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Human *growth hormone receptor* gene expression is regulated by Gfi-1/1b and GAGA *cis*-elements

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ABSTRACT

Human *growth hormone receptor* (hGHR) gene regulation is complex: mRNAs are transcribed from multiple variant (V) 5′UTR exons, several ubiquitously while others only in the postnatal hepatocyte. The liver-specific V1 exon promoter contains Gfi-1/1b repressor sites adjacent to a GAGA box, a GH response element (GHRE) in several mammalian genes. GAGA boxes are also present in the ubiquitously expressing V3 exon promoter. Heterologous sites in bovine, ovine and murine *GHR* genes suggest conserved roles. GAGA factor stimulated V1 and V3 promoters while Gfi-1/1b repressed basal and GAF-stimulated V1 transcription. HGH treatment of HepG2 cells resulted in a new complex forming with V3 GAGA elements, suggesting a functional GHRE. Data suggest liver-specific V1 transcription is regulated by inhibitory Gfi-1/1b and stimulatory GAGA *cis*-elements and Gfi-1/1b may control the lack of V1 expression in fetal liver, hepatic tumours and non-hepatic tissues. In addition, hGH may regulate *hGHR* expression through V3 GAGA boxes.

1. Introduction

Growth hormone (hGH) is essential for normal growth as well as protein, carbohydrate and lipid metabolism (Veldhuis et al., 2005). It acts on target cells by binding to its dimerized cell surface receptor (hGHR), leading to phosphorylation of the associated tyrosine kinase, JAK2, as well as the receptor itself, and subsequent activation of specific signalling pathways that mediate the hGH actions (Brooks et al., 2007; Lichanska and Waters, 2008b; Waters et al., 2006). As clearly evidenced in individuals with Laron syndrome, the level of hGHR expressed is the initial factor influencing hGH effectiveness at the target cell. Many developmental- and tissue-specific changes in hGHR expression have been observed (Goodyer et al., 2001a; Hill et al., 1992; Simard et al., 1996; Wei et al., 2006; Werther et al., 1993; Zogopoulos et al., 1996b,a). One of the most striking is a 4–6 fold increase in *hGHR* mRNA in the postnatal compared to the fetal liver paralleled by a 4 fold increase in ^{125}I-hGH binding; in contrast, there are postnatal decreases in *hGHR* transcripts in the lung, kidney and small intestine (Goodyer et al., 2001a).

The *hGHR* gene is located on the short arm of chromosome 5 (Barton et al., 1989; Godowski et al., 1989; Leung et al., 1987). There are nine coding exons (exons 2–10) while the 5′ flanking region contains thirteen 5′ untranslated region (5′UTR) first exons (Supplementary Fig. 1A) (Godowski et al., 1989; Goodyer et al., 2001b; Pekhletsky et al., 1992; Wei et al., 2006). Transcripts arising from these variant (V) 5′UTR exons all splice into the same site 11 bp upstream of the translation start site in exon 2 and, thus, code for the same protein. Seven of the 5′UTR exons are clustered in two small (<2 kb) regions: Module A contains V2, V3 and V9 while Module B has V1, V4, V7 and V8 (Supplementary Fig. 1A) (Goodyer et al., 2001b; Orlovskii et al., 2004; Pekhletsky et al., 1992; Wei et al., 2006). The mRNA expression profiles of the exons in these two clusters are strikingly different. Module A-derived *hGHR* transcripts (as well as those from V5 and V_A–V_E) are detectable in multiple human tissues by the third month of fetal life and are ubiquitously expressed in postnatal tissues. In contrast, the four Module B-derived mRNAs are restricted to the normal postnatal liver, first detectable ∼3–4 months after birth (Goodyer et al., 2001b; Wei et al., 2006; Zogopoulos et al., 1996a). The induction of Module B transcripts in the postnatal liver may, in fact, play a major role in the dramatic increase in both mRNA and protein levels of hGHR in hepatocytes after birth (Goodyer et al., 2001a). Therefore, we

Abbreviations: hGHR, human growth hormone receptor; GHRE, growth hormone response element; GAF, GAGA binding factor; GAF-519, drosophila GAF; 5′UTR, 5′untranslated region; V, variant; TSS, transcriptional start sites; Gfi-1, growth factor independence factor 1; β2m, beta-2 microglobulin; EMSA/EMSSA, electrophoretic mobility shift/supershift assays; ChIP, chromatin immunoprecipitation.

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have been very interested in what regulates the developmental- and tissue-specific expression pattern of the Module B transcripts.

V1 is the most highly expressing Module B exon in the human adult liver, likely because it has two active TATA boxes and transcriptional start sites (TSS); the other three Module B exons have non-consensus TATA elements (Goodyer et al., 2001b, 2008; Pekhletsky et al., 1992; Wei et al., 2006). Previous studies have shown that the region ~300 bp upstream of the second V1 TSS has significant promoter activity and that an HNF4α site within this region regulates V1 hGHR expression (Goodyer et al., 2008). However, we also observed a striking increase in V1 transcriptional activity when this 300 bp region was removed, suggesting the presence of inhibitory *cis*-elements close to the two TSS (Goodyer et al., 2008). Using the transcription factor scanning program, MatInspector, we identified in this region two putative binding sites for the transcriptional repressors, growth factor independence factor 1 (Gfi-1) and Gfi-1b, and one putative GAGA box, a known GH response element (GHRE) in several mammalian genes (Grimes et al., 1996; Kazanjian et al., 2006; Le Cam et al., 1994; McGhee et al., 2003; Wyse et al., 2000). The most proximal Gfi-1/1b site as well as the GAGA element are conserved in the ovine and bovine *GHR* genes while the mouse *GHR* has different transcriptional repressors but also GAGA elements in the same region (Supplementary Fig. 2A and Table 1A).

No Gfi-1/1b response elements were detected in the Module A promoter regions. However, the V3 promoter and exon were found to contain three putative GAGA boxes (Supplementary Fig. 2B and Table 1B). We observed a GAGA element in the mouse *GHR* gene, in a region homologous to the first two GAGA sites in the V3 promoter (Moffat et al., 2000), suggesting that this element may also be conserved; unfortunately, no corresponding genomic sequences are available for the ovine and bovine *GHR* genes. hGH is well-known to positively regulate expression of its receptor (Carter-Su et al., 1996; Mullis et al., 1991; Nuoffer et al., 2000). However, several transcription factor scanning programs have been unable to detect consensus STAT5 binding sites in up to 2 kb of promoter regions of the major V1, V2, V3 or V9 exons in Modules A and B. Because of increasing evidence that many hepatic genes are regulated by GH independently of STAT5b (Lichanska and Waters, 2008a; Rowland et al., 2005; Verma et al., 2005; Vidal et al., 2007), we speculated that hGH might act through alternative *cis*-elements, including these GAGA boxes. In the present study, the V1 and V3 promoters were examined in parallel to determine the functional relevance of the GAGA response elements in regulating either tissue-restricted (V1) or ubiquitous (V3) *hGHR* expression.

GAGA elements were identified first in drosophila gene promoters and subsequently in the gene promoter regions of other species, including mammals (Adkins et al., 2006; Lehmann, 2004). A BTB-POZ/single zinc finger protein called GAGA binding factor (GAF) binds to the GAGA boxes in drosophila genes and regulates both chromatin remodelling and transcriptional activation (Nakayama et al., 2007; Omichinski et al., 1997; Pedone et al., 1996; Shimojima et al., 2003; Wilkins and Lis, 1998). Functional GAGA elements have been identified on the promoters of several vertebrate genes (e.g. rat *Spi 2.1* (Le Cam et al., 1994), human and rat *Type I angiotensin receptor* (Wyse et al., 2000), rat *vasopressin V1b receptor* (Volpi et al., 2002), rat *carbamyl phosphate synthetase I* (Goping et al., 1995), human *α1(V) collagen* (Lee and Greenspan, 1995) and rat *CD44* (Kim et al., 2005)). In the case of the *vasopressin V1b receptor* gene, this element is activated by drosophila GAF (GAF-519), while in the *Spi 2.1* and the *Type I angiotensin receptor* genes, the GAGA box acts as a GHRE.

Gfi-1 and Gfi-1b are related transcriptional repressors expressed in numerous tissues (Grimes et al., 1996; Kazanjian et al., 2004; Kazanjian et al., 2006; McGhee et al., 2003; Moroy, 2005; Phelan et al., 2010; Shroyer et al., 2005). They regulate cell fate determination and differentiation and play an important role linking cytokine signalling and transcriptional regulation, especially within the hematopoietic system (Bjerknes and Cheng, 2010; De La Luz et al., 2007; Hock and Orkin, 2006; Phelan et al., 2010; Rathinam and Klein, 2007; Zhu et al., 2002, 2006). These zinc finger transcription factors bind as monomers to a common response element in their target gene promoters and repress transcription via the interaction of their SNAG domains with corepressors, histone deacetylases and histone demethylases (Kazanjian et al., 2006; Lee et al., 2010; McGhee et al., 2003; Saleque et al., 2007; Vassen et al., 2005; Vassen et al., 2006). Known targets include *SOCS1* and *SOCS3*, negative regulators of GH signalling (Doan et al., 2004; Jegalian and Wu, 2002; Rathinam and Klein, 2007).

In the present study, using co-transfection, site-directed mutagenesis, EMSA/EMSSA, ChIP and qRT-PCR assays, we have demonstrated that the Gfi-1/1b and GAGA *cis*-elements in the V1 and V3 promoters are functional. Our data suggest that: (1) the liver-specific *hGHR* V1 promoter is regulated by both inhibitory Gfi-1/1b and stimulatory GAGA response elements; (2) Gfi-1 and/or Gfi-1b may be responsible for the lack of *hGHR* V1 expression in fetal liver, hepatic tumours and non-hepatic tissues; and (3) hGH may regulate ubiquitous *hGHR* expression by V3 GAGA boxes.

2. Materials and methods

2.1. Tissues

Human fetal liver tissue (13.75 wk fetal age) was obtained at the time of therapeutic abortion following written consent and with the approval of the local ethics committees in compliance with Canadian Institutes of Health Research guidelines. Isolated fetal hepatocytes and blood cells were prepared as previously described (Zogopoulos et al., 1996a; Goodyer et al., 2001a).

2.2. Plasmid construction

Human genomic DNA was isolated from a Bac clone (hcit. 102E14) (Goodyer et al., 2001b). Specific DNA regions were isolated using restriction enzymes and cloned upstream of a luciferase vector, pA_3luc (Supplementary Fig. 1B) (Goodyer et al., 2003; Goodyer et al., 2008). All constructs were verified by sequencing prior to use (RSVS, Université de Laval, QC, Canada). The Gfi-1 and Gfi1b expression vectors were obtained from Dr. H.L. Grimes (University of Louisville, KY) (Grimes et al., 1996), the GAF-519 expression vector from Dr. G. Aguilera (NICHD, NIH, Bethesda, MD) (Volpi et al., 2002), the *Spi 2.1*-luc reporter vector from Dr. Nils Billestrup (Novo-Nordisk, Bagsvaerd, Denmark), the STAT5B expression vector from Dr. Vivian Hwa (Oregon Health and Sciences University, Portland OR) and the Myc/His-hGHR expression vector from Dr. Françoise Conte (INSERM U563, Toulouse, France).

2.3. Site-directed mutagenesis

Mutations were introduced into Gfi-1b sites (in the V1P4 and V1P5 constructs and V1 EMSA probes) and into GAGA elements (in the V1P5 and V3P3 constructs and V1 and V3 EMSA probes), using the Quikchange Site Directed Mutagenesis kit (Stratagene, La Jolla, CA) and the primers listed in Supplementary Tables 2 and 3. Presence of the mutations was confirmed by sequencing prior to use.

2.4. Cell culture and transfections

HEK293 and HepG2 cells (ATCC, Bethesda, MD) were cultured in DMEM (Invitrogen Corporation, Mississauga, ON) supplemented with 10% FBS and antibiotics and maintained at 37 °C in 5% CO_2. 1×10^5 cells were seeded in 12 well plates 24 h prior to transfection. Cells were transfected with 0.5 μg of *hGHR* promoter/reporter, *Spi 2.1* promoter/reporter or empty reporter vectors, 0.1 μg of pSV-β-galactosidase (Promega, Beverly, MA), 25 or 100 ng of the STAT5B expression vector, 1–20 ng of Gfi-1 and Gfi-1b expression vectors or 0.001–0.1 μg of the GAF-519 expression vector; total DNA per well was made up to 1 μg with sp64 and transfected with Polyfect (Qiagen, Mississauga, ON). Cells were harvested 48 h later and the EG&G MicroLumatPlus bioluminometer used to measure luciferase and β-galactosidase activities (Tropix Galacton-Star, Bedford, MA). Data are expressed as a ratio of luciferase over β-galactosidase activity and normalized to the empty reporter vector.

HEK293 and HepG2 cells were treated with hGH (50 or 150 ng/ml; Sigma, Mississauga, ON), insulin (10 and 100 nM; Novo-Nordisk, Mississauga, ON) or IGF-1 (10 or 100 nM; Sigma) for 10 min before harvesting for nuclear extracts and EMSA/EMSSA assays. Although both cell lines expressed *hGHR* mRNA and protein, as determined by RT-PCR assays and Western blots, respectively (data not shown), the level of GHR in the HEK293 cells was low. Therefore, when we studied the effect of hGH on *hGHR*