Transcription factor FOXF1 regulates growth hormone variant gene expression

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Lomenick, Jefferson P., Michael A. Hubert, and Stuart Handwerger. Transcription factor FOXF1 regulates growth hormone variant gene expression. Am J Physiol Endocrinol Metab 291: E947–E951, 2006.First published June 13, 2006; doi:10.1152/ajpendo.00128.2006.—Deletion analysis of the human growth hormone variant (GHV) promoter in transient transfection studies in BeWo choriocarcinoma and HepG2 cells indicated that the region extending from nt −158/−57 retained full transcriptional activity. DNase I footprint analysis of the fragment revealed a protected region at nt −82/−77, which is in a putative FOXF1/FOXF2 binding site. Supershift assays using an antiserum to human FOXF1 demonstrated that the protected region binds FOXF1. Overexpression of FOXF1 in BeWo and HepG2 cells induced the GHV promoter, whereas overexpression of FOXF2 was without effect. Mutagenesis of the FOXF1/FOXF2 site reduced basal promoter activity by 50–60% and markedly attenuated transactivation of the promoter by FOXF1. These studies indicate that FOXF1 induces GHV expression by interaction with a FOXF1/FOXF2 cis-element in the proximal promoter.

Forkhead box F1; placenta; transcription; promoter activity

The human growth hormone variant (GHV) gene is a member of a superfamily of closely related genes that includes pituitary growth hormone (hGH-N), the placental lactogens (hPL-A, hPL-B, and hPL-L), and prolactin (hPRL). Molecular studies suggest that the genes evolved from a common ancestral precursor (10). The hGH and hPL genes are arranged on a 66-kb locus of the long arm of chromosome 17 (q22-q24), whereas hPRL is located on chromosome 6. The genes of the hGH/hPL polymorphic locus are organized from 5′ to 3′ in the order hGH-N, hPL-L, hPL-A, GHV, and hPL-B. Each gene encodes a mature protein of ~200 amino acids. Gene expression is tissue specific: GHV and the three isoforms of hPL are expressed exclusively in the placenta, and hGH-N expression is restricted to the pituitary gland.

GHV is first detected in maternal blood at 10–12 wk gestation and increases gradually during the course of pregnancy. The hormone has both somatotropic and lactogenic actions, and numerous studies strongly suggest that GHV is important in the regulation of growth and development (10, 13). GHV binds to the growth hormone receptor with equal affinity as hGH-N (10), and the pattern of maternal plasma levels of insulin-like growth factor I (IGF-I) parallels the levels of GHV (4, 14, 15). hGH-N, however, is not detected in the fetal blood at any time during pregnancy, indicating that the physiological effects of the hormone on fetal growth and metabolism are mediated through actions on maternal or placental tissues (10).

At present, little is known about the transcriptional regulation of GHV gene expression. GHV transcript levels increase with differentiation of cytotrophoblast cells in vitro (8, 21), and agents that stimulate or inhibit cytotrophoblast differentiation modulate GHV expression. For example, GHV transcript levels and secretion increase after stimulation of trophoblast differentiation by cAMP (1), retinoids, or peroxisome proliferator-activated receptor (PPAR) ligand (21). Conversely, overexpression of copper/zinc superoxide dismutase, which inhibits trophoblast differentiation, decreases GHV expression (8). Glucose has been shown to inhibit GHV secretion in vitro in term placental explants and in trophoblast cells in culture (19). In addition, oral glucose administration to women with gestational diabetes decreases plasma GHV concentrations (2). The release of GHV is stimulated by thyroid hormone (17), but growth hormone-releasing hormone does not affect GHV secretion in vivo (6) or in vitro (7).

In this study, we used transient transfection studies, DNase I footprinting, gel shift and supershift assays, and site-directed mutagenesis to determine cis-elements and trans-acting factors that regulate GHV gene expression. The findings strongly suggest a role for FOXF1 (forkhead box F1, also called FREAC1) in trans-activation of the GHV promoter.

Materials and Methods

Cell cultures. BeWo human choriocarcinoma cells (American Type Culture Collection, Manassas, VA), which are known to express GHV (16), were grown in Ham’s F-12 medium with 2.0 mM l-glutamine and 15% fetal bovine serum (FBS). HepG2 cells (American Type Culture Collection) were grown in DMEM with 2.0 mM l-glutamine, 1.5 g/l sodium bicarbonate, 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate, and 10% FBS. A cell line of immortalized first-trimester human trophoblast cells (HTR-8/SV-neo), kindly provided by Dr. Charles Graham (University of Western Ontario, London, ON, Canada), was grown in RPMI with 5% FBS containing penicillin and streptomycin (9). Earlier studies demonstrated that first-trimester trophoblast cells express GHV (7). A highly enriched fraction of human cytotrophoblast cells was prepared by enzymatic digestion of a third-trimester placenta followed by purification with immunomagnetic beads coupled to an antiserum to human CD9 (5). The cells were cultured in DMEM with 10% FBS containing penicillin, streptomycin, and amphotericin B for 3 days, at which time >95% of the mononuclear cytotrophoblast cells had aggregated and fused to form a multinucleated syncytiotrophoblast.

Cloning of the 5′-flanking region of the GHV gene. The sequence of the GHV promoter has been previously described (18) and is highly

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trophoblast cells after 1 and 2 days of culture (40 mM EDTA, 75 mM KCl, 12% glycerol, 0.5 mM DTT, and 0.2 mM PMSF. DNA sequencing. The underlined sequence indicates the nucleotides in the consensus binding site. The sequence of the mutated binding site was confirmed by DNA sequence analysis. Mutations were created in the FOXF1/FOXF2 binding site of pGHV(−158/+57)-Luc using the Quick Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The FOXF1/FOXF2 site nt −82 to −77 was changed to TAAACAT to GCTCTGC. The underlined sequence indicates the nucleotides in the consensus binding site. The sequence of the mutated binding site was confirmed by DNA sequencing.

Expression plasmids. Expression plasmids for FOXF1 and FOXF2 (pEV-FOXF1 and pEV-FOXF2) were kindly provided by Dr. Peter Carlsson (Goethe University, Gothenburg, Sweden) (12).

Transfection studies. Transient transfection studies of human BeWo and HepG2 cells were performed in triplicate by the liposome method (5). Briefly, cells were incubated in a humidified atmosphere of 5% CO2 at 37°C with plasmid-liposome complexes composed of 4 independent transfection assays. Luciferase activities of the plasmids containing the GHV promoter fragments. pGHV(−610/+57)-Luc was created by excision of pGHV(−610/+57)-CR by double digestion with KpnI and XhoI and ligation into the KpnI and XhoI sites of pGL3 basic using T4 DNA ligase. Additional 5’ deletions of the GHV promoter were made by PCR of the 667-bp GHV promoter fragment. These fragments spannt −483/+57, −398/+57, −274/+57, and −158/+57. The PCR products were ligated into pCR2.1 and then into pGL3 basic. The orientation and sequence of each construct were confirmed by DNA sequence analysis.

Preparation of nuclear extract. Nuclear extracts were prepared from BeWo cells, HTR-8/SV-neo cells, HepG2 cells, and primary cultures of human trophoblast cells, as previously described (5). The protein concentration of each extract was determined by the Bradford assay (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin as a standard.

DNase I footprinting. DNase I footprinting was performed as previously described (3). Briefly, a 5’ end-labeled probe of the 215-bp GHV promoter region was generated by PCR using the 667-bp GHV promoter as a template. The DNA binding reaction was performed on ice in a buffer containing 10 mM Tris pH 7.5, 5 mM MgCl2, 50 mM EDTA, 75 mM KCl, 12% glycerol, 0.5 mM DTT, and 0.2 mM PMSF. A nuclear extract prepared from primary cultures of normal human trophoblast cells after 1 and 2 days of culture (40 μg), HTR-8/SV-neo cells (40 μg), BeWo cells (40 μg) or BSA (20 μg, Sigma, St. Louis, MO) was incubated with 2 μg of poly(dI-dC) (Roche, Basel, Switzerland) for 30 min. The DNA probe (20,000 dpm) was added, and the incubation was continued for 1 h. DNase I (Promega) digestion was then performed for 1.5 min at room temperature using different concentrations of DNase I (for BSA: 0.06, 0.12, and 0.3 units DNase I reaction; for nuclear extracts: 2.5 and 3.5 units/reaction), and the DNA fragments were separated on 6% polyacrylamide, 7 M urea sequencing gels. A G/A ladder of the same probe, cleaved at guanine and adenosine nucleotides with dimethyl sulfate and piperidine, was used as a size marker. Protected regions were detected by comparing the digestion patterns with the nuclear extract to that of control reactions using BSA.

RESULTS

Deletion analysis of the 5’-flanking region of the GHV gene. Transient transfection studies using deletion fragments of the 5’-flanking region of the GHV gene were used to identify putative DNA sequences on the GHV promoter that regulate GHV gene expression (Fig. 1). The studies were performed in BeWo and HepG2 cells with fragments of the 5’-flanking region of the GHV gene extending from nt −610, −483, −398, −274, or −158 to nt +57 (relative to the transcription start site) that were ligated upstream of a luciferase reporter gene. As shown in the experiment in BeWo cells depicted in Fig. 1, the nt −610/+57 fragment pGHV(−610/+57)-Luc supported a high level of reporter expression that was sixfold higher than that of other plasmids. A pGL3 control plasmid was used as an internal control.

![Fig. 1](http://ajpendo.physiology.org/)
higher than that of the promoterless vector alone (pGL3 basic). The transcriptional activity conferred by the nt −158/+57 fragment in three separate experiments in BeWo cells was 40.7% greater than that conferred by the nt −610/+57 fragment. The activities of the −398/+57 and −274/+57 fragments were almost identical to that of the −610/+57, and the luciferase activity of the −483/+57 fragment was −50% less than that of the −610/+57 fragment. These data indicate that the nt −158/+57 fragment retains full stimulatory activity for GHV gene expression and that the region of the promoter between nt −483 and −398 contains one or more inhibitory elements. Nearly identical results were observed with the deletion fragments in HepG2 cells (data not shown).

**DNase I footprinting of the proximal GHV promoter.** To identify specific regions of the 215-bp GHV promoter fragment that bind nuclear proteins, DNase I footprinting analyses were performed using a 32P-labeled fragment of the proximal GHV gene (nt −158/+57; Fig. 2) and nuclear extracts of human trophoblast cells, HTR-8/SV-neo immortalized human first-trimester trophoblast cells, and BeWo cells. One protected region (FP3) and one hypersensitive site (FP2) were observed in all three cell types. FP2 is located at nt −28/−23, and FP3 is located at nt −82/−77. A third protected region was detected using the nuclear extract from BeWo but not trophoblast cells. Computer analysis of the GHV proximal promoter region indicated that FP3 is located in a putative binding site for FOXF1/FOXF2 and that FP2 is located in a putative binding site for myocyte enhancer factor 2 (MEF2). The MEF2 binding site is known to bind MEF2A and other members of the MEF family, and the FOXF1/FOXF2 site is known to bind both FOXF1 and FOXF2. Subsequent studies focused on the FOXF1/FOXF2 site.

**Gel shift and supershift studies of the proximal GHV promoter.** To determine whether the putative binding site for FOXF1/FOXF2 binds transcription factors, gel shift assays were performed using 32P-labeled double-stranded oligonucleotides spanning the FP3 sequence (Fig. 3). A specific DNA-protein complex was formed when the 32P-labeled FP3 oligonucleotide was reacted with the nuclear extracts of BeWo and HepG2 cells. The formation of the complex was competed by an unlabeled FP3 oligonucleotide. Supershift analysis using a FOXF1 antiserum revealed the presence of a supershifted band with both nuclear extracts, whereas incubation of the nuclear extract-probe complexes with normal rabbit IgG did not result in a supershift.

**Transfection studies with FOXF1 and FOXF2.** To investigate whether FOXF1 trans-activates the proximal GHV promoter containing the putative FOXF1/FOXF2 binding site, transient transfection studies were performed in HepG2 cells using pGHV(−158/+57)-Luc alone and in combination with expression plasmids for FOXF1 (pEV-FOXF1) or FOXF2 (pEV-FOXF2) (Fig. 4). The cells cotransfected with pEV-FOXF1 at 0.4, 1.0, and 4.0 μg expressed 4.4-, 4.8-, and 8.4-fold more luciferase activity than the cells transfected with pGHV(−158/+57)-Luc alone (Fig. 4A). However, the luciferase activity of the cells cotransfected with FOXF2, which has been shown to bind to the same DNA sequence as FOXF1 (12), was not statistically different than that of cells transfected with pGHV(−158/+57)-Luc alone (Fig. 4B).

Mutation of the FOXF1/FOXF2 binding site in pGHV(−158/+57)-Luc caused a 26% decrease in basal promoter activity, and cotransfection of the mutated plasmid with the pEV-FOXF1 expression plasmid had little or no effect on luciferase activity (Fig. 5). These data strongly suggest that FOXF1 but not FOXF2 trans-activates the GHV promoter and that activation of the promoter by FOXF1 occurs by binding to a FOXF1/FOXF2 element.

**DISCUSSION**

The results of this study strongly suggest that cis-elements in the proximal 158 bases of the GHV promoter confer full basal activity. The region of the promoter that extends from nt −158/−610 confers less transcriptional activity (albeit still 3- to 6-fold greater than empty vector) than the nt −158/+57 fragment, suggesting that the region of the promoter upstream of nt −158 may contain one or more inhibitory elements. It is possible that such inhibitory elements render the FOXF1/FOXF2 site functionally inactive and are involved with repres-
sion of GHV in vivo in nonplacental cells that normally do not express this gene. However, the finding of similar patterns of transcriptional activity in the deletion analysis in BeWo cells (which express GHV) and HepG2 cells [which do not express GHV (Lomenick JP and Handwerger S, unpublished observation)] makes this less likely. Additional in vitro and in vivo studies are needed to determine the molecular mechanisms involved in cell-specific expression of GHV.

Several lines of evidence indicate that the FOXF1/FOXF2 binding site within the proximal promoter region of the GHV gene is essential for maximal basal gene expression. DNase I footprinting studies using nuclear extracts of trophoblast cells revealed that a putative binding site for FOXF1/FOXF2 was protected. In addition, gel shift assays indicated that an oligonucleotide containing a consensus FOXF1/FOXF2 motif bound one or more proteins present in nuclear extracts of BeWo and HepG2 cells. Supershift experiments revealed that the complex formed by interaction of the oligonucleotide and the nuclear extracts was supershifted by an antiserum to human FOXF1 but not by normal rabbit IgG. Furthermore, fragments of the GHV promoter containing a mutation of the FOXF1/FOXF2 motif that interfered with protein binding in gel mobility studies (data not shown) conferred less transcriptional activity than the wild-type promoter. Finally, transient transfection studies using expression plasmids that were shown to induce FOXF1 protein level in BeWo and HepG2 cells revealed that FOXF1 trans-activates the GHV promoter containing an intact FOXF1/FOXF2 binding site but not the promoter with a mutation in the FOXF1/FOXF2 binding site.

The forkhead family of transcription factors is a well-described group of DNA-binding proteins that are present in many organisms (11, 12, 20). Two family members, FOXF1 and FOXF2, are expressed in humans only in lung and placenta (20). The DNA binding sites for both transcription factors are very similar or identical (12) and share a core sequence of RTAAAYA (20). The DNA binding sites for both transcription factors are very similar or identical (12) and share a core sequence of RTAAAYA (20). Nevertheless, FOXF2 in our study did not trans-activate the GHV promoter.

Differential activation of a promoter by FOXF1, but not FOXF2, has been observed before. For example, the promoters for the lung-specific proteins surfactant protein B and Clara cell 10-kDa protein contain FOXF1 sites that are differentially regulated. Surfactant protein B is trans-activated by both FOXF1 and FOXF2, whereas the Clara cell 10-kDa protein is trans-activated only by FOXF1 (12). These findings emphasize the importance of interactions other than DNA binding for the specificity of FOX proteins.

In summary, we have shown that the proximal GHV promoter contains a FOXF1/FOXF2 binding site that is important for trans-activation. FOXF1, but not FOXF2, binds to the cis-element and plays a critical role in the regulation of the GHV gene in our in vitro experiments. Given that the GHV and hGH-N promoters are more than 95% homologous, it is possible that these same transcription factor binding sites are involved in the regulation of hGH-N as well. Indeed, the...
FOXF1/FOXF2 site on the two promoters differs in only one base. Additional experiments are needed to clarify the role of FOXF1 in hGH-N regulation, as well as in GHV expression in vivo.

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REFERENCES


