AP-1 and Oct-1 Transcription Factors Down-regulate the Expression of the Human PIT1/GHF1 Gene*

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The pituitary-specific transcription factor Pit-1/GHF1 is a member of the POU domain family of regulatory proteins. It is involved in the commitment and expansion of the somatotrop cell lineage and activates the transcription of a set of anterior pituitary genes. We have cloned the human PIT1/GHF1 gene and characterized the regulatory mechanisms controlling its promoter activation and regulation. A minimal promoter region (−102 to +15) contains the cis-acting elements that confer to the human PIT1/GHF1 gene a high basal transcriptional activity, the tissue-specific expression, and the autoregulation by Pit-1/GHF1 protein. The upstream promoter region contains a multiplicity of Pit-1/GHF1 binding sites that do not show any synergistic interaction with the minimal promoter. The transcriptional activity is negatively regulated by Oct-1 and mediated by an octamer-binding site (OTF). In addition, we have also identified a 12-O-tetradecanoylphorbol-13-acetate-responsive element, which overlaps with a Pit-1/GHF1 binding site. A mutually exclusive binding of the activator protein-1 (AP-1) and Pit-1/GHF1 has been observed on this composite site, and AP-1 was shown to down-regulate PIT1/GHF1 transcription.

Pit-1/GHF1 is a pituitary-specific transcription factor that belongs to the POU domain proteins (1–3). It was originally identified as a transcription factor responsible for the pituitary-specific activation of the growth hormone and the prolactin genes (4–6). Pit-1/GHF1 is also involved in the activation of the gene encoding the β subunit of the thyroid-stimulating hormone (7–10), of the gene encoding the receptor for the growth hormone-releasing hormone (11), and of its own gene (12, 13).

In addition to its role in gene activation, Pit-1/GHF1 is required for the specification, expansion, and survival of the somatotropes, lactotropes, and a subset of thyrotropes during anterior pituitary development (7, 10). In the mature pituitary, its expression is restricted to these three cell types (14, 15). Specific inhibition of Pit-1/GHF1 synthesis using antisense oligonucleotides leads to a decrease in proliferation of rat pituitary cell lines, GC and 235–1 cells (16).

In differentiated rat somatotropes and lactotropes, the cell type-specific expression of the PIT1/GHF1 gene is determined by a minimal promoter region centered around the TATA box, which interacts with a pituitary-specific TBP cofactor (17). The basal expression is controlled in a precise way by autoregulation, via two Pit-1/GHF1 binding sites, one activator at position −55 and one inhibitor at position +15 (12, 13). In addition, the rat PIT1/GHF1 gene transcription is regulated by factors affecting the intracellular level of cAMP. This regulation is mediated by two cAMP-responsive elements (CREs) localized in the proximal promoter region (12, 13). This region is also required for synergistic activation of PIT1/GHF1 gene expression by glucocorticoids and protein kinase C (18) and for transcriptional repression by the thyroid hormone T3 (19).

The mechanisms controlling the initial activation of PIT1/GHF1 gene during development remain poorly understood. Analyses performed in a mouse immortalized somatotropic progenitor cell (GHFT1) led to the identification of an enhancer located between −3.5 and −5.3 kb upstream to the promoter. This enhancer, functional at this early stage of development, is inactive in fully differentiated cells (20). The factors interacting with this stage-specific enhancer, however, remain to be identified. In transgenic mice, effective pituitary expression of the mouse PIT1/GHF1 gene requires the presence of a cell-specific enhancer located 10 kb upstream of the transcription start site. The regulatory elements identified in this enhancer indicate that vitamin D and members of the retinoic acid receptor family are likely to be involved in developmental regulation of the mouse PIT1/GHF1 gene (21).

In humans, mutations in the PIT1/GHF1 gene have been associated with pituitary dwarfism (22), and a contribution of Pit-1/GHF1 in the pathogenesis of pituitary adenomas has been proposed (23, 24). However, little is known about the regulatory mechanisms that control the expression of the human gene. Here we report the characterization of the regulatory elements involved in the control of the human PIT1/GHF1 promoter. We have identified alternative regulatory mechanisms that control the expression of this transcription factor. We show a role for Oct-1 and AP-1 in the down-regulation of the human PIT1/GHF1 gene expression.

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¶The abbreviations used are: CRE, cAMP-responsive element; kb, kilobase pairs; bp, base pair(s); CAT, chloramphenicol acetyltransferase; rhGHF1, recombinant human GHF-1; WCE, whole cell extract; TPA, 12-O-tetradecanoylphorbol-13-acetate; TRE, TPA-responsive element; EMSA, electromobility shift assay.

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Experimental Procedures

Isolation of Genomic Clones—Human PIT1/GHF1 genomic clones were isolated from a human placental genomic library in EMBL3 phage Lambda (CLONTECH, Palo Alto, CA). 10^6 plaque-forming units were plated, transferred onto nylon membranes, and hybridized in situ. The clone was hybridized under stringent conditions with a human Pit-1/GHF-1 cDNA probe (24). Positive plaques were identified and the recombinant DNA was purified from liquid culture using a Qiagen Lambda purification kit (Qiagen Inc., Chatsworth, CA). The genomic clones were analyzed by restriction mapping (25), and DNA fragments were subcloned into pBluescript SK+ Double-stranded DNA sequencing was performed using Sequenase version 2.0 sequencing kit (US Biochemical Corp.) and universal and gene-specific primers.

Primer Extension—Ten μg of total RNA were hybridized with 100 fmol of 32P-labeled human Pit-1/GHF-1-specific primer (5'-TTGGCACAATCTATTCCCAAGGAGT-3') in 20 μl of 50 mM Tris-HCl, pH 8.3, 50 mM KCl, 10 mM MgCl2, 10 mM dithiothreitol, 1 mM each dNTP, and 0.5 μg Escherichia coli RNA polymerase. The extension reaction was performed at 42 °C for 30 min in the presence of 1 unit of avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI). The primer extension products were purified by phenol/chloroform extraction and ethanol precipitation and loaded onto a 8% denaturing (7 M urea) polyacrylamide gel in TBE buffer.

Plasmid Constructions—A 1.5-kb EcoRI promoter fragment was subcloned into pBluescript SK+ to generate the plasmid pSKE35. This plasmid was used to construct by polymerase chain reaction amplification all the other recombinant plasmids required in this study. The universal T7 primer and the primer 5'-GCCGTACGGTATTATC-3' (814 to 800) were used to amplify from pSKE35 a 520-bp promoter fragment (1321 to 800). The primers 5'-GCGACTATCCGGAGACATCTTACGAATATGTTG (267 to 282) were used to amplify a 457-bp promoter fragment (1321 to 865) from pSKE35. The primers 5'-GCCGAAATTCTCATATCGACTCAAG-3' (595 to 580) and 5'-GCCTGACGCAGCAATATGG (267 to 282) were used to amplify a 241-bp fragment (814 to 800) from pSKE35. The universal T3 primer was used to amplify a 275-bp promoter fragment (102 to +173) from pSKE35. The four polymerase chain reaction fragments were then digested with SauI and EcoRI (restriction sites into the primer sequences) and inserted into pBluescript SK+ to generate the plasmids pSKPro550, pSKPro300, and pSKPro200, respectively. These constructs were used in DNA I footprinting experiments.

The −1321 LUC and −601 LUC plasmids were obtained by insertion into pG2basic (Promega) of KpnI-BglII fragments generated by polymerase chain reaction amplification starting from pSKE35 plasmid. For the −1321 LUC construct, the primer 5'-GCAGATCTCCAGGAAAGCTTC-3' (102 to +601) was used, and the universal T7 primer was used for polymerase chain reaction amplification. For the −601 LUC construct, the primers used were the following: 5'-GCAGATCTCCAGGAAAGCTTC-3' and 5'-GCCGTTACCACACTATCTACTACAC-3'. The −102/+15 LUC plasmid was constructed by insertion into pGL2basic of a KpnI-BglII fragment amplified from the pSKE35 plasmid using the primer 5'-GCAGATCTCGAGGCTTC-3' and the universal T7 primer. A 241-bp KpnI-HindIII fragment was prepared by restriction of pSKPro200 and inserted into pGL2basic to generate the −102/+140 LUC plasmid.

Site-directed Mutagenesis of OTF and GHF-1 Binding Sites—Site-directed mutagenesis was performed using the Altered Sites II in vitro Mutagenesis System (Promega). The Pit1/GHF1 promoter was subcloned into the vector pALTER1. Mutagenesis was performed with single-stranded DNA template in the presence of the mutagenic oligonucleotides, mutGHF-1 (5'-CCCTCTTCAGGATTATATGGA-3') or mutOTF (5'-AGTTTAATGTCGCCAATAC-3'). Selection of the mutated clones was performed by direct sequencing of the phagemid DNA isolated from transformed JM109 cells.

Cell Culture and Transfections—GH3 cells (ATCC) were grown in Ham's F-10 medium supplemented with 2.5% newborn calf serum and 12.5% fetal calf serum (Life Technologies, Inc.). HeLa cells (ECACC) were grown in BME supplemented with 1% nonessential amino acids, 20 mM glutamine, and 10% fetal calf serum (Life Technologies, Inc.).

For transfection experiments, cells were harvested by treatment with trypsin-EDTA followed by centrifugation at 1500 rpm. GH3 cells were resuspended in culture medium at a concentration of 4 × 10^6 cells/ml. HeLa cells were resuspended in Cytomix (120 mM KCl, 0.15 mM CaCl2, 10 mM KH2PO4/K2HPO4, pH 7.6, 25 mM Hepes, pH 7.6, 2 mM EGTA, 5 mM MgCl2, 2 mM CaCl2, and 5 μM dithiothreitol) and placed at a concentration of 4 × 10^6 cells/ml. The transfections were performed using the Electropore 2000 (Eurogentec, Seraing, Belgium). The transfected cells were immediately diluted with culture medium, transferred into 35-mm tissue culture dishes, and cultured for 24–48 h. When indicated, 10 μg forskolin and/or 50 ng/ml TPA were added 20 h after transfection, and the cells were further incubated for 5 h. Cells were then harvested, washed with phosphate-buffered saline, and lysed in the following buffer (25 mM Tris-phosphate, pH 7.8, 2 mM dithiothreitol, 2 mM 1,2-diaminocyclohexane-N,N′,N″-tetraacetic acid, 10% glycerol, and 1% Triton X-100).

Luciferase assays were performed using the luciferase detection kit (Promega), and the light emission was measured in a Lumac Biocounter M2000 (Perstorp Analytical). CAT activities were measured by enzyme-linked immunosorbent assays (CAT ELISA kit, Boehringer, Mannheim, Germany). The levels of luciferase activities were normalized according to the CAT activities. The results are presented as a fold change of the luciferase activities ± S.E. from single experiments performed in triplicate.

The experiments were repeated three times.

Protein and Antibody Preparations—A Ndel-Bcl I fragment containing the entire coding region of the human Pit-1/GHF-1 cDNA was inserted into the T7 expression vector pET11b to generate pETGHF-1. The recombinant protein (OTF/GHF-1) was expressed in the bacterial strain BL21 (DE3). Crude bacterial extract was prepared according to Pognonec et al. (26). For the generation of the human GHF-1 antisera, rhGHF-1 protein was partially purified by ammonium sulphate precipitation (5–33% (NH4)2SO4) and analyzed by SDS-polyacrylamide gel electrophoresis followed by Coomassie Blue staining. The band corresponding to the 35-kDa rhGHF-1 protein was cut out of the gel and injected to New Zealand White rabbits. The antisera was tested in Western blotting experiments on nuclear extracts from GH3 and HeLa cells and on rhGHF-1 protein. The detection was achieved using the GAR-gold kit (Bio-Rad).

GH3 and HeLa whole cell extracts (WCE) were prepared according to Manley (27) with a 5–60% (NH4)2SO4 cut. Protein concentrations were determined by the Bradford assay (Bio-Rad).

DNase I Footprinting—The genomic fragments purified from the plasmids pSKPro550, pSKPro520, pSKPro300, and pSKPro200 were dephosphorylated, 32P-end-labeled with T4 polynucleotide kinase, and then cut with restriction enzymes to obtain singly end-labeled probe. The labeled DNA fragments were incubated for 10 min at 4 °C with WCE or bacterially expressed recombinant proteins in a 50-μl reaction volume containing 25 mM Tris-HCl, pH 8.0, 50 mM KCl, 6 mM MgCl2, 0.5 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol, 2 μg of poly[dIdC], and 10 μg of bovine serum albumin. Fifty μl of CaCl2 (5 mM solution (5 mM CaCl2, 10 mM MgCl2 at room temperature) were added, and the samples were incubated for 1 min at room temperature. Three μl of diluted RQI DNA Nase (0.05 units/ml in 10 mM Tris-HCl, pH 8.0) were added for 1 min at room temperature. The reactions were stopped by the addition of 90 μl of stop solution (200 mM NaCl, 30 mM EDTA, pH 8.0, 1% SDS, and 100 μg/ml yeast RNA). The proteins were removed by phenol/chloroform extraction, and the DNA was purified by ethanol precipitation. The DNA fragments were resolved on a 8% polyacrylamide, 42% urea gel in TBE. In competition experiments, the following double-stranded oligonucleotides were used.

5′-AGTCATCTCACTACACATGCTGCAA 3′
3′-AGAGTTAGATATTACGATTTGCT 5′

Sequence 1, OTF (site DP)

5′-AGCTGATAGCTATGCTTGTTTATT 3′
3′-CCATCTACATTTGACTATTATG 5′

Sequence 2, tre/GHF1

5′-AGCTGTTAGATATTACGATTTGCTGCAA 3′
3′-ACACTCTAAACGTACTGTATTGCTAG 5′

Sequence 3, OTF

Electromobility Shift Assays (EMSA)—The following double-stranded oligonucleotides were used.

5′-AGTTTAATGTCGCCAATAC-3′
3′-CCATCTACATTTGACTATTATG 5′

Sequence 3, OTF

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Sequence 3, OTF
The oligonucleotides were labeled by filling with Klenow enzyme in the presence of \(^{32}\)P-dCTP and purified by chromatography on Sephadex G25. The binding reactions were performed for 20 min at 4°C in the presence of 0.1 ng of labeled probe and WCE or bacterially expressed recombinant proteins in a 15-ml solution of 25 mM Hepes, pH 7.9, 60 mM KCl, 12 mM MgCl\(_2\), 0.1 mM EDTA, 1 mM dithiothreitol, 12% glycerol, 100 µg/ml bovine serum albumin, and 2 µg of poly(dI-dC). Free DNA and DNA-protein complexes were resolved on a 4% nondenaturing polyacrylamide gel in 0.5 TBE buffer.

### RESULTS

The human PIT1/GHF1 promoter region contains multiple Pit-1/GHF-1 binding sites, an octamer binding site (OTF), and a TPA-responsive element (TRE)—We have examined the cis-regulatory elements and trans-acting factors involved in the control of the human PIT1/GHF1 promoter activity. For this purpose, four independent PIT1/GHF1 genomic clones were isolated from a human placental genomic library using the previously cloned human Pit-1/GHF-1 cDNA as probe (24). The extent of the clones was determined by restriction enzyme mapping and Southern blot analysis using primers specific to the different exons. One of the clones contained an 11-kb region encompassing the promoter and the 5' flanking sequences. A 1.5-kb EcoRI fragment containing the proximal promoter was sequenced (Fig. 1A). The sequence is available through the EMBL Data Bank (accession number X77223). The start site of transcription was determined by primer extension (Fig. 1B).

The cis-active elements within the proximal promoter were analyzed by DNase I footprinting. Bacterially expressed human Pit-1/GHF-1 protein (rhGHF-1) and GH3 WCE generated two footprints in a promoter fragment from \(-102\) to \(+173\), indicating the presence of two Pit-1/GHF-1 binding sites (GHF-1P at position \(-115\) and GHF-2P at position \(-255\)) (Fig. 2A). No DNase I protection was observed in the presence of HeLa WCE or bovine serum albumin. Five additional Pit-1/GHF-1 binding sites were identified in a promoter fragment from \(-281\) to \(-227\) (Fig. 2B). These sites were protected in the presence of low amounts (0.5 µg of crude extract) of rhGHF-1 as well as of GH3 WCE. Only a weak protection was observed on GHF-4P (position \(-378\)), indicating that this site may represent a low affinity Pit-1/GHF-1 binding site. Seven Pit-1/GHF-1 binding sites were identified in a distal promoter region extending from \(-1321\) to \(-800\) (Fig. 2, E and F). These sites were

![Fig. 1. Structure of the human PIT1/GHF1 promoter. A, nucleotide sequence of the human PIT1/GHF1 promoter region. The transcription start site (arrow), the TATA box (boldface and underlined), 5' AGCTCTTGATGAGTCAGCCGGAA 3' 3' GAACCTAGCTGCGGCTCTTAG 5' SEQUENCE 4. TRE 5' AGCTGAGTATGAATCATTAATT 3' 3' CTCACTACTTAGAATCT 5' SEQUENCE 5. TRE/GHF1 5' AGCTTCTGATTTGCAA 3' 3' AGAGTATAAATATGTAAACGTTCTAG 5' SEQUENCE 6. GHF-1 (site3P) The oligonucleotides were labeled by filling with Klenow enzyme in the presence of \(^{32}\)P-dCTP and purified by chromatography on Sephadex G25. The binding reactions were performed for 20 min at 4°C in the presence of 0.1 ng of labeled probe and WCE or bacterially expressed recombinant proteins in a 15-ml solution of 25 mM Hepes, pH 7.9, 60 mM KCl, 12 mM MgCl\(_2\), 0.1 mM EDTA, 1 mM dithiothreitol, 12% glycerol, 100 µg/ml bovine serum albumin, and 2 µg of poly(dI-dC). Free DNA and DNA-protein complexes were resolved on a 4% nondenaturing polyacrylamide gel in 0.5 TBE buffer.

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FIG. 2. Multiple Pit-1/GHF-1 binding sites and other cis-elements identified in the human PIT1/GHF1 promoter region by DNase I footprinting. Panel A, a PIT1/GHF1 DNA probe (−102 to +173), labeled at position −102, was incubated with 10 and 50 μg of GH3 or HeLa WCE or with 0.5 and 2.5 μg of bacterially expressed human Pit-1/GHF-1 protein (rhGHF-1) and subjected to DNase I footprinting analysis. Panel
protected in the presence of low amounts of rhGHF-1 (0.5 μg of crude extract) as well as of GH3 WCE except for GHF-1D and GHF-4D, which were only partially protected by GH3 WCE (Fig. 2, E and F). For all sites, the protection was abolished in the presence of a large excess of unlabeled oligonucleotide corresponding to the GHF-1 site 3P. Sequence analysis revealed that several sites (GHF-6P, GHF-7P, GHF-1D, GDH-3D, and GHF-4D) differed by one nucleotide from the consen-

sus Pit-1/GHF-1 high affinity binding sequence (28, 29). However, since sites 6P, 7P, and 3D were protected by low amounts of rhGHF-1, they represent new high affinity Pit-1/

GHF-1 binding sites. Interestingly, the site 7P (position -491) was also protected in the presence of HeLa WCE, indicating that a factor other than Pit-1/GHF-1 binds to that region (Fig. 2C). Nucleotide sequence analysis indicated the presence of a TRE at position -490 overlapping with the site GHF-7P. Indeed, this site was protected in the presence of recombinant AP-1 (c-Jun homodimers, Fig. 2C). The protected area was smaller than the area protected in the presence of GH3 and HeLa WCEs.

Footprinting analysis of the promoter fragment from -814 to -267 also revealed that protection of the OTF site (-783) was conferred by both GH3 and HeLa WCEs and was abolished when a large excess (100 ng) of oligonucleotides containing the OTF site were added as competitor (Fig. 2D). This site was also protected in the presence of large amounts of rhGHF-1, indicating that this factor weakly binds to the OTF site. A weak protection was observed around position -950 with both GH3 and HeLa WCEs (Fig. 2E). The ubiquitous protein binding on this site remains to be identified.

The Human PIT1/GHF1 Promoter is Pit-1/GHF-1-dependent and Autoregulated—To test whether these cis-DNA elements resulted in gene regulation, luciferase-reporter constructs containing progressive 5'-deletions of the human PIT1/

GHF1 promoter were transiently transfected into GH3 or HeLa cells (Fig. 3).

A promoter fragment from -102 to +15 (minimal promoter construct) containing the TATA box and the GHF-1 binding site 2P was sufficient to confer full activity to the promoter in GH3 cells, whereas this construct was inactive in HeLa cells (Fig. 3A). The promoter activity was specifically dependent on the expression of the pituitary Pit-1/GHF-1 protein, since the same construct inactive in HeLa cells was activated after cotransfection with the expression vector pH5VrGHF-1 (30) (Fig. 3B). A different promoter construct (-102/+140 LUC construct) containing the GHF-1P site located in the 5'-untranslated region of the gene resulted in a marked decrease (7-10-fold) of the promoter activity in GH3 cells (Fig. 3A). In addition, this construct was inactive in HeLa cells cotransfected with pH5VrGHF-1 (Fig. 3B). However, the -120/+140 LUC construct in which the GHF-1P site has been mutated to a non-Pit-1/GHF-1 binding site by site-directed mutagenesis (mut GHF-1P LUC construct) showed an increased transcriptional activity (Fig. 3A). These results indicated that the GHF-1P site located between position +15 and +22 has a negative effect on the transcriptional activity of the PIT1/GHF1 promoter.

Fig. 3. Basal expression of human PIT1/GHF1-LUC constructs in GH3 and HeLa cells. The constructs used in transient transfection experiments are described under "Experimental Procedures." GH3 cells (A) and HeLa cells (B) were transfected by electroporation with 40 μg of reporter plasmid, 5 μg of pCMVCAT (used as internal control), and 15 μg of pRSVrGHF-1 (when indicated by the shaded bars, for cotransfection in HeLa cells). The cells were harvested 40 h later, and the luciferase and CAT activities were determined. The levels of luciferase activity were normalized accordingly to the CAT activity. In each experiment, luciferase activities are expressed relatively to that measured with the promoterless pGL2 basic and are the means ± S.E. from single experiments performed in triplicate. The experiments were repeated three times, and similar results were obtained. The plasmid pGL2 control (Promega) contains the SV40 promoter and enhancer.

B, a PIT1/GHF1 DNA probe (~814 to -267), labeled at position -267, was incubated with 50 μg of GH3 WCE or with 25 and 50 μg of HeLa WCE or with 0.5 or 1.2 μg of rhGHF-1 protein and subjected to DNase I footprinting analysis. 100 ng of Pit-1/GHF-1 binding site 3P were added as competitor (C). Panel C, a PIT1/GHF1 DNA probe (~595 to -267), labeled at position -595, was incubated with 50 μg of GH3 or HeLa WCE or with 0.5, 1, and 2 μg of rhGHF-1 protein or three footprint units of recombinant human c-Jun (Promega) and subjected to DNase I footprinting analysis. Competition experiments were performed in the presence of 100 ng of TRE/GHF1 binding sites (C). Panel D, a PIT1/GHF1 DNA probe (~814 to -267), labeled at position -814, was incubated with 50 μg of GH3 or HeLa WCE or with 0.5, 1, and 2 μg of rhGHF-1 protein and subjected to DNase I footprinting analysis. 100 ng of OTF sites were added in competition experiments (C). Panels E and F, a PIT1/GHF1 DNA probe (~1321 to -800), labeled at position ~800 (E) or ~1321 (F) was incubated with 50 μg of GH3 or HeLa WCE or with 0.5, 1, and 2 μg of rhGHF-1 protein and subjected to DNase I footprinting analysis. 100 ng of GHF-1 site 3P were added in competition experiments (C). All of the control reactions were performed in the presence of 50 μg of bovine serum albumin (BSA). Maxam and Gilbert G + A sequencing ladders were used as size markers.
The transfection experiments using an upstream promoter fragment extending from -601 to +15 (-601 LUC construct), containing the five additional proximal Pit-1/GHF-1 binding sites and the TRE, did not further increase the transcriptional activity detected with the minimal promoter either in GH3 cells (Fig. 3A) or in HeLa cells cotransfected with pRSVrGHF-1 (Fig. 3B).

By contrast, transfection experiments using a promoter fragment extending from -1321 to +15 (-1321 LUC construct), resulted in a slight decrease (1.5–2 times) of the promoter activity in GH3 cells (Fig. 3A) or in HeLa cells cotransfected with pRSVrGHF-1 (Fig. 3B). This promoter construct could, however, be fully reactivated by site-directed mutagenesis of the OTF site to a non-octamer binding site (mut OTF LUC construct). This result suggested that the protein(s) binding to the OTF site is responsible for a negative modulation of the human PIT1/GHF1 promoter activity.

Pit-1/GHF-1 and AP-1 Compete for Binding to the Composite TRE/GHF1 Site of the human PIT1/GHF1 Promoter—The human PIT1/GHF1 proximal promoter region contains a TRE at position -490. DNase I footprinting analysis indicated that this site is recognized by AP-1 (Fig. 2C), a factor that mediates gene induction by phorbol esters, growth factors, and cytokines (31, 32). This site may also be recognized by CREB, a cAMP-responsive transcription factor whose activity is modulated by the protein kinase A pathway (33).

To determine which signal transduction pathway was involved in the control of the human PIT1/GHF1 promoter activity, reporter constructs (the -1321 LUC and the -601 LUC) containing the TRE site were transiently transfected into GH3 or HeLa cells and tested for cAMP and/or TPA induction. A moderate induction (15%) was observed in GH3 cells (Fig. 4A). This response was cell type-specific, since it was not observed in HeLa cells (Fig. 4B). No induction by TPA but rather an inhibition (45–55%) was observed in GH3 cells (Fig. 4, A and C). However, a 4–7-fold induction was observed in HeLa cells in the absence of Pit-1/GHF-1 (Fig. 4B). To analyze whether c-Jun was involved in down-regulation of PIT1/GHF1 transcription, the -1321 LUC and -601 LUC reporter constructs were transiently transfected into GH3 cells together with an expression vector for human c-Jun, pRSVhJun (34). A 50% reduction in the promoter activity was observed with both constructs (Fig. 4C). Cotransfection of GH3 cells with the minimal (-102/+15 LUC) promoter construct and pRSVhJun had no effect on the transcriptional activity (data not shown). The decrease in transcriptional activity was also observed (55% reduction) when, in addition to overexpression of c-Jun, endogenous AP-1 was activated by treatment of the cells with TPA (Fig. 4C).

As the TRE overlapped with the GHF-1 site 7P, a competition between AP-1 and Pit-1/GHF-1 for the binding on this composite TRE/GHF1 site was postulated.

To analyze which protein-DNA complexes were formed on the TRE/GHF1 site, we performed EMSA. Several complexes were specifically generated in the presence of GH3 WCE (Fig. 5A). Some of these complexes involved the transcription factor Pit-1/GHF-1, since they were completely shifted in the presence of rat GHF-1 antiserum (Fig. 5A). This result was confirmed by
specific binding of rhGHF-1 on the composite TRE/GHF1 site (Fig. 5B). Competition was observed either with unlabeled TRE/GHF1 or GHF-3P sites. The complex formed was partially shifted in the presence of human GHF-1 antiserum. No specific binding of rhGHF-1 was observed on consensus TRE sites (35) (Fig. 5B). In the presence of HeLa WCE, two complexes were specifically formed with TRE/GHF1 sites. The complex with the lowest mobility, which was also observed in the presence of GH3 WCE, involved the AP-1 factor, since the formation of this complex was abolished when an antiserum directed against the DNA-binding domain of the mouse c-Jun was added to the binding reaction mixture (Fig. 5A). Indeed, a complex with a similar mobility was observed when TRE/GHF1 sites were incubated with recombinant human AP-1 (c-Jun, Promega). Both unlabeled TRE/GHF1 and TRE sites competed for the formation of this complex. The same complex was generated by binding of AP-1 to TRE sites (Fig. 5B).

These results demonstrated that Pit-1/GHF-1 and AP-1 can both interact with the composite TRE/GHF1 site in vitro and are in agreement with the results obtained by DNase I footprinting. Since none of the complexes observed in the presence of GH3 WCE and shifted by recombinant GHF-1 antiserum was modified in the presence of c-Jun antiserum, it was unlikely that protein-protein interactions occurred between Pit-1/GHF-1 and the AP-1 factor on the composite TRE/GHF1 site. This hypothesis was assessed by incubating TRE/GHF1 sites with AP-1 (c-Jun) in the presence of increasing amounts of rhGHF-1 (Fig. 5C). A decrease in the formation of AP-1-DNA complexes was observed with a parallel increase in the formation of rhGHF-1-DNA complexes. Thus rhGHF-1 could displace AP-1 on the TRE/GHF1 sites. Conversely, increasing amounts of AP-1 inhibited the formation of rhGHF-1-DNA complexes (Fig. 5C). No ternary complex was observed, suggesting that AP-1 and Pit-1/GHF-1 do not interact simultaneously with the composite TRE/GHF1 site, but both bind on this site in a mutually exclusive way.

**DISCUSSION**

**Autoregulation of Human PIT1/GHF1 Gene Expression—**

This study was undertaken to determine the regulatory mechanisms responsible for the basal and induced expression of the human PIT1/GHF1 gene in pituitary cells as compared with non-pituitary cells. A 1.5-kb EcoRI fragment containing the proximal promoter region was analyzed. The nucleotide sequence comparison with that of the rat PIT1/GHF1 gene promoter (12, 13, 18) indicated a high similarity (88%) in the region extending from position +130 to position −110 relative to the transcription start site (data not shown).

Here we demonstrate that this region contains the cis-elements necessary for pituitary-specific expression and autoregulation of the human PIT1/GHF1 gene. A TATA box is localized at position −31 and conforms to the TATA box consensus sequence (36). A 15-bp region surrounding and including the TATA box of the rat PIT1/GHF1 gene was shown to bind a
pituitary transcription factor (called PTF) and to direct the pituitary-specific transcription of the gene (17). This cell type-specific TATA element seems not to be conserved in the human promoter, since DNase I footprinting experiments performed with GH3 and HeLa cell extracts failed to reveal any interaction between a pituitary-specific protein and the TATA box. This can be explained by the fact that three clustered bases located upstream of the TATA box are not conserved between the rat and the human genes. Indeed, point mutations affecting sequences upstream of the TATA box of the rat PIT1/GHF1 gene have been reported to affect the transcriptional efficiency (17).

In the case of the human gene, a promoter region from −102 to +15 (minimal promoter) was sufficient to direct its pituitary-specific expression. This region binds Pit-1/GHF-1 at a site (GHF-2P) located at position −55. The binding of Pit-1/GHF-1 to its own promoter (autoregulation) is responsible for the basal and pituitary-specific transcriptional activity of the promoter. Indeed, the minimal promoter could be activated in HeLa cells expressing transiently Pit-1/GHF-1. Human Pit-1/GHF-1 transcription is negatively autoregulated by Pit-1/GHF-1 binding to a site (GHF-1P) located in the 5′-untranslated region of the gene (position +15). Mutation of this site restored the basal transcriptional activity in GH3 cells. Taken together, these data indicate that PIT1/GHF1 transcription is autoregulated by an activating element at position −55 and an inhibitory element at position +15, which both bind Pit-1/GHF-1. These two elements may ensure a precise control of the basal level of expression of Pit-1/GHF-1 to maintain its steady-state expression (21). We also identified two elements may ensure a precise control of the basal level of expression of Pit-1/GHF-1 to maintain its steady-state expression (21). We also identified three Pit-1/GHF-1 binding sites in a superdistal region (−11 kb) of the human PIT1/GHF1 gene. In addition, eight Pit-1/ GHF-1 binding sites have also been described in the promoter and distal enhancer regions of the rat and human prolactin genes (6, 43). A high level of expression of the rat prolactin gene in lactotropes requires a synergistic interaction between the proximal promoter (−422 to −33 bp) and the distal enhancer (−1.8 to −1.5 kb). Both regions contain four high affinity Pit-1/GHF-1 binding sites indicating, in addition to their role in cell type-restricted expression, an involvement of these sites in regulating the transcriptional activity (44).

GHF-1D differs by two nucleotides from the consensus sequence and in fact more closely resembles an octamer site. However, we did not observe any binding of the ubiquitous Oct-1 factor to this site, at least with whole extracts from HeLa cells. Three of the high affinity binding sites (GHF-6P, GHF-7P, and GHF-3D) differ by one nucleotide from the consensus sequence reported for Pit-1/GHF-1 binding (28, 29). However, the first four nucleotides of the core recognition element (ATGN) are conserved. These nucleotides are responsible for the contact with the POU-specific domain of Pit-1/GHF-1 (28), which is required for high affinity and high specificity binding of Pit-1/GHF-1 to the target sites (30, 45). The high affinity Pit-1/GHF-1 binding sites identified in the prolactin and growth hormone promoters, although they contain a conserved consensus core sequence, also present divergences in the core flanking sequences (5, 6).

The ability to bind to diverse DNA sequences is a common feature among POU domain proteins. In addition to binding to the high affinity octamer sequence found in numerous different promoters, Oct-1 has been reported to bind a variety of degenerate octamer motifs (46–48). The neuronal POU domain factor Brn-2 also exhibits a high flexibility in DNA site recognition, tolerating core motifs presenting variable spacing (49). This property of POU domain proteins is an important aspect of their transcriptional regulatory functions, allowing them to discriminate their target DNA sequences. For example, the immunoglobulin heavy chain gene promoter IgH, containing high affinity octamer motifs, can be ubiquitously activated by Oct-1, while immunoglobulin light chain gene promoters, which contain low affinity octamer binding sites, require the B-specific Oct-2 factor to be fully activated. This restricts their expression to B-lymphocytes (47). The binding of POU domain proteins to different DNA sequences may also dictate their ability to interact with regulatory cofactors. It has recently been shown that synergistic activation of the prolactin gene by Pit-1/GHF-1 and the estrogen receptor requires a tyrosine-dependent synergy domain of Pit-1/GHF-1. This domain is accessible for interaction with the estrogen receptor only if Pit-1/ GHF-1 interacts with a dominant negative repressor (42).

**Multiplicity of Pit-1/GHF-1 Binding Sites in the Proximal and Distal Human PIT1/GHF1 Promoter Regions**—The human PIT1/GHF1 promoter contains a multiplicity of binding elements that were shown to bind the Pit-1/GHF-1 protein by DNase I footprinting. In addition to GHF-1P and GHF-2P involved in autoregulation, we have identified five binding sites (GHF-3P to GHF-7P) in a proximal region extending from −330 to −491 and seven binding sites in an upstream distal region from −889 to −1259 (GHF-1D to GHF-7D). All these sites were not equally protected by rhGHF-1 or GH3 cell extracts. We identified three low affinity binding sites (GHF-4P, GHF-1D, and GHF-4D) and nine high affinity binding sites.

A multiplicity of Pit-1/GHF-1 binding sites has been previously observed in the mouse PIT1/GHF1 enhancer located 10 kb 5′ to the transcription start site (21). We also identified three Pit-1/GHF-1 binding sites in a superdistal region (−11 kb) of the human PIT1/GHF1 gene. In addition, eight Pit-1/ GHF-1 binding sites have also been described in the promoter and distal enhancer regions of the rat and human prolactin genes (6, 43). A high level of expression of the rat prolactin gene in lactotropes requires a synergistic interaction between the proximal promoter (−422 to −33 bp) and the distal enhancer (−1.8 to −1.5 kb). Both regions contain four high affinity Pit-1/ GHF-1 binding sites indicating, in addition to their role in cell type-restricted expression, an involvement of these sites in regulating the transcriptional activity (44).

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In our study, we did not observe any synergistic interaction between the proximal promoter region and the upstream distal region of the human PIT1/GHF1 gene, which both contain a multiplicity of Pit-1/GHF-1 binding sites. The basal and cell type-specific expression of the human PIT1/GHF1 gene seems to be controlled by a minimal promoter region (−102 to +15). Although the function of each individual Pit-1/GHF-1 binding site has not been analyzed, it seems logical to speculate that they may participate in transcriptional control by recruiting cofactors to the Pit-1/GHF-1 binding sites. Indeed, Pit-1/GHF-1 cooperates with the estrogen receptor to activate the rat prolactin promoter (50, 51), with the thyroid hormone receptor to activate the rat growth hormone gene (52), and with an AP-1-
like factor to regulate the human TSHβ promoter activity (53), and it synergizes with P-Lim, a LIM homeodomain transcription factor, to activate several anterior pituitary genes (54). Taken together with our findings, these observations stress the need for the identification of co-activators and co-repressors interacting with Pit-1/GHF-1 in the regulation of pituitary-specific genes including its own transcription. Alternatively, the possibility is not excluded that multiple Pit-1/GHF-1 binding sites may participate in recruitment and positioning of different proteins to assemble an active transcriptional complex. It has indeed been shown that Pit-1/GHF-1 activates transcription by influencing the preinitiation transcription complex assembly, possibly by interacting with proteins from the basal transcription machinery (55). This mechanism would involve the binding of DNA induced by binding and multimerization of Pit-1/GHF-1. This hypothesis is supported by the finding that POU domain proteins including Pit-1/GHF-1 cause DNA bending when interacting with their DNA recognition sites (56).

Negative Regulation of the Human PIT1/GHF1 Gene Expression by AP-1 in Pituitary Cells—Besides autoregulation, we looked for additional regulatory mechanisms controlling the human PIT1/GHF1 proximal promoter activity. The rat PIT1/GHF1 gene transcription is positively regulated by an increase of the intracellular cAMP concentration. This regulation is mediated by two CREs located at positions -290 and -155 in the rat PIT1/GHF1 promoter and interacting with cAMP-activated transcription factors (12, 13). A similar regulatory mechanism is not conserved in the human PIT1/GHF1 gene promoter. The slight increase in transcriptional activity observed when transfected GH3 cells were treated with forskolin can be explained by cAMP stimulation of endogenous rat PIT1/GHF1 gene expression, which further stimulates our reporter construct. Furthermore, we could not identify any CRE within the proximal or distal promoter region. This indicates that the human PIT1/GHF1 promoter activity is not mainly regulated by activators of the protein kinase A pathway as it is the case in the rat gene.

However, activators of the protein kinase C pathway modulate expression of the human PIT1/GHF1 gene at a transcriptional level. Indeed, we have identified a TRE at position -490 in the proximal promoter region. This site differs by one nucleotide from the consensus AP-1 binding site (31) but has been shown by DNase I footprinting and EMSA to bind AP-1 with high affinity. An inhibition of the promoter activity in GH3 cells and a 4–7-fold induction in HeLa cells in the absence of Pit-1/GHF1 was observed after TPA induction, indicating that the response to TPA is tissue-specific. This TRE site overlaps with GHF-7P, and a mutually exclusive binding of AP-1 and Pit-1/GHF1 to this composite element was clearly demonstrated by EMSA. In addition, no direct protein–protein interaction was observed between these two factors. A similar effect to that induced by TPA could also be observed by cotransfecting the GH3 cells with pRSV-Cjun, indicating that the inhibitory effect is indeed mediated by AP-1.

This composite site represents, to our knowledge, the first example of a TRE associated with a binding site for a tissue-specific transcription factor. The implication of this composite site in the control of PIT1/GHF1 gene expression is puzzling. AP-1 and Pit-1/GHF1 bind to this site with high affinity. The preferential loading of AP-1 or Pit-1/GHF1 on this site may be influenced by their respective nuclear concentrations. Alternatively, post-translational modifications of these factors could induce a decrease in the binding affinity of one factor, facilitating the binding of the other. In vitro phosphorylation of Pit-1/GHF-1 by protein kinase A or C has been shown to inhibit its binding to high affinity binding sites (57). The phosphoacceptor residue, Thr-220 is located into the POU homeodomain. These results could not be confirmed in cultured cells (58). However, the Thr-220 is transiently phosphorylated during mitosis by a mitosis-specific kinase. This results in a transient decrease of the Pit-1/GHF-1 DNA binding activity during the M phase of the cell cycle (58). Therefore, a possible function for the TRE could be to mediate a complete transcriptional shut-off of the human PIT1/GHF1 gene when the DNA binding activity of the Pit-1/GHF-1 protein is inhibited by mitotic phosphorylation. This transient regulation could compensate for the defective autoregulation mechanism that is switched off during the M phase as Pit-1/GHF-1 DNA binding activity is inhibited by mitotic phosphorylation.

Role of Oct-1 in the Regulation of PIT1/GHF1 Gene Expression—An octamer site (OTF) was identified in the human PIT1/GHF1 promoter at position -783 by DNase I footprinting using pituitary and non-pituitary cell extracts. Transient transfection experiments show that this OTF site mediates a negative regulation of the transcription of the PIT1/GHF1 promoter. Indeed, an increase in the transcriptional activity of this promoter was observed when the OTF site was inactivated by site-directed mutagenesis. Although the protein(s) binding to this site has not yet been clearly identified, a good candidate is Oct-1. Indeed, in EMSA performed on the OTF site, we observed a specific binding of recombinant human Oct-1 and of rhGHF-1. Both proteins compete for binding to this site (data not shown).

In conclusion, we have analyzed the mechanism of transcription of the PIT1/GHF1 gene in humans. The data presented here demonstrate that except for a common autoregulatory mechanism controlling the basal expression of the gene in both humans and rats, the mechanism of activation substantially differs between the two species. The transcription of the PIT1/GHF1 gene is not positively controlled by activators of the protein kinase A pathway but is down-regulated by AP-1 and by Oct-1. However, further analysis is required to better understand the functional significance of these mechanisms.

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