (24). A series of dilutions of the chromatin input fraction were used to determine the linear region for each set of PCR amplifications. The relative enrichment was calculated as the ratio of the intensity of the band in the bound fraction to that of 1% of the input. Signals were determined from Sybr gold-stained agarose gels using a FL9500 Typhoon phosphor-imager system. The intensities of the bands were analyzed using the ImageQuant LT software. The primer sequences used in the ChIP assays are available upon request.

ChIP-3C assays. Nuclei were fixed as for the 3C assays, and the chromatin was briefly sonicated to shear it to <10 kb in size, a treatment found to decrease background. The partially sheared chromatin (50 µg) was then digested by BglII as in the 3C assays and analyzed by ChIP, using an antibody against Pit-1 (Santa Cruz) or normal IgG (Millipore). Antibody-bound chromatin was eluted in 50 µl of 1× NEB buffer 3.1 (NEB, Ipswich, MA) containing 1.6% SDS at 65°C for 30 min. The chromatin was diluted with 1 ml of 1× ligation buffer (NEB, Ipswich, MA) containing 1% Triton X-100. The samples were incubated at 37°C for 1 h, after which 2 µl of T4 DNA ligase (800 units; NEB, Ipswich, MA) was added to each sample and the samples were incubated at 16°C for 4 h. The cross-linking was reversed, and the DNA was purified as described for the 3C assays.

Lentivirus infections. TRC mPit-1 shRNA clones were purchased from Thermo Fisher Scientific (Waltham, MA). The lentivirus was packaged in 293T cells as described previously (26). A nontargeting shRNA clone was used as a control (Thermo Fisher Scientific, Waltham, MA). A lentivirus clone expressing the green fluorescent protein (GFP) marker was packaged in parallel to monitor transfection efficiency. The mPit-1-specific and control shRNAs were each transfected with Lipofectamine 2000 (Invitrogen), and the viral supernatants were collected after 2 days of incubation. Pit-1/Triple cells and GHFT1 cells were infected overnight with viral supernatant in the presence of 8 µg/ml of Polybrene, followed by removal of the viral suspension. The infected cells were washed with PBS and cultured for 5 days in DMEM and 10% FBS prior to analysis for Pit-1 depletion and 3C studies.

Western blots. Pit-1/Triple cells and GHFT1 cells were washed twice with cold PBS, followed by lysis in 200 µl of RIPA buffer for 30 min at 4°C with gentle rotation. The samples were then clarified by spinning at 10,000 × g for 20 min at 4°C. The supernatant was transferred to a fresh tube, and 35 µl of the protein lysate was added to 35 µl of 2× SDS loading buffer. The proteins were separated by SDS-PAGE on NuPAGE Novex 4 to 12% Bis-Tris protein gels and transferred to membranes. Pit-1 protein was detected using a goat anti-Pit-1 specific antibody (sc16288; Santa Cruz, Santa Cruz, CA). Lamin B protein was detected using a goat anti-lamin B antibody (sc6212; Santa Cruz, Santa Cruz, CA).

RESULTS

The *Pit-1* locus undergoes a shift in chromatin conformation between the initiation and maintenance phases of transcriptional regulation. The higher-order chromatin structure of the *Pit-1* locus was determined by 3C (11) in cells corresponding to the initiation and maintenance phases of *Pit-1* expression. The mouse pituitary cell line GHFT1 was used to model the initiation phase *Pit-1* expression. These cells, established by *in vivo* immortalization of mouse pituitary cells with a simian virus 40 (SV40) T-antigen transgene under Pit-1 regulatory control (21), constitute a validated model of the presomatotrope stage that immediately precedes terminal differentiation of the somatotrope and

FIG 1 The *Pit-1* locus assembles development-specific and tissue-specific chromatin configurations. (A) Map of a 33-kb segment of the mouse genome encompassing the *Pit-1* locus and surrounding regions. The *Pit-1* gene is shown as a single unit (introns are not displayed). The EE is indicated by the open oval. The Pit-1 binding site arrays at the DE and at the promoter are indicated by gray ovals. The BglII restriction map is indicated below the genomic diagram by tick marks. The positions of the PCR primers used for the 3C analysis are indicated by labeled directional arrows. (B) 3C analysis of the *Pit-1* locus using the *Pit-1* promoter as the anchor fragment. 3C assays were performed on the GHFT1 cell line and on primary somatotrope-enriched pituitary and brain tissues of adult mice. The Pit-1p (Pit-1 promoter primer) site served as the anchor for these studies and was paired with each of the five additional 5' primers in the PCR analysis of the 3C ligation products. The ligation frequency was normalized to the random ligation control (see Materials and Methods). Each thin vertical line represents the normalized relative ligation frequency (mean $\pm$ standard deviation [SD]) from four or more independent studies. The calculation of ligation frequencies is described in Materials and Methods. (C) 3C analysis of the *Pit-1* locus in the adult using the *Pit-1* promoter as the anchor fragment. Details of the diagram are as in panel B.

lactotrope lineages. These cells correspond to the progenitor cells for somatolactotropes in mouse pituitary gland development at E14.5 (17, 21, 27). Consistent with their developmental assignment, GHFT1 cells contain abundant Pit-1 mRNA but relatively low levels of Pit-1 protein (21, 27). The EE, located 5' to the Pit-1 locus (Fig. 1A), is active in these cells and is bound by the transcription factor Atbf1 (17, 21). Adult mouse pituitaries enriched for the somatotrope lineage by expression of a *GRF* transgene (23) were used to model the definitive (maintenance) phase of *Pit-1* expression.

The conformation of a 33-kb genomic region extending 18 kb upstream (kb -18) to 15 kb downstream (kb $+15$) of the *Pit-1* transcription start site (Fig. 1A) was analyzed by 3C analysis (8). This region encompasses the entire *Pit-1* gene and its contiguous EE and DE elements. Formaldehyde-fixed chromatin preparations of GHFT1 cells and of disaggregated cells from the somato-

trope-enriched adult mouse pituitary were each digested with BglII and ligated under highly dilute conditions (11). The 4.1-kb BglII chromatin fragment encompassing the *Pit-1* promoter served as the anchor (viewpoint) fragment for the 3C analyses (Fig. 1A).

Analyses of the GHFT1 cell chromatin revealed an elevated ligation frequency between the EE and the *Pit-1* promoter (Fig. 1B, solid line). A somewhat lower ligation frequency was observed between the *Pit-1* promoter and an EE-adjacent fragment at kb -8 . A parallel analysis of the adult pituitary chromatin revealed a peak of ligation frequency between the *Pit-1* promoter and the DE and a somewhat lower ligation frequency of the *Pit-1* promoter to the EE (Fig. 1B, dashed line). These 3C data reveal distinct chromatin conformations at the *Pit-1* locus in the GHFT1 cells and the adult pituitary, shifting from a singular interaction of the promoter with the EE in the former to a juxtaposition of the DE with the promoter in the latter.

The elevated ligation frequencies of the promoter with both the DE and EE in the adult pituitary (Fig. 1B, dashed line) might reflect either of two models. In the first model, the two enhancers are simultaneously in close proximity to the *Pit-1* promoter, approximating a chromatin hub, as described at the β -globin locus (28). In the second model, the locus exists in two mutually exclusive chromatin conformations, with the DE interacting with the *Pit-1* promoter in the majority of cells while the EE pairs with the *Pit-1* promoter in a less abundant population. To distinguish between these two models, we repeated the 3C analysis of adult pituitary chromatin, using the DE fragment rather than the *Pit-1* promoter as the anchor fragment (Fig. 1C). In this study, the definitive enhancer had an elevated ligation frequency with both the EE and the *Pit-1* promoter (Fig. 1C). Although we cannot completely rule out the model in which two distinct chromatin conformations of the *Pit-1* gene exist in the pituitary, the combined results of the 3C studies using the two anchor sites favor the model in which the DE, the EE, and the *Pit-1* promoter are all in close proximity, comprising a chromatin hub in the adult pituitary.

To assess the tissue specificity of the *Pit-1* locus conformation, we repeated the 3C analysis on chromatin isolated from the adult mouse brain (Fig. 1B, dotted line). This analysis failed to reveal any evidence of higher-order chromatin structure. Instead, the ligation frequencies of sites within the locus to the *Pit-1* promoter decreased in direct proportion to their intervening distances, a pattern consistent with a linear configuration (8, 11). Taken together, the 3C assays demonstrated a shift in enhancer proximities to the *Pit-1* promoter during pituitary differentiation.

The developmental shift in the chromatin conformation at the *Pit-1* locus is paralleled by alterations in histone acetylation and trans-factor occupancy. We next explored the correlation between the shift in the higher-order chromatin configuration at the *Pit-1* locus (Fig. 1) and covalent modifications across the region. Histone H3 lysine 27 acetylation (H3K27Ac) constitutes a well-documented marker of activating *cis*-regulatory elements and correlates in a positive manner with gene transcription (29, 30). H3K27Ac ChIP analysis of the *mPit-1* locus (Fig. 2A) in GHFT1 cells revealed that H3K27Ac enrichment was restricted to the EE region and the *Pit-1* promoter (Fig. 2B). Parallel analysis in the adult pituitary (Fig. 2C) revealed that H3K27 modifications at the EE and *Pit-1* promoter were maintained at levels similar to those seen in the GHFT1 cells, and in addition, the locus now

FIG 2 ChIP assays reveal the distinct distributions of H3K27 acetylation and dynamic recruitment of CBP, Pit-1, and Pol II at the *Pit-1* locus in GHFT1 cells and in the adult pituitary. (A) Diagram of the *Pit-1* locus (as in Fig. 1A). The positions of the amplicons used in the ChIP analyses are shown under the map. (B) Pattern of H3K27 acetylation at the *Pit-1* locus in GHFT1 cells. The ChIP assays were performed using antibody against acetylated H3K27 and preimmune IgG, respectively. The y axis represents the relative enrichment (the ratio of bound fraction to that of the input; see Materials and Methods for details). The histogram values represent the relative enrichment and SD from three independent ChIP assays. (C) Pattern of H3K27 acetylation at the *Pit-1* locus in the adult pituitary. The ChIP assays were performed as described for panel B, but on chromatin from adult somatotrope-enriched mouse pituitaries. (D) Distribution of CBP at the *Pit-1* locus in GHFT1 cells. The ChIP assays were performed using antibody specific for CBP or normal IgG. The analysis of the relative enrichment was as described for panel A. (E) Distribution of CBP at the *Pit-1* locus in the adult pituitary. The CBP ChIP assays were performed as described for panels A and C. (F) Pol II and Pit-1 occupancy at the *Pit-1* locus in GHFT1 cells. The ChIP assays were performed as described for panel A using antibodies to Pol II (Ser5P Pol II) and Pit-1, along with a preimmune IgG control. The relative enrichment was calculated as described in Materials and Methods, and 1% of the input was used for normalization. (G) Pol II and Pit-1 occupancy at the *Pit-1* locus in the adult pituitary. The ChIP assays were performed as described for panel A using antibody against Pit-1 and Ser5P Pol II. **, $P < 0.01$; *, $P < 0.05$.

contained an enhanced level of H3K27 acetylation at the DE compared to GHFT1 cells (10-fold and 3-fold above IgG controls, respectively). These data revealed a positive correlation between the shift in the chromatin conformation and the levels of H3K27Ac modification at the *Pit-1* locus.

CBP is the major HAT responsible for H3K27 modifications (31). With this in mind, we next determined the association of CBP with the *Pit-1* locus. CBP recruitment in the GHFT1 cells was localized to the early enhancer and the *Pit-1* promoter (modifications at the DE lacked statistical significance compared to IgG controls) (Fig. 2D). In contrast, analysis of the adult pituitary revealed that CBP was robustly recruited to the DE, as well as the *Pit-1* promoter (Fig. 2E). Significant accumulation appeared to be maintained at the EE, as well. This pattern of CBP recruitment at the *Pit-1* locus paralleled that of H3K27Ac enrichment (Fig. 2C) and is consistent with studies that link H3K27 acetylation to CBP HAT activity (31).

The analyses of the *Pit-1* locus were next extended to mapping *in vivo* occupancy of the Pit-1 protein (Fig. 2F and G). The low but significant level of Pit-1 binding at the *Pit-1* promoter in GHFT1 cells (Fig. 2F) and lack of occupancy at the EE are consistent with the overall low level of Pit-1 protein expression in these cells and the absence of *Pit-1* binding sites in the EE (21). In contrast, the analysis of the adult pituitary revealed robust peaks of Pit-1 occupancy at the DE, as well as at the *Pit-1* promoter (Fig. 2G). These data are consistent with the accumulation of Pit-1 at this definitive stage of pituitary development and the presence of functional Pit-1 binding sites within the DE (19, 20). The low level of Pit-1 binding at the EE in the adult pituitary (not significant relative to the IgG control) most likely reflects the proximity of the EE to the DE in a pituitary chromatin hub, as the EE lacks cognate Pit-1 binding sites (see above).

The distribution of engaged Pol II (serine-5-phosphorylated CTD) (32) was next determined (Fig. 2F and G). The low-level

FIG 3 Pattern of p300 distribution at the *Pit-1* locus. (A) Map of the *Pit-1* locus. (B) Distribution of p300 at the *Pit-1* locus in GHFT1 cells. The ChIP assays were performed as described for Fig. 2 using antibody specific for p300. (C) Distribution of p300 at the *Pit-1* locus in the adult pituitary. The ChIP assays were performed as described for Fig. 2 using antibody specific for p300. **, $P < 0.05$. The error bars represent SD.

occupancy of Pol II at the *Pit-1* promoter in the GHFT1 cells (Fig. 2F) (less than 3-fold higher than the IgG control) is markedly enhanced in the adult pituitary (10-fold greater than the IgG control), reflecting robust levels of maintenance transcription (Fig. 2G). These studies of Pit-1 and Pol II occupancy at the *Pit-1* locus are consistent with the shift to DE activity in the adult pituitary and with a role for Pit-1 binding at the DE in establishing a corresponding maintenance state for *Pit-1* expression (19, 33).

To further assess the basis for histone H3K27 acetylation, we also investigated the recruitment of the HAT p300. Both p300 and CBP can mediate H3K27 acetylation (31) (Fig. 3) and associate with active enhancers (13). The p300 ChIP study in GHFT1 cells revealed minimal levels of p300 (not reaching significance) in the EE region and an absence of occupancy in the promoter region in GHFT1 cells (Fig. 3B). In contrast, the recruitment of p300 at the *Pit-1* locus in the adult pituitary was detected at robust and highly

significant levels in both the DE and the promoter region (Fig. 3C). These data suggest that CBP and p300 play divergent roles in modification of the *Pit-1* chromatin locus at the induction and maintenance phases of transcriptional control.

The maintenance conformation at the *Pit-1* chromatin locus is dependent on autoregulation by Pit-1 protein. The cooccupancy of Pit-1 at the DE and the *Pit-1* promoter in the adult pituitary (Fig. 2C) correlates with their proximity as determined by 3C analysis (Fig. 1). These data are consistent with a model in which Pit-1 mediates and/or stabilizes a maintenance chromatin configuration of the *Pit-1* locus in the adult pituitary. To test this model, we first determined whether cross-linked chromatin fragments encompassing the DE and the *Pit-1* promoter were bound by Pit-1. In this Pit-1 ChIP-3C analysis (Fig. 4A), pituitary chromatin was sheared, digested with BglII, and immunoprecipitated with Pit-1 antibody, and the Pit-1-bound chromatin fragments were

FIG 4 Pit-1 is associated with the interacting distal enhancer and *Pit-1* promoter regions in the adult pituitary and in Pit-1/Triples cells. (A) Map of the *Pit-1* locus as described for Fig. 1A. (B) ChIP-3C analysis. ChIP-3C was performed on chromatin isolated from somatotrope-enriched adult pituitary and Pit-1/Triples cells (see Materials and Methods). The Pit-1 promoter primer (Pit-1p) was used as the anchor fragment in the assay. The PCR product between the DE and Pit-1p from the Pit-1 ChIP of adult pituitary and Pit-1/Triples cross-linked chromatin were redigested by BglII to confirm their identities as ligation products (not shown). (C) Pit-1 is recruited to the distal enhancer region and the promoter at the *Pit-1* locus in Pit-1/Triples cells. The ChIP assays were performed as described for Fig. 2F using an antibody specific to Pit-1. **, $P < 0.01$; *, $P < 0.05$. The error bars represent SD.

FIG 5 The definitive (maintenance) chromatin configuration at the *Pit-1* locus in Pit-1/Triple cells is Pit-1 dependent. (A) Pit-1 protein depletion in Pit-1/Triple cells. Cells transfected with the indicated shRNA-bearing plasmids were incubated for 5 days and then assayed for levels of Pit-1 by Western blotting. Quantification of the Western blot signals, normalized to a lamin B loading control (Cont), is shown in the histogram. The level of Pit-1 protein in the control sample was defined as 1.0. The histogram values are the averages from two different shRNA clones. The error bars represent SD. (B) Impact of Pit-1 protein depletion on the chromatin conformation of the *Pit-1* locus in Pit-1/Triple cells. The 3C assays were performed on chromatin from the Pit-1/Triple cells (see Materials and Methods). The positions of the PCR primers are labeled below the map of the *Pit-1* locus. The Pit-1p primer was used as the anchor primer for the assays. The relative ligation frequency was calculated after normalization to that of the random control and the ubiquitously expressed *Ercc3* locus. The primers for the *Ercc3* locus were located in two adjacent BglII fragments in the mouse genome (see Materials and Methods for details).

ligated under standard 3C conditions and ligation frequencies were assayed (Fig. 4A). This analysis revealed that the ligation products encompassing the DE and the *Pit-1* promoter were bound by Pit-1 (Fig. 3C). The absence of a signal corresponding to the EE in this study suggests that the EE interaction with the DE and the *Pit-1* promoter in the adult pituitary, as demonstrated in Fig. 1, may be mediated by a Pit-1-independent mechanism. Since the EE lacks function in the adult pituitary (18), the significance of the Pit-1-independent interaction of the EE with the Pit-1-bound DE and the *Pit-1* promoter remains unclear.

The association of Pit-1 with the DE-promoter ligation products could be of primary importance to the chromatin conformation in the adult pituitary or it could represent a secondary event. To test for a causative role, we assessed the impact of acute Pit-1 depletion on this structure. Since this targeted depletion of Pit-1 protein could not be done in the context of the adult pituitary, we used a surrogate model. We recently generated a series of cell lines from the pituitaries of mice carrying a T-antigen-expressing transgene under rat *Pit-1* promoter/enhancer control (22). One of these lines, Pit-1/Triple, expresses Pit-1, as well as all three Pit-1-dependent pituitary hormone genes (*GH*, *PRL*, and *TSHβ*). The *Pit-1* mRNA levels are similar in Pit-1/Triple cells and GHFT1 cells, but the Pit-1 protein levels in Pit-1/Triple cells are substantially higher (22). Furthermore, the expression of these hormone

genes in this cell line was demonstrated to be Pit-1 dependent (22). To validate the utility of the cell line for our studies, we confirmed by Pit-1 ChIP-3C that the chromatin conformation at the *Pit-1* locus in Pit-1/Triple cells recapitulated the conformation identified in the adult pituitary (Fig. 4B, Pit-1/Triple cells). We also performed Pit-1 ChIP assays to confirm the Pit-1 protein was present at the DE and promoter in Pit-1/Triple cells (Fig. 4C). Pit-1 was then depleted from the Pit-1/Triple cells (>60% at 5 days posttransfection) using each of two validated mPit-1 shRNAs (Pit-sh1 and Pit-sh2) (Fig. 5A). Analysis of these Pit-1-depleted cells compared with the cells transfected with the control shRNA revealed a significant decrease in 3C ligation of the *Pit-1* promoter site with the DE (Fig. 5B). The significant enrichment in the ligation frequency observed between the promoter and the two enhancers compared to that with the two intervening sites (−8 and −3.8) is completely lost in the Pit-1-depleted cells. The ChIP analysis in the Pit-1/Triple cells further revealed that the depletion of Pit-1 resulted in a corresponding decrease in the recruitment of CBP and Pol II at the promoter (Fig. 6A) and a decrease in CBP occupancy at the distal enhancer (Fig. 6B). These results were consistent with our previously published results showing that depletion of Pit-1 by small interfering RNA (siRNA) in the Pit-1/Triple cells significantly decreases *Pit-1* gene expression (22).

To demonstrate that the Pit-1-dependent looping is develop-

FIG 6 The recruitment of CBP and Pol II to the definitive (maintenance) *Pit-1* locus in Pit-1/Triple cells is dependent on the actions of Pit-1. (A) Pit-1 depletion by shRNA decreases recruitment of CBP and Pol II to the *Pit-1* promoter. ChIP assays were performed on chromatin from Pit-1/Triple cells transfected with plasmids carrying control shRNA (Cont.) or Pit-1 shRNA. The relative enrichment was calculated as the ratio of bound fraction to the 1% input control. The histogram values represent the averages and SD from three independent experiments. (B) Pit-1 depletion decreases recruitment of CBP to the DE of the *Pit-1* locus. Study details are as for panel A.

mental stage specific, we also performed Pit-1 depletion in GHFT1 cells (Fig. 7A). Our results confirmed that this depletion of Pit-1 fails to disrupt the chromatin configuration of the *Pit-1* locus in GHFT1 cells (Fig. 7B).

These results support the conclusion that Pit-1 is essential to the maintenance of close contacts between the DE and the *Pit-1* promoter in the adult pituitary, in recruitment of CBP to both of these sites, and to the overall transcriptional activity from the *Pit-1*

promoter as reflected in levels of engaged Pol II. These findings support the presence of a Pit-1-dependent autoregulatory loop involving the definitive enhancer that serves to maintain the *Pit-1* locus in a transcriptionally active state in the adult pituitary.

DISCUSSION

Pit-1 actions are essential, and in some settings sufficient, for expansion and differentiation of cell lineages in the mammalian pi-

FIG 7 The chromatin configuration at the *Pit-1* locus in GHFT1 cells is unaffected by Pit-1 depletion. (A) Pit-1 protein depletion in GHFT1 cells. Pit-1 shRNA transfection was as described for Fig. 5A. A Western blot analysis of Pit-1 and lamin B (loading control) is shown at the top, and a histogram of the band intensities ($n = 2$) is shown below the blot. The error bars represent SD. (B) 3C analysis of looping between the EE and the promoter at the *Pit-1* locus in GHFT1 cells with (dashed line) and without (solid line) Pit-1 depletion. The 3C assays were performed as described for Fig. 5B.

uitary (14). Activation of the *Pit-1* locus in the embryo and subsequent maintenance of expression throughout adult life are mediated by distinct enhancers (17, 18). The actions of the early enhancer at E13.5 are attributed to its binding by the *trans*-factor Atbf1 (17). This early developmental stage is marked by abundant *Pit-1* mRNA and trace levels of Pit-1 protein (27). The subsequent maintenance phase of *Pit-1* expression is initiated at E16.5 via actions of the DE. It has been hypothesized that this phase, marked by robust expression of Pit-1 protein, may be sustained by an autoregulatory pathway (19) in which Pit-1 binds to an array of sites at the *Pit-1* promoter and within the DE (19, 20). The structure of this Pit-1 binding site array at the DE is highly conserved between rodents and humans, suggesting that any delineated Pit-1 regulatory pathway would be correspondingly conserved (34). While transgenic studies confirm that the Pit-1 binding sites at the DE are critical for the temporal expression of the linked reporters (18), the presence of the putative autoregulatory pathway has not been directly tested, nor has the underlying mechanistic basis been further defined. Of note, autoregulatory pathways have been proposed for a number of genes encoding tissue-specific transcription factors, including the pancreas-specific transcription factor 1a (encoded by *Ptf1a*) and the basic helix-loop-helix (bHLH) transcription factor, encoded by *Sohlh1*, expressed in spermatogonia and oocytes (35–37). Such feed-forward self-sustaining loops may function to lock in durable and continuous expression of critical transcriptional control factors.

Whereas transcription factor recruitment to *cis*-regulatory elements has now been mapped in multiple genome-wide surveys, corresponding dynamic shifts of the chromatin architectures remain less well explored. The present analysis indicates that the switch from EE to DE action at the *Pit-1* locus is accompanied by a reconfiguration of the chromatin structure. In the embryonic stage, the early enhancer is proximal to the *Pit-1* promoter (Fig. 1B) and is accompanied by active histone marks (H3K27Ac) that are restricted to these two regions (Fig. 2B). The Pit-1 protein accumulates in the pituitary subsequent to this initiating event, and by E16.5, Pit-1 protein is abundant and binds to both the DE and the *Pit-1* promoter (Fig. 2C). The current data further demonstrate concurrent recruitment of the transcription coactivator CBP/p300 to the DE with corresponding acetylation of H3K27 at this site (Fig. 2D and G and 3). The binding of Pit-1 at the DE and *Pit-1* promoter is accompanied by a shift in the chromatin conformation, bringing the DE into close proximity to the promoter (Fig. 1 and 6). Interestingly, EE proximity to the promoter, established in the activation phase, is retained in the adult pituitary (Fig. 1B). This was somewhat unexpected, because EE function is not essential to maintenance of *Pit-1* expression in the adult (18). The net effect of these alterations in the chromatin configuration and structure at the *Pit-1* locus is to facilitate recruitment of Pol II (Fig. 2G) and to sustain robust *Pit-1* transcription. These data provide insight into the mechanisms underlying the maintenance of *Pit-1* expression throughout adult life.

Broad applications of 3C-based technologies (11, 38) have revealed that many distal enhancers interact with their target promoters via chromatin looping (12). Limited studies further indicate that such chromatin conformations can be dynamic and can mediate both activation and developmental silencing (39). During erythroid development, the active *Myb* promoter interacts with upstream enhancers to form an active chromatin hub (ACH) in undifferentiated cells. The loss of this ACH during erythroid dif-

ferentiation is associated with the downregulation of *Myb* expression (40). *Kit* repression during the same erythroid differentiation process is accompanied by a shift from an active configuration to a repressive configuration (41). The intensively studied set of DNase I hypersensitive sites (HSs) that constitute the β -globin locus control region (LCR) assemble in a developmentally conserved ACH (28) that shifts its promoter associations during the developmental process of *globin* gene switching (28). In this case, a subset of the long-range interactions are stabilized by LDB1, a component of the GATA1/Tal1 complex (42, 43). These and related studies have demonstrated the central connection between the chromatin conformation and gene transcriptional activity during cell differentiation.

It is clear that developmental controls over gene expression can track with alterations in the chromatin configuration, but the temporal and mechanistic controls that activate and stabilize these dynamic shifts remain poorly defined (3). Initial contacts may be nucleated via free diffusion, as chromatin undergoes rapid, locally constrained oscillations (3, 44). These random interactions may then be stabilized via interactions between transcription factors and architectural complexes recruited to these sites and by moving a locus from a repressive compartment to an active compartment within the interphase nuclei (45). A second, nonexclusive model proposes that binding of *trans*-factors, either architectural or sequence specific, may shorten the linear distance between two sites, thus enhancing the chances for substantive interactions (3). Studies in *Drosophila* support this model by showing that the binding of Nipped-B/NIPBL between the enhancer and target promoter facilitates enhancer-mediated long-range gene activation (46, 47). Thus, multiple pathways and mechanisms may be involved in the assembly of long-range enhancer-promoter interactions.

The interaction between the EE and the promoter at the *Pit-1* locus bridges 5.8 kb. This interaction activates *Pit-1* expression and recruits chromatin-modifying factors, such as CBP, to the EE and the *Pit-1* promoter. This initial configuration may further serve to facilitate subsequent looping of the DE to the *Pit-1* promoter by effectively shortening the distance separating the two sites. The retention of the EE, along with the DE and *Pit-1* promoter, in the adult pituitary chromatin hub (Fig. 1B and C) is consistent with this sequential-interaction model. The recruitment of CBP and p300 to the locus with the corresponding addition of activating histone modifications may further serve to open the *Pit-1* locus, facilitating the structural reconfigurations and loop formation. The mechanism underlying the Pit-1 dependence of the autoregulatory pathway may reflect dimerization of Pit-1 bound to the DE and to the promoter sites or some other stabilizing interaction (48). The combined impacts of these mechanisms and pathways lead to recruitment of Pol II to the *Pit-1* promoter and provide a robust model for sustained activation of this tissue-specific transcription factor throughout adult life.

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