GH is a regulator of *IGF2* promoter-specific transcription in human liver

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Abstract

The regulation of the insulin-like growth factor-II gene (*IGF2*) is complex and involves the usage of four promoters resulting in different 5' untranslated regions, but with a common translated product. The *IGF2* gene product is a mitogenic and survival factor that has been suggested to be important for a normal fetal development and cancer. In this paper we present evidence suggesting that the human *IGF2* gene is regulated by GH, and that this regulation occurs in a promoter-specific way. Three lines of evidence support this finding. First, *in vivo* data from patients treated with GH (one injection or daily injections for 5 consecutive days) showed an increase in the *IGF2* P2 promoter derived transcript after acute

treatment, and of the P4 promoter transcript after short-term treatment while the P1 promoter derived transcript did not show any significant change. Secondly, isolated human liver cells treated with GH for 2 h displayed an upregulation of the P2 promoter derived transcript. Thirdly, employing transfection experiments in GH-receptor positive CHO cells with P2 and P4 promoter-luciferase constructs, an upregulation by GH was evident, while a P1 promoter construct was unresponsive. We suggest that GH may be a physiological regulator of *IGF2* in humans.

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Introduction

The protein product of the *IGF2* gene, insulin-like growth factor-II (IGF-II), belongs to the same family of hormonally active polypeptides as insulin-like growth factor-I (IGF-I), proinsulin and relaxin, and circulates in serum concentrations approximately three times higher than IGF-I (Sara & Hall 1990). The production of the peptide persists in the adult human, in contrast to rats where IGF-II decreases after birth. The primary function of IGF-II in adult life remains to be elucidated. As a mitogen it is important for normal development of the fetus and placenta (Ohlsson *et al.* 1989). The cellular action of IGF-II is mediated through the tyrosine kinase receptors for insulin (insulin receptor A) and IGF-I (IGF type 1 receptor) (Frasca *et al.* 1999). It also binds to the 250 kDa IGF-II/mannose-6-phosphate receptor (M6P/IGF-IIR), which has been regarded as a concentration-lowering device for circulating IGF-II and mannoseylated proteins, and that recirculates between the plasma membrane and intracellular compartments (Brulke 1999). These receptors significantly regulate basal activity regarding

glucose homeostasis and growth-promoting, anabolic, activities (Jones & Clemmons 1995).

The *IGF2* gene is 30 kb in length, consists of nine exons and is transcribed from four promoters, P1–P4 (Sussenbach 1989, van Dijk *et al.* 1991). These are being used in a tissue- and developmental-specific manner. Four main transcripts with different 5' untranslated regions are generated, with a common translated sequence consisting of exons 7, 8 and the first part of exon 9. A fifth transcript generated from P2 including an exon called 4b has also been described (Ikejiri *et al.* 1991). The P2–P4 promoters are active throughout fetal development in most tissues (Gray *et al.* 1987, Ekström *et al.* 1995), including the liver where the P3 promoter is being highly expressed (Li *et al.* 1996). P3 is downregulated in the liver shortly after birth and P1 is turned on and gradually upregulated with age, and finally reaches a relative contribution of 50–70% to the total *IGF2* transcripts (Li *et al.* 1996). The activity of the P4 promoter is relatively high during human development (Li *et al.* 1996), and in the adult liver it accounts for the production of 25–60% of total *IGF2* mRNA. The relative contribution of the P2 promoter transcript is generally

quite low in human liver, approximately 10% (Li *et al.* 1996). Although four promoters are involved in the transcription of the *IGF2* gene, it appears that they all produce an identical final protein product.

The nascent protein product of the *IGF2* gene is the pre-pro form of IGF-II, which after processing, comprises 67 amino acids. The efficiency of translation is different for the multiple transcripts, and the mRNA from the P4 promoter has been shown to be constitutively translated, whereas the P3 generated transcript is subject to a growth-dependent translation, at least in some cell lines and in fetal liver (Moor *et al.* 1994, Nielsen & Christiansen 1995, Nielsen *et al.* 1995). In addition, the hepatocellular carcinoma cell line, Hep3B, shows a variability in *IGF2* transcription in relation to different growth conditions, i.e. serum deprivation leads to a marked increase in P3 transcription (Scheper *et al.* 1996).

The 'housekeeping' function at the translational level is also true for the transcriptional level, and certain features can be seen in the promoter nucleic acid composition. While the highly GC-rich P3 promoter contains both a TATA box and a CCAAT box in addition to other transcription factor binding sites, the P4 promoter region has four SP-1 sites but lacks both TATA or CCAAT boxes (van Dijk *et al.* 1991), a feature common among housekeeping promoters.

In vitro studies using reporter gene constructs have provided information regarding putative transcription factor binding sites in the promoters of *IGF2*. The P1 promoter was shown to be upregulated by CCAAT/enhancer binding protein (C/EBP) (van Dijk *et al.* 1992), and this effect requires the presence of a SP-1 binding site (Rodenburg *et al.* 1997). The P3 promoter contains proximal elements supporting general transcription, as well as several AP-2 binding sites which were shown to be able to confer both activation and repression (Rietveld *et al.* 1999), and upstream sequences that direct cell-specific expression (Holthuisen *et al.* 1993). The regulation of the P2 promoter is unclear. Previous reports regarding regulation of *IGF2* promoters *in vivo* so far, have described levels of the various transcripts under normal and pathological conditions.

No details regarding specific *in vivo* responses of transcription from autocrine, paracrine or endocrine effector molecules have previously been described for *IGF2*, in contrast to reports regarding the *IGF1* gene transcription, which is closely regulated by growth hormone (GH).

In the present study, we have investigated possible effects of GH administration on *IGF2* transcription in human liver. A statistically significant increase of the P2 promoter transcript was observed after acute GH treatment, while the P3 promoter transcript was still virtually undetectable. A longer treatment increased the amount of P4 transcripts significantly. In addition, two more lines of evidence using *in vitro* systems support the importance of GH for the promoter-specific regulation of *IGF2*.

Materials and Methods

Experimental subjects and experimental procedure

The study involved 31 patients submitted for laparoscopic cholecystectomy. None of the patients were markedly obese, and none had any clinical or laboratory signs of hepatic, renal or thyroid disease. Informed consent was obtained from each patient, and the ethical aspects of the study were approved by the ethics committee of the Karolinska Institute.

The patients were subdivided into three groups. The 5-day (short term) treatment group of 11 patients were treated with human recombinant GH (12 IU daily subcutaneously (Genotropin, Pharmacia & Upjohn, Stockholm, Sweden)) at 0800 h for 5 consecutive days, with a bolus injection on the night before surgery 12 h preoperatively. Ten patients (acute GH treatment group) received a single injection of GH (12 IU) in the morning, approximately 4–5 h before surgery. The remaining ten patients comprised the control group. After introduction of the laparoscopic instruments in the abdomen, a liver biopsy (200–300 mg) was taken from the left liver lobe. The biopsies were immediately frozen and kept in liquid nitrogen until analyzed. Cholecystectomy was then performed without complications.

RNA isolation

Total RNA was extracted from parts of the liver biopsies according to the guanidinium iso-thiocyanate/acid phenol procedure (Chomczynski & Sacchi 1987).

Probe generation and RNase protection analysis

Exon-specific antisense RNA probes for *IGF2* promoter transcripts P1 (exon 1), P2 (exon 4) and P4 (exon 6) were generated using T3 and T7 RNA polymerase (Life Technologies, Stockholm, Sweden) according to previously published procedures (Li *et al.* 1996).

IGF1 transcripts were detected by using a probe made from a 169 bp DdeI fragment of the human IGF-IA cDNA, covering the translated region of bases 316–484 (Jansen *et al.* 1983), blunt cloned into the SmaI site of pGEM 3Z vector (Promega, Madison, WI, USA). The construct was linearized with BamHI before being transcribed with T7 RNA polymerase.

The *GAPDH* antisense RNA probe was generated from a plasmid template (Ambion, Austin, TX, USA) using T7 RNA polymerase (Life Technologies). For the *IGF1* and *IGF2* probes, ³²P-UTP with a specific activity of 400 Ci/mmol was used and for the *GAPDH* (glyceraldehyde phosphate dehydrogenase) probe the ³²P-UTP was diluted with unlabeled UTP to 80 Ci/mmol.

RNase protection analysis was performed using the RPA II kit as described by the manufacturer (Ambion).

For quantitation of the various transcripts, phosphor imager analysis (Fuji BAS-1000) was performed after PAGE using GAPDH as internal control.

Isolation of human primary liver cells and treatment with GH

Human liver tissue was obtained from a patient undergoing liver transplantation. The patient suffered from a genetic disease named familial amyloidotic polyneuropathy (FAP). The deposited amyloid is caused by mutated transthyretin, synthesized in the liver, in which valine is replaced by methionine in position 30. The disease is characterized by a progressive polyneuropathy that affects both the peripheral and the autonomic neuron systems. Our patient had no history of liver disease and all liver function tests were normal. Approval to use human liver specimens from patients undergoing liver transplantation was given by the Ethics Committee at Huddinge University Hospital.

Hepatocytes were isolated using a two-step perfusion technique utilizing EGTA and collagenase type XI-S, 12.5 mg/100 ml as described by Li *et al.* (1992). They were seeded on 60 mm dishes at a density of 3.5×10^6 cells per dish as previously described (Ellis *et al.* 1998). Dishes were pre-coated with 0.2 ml matrigel. The cells were then incubated in William's E medium (3 ml per dish) supplemented with glutamine (292 µg/ml), Na₂SeO₃ (173 ng/ml), insulin (2 IU/ml), penicillin G sodium (100 U/ml), streptomycin sulfate (100 µg/ml) and gentamycin (85 µg/ml). The medium was replaced every 24 h.

GH (500 ng/ml final concentration) was supplied to the liver cells in PBS in three sets of experiments, each with triplicate plates, starting the same day, 1 day after or 2 days after isolation. Control plates were supplied with an equal volume of PBS. Treatments were for 2, 4 and 21 h for each experimental set. The triplicates were pooled during RNA isolation, after the given incubation times, using the iso-thiocyanate/acid phenol procedure (Chomczynski & Sacchi 1987).

Reporter plasmid constructs

The control plasmid (Spi-TK-Luc) containing the firefly-luciferase open reading frame driven by a Herpes simplex virus thymidine kinase promoter (TK) containing six growth hormone response elements (GHRE) from the serine protease inhibitor 2.1 gene promoter was used as a positive control for GH response (Wood *et al.* 1997).

The IGF2 P1-luciferase construct was made from a 1266 bp SphI–EagI fragment spanning from –38 in exon 1 and upstream. The SphI site was blunted and the fragment was cloned into Bluescript SKII+ cut with SmaI/EagI. This clone was cut with EagI, blunted and cut with KpnI. The resulting fragment was cloned into the KpnI and

blunted XhoI site of the pGL2 basic luciferase vector (Promega).

The IGF2 P2-luciferase construct was made by first inserting a 1169 bp EcoRI–PstI fragment from –31 in exon 4 and upstream, into Bluescript SKII+. The fragment was cut with PstI, blunted and cut with KpnI. This fragment was inserted into the pGL2 luciferase vector, which had been cut with BglII, blunted and cut with KpnI.

The IGF2 P4-luciferase construct was made from a blunted AvaII fragment going from 27 bp downstream of exon 6 and upstream to just 3' of exon 5. This was cloned into Bluescript SKII+ cut with EcoRV. The insert was cut out from SKII+ with KpnI–BamHI, and cloned into the KpnI and BglII site of the pGL2 basic luciferase vector.

As transfection control, a plasmid containing the β-galactosidase open reading frame driven by the SV40 promoter/enhancer was used.

The plasmids were purified by using the Qiagen plasmid purification kit (Qiagen, Hilden, Germany).

Cell culturing

CHO-A cells (Chinese hamster ovary cells) with a stable integrant of the human GH-receptor (Norstedt *et al.* 1994) were cultured in Ham's F12 medium with L-glutamine (cat. no. 21765–029, Life Technologies), penicillin, 50 units/ml, streptomycin 50 µg/ml and 10% fetal bovine serum (Life Technologies).

The cell cultures were incubated at 37 °C, in a humidified atmosphere containing 5% CO₂.

Transfection and reporter assays

Cells were split from confluent 100 mm plates 1:10 into six-well plates. At approximately 80% confluency, the cells were washed with PBS and the medium was changed to serum-free. Transfection was performed with Lipofectamine Plus (Life Technologies) in Optimem (Life Technologies) according to the manufacturer's protocol, with the same transfection master mix in two six-well plates for each promoter construct, one plate for control and one for GH treatment. The amount of plasmid DNA was 0.5 µg/well for the IGF2 promoter constructs and 0.1 µg for the Spi-TK-Luc plasmid. Co-transfections were done with 0.1 µg of the pSV40 β-galactosidase plasmid/well.

Transfections were left for 3 h after which the medium was replaced with fresh serum-containing medium for 4–5 h. The medium was again replaced with serum-free medium and left overnight. In the morning, GH (1 µg/ml) (Genotropin) was added and left for 6 h. After treatment, the plates were aspirated and washed with PBS. A 0.3 ml volume of passive lysis buffer (Promega) was added and after 15 min the extract was collected,

Figure 1 Exon organization of the human *IGF2* gene. Open boxes show the 5' untranslated regions of *IGF2*. Black boxes are the common translated exons (exon 9 is partially translated). P1, P2, P3 and P4 denote the promoter regions. The regions of the probes used for promoter-specific RNase protection analysis are indicated.

centrifuged for 1 min, supernatants collected and frozen at -70°C before analysis.

Luciferase activity was measured with 20 μl extract and 100 μl reaction solution according to the manufacturer (Promega) in a Monolight 401 luminometer.

β -Galactosidase activity was measured with 50 μl of a tenfold diluted extract in a phosphate buffer using *o*-nitrophenyl β -D-galactopyranoside (X-Gal) (Sigma-Aldrich, Stockholm, Sweden) as cleavable substrate. The absorbance at 415 nm was measured and used to normalize the luciferase values.

Protein was measured with the Micro BCA reagents (Pierce, Chester, Cheshire, UK) in microtiter plates.

Statistical analysis

Results from biopsy material, described in Fig. 3, were analyzed statistically with Kruskal–Wallis one-way ANOVA using SigmaStat (Jandel Scientific), for P1/tot, P2/tot and P4/tot separately. In P2/tot, Dunn's *post hoc* test was applied while normality of the data failed, and for P4/tot Dunnett's method was used as *post hoc* test. Transfection data presented in Fig. 5 were evaluated between the control group and treated group using Student's unpaired two-tailed *t*-test. $P < 0.05$ was taken as significant.

Results

IGF-II transcription *in vivo* induced by GH

The patients in the study were treated with GH as described in Materials and Methods and in Olivecrona *et al.* (1999). Liver biopsies were taken for RNA preparation, and RNase protection analysis (RPA) of total RNA combined with phosphor imaging was used to quantitate the various *IGF2* promoter transcripts.

An *IGF2* cDNA probe from exon 9 was initially used to measure the total transcript level. This probe encompasses a sequence contained within all known *IGF2* promoter transcripts. No effect of GH could be seen when RPA

analysis at the total *IGF2* transcript level was performed (Olivecrona *et al.* 1999). Since the total *IGF2* mRNA in adult liver originates almost exclusively from promoters P1, P2 and P4, with P2 being a minor contributor, there was a possibility that the absence of GH effects on the total mRNA level would be masked by reciprocal changes at the promoter activity level. We therefore tested the individual promoter transcripts with RPA by using probes for exon 1 (P1), exon 4 (P2) and exon 6 (P4). Figure 1 depicts the exon organization and the positions of the probes used. Initial assays were performed with separate RPAs for the different probes (Fig. 2A). In subsequent assays, RPAs with combined probes were utilized (Fig. 2B). Calculations were made from phosphor imager analysis by relating the promoter transcript levels to the *GAPDH* transcript level, as well as calculating the ratio of the individual transcripts to the sum of the P1, P2 and P4 transcripts. By using this approach, the risk of errors due to individual patient differences in *GAPDH* expression was minimized. Analysis of the RPAs revealed that *IGF2* transcription, normalized to *GAPDH* in the *in vivo* system used, did not display any significant effect by GH (data not shown). However, when the ratio of the individual promoter transcripts to the sum of total *IGF2* transcripts was calculated, significant effects were obvious. The acute GH treatment resulted in a significant increase in the P2 promoter transcript (ANOVA, $P = 0.0007$) (Fig. 3). The P1 transcript did not display any statistically significant change. GH treatment for 5 consecutive days resulted in a significant increase in the P4 transcript level (ANOVA, $P = 0.002$) (Fig. 3). Thus, the response of *IGF2* regulation by GH in the *in vivo* human liver is not a change in the level of total mRNA, but rather an internal change in the utilization of the three active promoters.

Effects of GH in isolated human liver cells

To further substantiate the finding that GH changes *in vivo* transcription of *IGF2*, we found it necessary to turn to a more controllable test system. Isolated human liver cells from material obtained in conjunction with a liver

Figure 2 Representative samples of RNase protection analysis of *IGF2* promoter-specific transcripts. (A) The P1, P2 and P4 promoter transcripts and the internal control *GAPDH* were probed separately using RNase protection. (B) The four probes were used together in the same RNase protection mixture.

transplantation was used. Due to difficulties in accessing liver material, the experiment was only performed once, as a control for the *in vivo* data presented above. After perfusion with collagenase, three sets of plates were used. The experiments were started during 3 consecutive days in order to find a time frame giving the best response to GH treatment. Treatments were carried out with 500 ng/ml GH for 2, 4 and 21 h, with non-treated controls for every time point. mRNA level for *IGF1* versus *GAPDH* was employed in order to obtain a measure for the GH response. The results from RPA showed that the liver cells responded best at day 2 after perfusion, resulting in an approximately fourfold induction of *IGF1* mRNA after a 4 h GH treatment, as measured by RPA and phosphor imaging analysis (data not shown). The levels of the *IGF2*

Figure 3 *In vivo* effects in the liver on the P1, P2 and P4 promoter transcripts after GH treatment. RNase protection analysis was performed on liver RNA isolated from patients treated with GH once 4 h preoperatively and once every day for 5 consecutive days with the last treatment 12 h preoperatively. After analysis using phosphor imaging, the respective promoter transcript was calculated as the ratio of the total (Tot) *IGF2* transcripts. The results are in arbitrary numbers from the phosphor imager analysis. The bars denote the average result from each patient group, and the error bars denote S.E.M. * $P < 0.05$, using ANOVA as described in Materials and Methods; *** $P < 0.001$; n.s. = not significant; C = untreated; GH1 = one treatment with GH (acute); GH5 = treatment with GH for 5 consecutive days (short term). The analysis is based on the three patient groups, which contained 11 individuals in the short-term treatment group, 10 individuals in the acute group, and 10 in the control group, according to the Experimental procedure section.

promoters P1, P2, P4 and *GAPDH* mRNA were then analyzed by RPA in a similar way as for the *in vivo* liver samples shown in Fig. 2. The data for *IGF2* is presented as the relative amount of P2 compared with the total amount of P1, P2 and P4 (Fig. 4A–C) and P2/*GAPDH* (Fig. 4D). It should be noted in Fig. 4D that, the P2 value has been multiplied 100 times in order to get integer values, since the P2 transcript is present in substantially lower amounts compared with *GAPDH*. Compensation for relative isotope label in the three probes (i.e. length of probes and number of uridines) has not been performed since it would not change obtained results of the GH effects. The response of P2 gave an induction on day 2 of approximately 1.7–2.3-fold depending on the method of calculation (Fig. 4B and D). Although these data are from liver cells prepared from only one patient, and no statistical analysis could be performed, the treatments and analyses were done in three rounds during 3 consecutive days, each in triplicate plates, which were pooled for RNA isolation. The Figure presents two of these rounds; treatments starting at day 1 and treatments starting at day 2. On

Figure 4 Induction of the *IGF2* P2 promoter by GH in isolated human liver cells. Liver cells were treated with 500 ng/ml of GH for 2 h or 4 h at day 1 or day 2 after isolation. The *IGF2* P1, P2, P4 and *GAPDH* transcripts were analyzed using RPA and phosphor imager. Since the triplicates were pooled during the RNA isolation, no variance can be deduced. (A) The P1 transcript level calculated as the ratio of P2 to the level of P1, P2 and P4 transcripts. (B) The P2 transcript level calculated as the ratio of P2 to the level of P1, P2 and P4 transcripts. (C) The P4 transcript level calculated as the ratio of P2 to the level of P1, P2 and P4 transcripts. (D) The level of P2 transcript is presented as the ratio to the *GAPDH* level. The P2 values have been multiplied by 100 to obtain integer values, since P2 mRNA is present in comparatively lower amounts.

day 3 post cell isolation, the GH-induced response was much poorer as measured by *IGF1* induction (data not shown).

Transfection analysis and GH-mediated *IGF2* promoter responses

As a third line of evidence showing the *IGF2* promoter responsiveness to GH, transfection strategies in the CHO-A cell line, with a stable integration of the human GH-receptor gene driven by the cytomegalovirus (CMV) promoter, was employed. The fragments upstream of the *IGF2* exon 1 (P1), exon 4 (P2) and exon 6 (P4) in front of the luciferase gene were used for transfections in the CHO-A cells. A second luciferase plasmid containing the herpes simplex thymidine kinase minimal basal promoter with six repetitive GHRE elements from the serine protease inhibitor gene promoter (Spi-TK-Luc) was used as a positive control for GH response. Transfections were performed with a small amount of a CMV- β -Gal expression vector to control for transfection efficiency. After transfection in hexaplicate experiments, using the promoter-luciferase constructs or the Spi-TK-Luc con-

struct, the cells were treated with GH (1 μ g/ml) for 6 h. This concentration has previously been used with CHO cells in the study of *IGF1* regulation (Norstedt *et al.* 1994). The ratio of the light units from the luminometer to the activity of β -galactosidase and amount of cellular protein is shown in Fig. 5. Addition of GH enhanced the Spi-TK-Luc vector activity twofold, while the P2 promoter responded significantly with a 37% increase ($P=0.006$). The P4 promoter construct displayed a substantially higher luciferase activity, and responded to GH with 28% induction ($P=0.0003$). The P1 construct did not show any significant GH induction ($P=0.26$). Thus, among the *IGF2* promoters, P2 and P4 displayed statistically significant induction by GH when calculated as light units/ β -Gal per protein. Lower concentrations of GH (0.01 and 0.1 μ g/ml) were also tested, but gave substantially lower effect (data not shown).

Discussion

This study demonstrates that GH can induce a promoter-specific response of the *IGF2* gene, in contrast to the

Figure 5 Transfection of P1-Luc, P2-Luc, P4-Luc and Spi-TK-Luc in CHO-A cells. The P1-, P2-, or P4-Luc or Spi-TK-Luc plasmids were co-transfected with a SV40-β-Gal plasmid into CHO-A cells. After serum-free conditions overnight, the cells were treated with 1 µg/ml of GH for 6 h. Luciferase activity was analyzed with a luminometer and calculated as the ratio of light units to β-Gal activity and amount of cellular protein. All transfections were done three times, each in hexaplicate. The error bars denote S.E.M.s. ** $P < 0.01$, *** $P < 0.001$, using Student's two-tailed *t*-test. The Figure depicts one of the three hexaplicate experiments. n.s., not significant.

general concept that GH is unimportant for the regulation of this gene. In this investigation we have studied the effects on the different promoters separately rather than analyzing the total amount of *IGF2* transcripts. All four promoter transcripts contain the same translatable sequence in their 3' end, including exons 7, 8 and part of exon 9. The latter part is often used for probing *IGF2* expression. In our hands, GH did not alter the total *IGF2* mRNA level. *In vivo* data from the patient groups used in this study showed that when analyzing *IGF2* transcription with a probe covering a part of the common translated region, no apparent response is demonstrated (Olivecrona *et al.* 1999). However, an acute effect could be observed on the transcript level from the P2 promoter, which was doubled. The rapid effect on P2 suggests a direct effect on the promoter, rather than an indirect effect, which may be true for the P4 promoter transcript after the 5-day treatment. Although we could not detect any statistically significant change in the P1 promoter transcript, the observed lowering of the average value from the 5-day treatment group may potentially explain the unchanged total transcript level. It should also be noted that in the short-term treatment, the final injection of GH was 12 h before taking biopsies as opposed to 4 h in the acute treatment. This means that it can not be determined whether the different GH effects on the *IGF2* promoters in the acute and short-term treatment groups were due to the treatment for 5 consecutive days or if the 4 vs 12 h treatments were responsible for this difference.

The results from the liver biopsy material were evaluated for each promoter in relation to the total *IGF2* transcripts, rather than to *GAPDH* since this transcript varied too much between the individuals. Using such an approach, we were able to demonstrate a GH induction which was statistically significant for P2 in the acute treatment and for P4 in the short-term treatment.

In order to gain more support for the observations obtained from the biopsy material from GH-treated patients, we also investigated isolated primary human liver cells. The difficulties in obtaining good quality GH-responsive human liver cells (as measured by *IGF1* induction) have prevented us from repeating this experiment using material from additional patients, and therefore no statistical analysis could be performed. We still feel that this information should be presented, since the response of the *IGF2* P2 promoter was quite clear and corroborated the *in vivo* data, i.e. the P2 transcript level increased about 40% and 60%, relative to the total transcript level and to *GAPDH* respectively, while the P1 and P4 promoter transcripts were not substantially affected.

The conclusion that GH has an effect on promoter-specific activity was further evaluated by the investigation of the *IGF2* promoter activities in reporter plasmid constructs. The P1, P2 and P4 luciferase constructs used in transfection experiments with CHO cells, stably transfected with the human GH receptor, showed that the P2 and P4 promoters are indeed responsive to the GH signal transduction pathway. The P1 promoter did not show any

significant induction. This is also supported by the *in vivo* data. In the transfections, the cells were incubated with GH for 6 h, thus putting the time frame in between the acute and short-term *in vivo* treatments, and this may explain the induction of the P4 promoter *in vitro*. It is important to note, however, that transfection experiments may only show that the potential of a given promoter to respond to a certain stimuli exists. The more complex situation that is presented *in vivo*, or even in primary liver cells, is not necessarily accurately reflected in a cell line.

The activity of the P4-luciferase construct in the transfection assays was substantially higher than the P1 and P2 constructs. The reason for this is unknown, but it is important to note that the P2 promoter *IGF2* mRNA is normally quite low in abundance in all tissues and cell lines, and one may ask what its physiological role might be. The low abundance may be a result of a lower transcriptional activity compared with the P1 and P4 promoter, but the turnover rate of the P2 transcript may be rapid, however, and the translational efficiency of its transcript high. Thus, a GH-induced doubling of the mRNA level, as shown *in vivo*, may in fact be of physiological significance. It has previously been shown that the different *IGF2* transcripts are subject to differential translational efficiencies, and that the rate of cell proliferation can switch, at least the P3 promoter derived transcript (which is not detected in adult liver), from a non-translatable to a translatable protein complex (Moor *et al.* 1994, Nielsen & Christiansen 1995, Nielsen *et al.* 1995). It is possible that one of the biological effects of GH is to induce a similar switch to P2-derived transcripts in addition to activation of the promoter. GH can stimulate general protein synthesis and an increase of P2 activity in such a scenario may be the physiological basis for promoter P2. A clinical indication that GH is involved in *IGF2* regulation was shown in acromegalic patients who displayed increased IGF-II content in cortical bone compared with normal (Ueland *et al.* 1999). We have previously shown that the GH treatment *in vivo* used in this study significantly decreases and increases serum levels of IGF-II and IGF-I respectively (Olivecrona *et al.* 1999). The obvious explanation for the decrease in IGF-II levels would be that GH primarily increases serum levels of IGF-I, which compete for the ternary complex (IGFBP-3/ALS/IGF-I), thus increasing the concentration of free IGF-II, making it more susceptible to proteolytic degradation. To explain the physiological relevance of switching *IGF2* promoters, one could speculate that there may be 5'-UTR directed differences in mRNA resulting in slightly different final protein product, final localization, i.e. autocrine/paracrine versus endocrine of the product, or both of these. The reduced serum levels of IGF-II caused by GH may thus not necessarily be reflected in interstitial locations, where it in fact may be higher and providing autocrine and/or paracrine stimulation.

This study provides the first evidence of GH as an effector of *IGF2* mRNA regulation. The fine details of this regulation is still unknown however. By using the PatSearch V1.1 program in the Transfac database (<http://transfac.gbf.de/cgi-bin/patSearch/patsearch.pl>) (Heinemeyer *et al.* 1998), we found the P2 promoter to contain a number of potentially interesting sequences previously known to be involved in gene regulation through GH signaling. Thus, we found several putative sites for binding of STATs. The presence of STAT consensus elements has been shown to be a hallmark for GH-directed gene activation. In the P2 upstream sequence there is a good STAT3 consensus at position -484-467 from the exon 4 start, as well as one at position -143-126. Another interesting site contains the consensus sequence for HNF1 at position -122. The HNF1 α transcription factor was shown to synergize with STAT5 in a GH-dependent way in an *IGF1* promoter construct (Meton *et al.* 1999), although co-transfection experiments in our hands using the CHO-A cells and a HNF1 α expression vector did not show any GH-dependent effects on the *IGF2* P2 promoter (data not shown). Also in the P4 upstream region a good consensus sequence for STAT3 is found at position -393-377 from the exon 5 start. The P1 promoter, however, showed no good consensus sequence for STATs, supporting the experimental findings in this paper. Binding studies and deletion constructs will have to be employed to verify these sites.

Little is known about the importance of IGF-II in adult life under normal conditions. Since the over-expression of the *IGF2* gene is implicated in pathological conditions, including cancer and over-growth syndromes, it is likely that a highly regulated expression of the gene is important for the normal phenotype. We suggest here that GH is one of several factors responsible for this regulation, and that previously established effects by GH on the IGF axis may synergize with the *IGF2* promoter-specific responses described here.

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