Identification of Sp1 as the transcription factor for the alternative promoter P2 of the bovine growth hormone receptor gene

H Jiang, C S Okamura, C K Boyd and M C Lucy

Department of Animal Sciences, University of Missouri, Columbia, Missouri 65211, USA

(Requests for offprints should be addressed to M C Lucy, 164 ASRC, Department of Animal Sciences, University of Missouri, Columbia, Missouri 65211, USA; Email: lucym@missouri.edu)

ABSTRACT

Growth hormone receptor (GHR) mRNA variants that differ in the 5'-untranslated regions (5'-UTR) have been isolated in various species. These 5'-UTR variants are generated from the use of alternative promoters and/or alternative splicing. The 5'-UTR 1B is one of the GHR 5'-UTR variants isolated in the bovine but its homologues are also present in other species. The 5'-UTR 1B is a predominant GHR 5'-UTR expressed in many tissues. In the present study, we screened a bovine genomic library and isolated a 1.7 kb bovine GHR genomic sequence including exon 1B and its 5' flanking region from which the GHR 5'-UTR 1B is generated. Using primer extension, two major transcription start sites were mapped in the bovine exon 1B. Transient transfection analysis of the 5' flanking region of exon 1B confirmed its promoter

activity (termed P2) in both Hep G2 and BHK-21 cells. Furthermore, analysis of deletion promoter-reporter constructs found that the basal activity of P2 resided in the proximal region of P2. DNase I footprinting analysis and electromobility shift assay (EMSA) identified the ubiquitous transcription factor Sp1 as the binding protein to a GC box-containing DNA element within the proximal P2. Deletion of the GC box greatly reduced the activity of P2 in cell lines. The GC box-containing site also appeared to bind Sp1 in the nuclear extracts from diverse bovine tissues. This suggests that interactions of Sp1 with the GC box-containing element in the proximal region of P2 may be part of the mechanism for the expression of the bovine GHR 5'-UTR 1B in diverse tissues.

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INTRODUCTION

The growth hormone receptor (GHR) is coded by a single GHR gene (Barton *et al.* 1989, Godowski *et al.* 1989). However, GHR mRNA variants that differ in the 5'-untranslated region (5'-UTR) have been isolated in various species (Pekhlitsky *et al.* 1992, O'Mahoney *et al.* 1994, Adams 1995, Baumbach & Bingham 1995, Domene *et al.* 1995, Southard *et al.* 1995, Jiang & Lucy 1998, Lucy *et al.* 1998, Jiang *et al.* 1999, Schwartzbauer *et al.* 1999). The GHR 5'-UTR 1B is one of the eight GHR 5'-UTRs that we previously isolated in the bovine (Jiang & Lucy 1998). It is ubiquitously expressed, with readily detectable levels in liver, fat, muscle, kidney, uterus, mammary gland and adrenal gland (Lucy *et al.* 1998). Homologues of the bovine GHR 5'-UTR 1B have been isolated in other species including sheep, humans, rats and mice, in which

they were named 1B (Adams 1995), V2 (Pekhlitsky *et al.* 1992), GHR2 (Baumbach & Bingham 1995) or V1 (Domene *et al.* 1995) and L2 (Southard *et al.* 1995) respectively (for a review, see Edens & Talamantes 1998). In these species, homologues of the bovine GHR 5'-UTR 1B are also expressed as a predominant form of GHR 5'-UTR in many tissues. Similarities in expression pattern and DNA sequence suggest that a common mechanism regulates the expression of the GHR 5'-UTR 1B and its homologues in the bovine and other species.

Growth hormone acts on GHR and stimulates the production of insulin-like growth factor I (IGF-I) (Zhou *et al.* 1997). In liver, where the highest levels of GHR mRNA are usually detected (Baumbach *et al.* 1989, Mathews *et al.* 1989, Hauser *et al.* 1990, Martini *et al.* 1997), GH-induced IGF-I enters the blood and accounts for the majority of the circulating IGF-I (Schwander *et al.* 1983, Yakar

et al. 1999). In non-hepatic tissues, GH acts on GHR and may stimulate the local production of IGF-I. The hepatic IGF-I was traditionally thought to be the major endocrine factor for postnatal growth (Daughaday & Rotwein 1989) and the local IGF-I was thought to affect tissue growth in a paracrine and/or autocrine manner (Isaksson *et al.* 1987). However, the importance of hepatic IGF-I to animal growth was challenged by a recent report that mice with a liver-specific deletion of the IGF-I gene grow normally despite a dramatic reduction in blood IGF-I (Yakar *et al.* 1999). Thus, the role of the local IGF-I in stimulating growth is probably more important than was originally realized. The GHR 5'-UTR 1B represents the majority of the GHR mRNA pool in many tissues. The expression of the GHR 5'-UTR 1B is, therefore, important for the local actions of GH.

The GHR 5'-UTR variants are generated by initiation of transcription from alternative promoters and/or alternative splicing. At least three alternative GHR promoters have been isolated. The GHR promoter 1 (P1), from which the liver-specific GHR 5'-UTR (named 1A in the bovine and ovine, and V1, L1 and V2 in humans, mice and rats respectively; Edens & Talamantes 1998) is initiated, has been characterized in various species (O'Mahoney *et al.* 1994, Menon *et al.* 1995, Zou *et al.* 1997, Schwartzbauer *et al.* 1998). Promoter 3 (P3), from which a second but less abundant ubiquitous GHR 5'-UTR (named 1C in the bovine, Jiang *et al.* 1999; V9 in humans, Schwartzbauer *et al.* 1999; V4 in rats, Domene *et al.* 1995) is initiated, has been characterized in the bovine (Jiang *et al.* 1999). Promoter 2 (P2), from which the 5'-UTR 1B is initiated, was first isolated and studied in the ovine (Adams 1995). More recently, sequence for the mouse homologue of the GHR P2 has also been reported (Moffat *et al.* 1999). In the present study, we isolated and characterized the bovine GHR P2. Our study demonstrated that binding of the ubiquitous transcription factor Sp1 contributed to the activity of the bovine GHR P2.

MATERIALS AND METHODS

Isolation of genomic clones

An 850 bp PCR product corresponding to -751 to +99 (Fig. 1) was amplified from bovine genomic DNA by using the 5' primer (5'-ATGACATTACA AGTGG-3') and the 3' primer (5'-GGTTCGA GACCCGCTG-3') that were designed from the

ovine GHR P2 sequence (Adams 1995). The PCR fragment was labeled with ^{32}P by the method of random priming and used as a probe to screen a bovine genomic library in the vector EMBL3 SP6/T7 (Clontech, Palo Alto, CA, USA). A standard screening procedure was used (Sambrook *et al.* 1989). Briefly, duplicate lifts from ten plates, each containing 5×10^4 plaque-forming units (pfu) of the library, were hybridized to the probe. Positive plaques from the initial screening were subjected to two additional rounds of amplification and screening using the same probe. Genomic inserts from positive clones of the tertiary screen were then characterized by restriction enzyme digestion mapping and Southern blot analysis. A 2.3 kb XhoI-XhoI genomic fragment containing exon 1B was subcloned into the pBluescript SK(-) vector (Stratagene, Torrey Pines, CA, USA). The subcloned plasmid was named pSK41-1-6. The insert in pSK41-1-6 was sequenced by the method of cycle sequencing (University of Missouri DNA Core, Columbia, MO, USA).

Primer extension analysis

Primer extension analysis was used to map the transcription start site within exon 1B. The experiment was based on published procedures with modification (Sambrook *et al.* 1989). Briefly, a 21 nucleotide (nt) oligonucleotide primer, 5'-CTGCA GCAGTCTCCGCTGCCG-3', which was complementary to nucleotide +337 to +358 of the bovine GHR DNA sequence (Fig. 1), was labeled at the 5' end with ^{32}P - γ -ATP (5000 Ci/mmol, DuPont NEN, Boston, MA, USA) and T4 polynucleotide kinase (Promega, Madison, WI, USA). Approximately 5×10^6 c.p.m. of ^{32}P -labeled primer was mixed with 10 μg of bovine total liver RNA (isolated from non-lactating cows) or equal amount of yeast tRNA (as a negative control) and heated at 70 °C for 10 min. The primer-RNA mixture was then added with dNTPs, RNase inhibitor, SuperScript II Reverse Transcriptase (Life Technologies, Gaithersburg, MD, USA), and appropriate buffers for the reverse transcription reaction and incubated at 50 °C for 1 h. Following incubation, the reaction was subjected to phenol/chloroform extraction and ethanol precipitation. The extension products were resolved by electrophoresis on a 6% denaturing polyacrylamide gel containing 8 M urea. A DNA sequencing product and a 100 bp DNA ladder were run in parallel as references to determine the size of the primer-extended products. The gel was dried and exposed to X-OMAT AR film (Kodak, Rochester, NY, USA) with an intensifying screen.

	<i>Xho</i> I				
-1217	CTCGAGGATC	AACACCCACC	TGCCTCAAGA	CGGGACAGTG	TGATCCACAC
-1167	CCCGTCTAGA	ATCTTCCCCC	TCCTTTCCTG	CTGGGTGTCT	TTGCCTTGAA
-1117	CAGTTGGCTT	GCTGAGCCAC	AGCTGAGCAC	CAGAAAGGAC	TCCAAGGAAC
-1067	AGAATTTTCC	ACTTCCTTGA	TTCTGTAAA	AACAGGGGTA	TCTGATGTGA
-1017	CATCTGCTGC	CCCCAAGTCC	TTGGTCCTGT	GTTTCTTCCA	TTCTTGCCCC
-967	ACCCACCCTC	CGGACTGTCC	CCTCATCCCC	TACTATTTTC	CATGCCCTGG
	<i>Hind</i> III				
-917	GGATTTTTGT	TTTATTCTAG	TGCTAAGAAG	CTTGGTTTCT	TTAAGTTGTT
-867	GCTTAAACAT	TTATCTCTGG	GTATAAATTT	GGCTTTGTGT	CTCGGAATCC
-817	TGTAAAATAC	ATACATACAT	ACACATATAT	ATATATGTAA	TTTAGTAAAC
-767	TCAAGCTGTT	AAATAAATGA	CATTACAAGT	GGCTGTGAGT	AATAAAGTTA
-717	AATAGTAAAA	AGATAGCATG	ACGCGCTGGT	CTGTCAAATC	TCAGACAACC
-667	TGGAAC TGGA	CTTTTGA ACT	TTCTGCTTAG	AAAAGCCTGT	GGTGACGAGA
-617	GTTTGATGCT	TTAACAACAT	GACGGGCTTA	TGCAGAACGA	CACTGACTTT
-567	CTAAAGCACG	TTGACAGCTT	TTCACTTTAT	TTTCCCTGCA	GTGGAATGGG
-517	GTGGAAAGGC	CCGTATTTGA	CAGATGTCAC	GCGCAGGACA	TTTAAAAGCC
-467	ACCTTCCCTA	GGTTGCACAC	AAGGATAATC	CGGGGGCTGA	GGCCTGTGTC
-417	TGAATACCCG	CCTCCCTGTG	CTCCAGGGTC	TGGACATACT	CCTATTCTAA
-367	CATGCCTTGT	GTCC TTGGTA	AATTACTTCA	TCTCTCCTGC	CTCTGTTTTT
-317	TAACCTCTAA	AATGAGGTTC	TTCTACTTTT	TAATATCTTT	TTCCACATGC
-267	CCGAGCAAGT	AGGTGTTTGG	AAATTGATTG	CAGGTGGAAA	GAGGCAGTGT
-217	TGGGTTTGCT	AATTACGCTT	TTCAAGTGGT	CTTGTCCTGT	TTCTCCCCCT
-167	TTCCAGCAGA	GAGCTAAGCG	TTCTCTTGAA	TTTGTTGACT	GCCACTTAGG
-117	CTCTTTTAGG	TCCTGCCTCC	TTTTTGGGAA	CAGGGGTGGG	CGGAGAGGAA
-67	GGAAGTGCCT	TGCAACTACC	AATATTTTCC	TCTAGGAGGA	GCCGCGCAGC
		*	*		
-17	CCAGATGAGA	GTGACACGCA	CCAAC TCCCA	ACTCCTCGCC	GGGGAACGCT
+34	TCATCCCAGC	TCCGCCGAAG	GC TCGGCCGG	GACGCGCCTC	GGCGCCTCCG
					<i>Not</i> I
+84	CAGCGGGTCT	CGAACCAGCC	GCCCTCAACT	CCCGCTCCCC	CTCGCTGCGG
		<i>Sma</i> I			
+134	CCGCCCGGCT	CCCGGGGGCG	GCTGGCCGGG	CCCGGCTTGA	CCCCTGGTGA
+184	ACGGCCGCGG	CCGGTTCCCA	CCCCTCCCCT	CCCAGCCTCC	CTTCCCGGCT
					<i>Sac</i> II
+234	CACCCCGCCC	CCTCCCCCCT	CCCCGAGCCT	GACAGCTTGC	GAGCCGCGGA
		<i>Nco</i> I			
+284	GCAGGGCTCA	GCCATGGGAA	GAGGAGGCGG	TCTGGGGAGC	AGCGGCGGCG
			<i>Pst</i> I		
+334	GCGGCGGCAG	CGGAGACTGC	TGCAGCGGCG	GCGGCGGCGG	CGGCGGCTGC
+384	TGAGCCGATG	CGGCGGCGGG	GACCCAGGGG	GCCGCAGGGC	CCCGCGCCCT
+434	GGGCCCCAGC	GCGGACGCGA	ACCGCGCTCT	CTCTCCCGAG	GCTCGGCTCG
+484	CAG				

FIGURE 1. The DNA sequence for the bovine GHR exon 1B and its 5' flanking region. A 2.3 kb genomic fragment of the bovine GHR gene was cloned from a bovine genomic library and sequenced completely. The complete 2.3 kb sequence was deposited in GenBank under accession number AF046861. The 1.5 kb sequence containing the exon 1B (indicated in bold) and its 5' flanking region is presented. Exon 1B contains two major transcription start sites (indicated with asterisks). Positions of nucleotides are numbered relative to the most 5' major start site that was designated as +1. Indicated restriction digestion sites for HindIII, NcoI, NotI, SmaI, SacII and PstI were used for constructing the promoter-reporter plasmids. The GC box is indicated (box).

Plasmid construction and deletion

All plasmid constructs were made by inserting the GHR genomic DNA fragment into the polycloning sites upstream from the firefly luciferase gene in the promoter-less plasmid pGL3-Basic (pGL3B, Promega). A 1.5 kb XhoI–NcoI fragment, corresponding to the 5' flanking region from –1217 to +300 (relative to the transcription start site +1 in Fig. 1), was digested from pSK41–1–6, blunted at the NcoI site, and then inserted into the XhoI and blunted HindIII sites in pGL3B. This construct was designated 1B(–1217/+300)-GL3. To construct plasmid 1B(–1217/–12)-GL3, in which the insert had a 311 bp deletion from the 3' end compared with that in construct 1B(–1217/+300)-GL3, a SacI-linked 5' primer 5'-GCTGGAGCTCCACCG CGGTGGC-3' specific for the vector sequence (SacI site underlined) and a NheI site-linked 3' primer 5'-TTCCGCTAGCATCTGGGCTGCGCGGCT CCT-3' specific for the GHR sequence (NheI site underlined) were used to amplify the –1217/–12 fragment from pSK41–1–6 by using standard PCR. The PCR products were digested with appropriate restriction enzymes, and then inserted into the SacI and NheI sites in pGL3B. The inserts in constructs 1B(–750/–12)-GL3, 1B(–191/–12)-GL3 and 1B(–104/–12)-GL3 contained serial 5' deletions and were generated using similar PCR procedures.

Construct 1B(–104/–12delGC)-GL3 had a deletion of the sequence GGGCGG (–80 to –75 in Fig. 1) compared with construct 1B(–104/–12)-GL3. The deletion was made by PCR-based site-directed mutagenesis (Fisher & Pei 1997). Briefly, 30 cycles of PCR were performed at 96 °C for 10 s (denaturation), 55 °C for 1 min (annealing) and 72 °C for 5 min (extension) with a forward primer 5'-AGAGGAAGGAAGTGCCTTGC-3' (–74 to –55 in Fig. 1), a reverse primer 5'-ACCCCTGTTCCCAAAAAGGA-3' (–81 to –100 in Fig. 1), plasmid 1B(–104/–12)-GL3 as a DNA template, 1 unit of Taq DNA polymerase, and 0.01 unit of Tli DNA polymerase. The PCR products were gel-purified, phosphorylated, circularized and cloned using standard procedures.

The insert–vector junctions and deletions in each plasmid were verified by DNA sequencing. Large-scale plasmid DNA purification was done by using the Qiagen Maxiprep kit (Qiagen, Valencia, CA, USA).

Cell culture and transfection

Approximately 3×10^5 Hep G2 cells or 1×10^5 BHK-21 cells (purchased from ATCC, Rockville, MD, USA) were plated onto 12-well plates,

maintained in Minimum Essential Medium (MEM) containing L-glutamine, 10% fetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin (Sigma, St Louis, MO, USA) at 37 °C under 5% CO₂. Following a 24 h incubation, 2 µg promoter–reporter construct and 0.02 µg pRLSV-40 plasmid were transfected in each well using the calcium phosphate method. The pRLSV-40 plasmid was co-transfected as the transfection efficiency control. Twelve hours after transfection, cells were washed with PBS and cultured in fresh medium for 36 h prior to luciferase assay. Each construct was transfected in duplicate in three or more experiments. In each experiment, the pGL3B plasmid was also transfected in separate wells in order to measure the basic activity of the promoter-less plasmid.

Luciferase activity assay

Following 48 h of culture after transfection, cells were washed twice with PBS and lysed in 200 µl Passive Lysis Buffer (Promega) at room temperature for 15 min. Activities of the firefly luciferase and Renilla luciferase in the cell lysate were measured sequentially by using the Dual-Luciferase Reporter Assay (DLR) system (Promega) essentially according to the manufacturer's instructions. The light output from the firefly luciferase or the Renilla luciferase was recorded for 15 s following a 3 s pre-measurement delay using a TD-20e Luminometer (Tuner Designs, Sunnyvale, CA, USA).

The variation in transfection efficiency was normalized by dividing the firefly luciferase activity with the Renilla luciferase activity. The transcriptional activity of the promoter–reporter construct was presented as relative values to that of the pGL3B plasmid. Data were expressed as mean ± s.e. of values from three or more independent experiments and tested for treatment differences by analysis of variance.

Extraction of nuclear proteins

The bovine liver, kidney and brain tissues were dissected from adult non-lactating cows at slaughter and transported to the laboratory on ice within 30 min. Extraction of nuclear proteins from the bovine tissues was performed essentially as described (Gorski *et al.* 1986) with the modification that 1 ml of protease inhibitor cocktail (Sigma) was added to the homogenization buffer for 20 g of tissue. Extractions of nuclear proteins from cultured Hep G2 and BHK-21 cells were carried out essentially as described (Ausubel *et al.* 1994). The

protein concentration of nuclear extracts was determined by the Lowry assay.

DNase I footprinting analysis

The DNase I footprinting analysis was done as described previously (Lakin 1994). Briefly, the DNA fragments containing the 5' flanking region (nt -191 to -12) of the bovine exon 1B were labeled at the 5' end of either the sense strand or the antisense strand with T4 polynucleotide kinase and ^{32}P - γ -ATP. The probe (2×10^4 c.p.m., approximately 0.06 ng) was incubated with the recombinant human Sp1 (Promega) on ice for 30 min in $1 \times$ binding buffer (50 mM Tris-HCl, 100 mM KCl, 12.5 mM MgCl_2 , 1 mM EDTA, 20% glycerol, 1 mM DTT, pH 8.0) containing 2 μg of poly d(A-T) (Sigma). The probe-protein mixture was then added with 50 μl of $\text{Ca}^{2+}/\text{Mg}^{2+}$ (5 mM CaCl_2 , 10 mM MgCl_2) and digested with 0.01 U/ μl DNase I (Promega) at room temperature for 1 min. The DNase I digestion was terminated by adding 90 μl of stop buffer (200 mM NaCl, 30 mM EDTA, 1% SDS, 40 $\mu\text{g}/\text{ml}$ yeast tRNA). The reaction was then extracted with 200 μl of phenol-chloroform-isoamyl alcohol and precipitated with ethanol followed by electrophoresis on a 6% polyacrylamide and 8 M urea sequencing gel in $1 \times$ TBE buffer (100 mM boric acid, 2 mM EDTA and 100 mM Tris-HCl, pH 8.4). The (G+A) Maxam-Gilbert sequencing ladders were prepared as described previously (Maxam & Gilbert 1980) and served as size markers. The gel was dried and subjected to autoradiography as described above.

Electromobility shift assay (EMSA)

The double-stranded oligonucleotide corresponding to the DNA fragment -87/-66 was generated by annealing complementary oligonucleotides in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA and 5 mM MgCl_2 by heating to 90 $^{\circ}\text{C}$ for 10 min and slowly cooling to 25 $^{\circ}\text{C}$ over 1 h. Both ends of the oligonucleotide were end-labeled with ^{32}P using T4 polynucleotide kinase and ^{32}P - γ -ATP. Approximately 2×10^4 c.p.m. (equal to 0.02 ng) probe was added to a mixture containing 5 μg nuclear extract, 2 μg of poly d(A-T), 2% Ficoll 400 and $1 \times$ binding buffer (as described before) in a total volume of 20 μl (the probe was added last). The binding reactions were further incubated on ice for 45 min and analyzed by electrophoresis on a native polyacrylamide gel in $0.5 \times$ TBE buffer. For competition or super-shift assays, the nuclear extract was incubated with the competitor oligonucleotides (unlabeled oligonucleotide or the Sp1

consensus oligonucleotide, Promega) or antibodies (anti-Sp1 and anti-Sp3, Santa Cruz Biotechnology, Santa Cruz, CA, USA) on ice for 2 h prior to the addition of the labeled probe.

RESULTS

Genomic sequence

Positive genomic clones were isolated by screening a total of 5×10^5 pfu of a bovine genomic library with an exon 1B-specific probe. Subcloning of a 2.3 kb XhoI-XhoI genomic fragment in the pBluescript SK(-) plasmid and subsequent sequencing revealed that the 2.3 kb fragment was composed of exon 1B, the 5' flanking region of exon 1B, and a 0.6 kb downstream region from exon 1B. The sequence of this 2.3 kb fragment has been deposited in GenBank under accession number AF046861. The 500 bp region from the 3' end, containing exon 1B, has 75% GC. Sequence comparison revealed that the 1.7 kb bovine GHR sequence was 95% identical to the ovine GHR sequence (GenBank accession S78252) and 90% identical to the human GHR sequence (GenBank accession AJ002175). Restriction site mapping of the 1.7 kb bovine GHR DNA sequence identified cleavage sites for common restriction enzymes such as HindIII, NcoI, NotI, SacII, SmaI and PstI. These sites were confirmed by restriction enzyme digestion of pSK41-1-6.

Transcription start sites in exon 1B

Based on a GenBank search, the reverse primer complementary to nt +337 to +358 (Fig. 1) of the bovine GHR DNA sequence was not complementary to other known DNA sequences. The primer extension analysis generated two major extended products that were 358 and 349 nt in length (Fig. 2). These two extended products corresponded to transcription initiation from two start sites, designated +1 and +10, in exon 1B of the bovine GHR gene (Fig. 1). Sequence analysis of the 5' proximal region of exon 1B using the MatInspector program (Heinemeyer *et al.* 1999) did not reveal a consensus TATA box that could define a transcription start site. However, the sequence surrounding the two start sites contained several sequence elements (CCAGATG, -17 to -11; TGAGAGT, -12 to -6; TGACACG, -6 to +1; CAACTCC, +5 to +11; CAACTCC, +12 to +18) that are similar to the consensus sequence ($\text{P}_y\text{P}_y\text{A}^{+1}\text{T}/\text{AP}_y\text{P}_y$) for initiator element (Inr), a

FIGURE 2. Mapping of the transcription start site in exon 1B of the bovine GHR gene. The mapping was done by using primer extension analysis. Ten micrograms of total liver RNA or yeast tRNA (as a negative control) were mixed with a ^{32}P -labeled primer complementary to the sequence from +337 to +358 within the bovine GHR gene (see Fig. 1 for locations) and extended by reverse transcription. Extended products were resolved on a 6% polyacrylamide sequencing gel. (A) Two major extended products were transcribed from the bovine liver RNA but not from the yeast tRNA. (B) The two major extended products were determined to be 358 and 349 nucleotides (nt) in length by comparison with a DNA sequencing ladder (lanes G, A, T and C) and an 100 bp DNA ladder (M). The two start sites corresponding to these two extended products were indicated in the bovine GHR genomic sequence presented in Fig. 1.

DNA sequence that defines the transcription start site in many TATA-less promoters (Javahery *et al.* 1994).

FIGURE 3. Transient transfection analysis of the 5' flanking region of exon 1B in Hep G2 and BHK-21 cells. The promoter-reporter plasmid constructs contain various 5' flanking regions of exon 1B inserted upstream of the firefly luciferase reporter gene in pGL3B. The numbers in each construct refer to the locations of nucleotides in the sequence presented in Fig. 1. Two micrograms of the plasmid construct and 0.02 μg pRL-SV40 (encoding the Renilla luciferase used as transfection efficiency control) were co-transfected into Hep G2 or BHK-21 cells using the calcium phosphate method. Activities of the firefly luciferase and Renilla luciferase were assayed by using the Dual-Luciferase Assay System (Promega). Activities of the promoter-reporter plasmids are expressed relative to that of the pGL3B plasmid after normalizing against the activity of pRL-SV40. Values are expressed as mean $\pm$ s.e. from three independent transfection analyses. Bars with different letters are significantly different ($P < 0.05$) from other groups within the same cell line.

Functional analysis of the 5' flanking region of exon 1B

The possibility that the 5' flanking region of exon 1B functioned as a promoter (designated P2) was suggested by the high level of GHR 1B mRNA expression *in vivo*. The transcriptional activity of P2 was examined in Hep G2 and BHK-21 cells. We initially tested the 1.5 kb fragment from the 5' end (-1217 in Fig. 1) of pSK41-1-6 to nt +300 in exon 1B (Fig. 1). As shown in Fig. 3, 1B($-1217/+300$)-GL3 had a sixfold greater activity than did the promoter-less plasmid pGL3B ($P < 0.01$). To identify the minimal regions that are required for

the basal promoter activity of P2, we next examined the transcriptional activity of various deletion constructs. Construct 1B(−1217/−12)-GL3 did not include the 300 bp downstream region from the transcription start site compared with construct 1B(−1217/+300)-GL3. Following transfection into cells, construct 1B(−1217/−12)-GL3 maintained a similar level of luciferase activity compared with construct 1B(−1217/+300)-GL3 ($P>0.05$) (Fig. 3), suggesting that the 300 bp exon 1B region was not important to the transcriptional activity of P2. Constructs 1B(−750/−12)-GL3, 1B(−191/−12)-GL3, and 1B(−104/−12)-GL3 had sequential deletions from the 5' end. Compared with 1B(−1217/−12)-GL3, construct 1B(−750/−12)-GL3 showed lower activity in BHK-21 cells ($P<0.01$) but maintained a similar level of activity in Hep G2 cells ($P>0.05$) (Fig. 3). However, further deletion of the 560 bp region between −750 and −191 resulted in a dramatic increase in the transcriptional activity of construct 1B(−191/−12)-GL3 in both cell types ($P<0.01$) (Fig. 3). This result suggested that the 560 bp region between −750 and −191 may contain negative element(s) for initiating transcription in exon 1B. Further deletion of an 87 bp region from the 5' end did not cause any significant reduction ($P>0.05$) of transcriptional activity in construct 1B(−104/−12)-GL3. Construct 1B(−104/−12)-GL3 conferred a 13- and 9-fold activity in Hep G2 and BHK-21 cells, respectively, compared with the promoter-less plasmid pGL3B ($P<0.01$) (Fig. 3). These results indicated that the basal promoter activity of P2 resides in the 93 bp proximal 5' flanking region.

Identification of a Sp1 binding site within nt −87 to −66 of P2

Computer analysis for transcription factor binding sites using the TFSEARCH program (at threshold score of 80.0) (Heinemeyer *et al.* 1999) and the MatInspector program (at optimized matrix similarity) revealed several putative protein binding sites including IK2, STAT, NGFIC, ETS, C-Ets and Sp1 within the 5' proximal region −104/−12. Among these putative binding proteins, Sp1 was of particular interest because Sp1 is a ubiquitous transcription factor interacting with many constitutive promoters (Jones *et al.* 1988, Latchman 1995). Therefore, Sp1 was the most likely candidate binding protein that activates the GHR promoter P2 in various tissues. To test this possibility, a DNase I footprinting analysis was performed to determine if the putative Sp1 binding site bound to Sp1 protein. A DNase I footprint was observed

when the recombinant human Sp1 protein was incubated with the 5' proximal DNA fragment (Fig. 4). The footprint spanned nt −87 to −66 (−87/−66). This region includes a GC box (GGGCGG) that represents a core binding sequence for Sp1. We next performed an EMSA analysis to determine if the same region bound to nuclear proteins isolated from Hep G2 and BHK-21 cells and to determine if the binding proteins from the nuclear extracts were indeed Sp1. Incubation of a 32 P-labeled double-stranded DNA oligonucleotide corresponding to the −87/−66 region with nuclear proteins extracted from Hep G2 and BHK-21 cells generated a distinct DNA–protein binding complex (Fig. 5). The specificity of this complex was demonstrated by the ability of an excess of the same unlabeled oligonucleotide to compete effectively with the probe for binding the nuclear protein. Addition of an excessive amount of the unlabeled Sp1 consensus oligonucleotide (5'-ATTTCGATCGGGGCGGGGCGAGC-3') also effectively competed with the probe for binding the nuclear protein. This suggested that Sp1 was present in the binding complex. The presence of Sp1 in the DNA–protein complex was further demonstrated by supershift experiments, in which anti-Sp1 antibodies retarded the DNA–protein complex formed with both Hep G2 and BHK-21 nuclear extracts (Fig. 5). The GC box is recognized by Sp1 as well as other members of the Sp1-family, i.e. Sp3, Sp4, BTEB and BTEB2 (Lania *et al.* 1997). However, the nearly complete shift of the DNA–protein complex by anti-Sp1 antibodies indicated that the DNA–protein complex was formed primarily with Sp1. This was further supported by the observation that anti-Sp3 antibodies had no effect on the mobility of the binding complex (Fig. 5).

Requirement of the Sp1 binding site for the transcriptional activity of P2

The functional importance of the Sp1 binding site to the promoter activity of P2 was demonstrated by deleting the GGGCGG sequence from the −104/−12 fragment and determining the transcriptional activity of the deleted fragment in transient transfection analysis. Deletion of the GC box abolished the binding of Sp1 to the −104/−12 fragment (demonstrated by DNase I footprinting analysis, data not shown). Deletion of the GC box significantly decreased the luciferase activity of deletion construct 1B(−104/−12delGC)-GL3 in both Hep G2 and BHK-21 cells, compared with the intact construct 1B(−104/−12)-GL3 ($P<0.05$) (Fig. 6). These results suggest that the −87/−66

site within P2 contributes to the promoter activity through interactions with the nuclear Sp1 protein.

Binding of the GC box-containing sequence by nuclear proteins in bovine tissues

The above experiments identified the interaction of Sp1 with the GC box-containing DNA element within the bovine GHR P2 in cell lines. To determine whether the GC box DNA element

found *in vitro* also bound to identical nuclear proteins in bovine tissues, we did additional EMSA analyses. The EMSA analyses of the GC box-containing DNA fragment in the bovine nuclear protein extracts demonstrated that the 22 bp –87/–66 DNA fragment formed a DNA–protein complex with the same mobility across different nuclear extracts (Fig. 7). The presence of Sp1 protein in the complexes was indicated by the competition of a Sp1 consensus oligonucleotide with the DNA fragment of –87/–66 for binding to the nuclear protein (Fig. 7). The DNA–protein complexes formed from nuclear protein of the bovine tissues and cell lines migrated with the same mobility (data not shown). Therefore, nuclear proteins in the bovine tissues and the non-bovine cell lines were either identical or closely related proteins. Furthermore, the observation of DNA–protein complexes in diverse bovine tissues (liver, kidney and brain) that express GHR 5'-UTR 1B suggests that these binding proteins may be ubiquitous in the bovine.

DISCUSSION

Numerous genes use multiple promoters to initiate transcription (Schibler & Sierra 1987, Ayoubi & Van De Ven 1996). Multiple variants of 5'-UTR have been isolated for the GHR mRNA in various species (Pekhletsy *et al.* 1992, O'Mahoney *et al.* 1994, Adams 1995, Baumbach & Bingham 1995, Domene *et al.* 1995, Southard *et al.* 1995, Jiang & Lucy 1998, Jiang *et al.* 1999, Schwartzbauer *et al.* 1999). Each 5'-UTR could be generated by transcription from an independent promoter. In the present study, we demonstrated that the bovine GHR 5'-UTR 1B is initiated from an alternative

FIGURE 4. DNase I footprinting analysis of the 5' flanking region of exon 1B in the presence of Sp1. A DNA fragment corresponding to the 5' flanking region –191/–12 of exon 1B (see Fig. 1 for locations) was end-labeled on the antisense strand using T4-polynucleotide kinase and ^{32}P - γ -ATP. Approximately 2×10^4 c.p.m. (corresponding to 0.06 ng) probe was partially digested with DNase I in the absence (–) or presence of 1, 2 and 4 footprint units of the recombinant human Sp1 protein (Promega). The (G+A) lanes contain a Maxam–Gilbert sequencing ladder of the same DNA fragment. The vertical line beside the autoradiograph indicates the DNase I protected site, which corresponds to the nt –87 to –66 sequence in Fig. 1. The same site was footprinted when the sense strand was analyzed (autoradiograph not shown).

FIGURE 5. Electromobility shift assay (EMSA) of the $-87/-66$ DNase I protected DNA fragment (GC box) in the nuclear extracts from Hep G2 and BHK-21 cells. Approximately 2×10^4 c.p.m. (0.02 ng) ^{32}P -labeled double-stranded oligonucleotide corresponding to the footprinting region in Fig. 4 (nt -87 to -66 in Fig. 1) was incubated with $5\text{ }\mu\text{g}$ of nuclear extracts on ice for 45 min. The DNA-protein complex was analyzed by gel electrophoresis and visualized by autoradiography. For the competition assay, the nuclear extract was pre-incubated with 100 -fold molar excess of unlabeled oligonucleotide (cold oligo) or the Sp1 consensus oligonucleotide (Sp1 oligo) for 45 min on ice prior to addition of the probe. For the supershift assay, the nuclear extract was pre-incubated with $2\text{ }\mu\text{l}$ of antibody anti-Sp1, anti-Sp3 or a nonspecific antiserum (NSA) on ice for 2 h prior to addition of the probe. (A) Detection of the DNA-protein complex and competition of the complex by excess unlabeled oligonucleotide or excess Sp1 consensus oligonucleotide. (B) Supershift of DNA-protein complex by anti-Sp1 but not by anti-Sp3 antibodies. The binding reactions were electrophoresed on a 6% polyacramide gel for 3 h at 130 V (upper panel) or on a 4% gel for 5 h at 130 V (lower panel). Letters B, F and SS denote the DNA-protein complex, free probe and the supershifted DNA-protein complex respectively.

promoter named P2 within the bovine GHR gene. While our study of the bovine P2 confirmed the findings of a previous study of the ovine GHR gene (Adams 1995), our study of the bovine P2 identified, for the first time, the ubiquitous transcription factor Sp1 as the binding protein to the bovine GHR P2.

FIGURE 6. Functional analysis of the GC box in the $-104/-12$ DNA fragment. Construct 1B($-104/-12\text{delGC}$)-GL3 had a deletion in the GGGCGG sequence (-80 to -75 in Fig. 1) compared with construct 1B($-104/-12$)-GL3. The transfection and luciferase assay were done as described in Fig. 3 and in the text. Values are mean $\pm$ s.e. from four experiments. Asterisks indicate the statistical difference (detected by analysis of variance) between two groups within the same cell line ($P < 0.05$).

The promoter activity of a DNA fragment is generally assessed by its ability to direct reporter gene expression in cultured cells. In the present study, we fused the $5'$ flanking region of the bovine GHR exon 1B with a luciferase reporter gene in the promoter-less plasmid pGL3B. Significant luciferase activity was observed when the promoter-reporter plasmids were transfected into the human hepatoma cell line Hep G2 and the baby hamster kidney cell line BHK-21. These *in vitro* results with cell lines that differ in both tissue and species origins support the previous *in vivo* studies of the GHR $5'$ -UTR 1B that showed a ubiquitous expression pattern (Adams 1995, Domene *et al.* 1995, Southard *et al.* 1995, Lucy *et al.* 1998).

The bovine GHR exon 1B and its proximal $5'$ flanking region have a high GC content and lack a TATA box. These features are common to many housekeeping gene promoters (Dynan 1986). Not surprisingly, the same structural features in the bovine GHR gene were found in the respective regions of the ovine (Adams 1995), human (GenBank accession AJ002175) and mouse (GenBank accession AF120480) GHR genes. Consistent

FIGURE 7. Electromobility shift assay of the $-87/-66$ DNA fragments in nuclear extracts isolated from the bovine liver, kidney and brain. The EMSA was done as described for Fig. 5 in the absence or presence (+) of excess unlabeled oligonucleotide (cold oligo) or Sp1 consensus oligonucleotide (Sp1 oligo).

with the similarity between the 5' flanking sequences of exon 1B in the GHR genes, the transcription start sites mapped in the bovine and ovine exon 1B were within a few nucleotides (Adams 1995). The mouse start site was not mapped to the same region, perhaps because of sequence differences between the mouse GHR P2 and the bovine and ovine P2 (Moffat *et al.* 1999). Two major bovine start sites in exon 1B were mapped in the present study and multiple start sites were also identified in the ovine exon 1B (Adams 1995). Mapping of multiple transcription start sites in both the bovine and the ovine sequences is consistent with the lack of a consensus TATA box in the 5' proximal region of exon 1B. In some TATA-less promoters, the transcription initiation site is determined by a DNA element called transcriptional initiator (Inr), which usually surrounds the transcriptional start site (Javahery *et al.* 1994). Sequences that are similar to the consensus Inr sequence were found around the mapped transcription start sites in the bovine, ovine and mouse GHR genes. An interesting question that remains to be answered is whether the transcription initiation sites are dictated by these Inr-like sequences.

Functional analysis of the 5' flanking region of exon 1B identified two major regulatory regions. The region between -750 and -191 contained negative element(s) for the transcription of exon 1B, whereas the 93 bp proximal region between -104 and -12 contained positive regulatory elements

necessary for basal promoter activity. DNase I footprinting analysis and EMSA of the 5' proximal region identified a DNA element (GC box containing site) that binds Sp1. The functional importance of the interaction between Sp1 and the GC box to the activity of P2 was demonstrated by deletion analysis. Deletion of the GC box decreased the activity of P2 in cells, presumably by preventing Sp1 binding. Sp1 is known as a ubiquitous transcription factor that transactivates many constitutive promoters (Latchman 1995, Jones *et al.* 1988). We demonstrated that nuclear extracts from diverse bovine tissues also bound the GC box. Therefore, interaction of Sp1 with the GHR promoter P2 is part of the mechanism for the expression of the bovine GHR 5'-UTR 1B in various tissues.

Deletion of the GC box only partially abolished the transcriptional activity of the $-104/-12$ region. This suggests that the maximal transcriptional activity of the fragment involves other regulatory elements. Using similar techniques, we identified a second DNA element containing a CCAAT box within nt -61 to -31 and this CCAAT box-containing site appeared to bind ubiquitous nuclear proteins. We tested for the ubiquitous CCAAT box binding factors CTF/NF-1 and NF-Y by using supershift analysis but found that neither protein was part of the DNA-protein complex. We are currently trying to identify the binding protein to this second DNA element by testing other candidate transcription factors as well as by using the yeast one-hybrid system. Another regulatory region of the GHR P2 may reside in the upstream region of P2 (nt -750 to -191). Deletion of this region increased the promoter activity of the 5' flanking region, suggesting that this region contains repressor elements for P2 activity. The binding protein(s) to this region remains to be characterized.

Although the 5'-UTR 1B initiated from P2 is ubiquitously expressed, GHR mRNA amounts in many tissues change in response to hormonal and physiological statuses (Harvey *et al.* 1995, Bennett *et al.* 1996, Florini *et al.* 1996, Li *et al.* 1996, Nguyen *et al.* 1996, Chen *et al.* 1997, Jux *et al.* 1998). Changes in the total GHR mRNA may at least in part result from the changes in the activity of P2 because the GHR 1B mRNA represents the majority of the GHR mRNA pool. Although Sp1 has traditionally been characterized as a constitutive transcription factor, increasing evidence has indicated that Sp1 binding and transactivation can be modulated by many hormones and growth factors (Jensen *et al.* 1995, Merchant *et al.* 1995, Porter *et al.* 1997, Ahlgren *et al.* 1999).

Identification of Sp1 as one of the transcription factors for the GHR P2 allows us to ask whether Sp1 mediates the hormonal regulation of the GHR P2 activity and therefore the levels of the GHR 1B mRNA. The changes in GHR 1B mRNA that are in part controlled by Sp1 as well as other transcription factors may ultimately determine the GH-responsiveness of most tissues.

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