Dynamic Interactions between Pit-1 and C/EBP α in the Pituitary Cell Nucleus[∇]

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The homeodomain (HD) transcription factors are a structurally conserved family of proteins that, through networks of interactions with other nuclear proteins, control patterns of gene expression during development. For example, the network interactions of the pituitary-specific HD protein Pit-1 control the development of anterior pituitary cells and regulate the expression of the hormone products in the adult cells. Inactivating mutations in Pit-1 disrupt these processes, giving rise to the syndrome of combined pituitary hormone deficiency. Pit-1 interacts with CCAAT/enhancer-binding protein alpha (C/EBP α) to regulate prolactin transcription. Here, we used the combination of biochemical analysis and live-cell microscopy to show that two different point mutations in Pit-1, which disrupted distinct activities, affected the dynamic interactions between Pit-1 and C/EBP α in different ways. The results showed that the first α -helix of the POU-S domain is critical for the assembly of Pit-1 with C/EBP α , and they showed that DNA-binding activity conferred by the HD is critical for the final intranuclear positioning of the metastable complex. This likely reflects more general mechanisms that govern cell-type-specific transcriptional control, and the results from the analysis of the point mutations could indicate an important link between the mislocalization of transcriptional complexes and disease processes.

The activation of transcription during development or in response to cell signals involves the transient assembly of multiprotein complexes at specific gene enhancers and promoters. The dynamic assembly of these complexes occurs through networks of protein interactions that are coordinated by sequence-specific DNA-binding factors (8, 25). It is through these combinatorial interactions that tissue-specific transcription factors orchestrate eukaryotic gene expression (35). These activities are typified by the homeodomain (HD) transcription factors, a family of structurally conserved proteins that play critical roles in directing cell differentiation pathways during development and in the regulation of programs of gene expression in the differentiated cell types. The central role of these proteins is illustrated by many human diseases, from developmental defects to metabolic disorders that are linked to mutations affecting the HD transcription factors (5). For example, the pituitary-specific HD transcription factor Pit-1 (also called GHF-1 and POU1F1) is a member of the POU family of transcription factors that directs the development of the anterior pituitary lactotrope, somatotrope, and thyrotrope cell lineages (9, 16). Many different point mutations have been identified in the gene encoding Pit-1, and all three Pit-1-dependent cell types fail to develop in patients with inactivating mutations. This results in the syndrome of combined pituitary hormone deficiency (CPHD), a disease characterized by the lack of prolactin (PRL), growth hormone (GH), and thyroid-stim-

ulating hormone beta produced by these cells (6, 31). A clear understanding of how these disease-causing point mutations affect protein activities can provide unique insight into protein structure and function.

In addition to its role in directing pituitary cell differentiation, Pit-1 also controls the transcription of the genes encoding the hormone products of the mature cell types (9, 22). Through its interactions with specific DNA elements in target gene promoters, Pit-1 recruits coregulatory proteins that alter histone acetylation and modify the chromatin structure, providing either a permissive or repressive environment for transcription (15, 37, 38, 42, 43, 45). In the permissive environment, Pit-1 mediates the transduction of cell signals at target promoters, but the direct phosphorylation of Pit-1 does not appear to play a critical role in these events (19, 30). Rather, precise homeostatic control is achieved through a network of interactions between Pit-1 and several different classes of transcription factors, including the nuclear receptors (10), other homeodomain proteins (14), Ets family proteins (2), and basic region-leucine zipper (B-Zip) transcription factors (37). The goal of our study was to determine how specific point mutations in Pit-1 affected its dynamic network interactions with other transcription factors.

Our earlier studies showed that Pit-1 and the B-Zip transcription factor C/EBP α cooperated in the regulation of pituitary gene expression (18, 37), and the present studies define the dynamic interactions between these transcription factors in the pituitary cell nucleus. C/EBP α acts to direct programs of cell differentiation and plays key roles in the regulation of genes involved in energy metabolism (24). Paradoxically, several studies showed that C/EBP family proteins localized to regions of centromeric heterochromatin (37, 40) which are typically associated with gene silencing (32). In an earlier study we showed that Pit-1 could recruit C/EBP α from the regions of

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centromeric heterochromatin to the intranuclear sites occupied by Pit-1 (18). These observations indicated a potential role for the HD transcription factor in organizing other gene regulatory proteins in transcriptionally permissive regions of the pituitary cell nucleus. In this study, we use the combination of biochemical analysis and live-cell microscopy to show that two different point mutations in Pit-1 linked to CPHD which disrupt distinct activities affect the dynamic interactions of Pit-1 with C/EBP α in different ways. Our studies emphasize that the interactions between transcription factors that regulate cell-specific gene expression are a dynamic process. Moreover, these studies show that the probability of these proteins assembling in a particular region of the cell nucleus is related to the dominant chromatin-binding activities of the different protein partners, supporting the view that the repositioning of transcription factors represents an important mechanism for directing changes in cell-specific gene expression.

MATERIALS AND METHODS

Construction of expression vectors, transfection of cell lines, and reporter gene assays. The construction of plasmids encoding Pit-1 and C/EBP α was described previously (18). The fluorescent protein (FP) fusion proteins were generated using expression vectors encoding the monomeric (A206K [44]) forms of the enhanced yellow or cyan fluorescent proteins (EYFP and ECFP; Clontech Takara Bio, Mountain View, CA). The mutations in Pit-1 were generated by site-directed mutagenesis (QuikChange; Stratagene, La Jolla, CA), and all expression and reporter vectors were verified by automated nucleotide sequencing. The mouse GHFT1 (27) or human HeLa (ATCC CCL-2) cell lines were maintained as monolayer cultures in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. The harvested cells were transfected with the indicated plasmid DNA(s) by electroporation as described previously (12). The total amount of DNA was kept constant by using the empty plasmid DNA. For reporter gene analysis, the transfected cells were transferred to 35-mm dishes and maintained in culture. Extracts were prepared from the cells after 24 h, and luciferase (Luc) activity was determined as described by the manufacturer (Promega Corporation, Madison, WI). Each experiment was performed a minimum of three times, and Luc activity, corrected for total protein, was expressed as the mean $\pm$ standard error of the mean (SEM).

Western blotting, electrophoretic mobility shift assay, immunocytochemical staining, and immunoprecipitation. The Western blot analysis of the expressed proteins was described previously (11, 18). The electrophoretic mobility shift assays (EMSAs) were performed on whole-cell extracts prepared from transiently transfected HeLa cells as described previously (11). A duplex oligonucleotide corresponding to a consensus Pit-1-binding site, 5'-GATCCGATTACATGAATATTC, was end labeled using [γ -³²P]ATP and T4 polynucleotide kinase, purified, and used as a probe. Simon Rhodes (Indiana University School of Medicine) provided the Pit-1 antibody used in the EMSAs. Indirect immunocytochemical detection of endogenous Pit-1 protein in fixed GHFT1 cells was performed as reported earlier (42). For the coimmunoprecipitation assays, Pit-1 and the mutated variants were epitope tagged with hemagglutinin (HA). HeLa cells were transfected with CFP-C/EBP α alone or in combination with HA-Pit-1, HA-Pit-1^{W261C}, or HA-Pit-1^{F135C}. The whole-cell lysates were prepared after 24 h and precleared with agarose beads, followed by incubation with an HA-specific antibody conjugated to agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA). The beads were washed several times by centrifugation, and the bound proteins were eluted with denaturing sample buffer (Invitrogen Life Technologies, Carlsbad, CA) and analyzed by Western blotting. The chemiluminescence detection was performed using anti-green fluorescent protein (GFP) primary antibody (Molecular Probes, Invitrogen Life Technologies, Carlsbad, CA) and a horseradish peroxidase-conjugated anti-rabbit secondary antibody (Pierce Biotechnology, Rockford, IL).

ChIP. Two days following transfection with FuGENE (Roche, Indianapolis, IN) with the HA-Pit-1 expression plasmid, approximately 5×10^7 GHFT1 cells in 150-mm dishes were incubated in Dulbecco's modified Eagle's medium containing 1% formaldehyde for 10 min. The cells were then rinsed with phosphate-buffered saline, and the cross-linking was stopped by the addition of 0.125 M glycine for 5 min. The cells were washed twice with ice-cold phosphate-buffered saline and then collected by scraping and pooled by centrifugation. The cell

pellet was resuspended in ice-cold lysis buffer supplemented with protease inhibitors and Dounce homogenized. The nuclei were recovered by centrifugation, and the genomic DNA was sheared to an average length of 500 bp by enzymatic digestion as described for the ChIP-It kit (Active Motif, Carlsbad, CA). The efficiency of chromatin shearing was verified by gel electrophoresis. The chromatin was precleared by treatment with protein G beads, and the chromatin immunoprecipitation (ChIP) assays were then performed with the indicated antibodies. Following immunoprecipitation, cross-linking was reversed by incubation at 65°C, the proteins were removed by treatment with proteinase K, and the DNA was purified. ChIP DNA was detected using standard PCR with the following primer pairs: mouse Pit-1 enhancer (E) at kb -10.4 , GCCTGTGTGACATATACTTCAG and GGAGATTAACATGTAAGCACCG; Pit-1 intervening sequence at kb -4.7 (C), CCTGAAGCTGCGAGAGAAGC and GCCTGAGTAAGTGAAGAAGC; Pit-1 promoter (P) at kb -0.2 , GCCTGCTCC TCACTTTGTACG and GACCCGCTGCTTGCCAGTTCAC; mouse *c-fos* serum response element (SRE) at kb -0.35 , GCGAGCTGTTCCTGCAATC and GGATGGACTTCTACGTCAC. The ChIP results were analyzed by ethidium bromide staining of 3% agarose gels containing the PCR-amplified DNA and compared to reactions containing 5% of the input DNA.

Live-cell microscopy and FRAP. Pituitary GHFT1 cells were transfected with the indicated plasmid DNA(s) encoding the FP fusion proteins and inoculated into culture dishes containing a 42-mm cover glass (ProSciTech, Queensland, Australia). On the following day, the cover glass with the monolayer of cells was transferred to a medium-filled chamber fitted to the microscope stage (12). The temperature of the stage was maintained between 35 and 37°C using a Nevtek airstream stage incubator (Nevtek, Burnsville, VA). The fluorescence recovery after photobleaching (FRAP) experiments were performed using a Zeiss LSM 510 confocal microscope equipped with a 25-mW diode laser generating the 405-nm line. For these experiments, prebleach images were collected using 1.5- μ W laser power, followed by a 500-ms bleach pulse at 135- μ W laser power delivered to a 2- μ m-diameter spot. The shape and size of the bleach spot were kept constant for all experiments. Single images were then collected with the 405-nm laser line (at 1.5- μ W power) every 500 ms for 20-s-duration experiments or every 2 s for 80-s-duration experiments. The fluorescence intensity in the bleached area was normalized to the initial fluorescence in that same area (intensity of 1). Typically, a second cell in the same field of view was used to correct for any bleaching that occurred during the repetitive scanning. The individual experiments involved the analysis of 5 to 10 different cells, and each experiment was repeated at least three times. The Student *t* test was used to determine significance.

FRET microscopy and image analysis. The Förster resonance energy transfer (FRET) data were collected with a wide-field Olympus inverted IX-70 microscope equipped with a 60 $\times$ 1.2-numerical-aperture water-immersion objective lens. The filter combinations were 500/20-nm excitation, 515-nm beam splitter, and 535/30-nm emission for YFP and 436/20-nm excitation, 455-nm beam splitter, and 470/30-nm emission for CFP (Chroma Technology Corporation, Brattleboro, VT). The 12-bit-depth images with no saturated pixels were obtained using a cooled digital interline camera (Orca-200; Hamamatsu, Bridgewater, NJ). All images were collected at a similar gray-level intensity by controlling the excitation intensity using neutral density filtration and by varying the on-camera integration time. The acceptor photobleaching FRET (apFRET) method used here was described in detail previously (12, 13). To quantify the changes in donor fluorescence after acceptor photobleaching in specific regions of the nucleus, an intensity profile comparison between the pre- and postacceptor bleach donor images was obtained and plotted as the pixel-by-pixel efficiency of donor dequenching (*E*%). For cell population analysis, the ISee graphical software was used to integrate a series of computerized image analysis functions into a single algorithm that could be applied to sets of images in a consistent and unbiased way. First, a histogram-based statistical method was used to determine the optimal threshold of the acquired images. The mean intensity of a defined area outside the whole-nucleus region of interest (ROI) was measured to define the background fluorescence, and this value was subtracted from each particular image. The algorithm then measured the mean intensity of background-subtracted nucleus ROI. All values measured were then exported for further analysis using the Excel spreadsheet software (Microsoft, Redmond, WA). This method automatically corrected for donor bleaching and calculated the donor/acceptor ratio and the efficiency of donor dequenching for each set of images acquired from the population of transfected cells.

For the printed images, the background was subtracted and the resulting image files were processed for presentation using Canvas 8.0 (Deneba, Inc., Miami, FL) and rendered at 300 dots per inch.

FIG. 1. (A) Subnuclear distribution of the endogenous Pit-1 protein in mouse GHFT1 cells. The GHFT1 cells were fixed and permeabilized and then incubated with an anti-Pit-1 antibody followed by a Texas Red-conjugated secondary antibody. (B) Diagrams of the Pit-1 and *c-fos* gene regulatory regions, showing the binding sites for Pit-1 (vertical bars) and the annealing sites for the different primer sets used in the ChIP assays (dashes). For the *Pit-1* 5'-flanking sequence, the primers annealed to the enhancer (E) and the promoter (P) regions, as well as an intermediate region (C) as a negative control. For the *c-fos* 5'-flanking region, the primers annealed to sequences that encompassed the Pit-1-responsive SRE. (C) ChIP analysis was done using GHFT1 cells transfected with HA-tagged Pit-1 (lanes 1 to 3, 6, and 7) or the empty HA vector (Vec; lanes 4 and 5). The nuclear extracts were incubated with an anti-HA antibody (α -HA; lanes 1 to 5) or a control immunoglobulin G (IgG; lanes 6 and 7), and the precipitated DNA was amplified by PCR with the *Pit-1*-specific primers indicated in panel B. The bottom panel shows the reaction controls using 5% of the input DNA. Note that the input for the IgG control and the α -HA was the same (lanes 1 to 3). (D) Pit-1 activates transcription from the *c-fos* SRE. HeLa cells were transfected with a *c-fos* SRE-Luc construct in the absence or presence of a *Pit-1*-carrying plasmid (top panel). The results are plotted as fold activation of Luc expression $\pm$ SEM, determined for two independent experiments, each done in triplicate. ChIP analysis showed that HA-Pit-1 binds to the endogenous *c-fos* SRE region (bottom panel). The PCR used the SRE-specific primers indicated in panel B, and the precipitated DNA (lanes 1 to 3) or the input DNA (lanes 4 and 5) from the ChIP assay are shown as described for panel C.

RESULTS

In this study, we used the mouse pituitary GHFT1 cell line as a model to determine how specific point mutations in Pit-1 affect its dynamic network interactions with other transcription factors. These cells have the characteristics of a pituitary somatotrope progenitor cell that expresses low levels of Pit-1. Unlike mature pituitary somatolactotrope cells, GHFT1 cells do not express endogenous C/EBP α and have not differentiated to the point of expressing the hormone products PRL and GH (27, 37). Immunocytochemical staining of the endogenous Pit-1 protein in the mouse GHFT1 cells showed that it was

distributed in a reticular pattern throughout the nucleus (Fig. 1A), and we showed earlier that exogenous Pit-1 expressed in these cells has a similar pattern (18). Although some Pit-1-dependent gene promoters, including the PRL and GH promoters, are inactive in GHFT1 cells, Pit-1 does regulate its own transcription (36) as well as transcription of the *c-fos* gene (21) (Fig. 1B). We used ChIP to demonstrate that the expressed Pit-1 protein occupied the regulatory regions of these specific target genes in GHFT1 cells. The results shown in Fig. 1C demonstrate that the expressed HA-Pit-1 bound to the endogenous mouse *Pit-1* enhancer, located -10 kb upstream of the

FIG. 2. (A) Schematic representation of the Pit-1 and the CPHD proteins; the positions of the mutations are indicated by arrows. The relative DNA-binding activity and activation of PRL transcription for each protein are shown on the right. (B) DNA-binding and reporter gene activities of Pit-1 and the CPHD mutant Pit-1^{W261C}. The top panel shows an EMSA using a consensus Pit-1 element. The DNA-protein complexes for Pit-1 (arrowhead) and an Ets protein were observed, but no complex was formed by Pit-1^{W261C} (lane 5). Binding specificity was demonstrated by competition with two different concentrations of the unlabeled oligonucleotide (wedge; lanes 2, 3, 6, and 7), and the presence of Pit-1 in the complex was verified by the addition of an antibody specific for Pit-1 that cleared the protein from the reaction mixture (lane 4). The reporter gene analysis (lower panel) showed that Pit-1^{W261C} was inactive. The results are plotted as fold activation of Luc expression $\pm$ SEM, determined for three independent experiments, each done in triplicate. Western blot analysis showed that Pit-1 and Pit-1^{W261C} were expressed at similar levels in the HeLa cells (inset). (C) DNA-binding and reporter gene activities of the CPHD mutant Pit-1^{F135C}. The top panel shows an EMSA demonstrating that the F135C mutation retained DNA-binding activity (lane 5) similar to Pit-1 (arrowhead). Binding specificity was demonstrated by competition with unlabeled oligonucleotide, and the presence of Pit-1 or Pit-1^{F135C} was verified by the addition of an antibody specific for Pit-1 to clear the proteins (lanes 4 and 8). Western blot analysis showed that Pit-1 and Pit-1^{F135C} were expressed at similar levels in the HeLa cells (inset). The reporter gene analysis (lower panel) showed that Pit-1^{F135C} retained some transcriptional activity.

transcription initiation site, as well as to the *Pit-1* promoter. Importantly, no HA-Pit-1 binding was detected at a sequence between the enhancer and promoter regions at kb -5 (Fig. 1C). An earlier study showed that Pit-1 also interacted with the SRE of the *c-fos* gene and that it could induce transcription of that gene (21). We show here that Pit-1 activated expression of a Luc reporter gene under the control of the *c-fos* SRE, and the ChIP analysis demonstrated that HA-Pit-1 occupied the endogenous DNA element (Fig. 1D). These results indicated that although Pit-1 is distributed throughout the pituitary cell nucleus, it occupies the enhancer and promoters of specific target genes.

We next determined how specific point mutations in either the POU-HD or the POU-S domain of Pit-1 (Fig. 2A) affected its activity, subnuclear distribution, and interactions with another transcription factor. A mutation located in the DNA recognition helix of the Pit-1 HD, W261C, is responsible for the Snell dwarf mouse phenotype (28). In agreement with this earlier report (28), we demonstrate here that the binding of Pit-1 to a consensus response element was prevented by the Pit-1^{W261C} mutation (Fig. 2B, top panel). Pit-1^{W261C} was also deficient in the transcriptional activation of a Luc reporter gene under the control of the Pit-1-dependent PRL promoter (Fig. 2B). A second CPHD mutation, Pit-1^{F135C},

FIG. 3. The W261C mutation disrupts the intranuclear organization of Pit-1. Wide-field microscope images show the nuclei of pituitary GHFT1 cells that expressed YFP-Pit-1 (A), YFP-Pit-1^{W261C} (B), or YFP-Pit-1^{F135C} (C). In each case, the cells were stained briefly with the cell-permeant DNA dye H33342 before imaging. The profile plots quantify the relative intensities of the YFP-labeled proteins and the H33342-stained chromatin at the position along the red line in each overlay image. (D) Effects of the different CPHD mutants on intranuclear mobility of Pit-1. GHFT1 cells expressing the indicated CFP-labeled proteins were subjected to FRAP analysis. The recovery plots show the mean change in relative fluorescence intensity over a 90-second time frame, normalized to the prebleach levels. The results are the averages for 12 to 15 different cells, and the arrows indicate the t_{50} recoveries for each group. (E) Western blot analysis of proteins extracted from GHFT1 cells (lane 1) or from GHFT1 cells transfected with 10 μ g of a CFP-Pit-1 expression plasmid (lane 2). Equal amounts of total protein were loaded, and the resulting blot was probed with an antibody against Pit-1. The endogenous Pit-1 is the doublet running at 31 and 33 kDa (double arrowhead), and the expressed CFP-Pit-1 ran at 60 kDa (full arrowhead). NS (open arrowhead) indicates a nonspecific band detected by the Pit-1 antibody.

which disrupts the first α -helix of the POU-S domain (Fig. 2A), was shown earlier to bind to DNA (41). Here, we showed that Pit-1^{F135C} retained the DNA-binding activity (Fig. 2C, top panel), but the mutated protein was greatly impaired in its ability to activate the Pit-1-dependent PRL promoter (Fig. 2C).

The effects of these two different CPHD mutations on the intranuclear distribution of Pit-1 in the GHFT1 pituitary cells were then characterized. The results showed that YFP-labeled Pit-1 expressed in the mouse GHFT1 cells adopted a pattern of subnuclear distribution similar to that of the endogenous protein (compare Fig. 1A and 3A). In contrast, the YFP-labeled CPHD mutation, Pit-1^{W261C}, which failed to bind to specific

DNA elements (Fig. 2B), did not adopt this pattern of subnuclear distribution (Fig. 3B, intensity profile), while Pit-1^{F135C}, which has DNA-binding activity (Fig. 2C), distributed similarly to the wild-type Pit-1 (Fig. 3C, intensity profile). These results argued that the DNA-binding activity of Pit-1 directs its positioning within the nucleus. The FRAP approach was then used to characterize the effects of the Pit-1 CPHD mutations on the intranuclear mobility of Pit-1 in the pituitary cell nucleus. Pituitary GHFT1 cells expressing the different CFP fusion proteins were identified using low-intensity illumination with the 405-nm laser line. Sharp and colleagues (39) showed that the mobility of GFP-Pit-1 in HeLa cells was not significantly different over a broad range of expression levels,

TABLE 1. Intranuclear mobilities of Pit-1, CPHD mutants, and C/EBP α , expressed alone and in combinations in pituitary cells

Method and protein analyzed	Avg recovery time (s) $\pm$ SD	
	t_{50}	t_{80}
FRAP with CFP-Pit-1		
Pit-1	22.32 $\pm$ 2.77	96.9 $\pm$ 3.51
Pit-1 ^{W261C}	1.66 $\pm$ 0.34	6.91 $\pm$ 1.39
Pit-1 ^{F135C}	8.17 $\pm$ 0.31	26.27 $\pm$ 1.50
FRAP with CFP- C/EBP α		
C/EBP α (euchromatin)	4.24 $\pm$ 0.65	17.52 $\pm$ 2.90
C/EBP α (heterochromatin)	4.50 $\pm$ 0.70	20.70 $\pm$ 3.34
C/EBP α + Pit-1	15.03 $\pm$ 1.33 ^b	79.03 $\pm$ 3.73
C/EBP α + Pit-1 ^{W261C}	5.25 $\pm$ 0.61	24.48 $\pm$ 3.57
C/EBP α + Pit-1 ^{F135C}	4.93 $\pm$ 0.56	28.30 $\pm$ 1.09

^a Significantly different from Pit-1^{W261C} alone ($P < 0.05$).^b Significantly different from C/EBP α alone ($P < 0.05$).

but it is recognized that the level of protein expression can influence protein mobilities. We compared the relative expression levels of CFP-Pit-1 to the endogenous Pit-1 in GHFT1 cells (Fig. 3E). GHFT1 cells express Pit-1 at levels about 10 to 30% of that present in mature, PRL-secreting pituitary cells (37). Electroporation typically yields 40 to 60% transfection efficiency, determined by comparison fluorescence and bright-field images (data not shown). The expressed CFP-Pit-1, detected with an antibody directed against Pit-1, was similar in abundance to the endogenous Pit-1 protein in the GHFT1 cell extracts (Fig. 3E, compare endogenous Pit-1 in lane 1 with expressed Pit-1 in lane 2). Although there is cell-to-cell variation in the expression level of the CFP fusion proteins, these results showed that, on average, the fusion proteins were not grossly overexpressed relative to the endogenous Pit-1 protein. Because of this concern, however, only cells expressing low levels of the CFP-labeled proteins were selected for FRAP measurements. The results of the FRAP analysis, summarized in Fig. 3D and Table 1, show that the time necessary for the recovery of fluorescence for CFP-Pit-1 in the bleached region, represented as 50% recovery (t_{50}) and 80% recovery (t_{80}), was about 20 s and 97 s, respectively. The CPHD mutation Pit-1^{W261C}, in contrast to the wild-type protein, had significantly higher mobility ($t_{50} = 1.6$ s) than the wild-type protein, indicating that Pit-1 mobility is strongly affected by its DNA-binding activity (Fig. 3D; Table 1). When compared to the mobility of unfused CFP ($t_{50} \sim 0.4$ s) (Fig. 3D), Pit-1^{W261C} moved significantly slower, suggesting that this mutant may still be interacting with either chromatin or other nuclear proteins to some degree. Interestingly, the mobility of the other CPHD mutation, Pit-1^{F135C}, which retains DNA-binding activity, was intermediate between Pit-1 and Pit-1^{W261C} (Fig. 3D; Table 1). These protein mobilities represent the averages of many different interactions, including the transient binding to chromatin and the cooperative interactions with other protein partners.

The next studies were designed to determine how the coop-

erative interactions between Pit-1 and C/EBP α were affected by the two different CPHD point mutations. Earlier studies had shown that the endogenous C/EBP family proteins were localized to regions of centromeric heterochromatin in mouse cells (18, 29, 34, 37, 40, 46). When YFP-labeled C/EBP α was expressed in GHFT1 cells, it accumulated in regions of centromeric heterochromatin, here identified by the preferential staining with the cell-permeant DNA dye H33342 (18). We used FRAP analysis to characterize the mobility of CFP-labeled C/EBP α in both the heterochromatin and areas of the nucleus outside the heterochromatin. This analysis demonstrated that CFP-C/EBP α had similar mobility kinetics in both regions of the nucleus ($t_{50} = 4.5$ s; $t_{80} = 20.7$ s), indicating that there is an exchange of C/EBP α between heterochromatic and euchromatic regions (Table 1). In earlier studies we showed that Pit-1 and C/EBP α cooperated in the activation of both PRL and GH transcription (18, 37). In agreement with these earlier observations, we show here that a Pit-1-dependent PRL-promoter luciferase reporter gene (~ 2.5 PRL-Luc), which has minimal activity in HeLa cells, was induced 20-fold by the expression of Pit-1 (Fig. 2 and 4A). Whereas expression of C/EBP α alone induced the reporter gene activity less than 5-fold, the combination of Pit-1 and C/EBP α resulted in approximately 100-fold activation, demonstrating the cooperativity of these factors at the PRL gene promoter (Fig. 4A and B). The CPHD mutation Pit-1^{W261C}, which had no detectable transcriptional activity on its own, also had no activity when coexpressed with C/EBP α (Fig. 4A). The CPHD mutation Pit-1^{F135C}, in contrast, retained some transcriptional activity at the PRL promoter, although it was significantly impaired compared to the wild-type protein (Fig. 4B). Importantly, Pit-1^{F135C} failed to cooperate with C/EBP α at the PRL promoter (Fig. 4B). Since the mutated protein retained some transcriptional activity (Fig. 2C) but was defective in cooperativity with C/EBP α (Fig. 4B), this result raised the question of whether the F135C point mutation disrupted the interactions between Pit-1 and C/EBP α .

To address this question, we characterized the effect of the CPHD mutations on the interactions between Pit-1 and C/EBP α by coimmunoprecipitation. The results demonstrated that the CFP-C/EBP α protein coprecipitated with the HA-tagged Pit-1 (Fig. 4C and D), indicating that these proteins associate as part of a metastable macromolecular complex. Similarly, C/EBP α was also pulled down as part of a macromolecular complex with the CPHD mutation Pit-1^{W261C} (Fig. 4C). This result indicated that despite the lack of binding to specific DNA elements, Pit-1^{W261C} retained the ability to associate with the protein complex that contained C/EBP α . On the contrary, C/EBP α was not coimmunoprecipitated with Pit-1^{F135C} (Fig. 4D). This result demonstrated that the F135C point mutation disrupted the ability of Pit-1 to interact with the protein complex that included C/EBP α .

When C/EBP α and Pit-1 were coexpressed in the GHFT1 cells, we observed that Pit-1 recruited C/EBP α from the heterochromatic regions (18), and this activity is demonstrated here (Fig. 5, compare panels A and B). We then used the FRAP approach to determine whether the coexpression of Pit-1 influenced the intranuclear mobility of CFP-C/EBP α . Here, we show that when Pit-1 recruited C/EBP α from the heterochromatic regions, the mobility of CFP-C/EBP α was

FIG. 4. Cooperative interactions between Pit-1 and C/EBPα in activation of the PRL promoter are prevented by the CPHD mutations. (A and B) HeLa cells were transfected with the reporter gene alone or with expression vectors encoding Pit-1, Pit-1^{W261C}, Pit-1^{F135C}, or C/EBPα or in the indicated combinations. The Luc activity, corrected for total cellular protein, was normalized to the activity of the reporter alone, and errors are the SEM for five independent experiments, each done in triplicate. (C) Coimmunoprecipitation analysis showed that Pit-1 and C/EBPα assemble in the same macromolecular complex. The lysates from cells coexpressing the indicated HA-tagged Pit-1 or Pit-1^{W261C} and CFP-C/EBPα were incubated with an HA-specific antibody conjugated to agarose beads. The bound proteins were eluted, and the CFP-labeled C/EBPα was detected by Western blotting; the left panel shows the input reaction. (D) In contrast to HA-Pit-1, HA-Pit-1^{F135C} failed to pull down CFP-C/EBPα.

significantly reduced, as demonstrated by an increase in the average recovery time (t_{50}) for CFP-C/EBPα from approximately 4.5 s to 15 s ($P < 0.05$) (Fig. 5C; Table 1). The mobility of CFP-Pit-1, however, was not affected by the coexpression of C/EBPα, and the average recovery time was not significantly changed (17 s compared to 22 s alone) (Table 1). This result could indicate that the stochastic association of C/EBPα with Pit-1 was stabilized by the interaction of Pit-1 with its chromatin-binding sites, thereby reducing the mobility of C/EBPα. This view was supported by the observation that C/EBPα, which interacts at heterochromatic sites, significantly reduced the mobility of the DNA-binding-deficient CFP-Pit-1^{W261C} ($P < 0.05$) (Table 1), whereas the mobility of CFP-C/EBPα was not significantly changed by Pit-1^{W261C} (Fig. 5D; Table 1). In contrast, there was no effect of C/EBPα on the mobility of the CFP-Pit-1^{F135C} mutation (Table 1), which was consistent with their failure to associate. Together, these results indicate that dynamic processes drive the assembly of these proteins

and that the interactions of these transcription factors with chromatin stabilize the protein complexes at those sites. The coimmunoprecipitation studies (Fig. 4C and D) demonstrated that C/EBPα and Pit-1 could associate in a common macromolecular complex, but what was missed by the *in vitro* analysis was where the proteins were assembled within the cell nucleus. To define the spatial relationships between Pit-1 and C/EBPα in the nuclei of living cells, we used the technique of *apFRET* microscopy, which provides sensitive and consistent measurements that do not require the correction for spectral cross talk (12, 26). This approach uses specific photobleaching of the acceptor fluorophore in the entire field of view and then measures the change in the donor fluorophore signal after destruction of the acceptor fluorophore. An increase or dequenching of the donor fluorophore signal provided a direct measure of FRET, demonstrating that the average distance between the fluorophores was less than about 80 Å. Figure 6A shows that when CFP-C/EBPα was recruited by

FIG. 5. When C/EBPα and Pit-1 were coexpressed in pituitary cells, C/EBPα was recruited from the heterochromatic regions to the intranuclear regions occupied by Pit-1. (A and B) Nucleus of a GHFT1 cell that coexpressed YFP-C/EBPα and mRFP-HP1α (A) or YFP-C/EBPα and mRFP-Pit-1 (B) and was stained with H33342 just before imaging. (C) FRAP analysis of cells that expressed CFP-C/EBPα alone or with YFP-Pit-1. The results compare the average recovery after photobleaching for CFP-C/EBPα alone or for CFP-C/EBPα that was coexpressed with YFP-Pit-1, and the arrows indicate the t_{50} recoveries for each group. (D) FRAP analysis of cells that coexpressed CFP-C/EBPα alone or with YFP-labeled Pit-1^{W261C} or Pit-1^{F135C}. Neither of the CPHD mutants affected the mobility kinetics of C/EBPα, as indicated by the t_{50} recoveries for each group (arrows).

using YFP-Pit-1, the proteins were in close proximity in specific regions of the pituitary cell nucleus, confirming our earlier studies (13). Here, images of the YFP-labeled Pit-1 (Acc1) and the colocalized CFP-C/EBPα (not shown) were acquired, and then the YFP labeling Pit-1 was selectively bleached in the entire field of view. A second image of CFP-C/EBPα (Don2)

was acquired under identical conditions to the first, and the change in the donor signal was measured using an intensity profile to characterize the efficiency of donor dequenching (Fig. 6A, E% profile). In addition, Fig. 6B shows that C/EBPα and Pit-1^{W261C} were also in close proximity in the intact pituitary cell nucleus (Fig. 6B, E% profile). However, these results

FIG. 6. Acceptor photobleaching FRET analysis in GHFT1 cells expressing YFP-Pit-1 and CFP-C/EBP α (A), YFP-Pit-1^{W261C} and CFP-C/EBP α (B), or YFP-Pit-1^{F135C} and CFP-C/EBP α (C). The images were acquired in the YFP (acceptor) and CFP (donor) channels before and after selective photobleaching of the YFP (see Materials and Methods). Here, the images of the acceptor before photobleaching (Acc 1) and of the donor after photobleaching of the acceptor (Don2) are shown. Bar, 10 μ m. Changes in relative CFP intensity after selective acceptor photobleaching were measured along the yellow lines, and the results were plotted as the efficiency of donor dequenching (E%) as a function of the relative pixel position (right panels). (D) FRET analysis in GHFT1 cell populations. Data were collected as described for panels A to C. Twenty cells expressing different YFP/CFP intensity ratios were picked for each combination of expression vectors, and the analysis was done in an automated way, as described in Materials and Methods. E% for the whole-nuclei ROI was plotted as a function of the YFP/CFP intensity ratio for each DNA condition.

also clearly show that the intranuclear positioning of the protein assembly involving Pit-1^{W261C} was different from that of the wild-type protein (compare Fig. 6A and B). The apFRET revealed that the interactions between Pit-1^{W261C} and C/EBP α

occur in regions of heterochromatin (Fig. 6B). These results, supported by the FRAP analysis (Fig. 5; Table 1), suggest that the chromatin-binding activities of these transcription factors stabilized the association of these transcription factors in par-

ticular regions of the cell nucleus. In striking contrast, when the CPHD mutation Pit-1^{F135C} was coexpressed with CFP-C/EBP α , there was no evident recruitment of either protein, and CFP-C/EBP α remained localized to sites of heterochromatin (Fig. 6C). Although the proteins were colocalized in many regions of the cell nucleus, the selective bleaching of the YFP labeling Pit-1^{F135C} did not lead to dequenching of the CFP labeling C/EBP α (Fig. 6C, E% profile).

Because these FP-labeled proteins were expressed from independent plasmids, the ratio of donor and acceptor proteins varied between cells within the transfected cell population. The efficiency of donor dequenching, which is proportional to FRET efficiency, is influenced by the ratio of donor- to acceptor-labeled proteins. When FRET occurs, the efficiency of donor dequenching will increase with increasing amounts of available acceptor until the donor population is saturated. If FRET does not occur, there will be no donor dequenching, regardless of the acceptor concentration. Therefore, quantitative measurements of the spatial relationships between C/EBP α and Pit-1 or the CPHD mutations required the analysis of populations of transfected cells. To collect apFRET measurements from cell populations, we developed an automated computer algorithm that provided consistent and unbiased measurements from populations of cells (see Materials and Methods).

Here, the apFRET analysis determined the average donor dequenching efficiency within the nucleus of each cell in the population, and this was plotted as a function of the acceptor-to-donor ratio within those cells (Fig. 6D). The results showed a similar relationship of donor dequenching to the acceptor-to-donor ratio for the cell populations that coexpressed CFP-C/EBP α with either YFP-Pit-1 or YFP-Pit-1^{W261C} (Fig. 6D), with maximum average dequenching efficiencies of about 15%. In contrast, there was no significant donor dequenching at any of the donor/acceptor ratios tested for the pituitary cells that coexpressed CFP-C/EBP α and YFP-Pit-1^{F135C}. The cell population results validated those shown for the representative cells (Fig. 6A, B, and C) and demonstrated that both Pit-1 and Pit-1^{W261C} were closely associated in the intact cell nucleus. It is important to point out, however, that the value of the representative cell images is that they illustrate the differences in the subnuclear locations of the associated proteins. The apFRET analysis of the cell populations also confirmed the *in vitro* analysis, demonstrating that the CPHD mutation Pit-1^{F135C} failed to interact with C/EBP α in the intact pituitary cell nucleus. Taken together, these results showed that the first α -helix of the POU-S domain is critical for the interactions of Pit-1 with C/EBP α and showed that DNA-binding activity conferred by the HD is critical for the final intranuclear positioning of this protein complex.

DISCUSSION

In sum, our findings have provided new insight into the dynamic interactions between transcription factors in the cell nucleus. Aside from the core histone proteins, most chromatin-associated proteins are dynamic within the nuclear compartment, and their mobilities reflect their transient interactions with chromatin and with a variety of different protein partners (for example, see reference 33). Here, we showed that two

different transcription factors that function together in the control of pituitary cell-specific gene expression interacted as part of a metastable protein complex. The results indicate that the assembly of the protein complex is stabilized by the chromatin-binding activities of these transcription factors, which position those proteins in particular regions of the cell nucleus. Our study emphasizes how disease-causing point mutations can affect these processes, showing that a point mutation disrupting DNA-binding activity of one partner shifted the intranuclear location where the proteins assembled. Although our studies focused on the protein interactions involving the pituitary-specific HD transcription factor Pit-1, they are germane to understanding the molecular mechanisms that control the intranuclear organization of different classes of transcription factors.

The three-dimensional structure of the HD protein family is remarkably conserved, indicating the importance of this structure for DNA site recognition and the interactions with protein partners. The DNA-binding domain of Pit-1 is formed by both the POU-S and POU-HD, which are separated by a flexible linker region that allows them to adopt various conformations on DNA (23, 38). The Pit-1^{W261C} mutation, which causes the Snell dwarf mouse phenotype, disrupts DNA binding by the POU-HD (28). The DNA-contacting surface within the third α -helix of the HD is formed by the highly conserved V260 and C263 residues, and the ability of this surface to hydrogen bond with nucleotides of the target DNA elements is prevented by the W261C mutation (23). The failure of the mutated protein to bind specific DNA elements might adequately explain the lack of transcriptional activity, but there are examples of transcription factors with important functions that do not require their typical binding to specific DNA elements. For instance, the steroid hormone receptors, including the estrogen receptor, can regulate gene expression without binding to DNA elements through their interactions with other classes of transcription factors. Therefore, it was important to characterize the intranuclear dynamics and interactions of the Pit-1^{W261C} mutation.

Our findings indicated that disruption of the DNA-binding surface in the Pit-1 HD prevented the mutated protein from adopting the subnuclear organization associated with the wild-type protein. This result argues that the DNA-binding activity of the HD specifies the organization of Pit-1 in the cell nucleus. Furthermore, the FRAP analysis showed that the Pit-1^{W261C} mutation had significantly higher intranuclear mobility than the wild-type protein. This result generally agreed with an earlier study in nonpituitary cells (39), although the mobilities we observed here in pituitary cells were much slower than those reported for the HeLa cells. Since the FRAP results represent the averages of many different interactions, including specific interactions with chromatin and the cooperative associations with protein partners, it seems likely that the slower mobilities observed here could reflect these pituitary-specific interactions.

The slower mobility of the wild-type Pit-1 compared to Pit-1^{W261C} cannot be explained on the sole basis of DNA binding, however, since the second CPHD mutation, Pit-1^{F135C}, which can bind to DNA elements, had an intermediate mobility. In an earlier study, Vallette-Kasic et al. (41) showed that the substitution of the bulky phenylalanine for cysteine at position

135 filled a cavity between the first and fourth helices that is normally present in the POU-S domain. It was suggested that this cavity might function to mediate the interactions with other proteins. Earlier studies showed that Pit-1 interacts with several different coregulatory proteins, including the CREB-binding protein (CBP/p300) coactivator complex (43, 45). Recently, Cohen et al. (7) showed that the Pit-1^{F135C} protein bound to DNA was still capable of recruiting CBP, indicating that the cavity in the POU-S domain was unimportant for the interaction with that coactivator protein. However, similar to our results, they observed that the transcriptional responses conferred by the mutated protein were impaired in pituitary cells, suggesting that interactions with other nuclear proteins might be prevented by the F135C mutation (7).

The activation of Pit-1-dependent transcription requires the combinatorial interactions of Pit-1 with several different transcription factors, including the B-Zip protein C/EBP α . Surprisingly, several studies have shown that some C/EBP family proteins preferentially localize to regions of centromeric heterochromatin (29, 34, 37, 40, 46), regions that are typically associated with transcriptional silencing (32). For example, the β -globin gene is located in regions of heterochromatin in cell types where it is not expressed, but during erythroid cell differentiation it is repositioned outside the heterochromatin to nuclear regions that contain the erythroid-specific transcription factor NF-E2 (3, 20). Here, in an analogous mechanism at the protein level, we showed that Pit-1 functioned to reposition C/EBP α from sites of heterochromatin to the intranuclear sites occupied by Pit-1. The recruitment activity correlated with the cooperative transcriptional responses conferred by the coexpressed proteins, suggesting that the recruitment may be essential for this activity at the PRL gene promoter.

Using FRAP analysis, we demonstrated that C/EBP α was very mobile in both heterochromatic and euchromatic regions of the pituitary cell nucleus, suggesting a dynamic equilibrium between these two regions. Importantly, the coexpression of Pit-1, which led to the recruitment of C/EBP α to the intranuclear sites occupied by Pit-1, resulted in a significant reduction in the mobility of CFP-C/EBP α . This result could indicate that the interaction of Pit-1 with chromatin-binding sites functions to stabilize the complex with C/EBP α , reducing its mobility. This activity appears predominant, since C/EBP α had little effect on the mobility of Pit-1 (Table 1). This view was supported further by the observation that C/EBP α , which interacts at heterochromatic sites, significantly reduced the mobility of the DNA-binding-deficient CFP-Pit-1^{W261C}. These results suggest that the transient interactions of these transcription factors with chromatin are the dominant activity and that dynamic processes drive the assembly of these proteins in metastable complexes at those sites. These observations support a model where proteins move independently within the nuclear compartment and stochastically assemble at specific intranuclear sites, as was used to describe the network of interactions between histone H1 and high-mobility-group (HMG) proteins (4). Another recent study describing the interaction between HMGB1 and the glucocorticoid receptor found that the residence time of the glucocorticoid receptor bound to chromatin was increased in the presence of HMGB1 (1), suggesting that kinetic cooperativity of transcription factors may be a common feature in transcriptional activation. Finally, a similar model

was also used to describe the assembly of the RNA polymerase I complex, which was envisioned as a series of sequential intermediate subunit interactions that give rise to progressively more stable complexes (17).

Our observations indicate that the assembly process is highly specific, since the Pit-1^{F135C} mutation failed to associate with C/EBP α (Table 1). The failure of the mutated protein to associate with C/EBP α was borne out by the coimmunoprecipitation and reporter gene studies. Moreover, the apFRET studies demonstrated that, although Pit-1^{F135C} and C/EBP α were colocalized in many regions of the cell nucleus, there was no evidence for the interaction of these proteins. This contrasted with the apFRET results for Pit-1 and C/EBP α , which provided direct evidence that, on average, less than 80 Å separated the fluorophores that labeled these proteins. The assembled proteins were not distributed uniformly in the nucleus, but rather occurred in discrete regions. These studies also confirmed the coimmunoprecipitation results demonstrating the interactions between C/EBP α and Pit-1^{W261C} but, importantly, extended the results to show that the interactions involving Pit-1^{W261C} and C/EBP α occur at heterochromatic sites. Further, the association of Pit-1^{W261C} and C/EBP α in heterochromatic regions, also confirmed by the population analysis, may provide a clue to the dominant inhibitory activity of the CPHD mutation. It is likely that other proteins that normally associate with Pit-1 were similarly positioned to transcriptionally silent regions of heterochromatin. Together, the results demonstrate a network of dynamic interactions between Pit-1 and C/EBP α in the intact pituitary cell nucleus and indicate that the DNA-binding activity of Pit-1 directs the intranuclear positioning of the protein assemblies. In the absence of this dominant activity, as with the Pit-1^{W261C} mutation, the proteins assemble at the heterochromatic sites bound by C/EBP α .

Changes in the nuclear distribution of proteins are known to accompany stages in cell differentiation, suggesting that nuclear organization may participate in establishing transcriptional networks that lead to cell-specific patterns of gene expression (20). The identification of the protein-protein interaction networks that govern these processes will be critical for understanding gene expression control at the structural level (35). This goal is being achieved through the application of *in vitro* and *in silico* approaches, but these studies must be supported by the quantitative analysis of protein-protein interactions in living cells. Furthermore, the analysis of how these protein-protein interaction networks are disrupted by disease-causing mutations will be crucial for understanding these complex processes. Here, we used kinetic and quantitative microscopy approaches to confirm and extend the biochemical analysis of protein-protein interactions in the cell nucleus. The example we provide here likely reflects more general mechanisms that govern cell-type-specific transcriptional control, and the results obtained with the CPHD mutations could indicate an important link between the mislocalization of transcription factors or transcriptional complexes and disease processes.

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