Transcription from the P2 promoter of the growth hormone receptor gene involves members of the Sp transcription factor family

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The P2 promoter of the gene for growth hormone receptor is developmentally regulated and is differentially active in a number of tissues. Little is known about the identity of the transcription factors that participate to effect this pattern of transcription. Deletion analysis and transient transfection were used to localize a previously identified cis-acting element within the sheep P2 promoter to between positions −99 and −87. Gel mobility-shift assays with nuclear extracts from Chinese hamster ovary (CHO-K1) fibroblasts revealed that this sequence encompasses an atypical binding site for both Sp1 and two isoforms of Sp3. A gel mobility-shift scan of promoter sequences between −88 and −21 indicated the existence of three other binding sites for Sp1 and Sp3. One of these, designated site II and found by using a probe spanning −74 to −54, corresponds to a classical GC box consensus sequence. Site III (−63 to −41) and site IV (−27 to −5) harbour atypical Sp1/Sp3-binding sequences. Site-directed mutagenesis of site II or site IV decreased promoter activity by approx. 40%, whereas a promoter construct incorporating both mutations exhibited negligible (approx. 1%) activity. Co-transfection of expression plasmids encoding either Sp1 or Sp3 significantly transactivated reporter gene activity from a P2 promoter construct carrying all four Sp1/Sp3-binding sites (8-fold compared with 7.1-fold induction respectively). Sp1 is known to interact with a variety of other transcription factors to regulate the transcription of a number of differentially expressed genes. The identification of four binding sites for Sp1 and Sp3 within the P2 promoter of the gene for growth hormone receptor might point to other factors that interact to regulate the activity of this promoter in different tissues during foetal and post-natal development.

Key words: binding sites, ovine, Sp1/3, transactivation.

INTRODUCTION

The onset of growth hormone (GH)-dependent somatic growth in mammals is developmentally regulated. Foetal growth is essentially independent of the influence of GH, even in those species in which there is an excess of circulating foetal GH [1]. After birth, postnatal growth becomes progressively dependent on GH owing to the appearance of high-affinity membrane-bound receptors for GH on the liver and other tissues [2,3]. Structural defects within the GH receptor (GHR) gene that render it non-functional, as has been observed in individuals with the autosomal recessive disorder Laron syndrome [4,5], result in pronounced growth retardation in postnatal life.

The expression of the gene for GHR is regulated temporally and in a tissue-specific manner. GHR transcripts can be detected at low levels in a number of foetal tissues [6–8]. During late gestation and early postnatal life there is a marked increase in GHR mRNA expression, such that GHR transcripts can be found in most, if not all, tissues assayed [9]. However, there are significant differences in the level of GHR mRNA expressed between different tissues: liver, kidney and adipose tissue express the highest levels of GHR transcripts. In addition, the expression of GHR mRNA in individual tissues can exhibit tissue-specific modulation in response to a given hormonal stimulus [10–13] or changes in physiological status (i.e. pregnancy [14,15]). These differences seem to arise in part through the utilization of different promoters. Multiple unique 5’ untranslated regions alternatively spliced to a common acceptor site upstream of the initiating AUG codon are a characteristic of GHR cDNA sequences isolated from all mammalian species studied so far. Seven variant (V) 5’ non-coding sequences have been identified from human GHR mRNA [16]; the sheep counterparts for two of these sequences, V1 and V2, have been isolated and the corresponding genomic non-coding exons, designated 1A and 1B respectively, have been cloned and the flanking promoter sequences characterized [17–19]. Exon 1A-GHR mRNA is transcribed from the liver-specific P1 promoter and comprises the bulk of GHR mRNA expressed in this tissue [18]. In contrast, exon 1B-GHR mRNA is transcribed in a number of tissues under the control of a second promoter, the P2 promoter [19]. Transcribed sequences analogous to exon 1B-GHR mRNA have been described in several species [20]; expression of these transcripts exhibits tissue-specific, developmental and physiological influences. However, nothing is known at a cellular level about the role of specific transcription factors in regulating this profile of expression.

In a previous study from this laboratory, deletion analysis was used to delineate a proximal fragment of the P2 promoter, from −134 to +87, that promoted strong reporter gene activity following transient transfection of a number of cell lines [19]. Within this core promoter region it was observed that the removal of the region upstream of −87 significantly decreased promoter activity, indicating the presence of a critical cis-acting element. In the present study, multiple binding sites for members of the Sp family of transcription factors are identified within the core P2 promoter, including a site immediately upstream of −87, and the role of these factors in mediating P2 promoter function in cultured cells is addressed.

Abbreviations used: CHO, Chinese hamster ovary; CMV, cytomegalovirus; GH, growth hormone; GHR, growth hormone receptor; α-MEM, α-minimal essential medium; C/EBP, CCAAT/enhancer binding protein.

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EXPERIMENTAL

Plasmid construction

A series of GHR P2 promoter deletions was assembled in the luciferase reporter plasmid, pGL2-Basic (Promega), by a combination of truncation with exonuclease III and digestion with appropriate restriction enzymes [19]. Plasmids were purified using the Wizard Plus system (Promega).

To monitor transfection efficiency, the plasmid expression vectors pCMV-β-galactosidase (CMV-β-gal; Promega) (in which CMV stands for cytomegalovirus) and pSEAP Control, encoding a secreted alkaline phosphatase (Clontech), were used. The Sp expression constructs CMV-Sp1 and CMV-Sp3 have been described [21] and were kindly provided by Dr. Guntram Suske.

Cell culture and transfection

Chinese hamster ovary (CHO-K1) cells were maintained at 37 °C in α-minimal essential medium (α-MEM; Gibco-BRL) supplemented with 5 % (v/v) foetal calf serum, penicillin (50 i.u./ml), streptomycin (50 μg/ml) and glucose (2 mM). Transient transfection of CHO-K1 cells was performed with LipofectAMINE (Gibco-BRL). In brief, cells were seeded at 8 × 10⁴ cells in each well of a 24-well tissue culture plate 24 h before transfection.Reporter gene constructs were complexed with lipid (1 μl of LipofectAMINE) in 200 μl of serum-free and antibiotic-free α-MEM for 15-30 min, and subsequently diluted to 1 ml with the same medium. Where co-transfection experiments were performed, a ratio of 19:1 (P2 luciferase reporter to internal control or transactivator plasmid) was maintained. Cell cultures were washed twice with PBS, after which 300 μl of DNA/lipid cocktail was added to each well. After 5 h of incubation at 37 °C, each well was aspirated, washed once with complete growth medium and re-fed; 48 h after transfection, cells were washed twice in PBS containing 1 mM Ca²⁺ and Mg²⁺, then lysed with 200 μl of LucLite (Canberra-Packard) in accordance with the manufacturer’s instructions. Aliquots were taken and chemiluminescent detection of luciferase reporter gene activity was performed with a Packard TopCount Microplate Scintillation and Luminescence Counter. In those experiments in which pSEAP-Control was included to ‘normalize’ for transfection efficiency, an aliquot of cell culture supernatant was taken and chemiluminescent detection of secreted alkaline phosphatase activity was performed with a Great EscAPE Detection Kit (Clontech).

Gel mobility-shift assays

Nuclear extracts from cultured cells were prepared as described by Dignam et al. [22]. End-labelled probes were prepared by annealing complementary oligonucleotides (synthesized by DNAGency) containing 5ʹ overhangs of one to four bases and filling in the ends with the Klenow fragment of DNA polymerase I, [α-³²P]dCTP, dATP, dGTP and dTTP. Radiolabelled probes were purified on Bio-Spin P-6 chromatography columns (Bio-Rad Laboratories). Nuclear extracts (5-10 μg) were preincubated for 20 min at room temperature with 2 μg of poly(dI-dC) and binding buffer [12.5 mM Hepes/KOH (pH 7.9)/12.5 % (v/v) glycerol/0.4 mM MgCl₂/80 mM KCl/0.075 mM EDTA/0.25 mM dithiothreitol/0.25 mM PMSE/0.1 % (v/v) Nonidet P40]. Labelled probe (20000 c.p.m.; approx. 1 ng) was then added and the binding reaction was incubated for a further 20 min at room temperature.

Unlabelled competitor oligonucleotides (100-fold molar excess) were added to binding reactions at the start of the preincubation period. For supershift assays, 1 μl of anti-Sp1 or anti-Sp3 serum (Santa Cruz Biotechnology) or rabbit anti-mouse IgG was added to binding reactions at the start of the preincubation step. Protein-DNA complexes were resolved on 5 % (w/v) native polyacrylamide gels in 0.5 × Tris/borate/EDTA run at 175 V at 4 °C. Gels were then dried and autoradiographed at −70 °C.

Mutagenesis

Site-directed mutagenesis of Sp1/Sp3-binding sites was performed with the method of Kunkel et al. [23]. Mutagenic oligonucleotides containing three nucleotide substitutions (underlined) were synthesized as follows: SpII, 5ʹ-GAACAGGGGTGG-AAAGAGAGGAAGAA-3ʹ; SpIV, 5ʹ-TTTTCTCTAGGGCTTACGCAGGCCGCAGCC-3ʹ.

Both oligonucleotides were purified by reverse-phase chromatography on Sep-Pak C₁₈ columns (Waters) before use. P2 promoter fragments were subcloned into the phagemid vector pGEM3Zf(+) (Promega) and uracil-containing single-stranded template DNA was prepared after the infection of transformed CJ236 cells by M13K07 helper phage. Plasmid templates harbouring mutations were identified by Southern blotting with mutant oligonucleotides and confirmed by DNA sequencing. Mutated promoter fragments were excised and subcloned back into pGGL2-Basic.

Statistical analysis

The paired Student’s t-test was used to compare the significance of the differences between data.

RESULTS

Cis-acting element adjacent to −87 of the GHR P2 promoter incorporates an Sp1/3-binding site

To delineate the functional boundary of the putative cis-acting element between −134 and −87 of the GHR P2 promoter, a nested set of promoter deletions covering this region were assembled upstream of the luciferase reporter gene and trans-
Table 1 Nucleotide sequence of oligonucleotide probes used in gel mobility-shift assays

<table>
<thead>
<tr>
<th>Position</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>−107/−85</td>
<td>GGGTTTTATTAGTCCTGCCTCCC</td>
</tr>
<tr>
<td>−74/−54</td>
<td>AGGGCTGGCGGAGGAGAAGG</td>
</tr>
<tr>
<td>−63/−41</td>
<td>GAGAGGAGGAGGAGTGGCTCAGCA</td>
</tr>
<tr>
<td>−27/−5</td>
<td>CCTCTAGAGAGGAGGCGGAGCC</td>
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Regulation of the growth hormone receptor gene by Sp family members

Table 1 Nucleotide sequence of oligonucleotide probes used in gel mobility-shift assays

The position of each oligonucleotide is indicated relative to the major transcriptional start site of the GHR P2 promoter [23].

<table>
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Figure 2 Cis-acting element adjacent to −87 is a binding site for Sp1 and Sp3

(A) Gel mobility-shift assays were performed with a 32P-labelled probe spanning −107 to −85 and nuclear extracts from CHO-K1 cells. For oligonucleotide competition experiments (lanes 2–5), a 100-fold excess of unlabelled competitor was included in the reaction before the addition of labelled probe. Lane 1, probe plus extract; lane 2, WT (wild-type) −107/−85; lane 3, Sp1 consensus sequence (Promega); lane 4, −113/−100; lane 5, −88/−69. For antibody supershift experiments (lanes 7–9), 1 μl of rabbit antiserum was added to the reactions before the addition of probe. Lane 6, probe plus extract; lane 7, rabbit anti-mouse IgG serum; lane 8, rabbit anti-Sp1 serum; lane 9, rabbit anti-Sp3 serum. I, II and III refer to specific DNA–protein complexes that can be distinguished by anti-Sp1 (I) and anti-Sp3 (II, III) antibodies. (B) Gel mobility-shift assays were performed with nuclear extract and −107/−85 probe alone, or after the addition of a 100-fold excess of the indicated unlabelled competitor oligonucleotide. WT refers to the wild-type sequence spanning −101/−85; whereas Sp1 contains the consensus binding site. The mutant series of oligonucleotides (M1–4) incorporate a series of 3 bp substitutions, as indicated, in the wild-type sequence.
competition experiments revealed that an unlabelled excess of −74/−54 was most efficient at inhibiting DNA–protein complex formation by the other two probes, whereas the sequence −64/−41 was least efficient (results not shown).

Taken together with results from the previous section, these results indicate the existence of at least four binding sites for Sp1/3 within the basal GHR P2 promoter. Between −74 and −54 the obvious candidate Sp1/3-binding site is the conserved GC box, GGCGCGG, found at −68/−63 (Table 1). A mutant oligonucleotide bearing a 3 bp substitution (GCG to AAA) within this GC box failed to inhibit the formation of Sp1/3 complexes with the wild-type probe (results not shown), confirming the role of this element in mediating Sp1/3 binding within the sequence −74/−54. Within the oligonucleotide (−27/−5) designated as containing an Sp1/3-binding site of intermediate affinity there is the sequence AGGAGGAGC (−22 to −14; Table 1) that matches, in eight of nine nucleotides, the GGAGAGG box element of the human GH gene that is known to bind Sp1 [23]. Indeed, mutated oligonucleotides carrying substitutions in either of the AGG repeats were significantly impaired in their ability to block the binding of Sp1 and Sp3 to the wild-type probe, whereas substitutions introduced into the 3′ flanking sequences had a much less pronounced effect (results not shown). The nature of the sequence between −63 and −41 that acts as a low-affinity binding site for Sp1 and Sp3 has not yet been established.

Multiple Sp1/3-binding sites are required for basal function of the GHR P2 promoter

Given that the disruption of the Sp1/3-binding site near −87 of the P2 promoter (designated Sp1/3-binding site I; see Figure 4A) significantly impairs promoter function, mutations were introduced into the high-affinity (site II) and intermediate-affinity (site IV) Sp1/3-binding sites to assess their impact on promoter function. Luciferase reporter gene constructs were assembled by using the −99 to +87 P2 promoter fragment bearing null mutations (as determined in gel-shift assays) at site II or site IV or both sites II and IV, and transiently transfected into CHO-K1 cells (Figure 4A). The introduction of mutations at either site significantly decreased the promoter activity of each construct to similar extents, to approx. 60%, relative to the wild-type P2−99luc reporter plasmid. The loss of activity accompanying the mutation of site II or site IV was similar to that obtained after the deletion of site I in P2−87luc (see Figure 1; compare P2−99luc with P2−87luc). However, what is striking is the effect of a double mutation (sites II/IV) on the activity of the basal P2 promoter fragment, decreasing reporter gene activity to approx. 1% of that obtained with the wild-type promoter (Figure 4A). Thus, despite the presence of two intact Sp1/3-binding sites (sites I and III) within the P2 promoter fragment carrying the II/IV mutations, promoter function was essentially ablated by these two mutations.

To establish what effect the overexpression of either Sp1 or Sp3 might have on basal GHR P2 promoter activity, the P2−99luc reporter plasmid was co-transfected together with expression vectors encoding Sp1 (CMV-Sp1) or Sp3 (CMV-Sp3) into CHO-K1 cells. Co-expression of either Sp1 or Sp3 resulted in a significant transactivation of GHR P2 promoter function (Figure 4B), with both transcription factors mediating similar increases (8-fold compared with 7.1-fold) in reporter gene activity. This last result would imply that changes in the cellular levels of Sp1 or Sp3 or both factors might influence the transcriptional activity of the GHR P2 promoter in vivo.
DISCUSSION

Transcription from the P2 promoter of the gene for GHR exhibits developmental, physiological and tissue-specific regulation [20]. Until the present study, the identity of any transcription factors regulating P2 promoter function was unknown. In establishing a role for multiple Sp1/Sp3-binding sites in achieving optimum levels of GHR P2 function in transfected cells, as well as documenting the ability of Sp1 and Sp3 to act as transcriptional activators of the same promoter, the current study raises obvious questions of how these two members of the same transcription factor family might participate to regulate P2 promoter activity.

Multiple Sp1-binding sites within a given promoter might interact to influence transcription in a synergistic [30,31], additive [32] or even antagonistic [33] manner. The individual deletion/mutation of three Sp1/Sp3-binding sites (I, II and IV) within the GHR P2 promoter decreased promoter function by equivalent degrees. This suggests that within the context of the promoter fragment used for transfection (−99/+87), all three sites are functional in maximizing promoter activity. That Sp1 and/or Sp3 are essential for GHR P2 transcriptional activity is borne out by the observation that the mutation of both sites II and IV decreased reporter gene activity to 1% of wild-type (Figure 4). Whether this reflects a pivotal role of Sp1 or Sp3 alone in regulating basal transcription from the P2 promoter is not known. It has been shown that for promoter elements that lack TATA boxes, of which the GHR P2 promoter is an example [19], Sp1 can interact directly with the transcriptional co-activator TAF199 to initiate transcription via the TFIID protein complex [34,35]. However, other cellular factors have been identified that can interact directly with both Sp1 and components of TFIID, including YY1 [36] and several members of the E2F transcription factor family [37]. The possible role of these proteins in regulating basal transcription from the GHR P2 promoter remains to be established.

Although studies from cultured cell lines suggest otherwise, there are striking differences in the levels of Sp1 expression between different tissues [38]. Although this seems to be manifested at the level of transcription [38], other mechanisms, such as the rapid proteolytic degradation of Sp1 in the liver, kidney and pancreas [39], might be contributory. However, there is little correlation between the expression of GHR P2-derived transcripts in different tissues and that of Sp1; the tissues in which P2 transcripts are preferentially expressed in rodents (liver, skeletal muscle and kidney) are those in which the levels of Sp1 mRNA and protein are among the lowest of the tissue surveyed [38,39]. Clearly, the levels of Sp1 expression in these tissues are sufficient for Sp1-dependent gene expression: for the liver-specific P1 promoter of the human insulin-like growth factor II gene, P1-derived transcripts are highly expressed after birth and transcriptional activation of this promoter in response to the liver-enriched transcription factors CCAAT/enhancer binding protein β (C/EBPβ) and hepatocyte nuclear factor 3β requires a functional Sp1-binding site within the proximal promoter region [40]. However, the presence of Sp1 protein is not itself necessarily indicative of functional status, because its ability to bind to, and activate, promoter elements can be
influenced by post-translational modification such as phosphorylation [41,42].

All Sp-binding sites identified within the GHR P2 promoter bound both Sp1 and the two isoforms of Sp3. This was observed consistently for all sources of nuclear extract, which included CHO-K1 fibroblast and HepG2 hepatoma cell lines, and sheep foetal and postnatal liver (results not shown). Sp1 and Sp3 are members of an expanding family of transcription factors that exhibit structural and sequence similarity [43–45]. Sp3 is expressed ubiquitously [43,44] and binds both the consensus GC box and the variant GT box with the same affinity as Sp1 [43]. Whereas Sp1 is characteristically associated with transcriptional activation, Sp3 was originally described as a transcriptional repressor [21]. Subsequent studies have demonstrated that Sp3 can act not only as a transcriptional repressor but also as a transcriptional activator [24]. With respect to the −99/+87 GHR P2 promoter used in this paper, the overexpression of Sp3 transactivated reporter gene activity to the same extent as that of Sp1 (7.1-fold compared with 8-fold induction).

In ruminants, exon 1B mRNA derived from P2 is the most commonly expressed GHR transcript found in non-hepatic tissues [46]. There are striking differences in exon 1B mRNA expression between tissues: liver, muscle and adipose tissue express the highest levels of transcript. Furthermore, exon 1B transcripts are found in a number of foetal tissues [46]. The exact roles of members of the Sp family of transcription factors in regulating transcription from the P2 promoter in a number of genes [40,47,48], it is likely that a similar interaction occurs to the developmental and tissue-specific activation of a number of genes [49].

Whereas Sp1 is characteristically associated with transcriptional activation, Sp3 is shown to exhibit structural and sequence similarity [43–45]. Sp3 is commonly expressed GHR transcript found in non-hepatic tissues [46]. Also of some interest is the possible role of transcription factors in influencing this pattern of expression are not known. In preliminary experiments, I have found that the P2 promoter can be transactivated by members of the C/EBP transcription factor family, in particular C/EBPα (T. E. Adams, unpublished work). Given the emerging interplay between Sp1 and C/EBP factors in the developmental and tissue-specific activation of a number of genes [40,47,48], it is likely that a similar interaction occurs to regulate transcription from the P2 promoter in a number of tissues. Also of some interest is the possible role of transcription factors binding within P2 promoter sequences in influencing transcription from the recently identified P3 promoter, which lies 700 bp downstream of exon 1B of the gene for bovine GHR [49]. Inclusion of a P2 promoter fragment upstream of the P3 promoter significantly enhanced the activity of a linked reporter gene. P2-and P3-derived GHR mRNA species are co-expressed at consistent ratios in a variety of foetal and postnatal tissues, raising the possibility of co-ordinate regulation of distinct promoters through one or more common elements [49].

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REFERENCES


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