Nuclear factor 1 regulates the distal silencer of the human *PIT1/GHF1* gene

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Here we report the characterization of 12 kb genomic DNA upstream of the human *PIT1/GHF1* promoter. Different regions involved in the modulation of human *PIT1/GHF1* gene expression were defined by transient transfection studies. Two regions, one proximal (−7.1/−2.3) and one distal (−11.8/−10.9), presented an enhancer activity in pituitary cells when placed upstream of the SV40 promoter. The 0.9 kb distal region was analysed further and found to decrease the basal transcriptional activity of the human *PIT1/GHF1* minimal

promoter, indicating that this region behaves as a silencer for its own promoter. Three Pit-1/GHF-1-binding sites and two ubiquitous nuclear factor 1 (NF-1)-binding sites were identified by DNase I footprinting in the distal regulatory region. Deletion analysis indicated that NF-1 or NF-1-related protein(s) participate in the down-regulation of human *PIT1/GHF1* gene expression by interacting with an NF-1-binding site within the distal regulatory region.

INTRODUCTION

Pit-1/GHF-1 is a pituitary-specific transcription factor that binds to and transactivates the promoter of the genes for growth hormone, prolactin and thyroid-stimulating hormone [1–5]. The expression of Pit-1/GHF-1 is restricted to somatotropes, lactotropes and thyrotropes. Pit-1/GHF-1, in synergy with other transcription factors, is essential for the development of the anterior pituitary gland and for the differentiation of these three cell types [3,6–9]. Indeed, mice with mutations or deletions of the *PIT1/GHF1* gene fail to generate somatotropes, lactotropes and thyrotropes and consequently exhibit anterior pituitary hypoplasia and dwarfism [10], demonstrating the importance of Pit-1/GHF-1 in the ontogeny of the pituitary gland. Mechanisms involved in the activation of this gene are therefore of considerable interest. Studies performed in transgenic mice have shown that a 15 kb of the 5' flanking region of the *PIT1/GHF1* gene is necessary to target gene expression to the anterior pituitary gland [11,12]. Deletion analyses of this region led to the identification of a potent 390 bp cell-specific enhancer located approx. 10 kb upstream of the *PIT1/GHF1* transcription start site. The enhancer comprises Pit-1/GHF-1 autoregulatory sites, a cell-specific *cis*-element and morphogen-responsive elements [12,13]. Transgenic mice in which the SV40 large T antigen expression was targeted to the pituitary by 15 kb of the *PIT1/GHF1* gene 5' flanking region were dwarfs and developed pituitary tumours [11]. These tumours expressed Pit-1/GHF-1 but failed to express growth hormone and prolactin. Studies *in vitro* with the GHFT1 cell line derived from this tumour led to the identification of a stage-specific enhancer located between

−3.1 and −5.3 kb that activated *PIT1/GHF1* at an early stage of pituitary development but was inactive in fully differentiated cells [11].

In addition to its role in the regulation of the gene expression for growth hormone and prolactin, Pit-1/GHF-1 is important for the regulation of its own gene expression. Indeed, two autoregulatory Pit-1/GHF-1-binding sites have been described on the proximal promoter of the rat *PIT1/GHF1* gene: a positive Pit-1/GHF-1-responsive element located at positions −60 to −45 and an inhibitory Pit-1/GHF-1-responsive element located in the 5' untranslated region [14,15]. In addition to autoregulation, the *PIT1/GHF1* gene expression in rat is modulated by variations in the intracellular cAMP level [14,15].

In a previous study we analysed the regulatory elements controlling *PIT1/GHF1* (*hPIT1/GHF1*) gene expression in human [16]. A minimal promoter region (−102 to +15) was shown to contain the elements responsible for high basal transcriptional activity, autoregulation and cell-specific expression. The transcriptional activity is negatively regulated by Oct-1, which binds to an octamer-binding site, and by AP-1, which competes with Pit-1/GHF-1 for the binding to a composite PMA response element (TRE)/GHF1 element [16].

Here we report the analysis of the 5' flanking region of the *hPIT1/GHF1* promoter. A distal regulatory region that contains three Pit-1/GHF-1 autoregulatory elements and two ubiquitous nuclear factor 1 (NF-1)-binding sites were identified and characterized. We provide evidence that NF-1 or NF-1-related protein(s) participate in the down-regulation of *hPIT1/GHF1* gene expression.

Abbreviations used: CAT, chloramphenicol acetyltransferase; GRE, glucocorticoid response element; LUC, luciferase; NF-1, nuclear factor 1; RAR, retinoic acid-responsive; TK, thymidine kinase; UFE, ubiquitous factor element; vNF1BD, baculovirus-expressed recombinant NF1 DNA-binding domain; WCE, whole cell extract.

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The nucleotide sequence data reported will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number X97489.

EXPERIMENTAL

Cloning and plasmid constructions

A genomic fragment containing exon 1 and the upstream regulatory sequences (11 kb) of the *PIT1/GHF1* gene was isolated from a human placental genomic library using hPit-1/GHF-1 cDNA as probe [17]. *EcoRI* fragments were subcloned into pBluescript SK+ to generate p(*PIT1/GHF1*)SK constructs. Restriction fragments from the upstream *hPIT1/GHF1* promoter region were subcloned into the pCATpromoter vector (Promega, Madison, WI, U.S.A.) containing the chloramphenicol acetyltransferase (CAT) reporter gene under the control of the SV40 promoter to generate (PIT1/GHF1)SV/CAT constructs. The (−11.8/−10.9)TK/CAT construct was made by subcloning a *BamHI*–*SalI* fragment isolated from p(−11.8/−10.9)SK+ upstream of the thymidine kinase (TK) promoter in pBLCAT2. To generate the (−11.8/−10.9)PIT1–LUC constructs (in which LUC stands for luciferase), the *BamHI*–*SalI* or *KpnI*/SalI fragments isolated from p(−11.8/−10.9)SK+ were subcloned in both orientations into the −102/+15 LUC and the −601 LUC constructs (previously described in [16]) upstream of the *hPIT1/GHF1* promoter (see Figure 2B). Deletion mutants of the −11.8/−10.9 regulatory region were generated by PCR and subcloned into the −102/+15 LUC construct. Restriction fragments (*EcoRI*/HindIII 1–253, HindIII/*XbaI* 253–591 and *EcoRV*/*EcoRI* 483–879) were subcloned into pBluescript SK+ to generate pΔ(1–253)SK, pΔ(253–591)SK and pΔ(483–879)SK plasmids respectively. These plasmids were used in DNase I footprinting experiments. The −11.8/−10.9 *PIT1/GHF1* fragment was sequenced on both strands with the Sequenase Version 2.0 sequencing kit (USB, Cleveland, OH, U.S.A.).

Transient transfection experiments

GH3 rat pituitary tumour cells (from ATCC) and human cervical carcinoma HeLa cells (from ECACC) were transfected as described previously [16]. Cells [(4–5) × 10⁶] were electroporated with 40 µg of reporter plasmid and 10 µg of pCMVβGAL, or with 10 µg of pCMVCAT used as an internal control for transfection efficiency. The cells were then diluted into 5 ml of culture medium and cultured in two 35 mm Petri dishes. Cells were harvested after 48 h, lysed in 400 µl of reporter lysis buffer (Promega) for 30 min at room temperature and centrifuged at 10000 g for 10 min. Aliquots (25–50 µl) of supernatant were assayed for CAT, luciferase and/or β-galactosidase activities. CAT assays were performed by ELISA (CAT ELISA kit; Boehringer Mannheim, Germany) and β-galactosidase activities were determined with a colorimetric assay (Promega) and measured on an ELISA reader. Luciferase assays were performed with the Luciferase detection kit (Promega) and the light emission was measured with a Lumac Biocounter M2000 (Perstorp Analytical).

DNase I footprinting analysis

All oligonucleotides were purchased from Eurogentec (Liège, Belgium) except for NF-1, which was a gift from F. Lemaigre (ICP, Brussels, Belgium). The following double-stranded oligonucleotides were used: sequence 1 (GHF1-3P), 5'-AGCTTCTCATATTTATACATTTGCAA-3'/3'-AGAGTATAAATATGTAAACGTTCTAG-5'; sequence 2 (UFE), 5'-AGCTTGGATGTTCTTGGCACCTTTGTCAAGT-3'/3'-ACCTACAAGAACCGTGGAACAGTTCACCTAG-5'; sequence 3 (NF1), 5'-AGCTATTTTGGCTACAAGCCAATATGAT-3'/3'-TAAACCGCTGTTCCGGTTATACTACTAG-5'.

Oligonucleotide GHF1-3P is the −337/−317 fragment comprising the GHF-1-binding site of the *hPIT1/GHF1* promoter [16]. Oligonucleotide UFE corresponds to the sequence 534–560 of the (−11.8/−10.9) *PIT1/GHF1* distal regulatory region. Oligonucleotide NF-1 corresponds to the high-affinity binding site (+21 to +44) in the adenovirus origin of replication, modified as described by Courtois et al. [18].

Crude cell extracts were prepared from GC, GH3 and HeLa cells as described by Manley [19] with a 5–60% (NH₄)₂SO₄ precipitation. Recombinant hGHF-1 (rhGHF-1) crude bacterial extracts were prepared by the method of Pognonec et al. [20]. Recombinant NF1 protein (vNF1BD), corresponding to the DNA-binding domain (residues 4–240) of the rat liver NF1 protein, was a gift from F. Lemaigre and was expressed in eukaryotic cells by using a vaccinia virus expression vector [21]. Protein concentrations were determined by the Bradford assay (Bio-Rad, Richmond, CA, U.S.A.).

DNase I footprinting analyses were performed as described previously [16]. Restriction fragments obtained from pΔ(1–253)SK, pΔ(253–591)SK and pΔ(483–879)SK constructs were end-labelled with [γ-³²P]ATP by using T4 polynucleotide kinase and then digested with restriction enzymes to obtain single end-labelled probes. DNA probes were incubated with either BSA or proteins prepared from whole cell extracts (WCEs), rhGHF-1 or vNF1BD in binding buffer supplemented with 2 µg of poly(dI-dC) and were further digested with DNase I. For competition footprinting assays, double-stranded oligonucleotides were added to the binding reactions. DNA fragments were analysed on a denaturing 8% (w/v) polyacrylamide sequencing gel. DNA ladders were generated by A + G Maxam and Gilbert sequencing.

RESULTS

Identification of regulatory sequences upstream of the human *PIT1/GHF1* promoter

To identify regulatory regions in the *hPIT1/GHF1* gene, genomic DNA fragments extending from −1.4 to −11.8 kb upstream of the transcription start site (Figure 1A) were subcloned into a reporter vector containing the CAT gene under the control of the SV40 promoter (pCAT promoter). These constructs were transiently transfected into rat pituitary GH3 cells and human non-pituitary HeLa cells (Figure 1B). Two constructs induced an increase in the basal SV40 promoter activity in pituitary GH3 cells, identifying two potential enhancer regions: a large proximal regulatory region located upstream of the proximal promoter (−2.3 to −7.1 kb) and a distal regulatory region of 0.9 kb located at approx. −12.0 kb (−11.8 to −10.9 kb). These fragments enhanced the SV40 promoter reporter gene activity in GH3 cells 5-fold and 9-fold respectively (Figure 1B). The 0.9 kb distal enhancer also increased 4-fold the SV40 promoter reporter gene expression in HeLa cells. Two other regions, −2.3/−1.4 kb and −10.0/−7.1 kb, exhibited a very low enhancer activity in GH3 cells (Figure 1B). The construct containing the region between −10.9 and −10.0 kb did not enhance the promoter activity in GH3 cells but did so in HeLa cells (Figure 1B). We focused our further study on the characterization of the *PIT1/GHF1* distal regulatory region, which presented differential activities in GH3 and HeLa cells, suggesting the presence of pituitary and non-pituitary specific regulatory elements.

The 0.9 kb distal region also displayed a 9–10-fold enhancer activity on the SV40 promoter in GH3 cells when placed in an inverted position (anti-sense) (Figure 2A). Surprisingly, the same regulatory region did not function as an enhancer when cloned upstream of the heterologous TK promoter. On the contrary, a 1.5-fold decrease in basal TK promoter activity was observed

Figure 1 Identification of transcriptional regulatory regions 5' of the human *PIT1/GHF1* promoter

(A) Schematic representation of the six exons of the human *PIT1/GHF1* gene (top line) and the 5' regulatory regions upstream of the transcription start site (bottom). (B) Activities of CAT reporter constructs containing the SV40 promoter and different upstream regions of the human *PIT1/GHF1* promoter. The numbers in brackets represent the position (in kb) relative to the transcription start site. GH3 cells (filled bars) and HeLa cells (hatched bars) were transiently transfected with the constructs depicted in the figure. pCMV/βgal was used as an internal control. Levels of expression were determined 48 h after electroporation and were normalized to the level of β-galactosidase activity. Fold stimulations over basal SV40 promoter activity were obtained from four independent experiments and are means ± S.E.M.

(Figure 2B). The activity of the distal regulatory region was further assessed on the *hPIT1/GHF1* promoter. A significant decrease in the activity of both the minimal (−102/+15) promoter (2–2.5-fold) and the larger (−601/+15) promoter (3.5–6.5-fold) was observed independently of the orientation of the distal region (Figure 2B). Moreover, the distance between the distal region and the *PIT1/GHF1* promoter had no influence on the silencer activity of the distal regulatory region (Figure 2B). These results indicate that the distal regulatory region of the *hPIT1/GHF1* gene could function either as an enhancer or as a silencer depending on the promoter on which its activity is assessed. This suggested that differential crosstalk might occur between factors interacting with the distal regulatory elements and factors controlling the basal promoter activity.

Distal regulatory region of the human *PIT1/GHF1* gene contains Pit-1/GHF-1-binding sites and ubiquitous *cis*-active elements

The nucleotide sequence of the 0.9 kb distal regulatory region is shown in Figure 3(A) (sequence 1–879 from the 5' *EcoRI* site).

To map the *cis*-active elements present in this region, we performed DNase I footprinting experiments (Figure 4). In the presence of bacterially expressed hPit1/GHF-1 (rhGHF-1) or

GH3 WCE, we observed three specific footprints identifying three Pit-1/GHF-1-binding sites (GHF-1a, GHF-1b and GHF-1c; Figure 4). These sites were not protected by HeLa WCE nor by BSA. Pit-1/GHF-1 binding to these sites was abolished in competition experiments in the presence of an excess of oligonucleotide containing Pit-1/GHF-1-binding site (GHF-1-3P site [16]). The GHF-1a site, located at position 218, was protected in the presence of small amounts (0.5 μg) of rhGHF-1 (Figure 4A) but only a weak footprint was observed in the presence of GH3 WCE (results not shown). The sites GHF-1b and GHF-1c, located at positions 466 and 574 respectively, were protected in the presence of small amounts (0.5 μg) of rhGHF-1 as well as GH3 WCE (Figures 4B and 4C). These results indicate that the GHF-1a site might display a lower affinity than the two other Pit-1/GHF-1-binding sites for the Pit-1/GHF-1 protein.

Footprinting analysis of the distal regulatory region from positions 483–879 (Figure 4C) revealed protection by both GH3 and HeLa WCEs at positions 543–560 and 632–647 and the appearance of a DNase I-hypersensitive site upstream of position 560. No footprint was observed in the presence of rhGHF-1 protein. These regions were designated as UFEs, for 'ubiquitous factor elements'. Sequence analysis of the UFEs showed that these sites contained potential NF-1-binding sites with one base

Figure 2 Transcriptional effect of the human *PIT1/GHF1* distal regulatory region on different promoters

(A) Activities of CAT reporter constructs containing the *hPIT1/GHF1* (–11.8/–10.9) distal region (879 bp fragment) in both orientations upstream of the SV40 promoter. (B) Activities of CAT and LUC reporter constructs containing the same distal fragment subcloned upstream of the herpes simplex virus TK promoter, or upstream of the minimal (–102/+15) *hPIT1/GHF1* promoters in both orientations. Hatched bars represent the CAT activity under the TK promoter, open bars the LUC activity with the –102/+15 *hPIT1/GHF1* promoter and filled bars the LUC activity with the –601/+15 *hPIT1/GHF1* promoter. The broken line representing the 2730 bp fragment of the pGL2basic plasmid is located between the minimal *hPIT1/GHF1* promoter and the distal PIT fragment. GH3 cells were transiently transfected as described in the legend to Figure 1(B). CAT and LUC activities are reported relative to basal promoter activities of their respective promoter taken as 100%, and are means $\pm$ S.E.M. for three experiments.

difference from the consensus sequence (TTGGA/C N6 GCCAA) [22,23] for the distal UFE (TTGGC N6 GtCAA) and three base differences (TTtGt N6 GCCAt) for the proximal UFE.

We therefore investigated whether NF-1 could bind to these UFE sites. Footprinting analyses shown in Figure 4(C) demonstrated that recombinant baculovirus expressed NF1 protein (vNF-1BD), corresponding to an NF-1 isoform mainly expressed in the liver [21], could specifically bind both UFE sequences with high affinity. Indeed, the binding of NF-1 was prevented in competition experiments performed with a large excess of UFE or NF-1 oligonucleotides. Unrelated oligonucleotides such as GHF1-3P did not compete for the binding of NF-1 to UFEs. The hypersensitive site also disappeared in the presence of the specific competitors. These results suggest that the UFE sequences bound NF-1 or an NF-1-like protein.

By sequence analysis we also identified in the distal regulatory region a putative glucocorticoid response element (GRE) located at positions 765–780 (Figure 3). In DNase I footprinting experiments, no footprint was observed in the region containing the potential GRE in the presence of either GH3 or HeLa WCEs, even after the induction of the glucocorticoid receptor by treatment of the cells with dexamethasone (results not shown). These results suggest that the GRE site might not be functional.

To test whether the –11.8/–10.9 regulatory region could, however, be regulated by glucocorticoid, the (–11.8/–10.9)SV/CAT construct (described in Figure 1B) was transiently transfected into dexamethasone-treated GH3 and HeLa cells (results not shown). We observed no induction of this reporter construct by dexamethasone. To demonstrate that the absence of response

by dexamethasone was not due to the absence (or very small amounts) of glucocorticoid receptor in GH3 and HeLa cells, cells were co-transfected with a human glucocorticoid receptor expression vector (HGo plasmid [24]); no induction was observed (results not shown). The pMAMneo–LUC plasmid (containing the inducible MMTV–LTR promoter; Clontech, Palo Alto, CA, U.S.A.) was used as a positive control for dexamethasone induction (results not shown). We therefore conclude that the GRE site was indeed not functional.

Deletion analysis of the –11.8/–10.9 *PIT1/GHF1* distal regulatory region

To characterize further the *cis*-active elements in the –11.8/–10.9 *PIT1/GHF1* region responsible for the silencer activity on the minimal *hPIT1/GHF1* promoter (–102/+15), reporter constructs containing progressive 5'-end deletions of this region were generated. These constructs were transiently transfected into GH3 cells. The luciferase activities were compared with the basal activity of the minimal promoter (Figure 5). A 60% decrease in the basal promoter activity was observed in GH3 cells when either the full-length silencer or a fragment carrying a deletion from 1 to 483 including the Pit-1/GHF-1-binding sites a and b (SAa construct) was placed downstream of the minimal *PIT1/GHF1* promoter (Figure 5). However, deletion of the distal NF-1-binding site (SAb construct) resulted in the recovery of 80% of the basal promoter activity, which confirmed the negative regulatory effect of this NF-1-binding site. Full recovery of promoter activity was observed with further deletion of the Pit-1/GHF-1 c site (SAc construct), indicating that this element

A

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5'
GAATTCCTGGGAGATACAATTCGAATTGAGATTTTCAGTGGGGACACAGACAAAACATATCA 60
TACTTGTGGCCATTTTATGTTTCTTTTGGAGAAATATGCGTGTCTCTGTGCCACTTT 120
TTTGTACTGTTGAGTTGTTCAAGTTCCTGTATATCTGGATATTAGTCCCATGTTGGATA 180
AATATTTGCAACATTTTCTTACATTCAACAGGATACCTATTTCATCTACTGAATGTTTC 240
TTTGTGTGCAGAAGCTTTTATGTTTTCATATAGTCTATTTGTCTATTTTGTCTTCTGT 300
TGCTTGTGCTTTTGGAGTCATAGCTGTAACCTTTGCCTAGACCATTGTTCTGAAGTAT 360
TTTCCCTGTTTCTCTAGTAGTTTACAGTTTCAGGTCTCATGTTTAAATATTAAATCC 420
ATCTTGAGTTGATTTTGCATATGGTGAGAGATAGGGTCTTGTCTATTTCATCTGCATA 480
TAGATATCCAATTTTCTCAGCACCATTATTGAAGAGACTGTCTTTGCCCATTTGGATGT 540
TCTTGGCACCTTTGTCAAATATCTATTAGCTGCAATACATTCATTTATTCTAGATTCT 600
CTATTATGTTCTATTGCTATATGCTGTCTTTTGTACTAATGCCATGCTGCTTTGGTTA 660
CTATGACCTAGCGATATATTTTAAAGTCAGATAGTTTGTAGCCCTTTCAGCTTTATTCTTTT 720
TACTTGAGATTGCTTTGGCTCTTTGGGGTATTTTGTGATTGCTTGGGGCTACTGTTCT 780
TGAATCTGAACATTATCTGTCAGGAATCTAGAAATTTCTTGCCATCATATATTGAAT 840
ATTGCTCTCCAGATTTTATGTTCTTTTCTAGAAATTC -3' 879

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B

Figure 3 Structure of the distal regulatory region of the human *PIT1/GHF1* gene

(A) Nucleotide sequence of the (−11.8/−10.9) distal silencer region of the *hPIT1/GHF1* gene. The base pairs are numbered from the 5′ *EcoRI* site. Binding sites for Pit-1/GHF-1 and NF-1 transcription factors are delineated. A potential GRE is also indicated. (B) Schematic representation of transcription factor-binding sites in the (−11.8/−10.9) distal regulatory region. Binding sites for Pit-1/GHF-1 (open ovals), NF-1 (filled boxes) and glucocorticoid receptor (hatched oval) are depicted.

is also involved in the silencer activity (Figure 5). Deletion of the proximal NF-1-like site (SΔd construct) did not further influence the basal promoter activity (Figure 5).

DISCUSSION

To determine the molecular mechanisms responsible for the activation and the tissue-specific expression of the *hPIT1/GHF1* gene, we have analysed 12 kb 5′ flanking regions of the gene. In a previous study we showed that basal transcription of the human *PIT1/GHF1* gene is controlled by a minimal promoter region (−102 to +15) containing the *cis*-acting elements that confer pituitary-specific expression and autoregulation by its own gene product. The transcriptional activity is negatively regulated by Oct-1 and AP-1 transcription factors. AP-1 competes with Pit-1/GHF-1 for the binding to a composite PMA response element (TRE)/GHF1 element [16]. We here report the characterization of a 5′ distal silencer region of the human *PIT1/GHF1* basal promoter. This region is involved in the modulation of the human *PIT1/GHF1* gene expression by Pit-1/GHF-1 itself and NF-1 transcription factor.

Transient transfection experiments into pituitary GH3 cells have demonstrated the presence of two regulatory regions, one distal (−11.8/−10.9) and one proximal (−7.1/−2.3). Both regions enhanced the transcriptional activity of SV40 promoter in GH3 cells. In addition, the distal regulatory region weakly enhanced transcription from the SV40 promoter in HeLa cells, which indicates the presence of potential ubiquitous regulatory elements. These two regulatory regions identified in the *hPIT1/GHF1* gene correspond in position to the enhancer regions previously described in the *PIT1/GHF1* gene from rodents [11–13]. Indeed, a potent enhancer located between −3.1 and −5.1 kb of the rat *PIT1/GHF1* gene has been identified [11]. This stage-specific enhancer is active only in the somatotrophic progenitor GHFT1 cells, not in differentiated pituitary GH3 or GC cells, which suggests that this enhancer is important for early activation of the *PIT1/GHF1* gene during development [11]. In our study the proximal regulatory region located between −7.1 and −2.3 exhibited an enhancer activity in GH3 cells but its activity in GHFT1 cells has not yet been investigated and the factors interacting with this enhancer region remain to be identified in both rodents and human. The distal regulatory region (−11.8/−10.9) of the *hPIT1/GHF1* gene corresponds in position to a strong *PIT1/GHF1* enhancer identified in rodents [12,13]. This enhancer, located more than 10 kb from the *PIT1/GHF1* open reading frame, is highly conserved in rodents [13], suggesting a crucial role in *PIT1/GHF1* gene regulation. However, related sequences were not identified in guinea pig [13], which suggests that this regulatory region is not conserved throughout mammalian evolution [13]. In addition, sequence analysis did not reveal any similarity between the distal human *PIT1/GHF1* regulatory region and the mouse/rat *PIT1/GHF1* distal enhancer. These two regions also differ in their structures. The distal human *PIT1/GHF1* regulatory region contains three Pit-1/GHF-1-binding sites, two sites for the ubiquitous NF-1 transcription factor and one for a potential GRE. These regulatory elements differ from those found in the mouse/rat *PIT1/GHF1* upstream enhancer, which contains five Pit-1/GHF-1-binding sites, a vitamin D₃-responsive element and a retinoic acid-responsive (RAR) element [12,13]. The difference in structure was associated with a difference in function. Although the human *PIT1/GHF1* distal regulatory region increased the SV40 promoter activity (9–10-fold), a 2–2.5-fold and a 3.5–6.5-fold decrease in respectively the minimal and the proximal *hPIT1/GHF1* promoter activity as well as a 1.5-fold decrease in the basal TK promoter activity were observed in GH3 cells. Those results demonstrate clearly that the distal regulatory region functioned here as a silencer. The differences in inhibition by the distal regulatory region on respectively the minimal and proximal promoters suggest that DNA-binding sites located on the 602 *hPIT1/GHF1* promoter are implicated in this response.

The prevalence of Pit-1/GHF-1-binding sites in the distal regulatory region and in the promoter of the *hPIT1/GHF1* gene suggests that autoregulation might have an important role in the control of *hPIT1/GHF1* gene expression. Of the three Pit-1/GHF-1 autoregulatory sites identified in the *hPIT1/GHF1* distal regulatory region, one site (GHF-1b) conformed to the consensus sequence for high-affinity Pit-1/GHF-1-binding sites (WWWTATNCAT) [6,8]. The GHF-1a site seemed to display a lower affinity but contained the core consensus sequence (TATNCAT). The GHF-1c site might contain two overlapping Pit-1/GHF-1-binding sites (gcAaATNCAT and ATAcATNCAT). GHF-1c differs from the consensus core but seemed to display a high binding affinity for rhGHF-1. Interestingly, the three nucleotides (CAT) of the core motif responsible for POU-specific domain binding with DNA are

Figure 4 Multiple Pit-1/GHF-1 and NF-1-binding sites identified in the human *PIT1/GHF1* silencer by DNase I footprinting

(A) Analysis of fragment 1 to 253, labelled at position 253. (B) Footprinting analysis of fragment 253–591, labelled at position 591. (C) Footprinting analysis of fragment 483–879, labelled at position 879. Labelled fragments were incubated with either 0.5 or 1.25 μ g of bacterially expressed *hPIT1/GHF1* protein (rhGHF-1), 0.5 μ g of baculovirus-expressed recombinant NF1 protein (vNF1BD), 50 μ g of GH3 WCE (GH3) or HeLa WCE (HeLa), before digestion with DNase I. Control experiments were performed in the presence of 50 μ g of BSA. Competition experiments were performed with a 100-fold excess of unlabelled GHF1-3P (C1), UFE (C2) or NF-1 (C3)-binding sites added to the binding reaction as indicated above the lanes. The A/T box represents an A/T-rich region which, however, differs from the consensus *PIT1/GHF1*-binding site but binds rhGHF-1 protein [6]. Maxam and Gilbert G + A sequencing ladders were used as size markers.

Figure 5 Deletion analysis of the human *PIT1/GHF1* distal regulatory region

GH3 cells were transiently transfected with luciferase reporter constructs containing progressive 5'-end deletions (Δ) of the silencer region (S), placed at 2730 bp (broken line) upstream of the minimal *PIT1/GHF1* promoter (P). The Pit-1/GHF-1-binding sites (open ovals), the NF-1-binding sites (filled boxes) and the potential GRE (tinted box) are indicated. pCMVCAT was used as internal control for transfection efficiency. Luciferase activities are reported relative to the basal promoter activity taken as 100%, and are means $\pm$ S.E.M. for three experiments.

conserved [25]. The mechanism by which Pit-1/GHF-1 molecules can bind to this composite site has not yet been studied. It has been shown that Pit-1/GHF-1 can bind DNA as a dimer and that protein-protein interactions also depend on the POU-specific domain [25]. The regulatory function of each of the three sites found in the *hPIT1/GHF1* distal regulatory region seems to be different. Deletion of both GHF-1a and GHF-1b sites had no effect on the transcriptional activity in transient transfection experiments. In contrast, GHF-1c seemed to be required for regulation. In the *hPIT1/GHF1* promoter, three high-affinity Pit-1/GHF-1-binding sites were also described that showed differences from the consensus sequence [16]. We hypothesize that the binding of Pit-1/GHF-1 to slightly different DNA sequences might dictate its ability to interact with other transcription factors [16]. Alternatively the interaction of Pit-1/GHF-1 with specific DNA sequences could be the consequence of its interaction with regulatory cofactors.

Multiple Pit-1/GHF-1-binding sites were also observed in the proximal and distal regions of the mouse *PIT1/GHF1* gene [12,13] and of the rat and human prolactin genes [4,26,27]. Some of them are required as co-regulators in mediating hormonal effect. Indeed, the major RAR element of the mouse/rat *PIT1/GHF1* enhancer reveals a synergistic interaction between Pit-1/GHF-1 and RAR to mediate the pituitary-specific effects of retinoic acid [12,13]. Pit-1/GHF-1 co-operates with the oestrogen receptor to activate the rat prolactin gene [28,29]. Interestingly, the nucleotide sequence of the human distal silencer suggested the presence of a potential GRE. However, we failed to detect any functional GRE in this region. Lack of functionality might be due to one base difference (TGGGgCCN3TGTCT) detected in the 5' half region of the palindromic consensus sequence required for the binding of the glucocorticoid receptor. The conversion of an adenine residue into a guanine might be responsible for the loss of the activity [30].

Dissection analysis of the human *PIT1/GHF1* silencer showed that its activity essentially implies the binding of NF-1 or NF-1-like transcription factors to the upstream NF-1 element. The downstream degenerated NF-1-binding site contributed only slightly to the silencer activity. NF-1, also designated CTF [23], consists of a family of CCAAT box-binding proteins that are differentially expressed depending on the tissue and species [31,32]. The function of NF-1 proteins as transcriptional activators is well documented [22,23,33,34]. NF-1 can also act as a repressor of transcription [35–38] but the involvement of NF-1 proteins in negative transcriptional regulation is still poorly understood. NF-1 was shown to mediate the repression of the rat GH promoter activity by interacting with a proximal silencer [36,39]. However, this single factor was not sufficient by itself to confer pituitary-specific repression, which required the presence of additional proteins that are as yet unidentified [40]. Szabó et al. [38] have suggested the existence of isoforms lacking a functional activation domain or containing a repressor domain. Members of the NF-1 family were indeed reported to interact co-operatively or negatively with other transcriptional modulators [41,42]. A similar NF-1 site has been identified in the first intron of the rat *PIT1/GHF1* gene (results not shown). Further experiments will be necessary to clarify its transcription effect on the rat *PIT1/GHF1* promoter.

The silencer activity in the human *PIT1/GHF1* gene is not mediated solely by NF-1, as deletion of the upstream NF-1-binding site did not completely abolish the repression activity. It is most likely that NF-1 interacts with proteins binding to regulatory elements located in the immediate vicinity of the NF-1-binding sites (degenerate GRE or Pit-1/GHF-1-binding sites) or located far downstream in the proximal regulatory region or

in the promoter region. A further analysis of Pit-1/GHF-1 binding to the composite GHF-1c site and the identification of adaptor proteins interacting with Pit-1/GHF-1 would permit a better understanding of the functional mechanism of this silencer in the control of *hPIT1/GHF1* gene expression. This mechanism might require a combined interaction between activators or repressors of transcription, chromatin-associated proteins and components of the basal transcription machinery. Implication of nucleosomal structure in the regulation of the MMTV promoter by NF-1 has been widely documented [42–45]. Our results allow us to hypothesize that the transcription of the *hPIT1/GHF1* gene might be controlled by at least two regulatory regions, a proximal promoter and a distal silencer. The formation of chromatin loops between these two regions, approx. 10 kb distant, might be required for the effective transcriptional control of *hPIT1/GHF1* gene expression. The analysis of the structure and function of the proximal regulatory region should contribute further to the elucidation of the complex mechanism controlling the tissue-specific expression of the human *PIT1/GHF1* gene.

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