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A Distal Regulatory Region of the Insulin-Like Growth Factor Binding Protein-2 (IGFBP-2) Gene Interacts with the Basic Helix–Loop–Helix Transcription Factor, AP-4

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Insulin-like growth factor binding protein-2 (IGFBP-2), the predominant IGFBP in the fetal circulation and an induced protein during several types of malignancies, belongs to a family of structurally related proteins that bind the mitogens, IGF-1 and IGF-2. The present study focused on functional analysis of the 5'-flanking region (~1.3 kb) of the IGFBP-2 gene to identify nuclear factors that mediate hepatic transcription of this gene. Luciferase (LUC) reporter constructs containing progressive deletions of 5'-flanking DNA and the intact promoter of the porcine IGFBP-2 gene were examined for functional activity by transient transfection of human HepG2 liver cells. LUC activity of the transfected reporter gene driven by the IGFBP-2 promoter and flanking sequences to –1397 (numbering relative to initiation codon at +1) was 22-fold higher than that of promoterless parent LUC vector. This activity was decreased by 60% with deletion of sequences to –874 bp, and dropped to basal levels with further truncation to –764 bp. The region between –874 and –765 bp (110 bp) functioned as a potent stimulator of heterologous SV40 promoter activity (110 bp/SV40-LUC construct) and was found to contain two noncontiguous basic helix–loop–helix (bHLH) transcription factor binding motifs (E-boxes [CANNTG]: CACCTG and CAAATG). In electrophoretic mobility shift assays, nuclear proteins prepared from HepG2 cells formed two complexes (C1, C2) with double-stranded oligonucleotides containing either HLH sequence, mutations of which resulted in loss of complex formation. Southwestern blot analysis identified an HepG2 nuclear protein with molecular mass of 48 kDa, similar to that of the bHLH transcription factor AP-4, which bound the CACCTG motif. Cotransfection of HepG2 cells with the 110-bp/SV40-LUC construct and an expression vector encoding human AP-4 increased IGFBP-2 fragment-dependent SV40 promoter activity by 16-fold. This AP-4-mediated stimulation was lost following block mutation of both bHLH motifs within the IGFBP-2 110-bp fragment. Results demonstrate the functional importance of sequences upstream of the promoter in IGFBP-2 gene transcription and identify a novel mechanism by which bHLH proteins potentially may affect cell proliferation and differentiation via induction of IGFBP-2 synthesis.

Key Words: IGFBP-2; transcription; AP-4; Hep G2.

Introduction

The insulin-like growth factor binding proteins (IGFBPs) are a family of secreted proteins that share the ability to bind IGF-1 and IGF-2 *(1–4)*. As a consequence of this high-affinity binding, IGFBPs modulate IGF bioavailability and interactions of IGFs with cell membrane IGF receptors. IGFBPs also may have IGF-independent functions *(3,4)*. IGFBP-2, an evolutionarily well-conserved protein with molecular mass of 34–36 kDa, is the predominant circulating IGFBP in developing fetuses and is elevated during several human malignancy states *(5–13)*. The mRNA encoding IGFBP-2 is expressed during early embryogenesis, in a number of discrete fetal cell populations of ectodermal and endodermal origins, in fetal liver and kidney tissues, and in uterine endometrium during pregnancy *(6,11,12,14–16)*. In rodents, this gene's mRNA expression declines to very low levels after birth in all tissues, except for the brain, where it continues to be expressed at high levels in the choroid plexus *(17)*. Pathophysiological states known to affect hepatic IGFBP-2 gene expression include fasting *(14,18)*, hypophysectomy *(18,19)*, somatotropin

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excess *(20)*, and diabetes *(21)*. An understanding of the mechanisms by which liver IGFBP-2 biosynthesis is regulated is essential to an understanding of the physiological control of this IGFBP and its ligand(s) during development and other normal and abnormal physiological states.

The genomic organization and functional promoter of the IGFBP-2 gene have been elucidated for the mouse *(10)*, rat *(22)*, human *(23)*, pig *(16)*, and chicken *(24)*. The promoter and immediate 5'-flanking region are G/C-rich (CpG island) and devoid of TATA and CAAT motifs. Several G/C boxes identified in the rat IGFBP-2 promoter are conserved in the other mammalian IGFBP-2 promoter regions, and these have been shown to bind Sp1 and to mediate basal IGFBP-2 promoter activity *(25,26)*. However, based on the relatively ubiquitous expression of Sp1 and related Sp family members that bind G/C boxes, it is anticipated that tissue-specific transcription factors additionally might mediate IGFBP-2 gene expression. In this study, we have searched for upstream regions that contribute to hepatic IGFBP-2 gene transcriptional activity. We demonstrate that sequences distal to the promoter can augment native (IGFBP-2) as well as heterologous (SV40) promoter activity, and that one such region contains two bHLH protein recognition motifs that are *trans*-activated by the AP-4 transcription factor and that may mediate IGFBP-2 gene transcription in HepG2 liver cells.

Results

IGFBP-2 Expression in HepG2 Cells

In order to validate its use as a recipient cell line for IGFBP-2 gene promoter transfection experiments, the human hepatoma cell line HepG2 was characterized for endogenous expression of the IGFBP-2 gene. Northern analysis of total cellular RNA isolated from these cells identified a diffuse IGFBP-2 transcript of ~4.2 kb in length (Fig. 1A). Moreover, HepG2 cells secreted a 36-kDa protein consistent with the size of human IGFBP-2 *(5)* and was recognized on Western blots by rabbit polyclonal antiserum specific for IGFBP-2 (Fig. 1B).

Identification of Upstream Activating Regions in the IGFBP-2 Gene

We previously reported the structure of the porcine IGFBP-2 gene and the nucleotide sequence of 1.3 kb of its 5'-flanking region *(16)*. To determine whether the upstream region is functionally relevant to hepatic IGFBP-2 expression, 5'-deletions (*Hin*dIII, –1397; *Sac*I, –874; *Bgl*II, –764; and *Sma*I, –305; relative to the translation initiation codon, +1) were fused to an LUC enzyme reporter gene (Materials and Methods) and transiently transfected into HepG2 cells (Fig. 2). The –1397 bp fragment stimulated LUC activity ~22-fold over the parent promoterless LUC vector (Fig. 2). Removal of the region located between nt –1397 and nt –875 decreased the IGFBP-2 promoter activity by ~57%. Further deletion to nt –765 decreased IGFBP-2 promoter activity to basal levels (~5% of the longest construct), but no additional change was detected with removal of sequences extending from nt –764 to nt –306 (Fig. 2). These results indicate that the region between –1397 and –765 contains sequences that stimulate IGFBP-2 gene transcription in HepG2 cells.

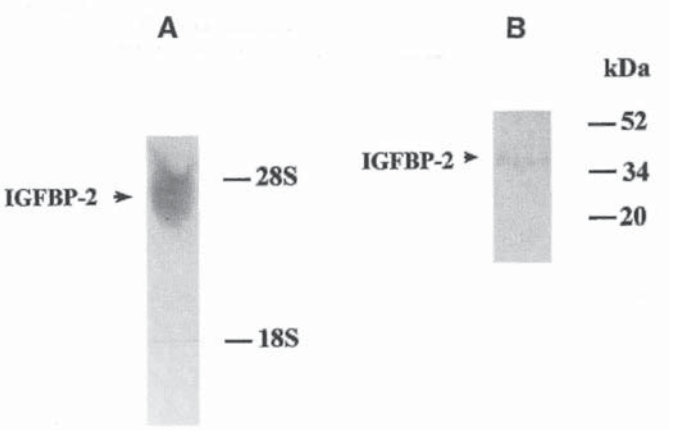

Fig. 1. Characterization of HepG2 cellular IGFBP-2 mRNA and secreted IGFBP-2 protein. **(A)** Northern blot of IGFBP-2 mRNA. Thirty micrograms of total cellular RNA isolated from confluent HepG2 cells were subjected to Northern analysis using a porcine IGFBP-2 cDNA probe. **(B)** Western blot of IGFBP-2 in HepG2 conditioned medium (CM). Approximately 100 µg of CM proteins were subjected to Western blot analysis using a rabbit polyclonal antibody to porcine recombinant IGFBP-2. The migration positions of the prestained M_r standards are indicated.

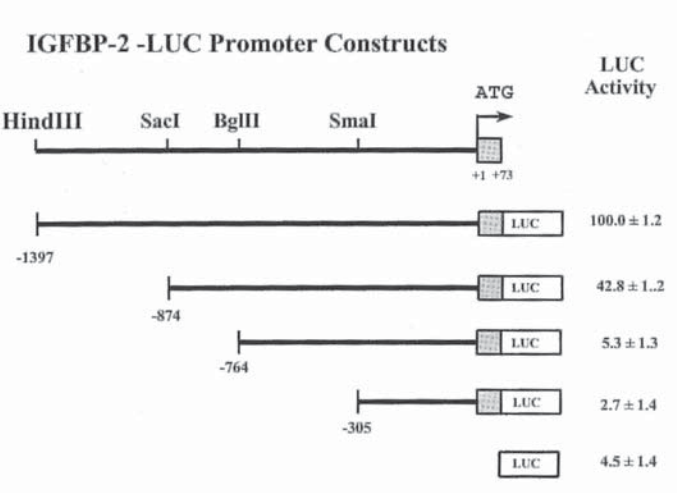

Fig. 2. Analysis of IGFBP-2 promoter activity in HepG2 cells. Subconfluent HepG2 cells were transiently transfected with plasmid DNA (20 µg) containing varying lengths of IGFBP-2 sequence inserted upstream of the LUC reporter gene. Forty-eight hours posttransfection, cells were lysed and assayed for LUC enzyme activity. LUC activity is expressed relative to that of the longest DNA construct, and the results are means ± SEM of four independent transfection experiments (n = 3 replicates/construct/experiment).

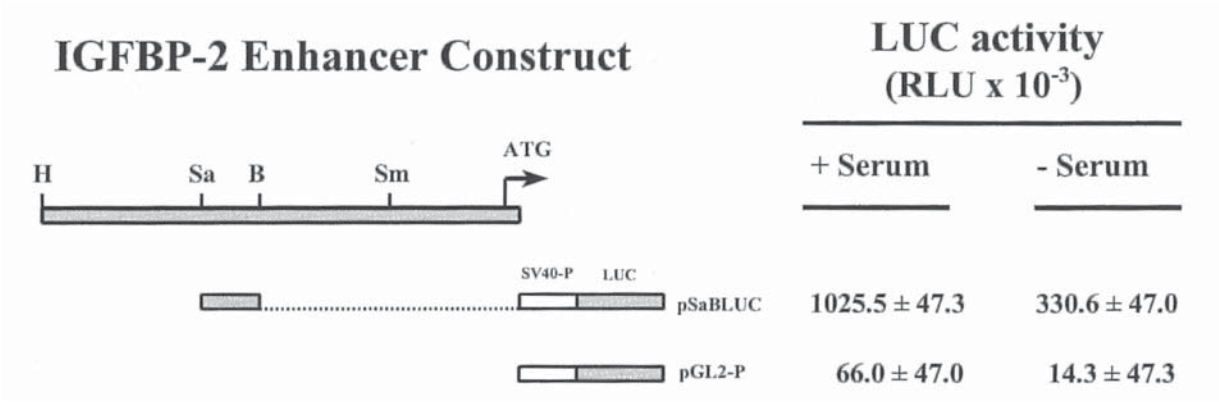

Fig. 3. Functional analysis of an IGFBP-2 gene upstream region. HepG2 cells were transiently transfected with plasmid DNAs (20 μg) representing the 110-bp upstream DNA fragment linked to the pGL2-promoter vector (pSaBLUC) or the parental pGL2-promoter vector. Cells were incubated with or without fetal bovine serum for 48 h posttransfection and assayed for LUC enzyme activity. Results are expressed as relative light units (RLU) and represent means ± SEM of four independent transfection experiments ($n = 3$ replicates/construct/experiment).

Stimulatory Activity of the IGFBP-2 Upstream Region

The above studies implicated a region between –874 and –765 as a positive effector of IGFBP-2 promoter activity in HepG2 cells. To examine further the functional activity of this region, the –874 construct was cleaved with *Sac*I and *Bgl*II to yield the 110-bp fragment spanning from nt –874 to nt –765. This DNA fragment was inserted, in sense orientation, immediately adjacent to the SV40 promoter-LUC region of the pGL2-promoter (pGL2-P) vector (Fig. 3). This construct, designated as pSaBLUC, had 16-fold greater LUC activity than the parent pGL2-P vector when transfected into HepG2 cells (Fig. 3). Interestingly, the 110-bp-dependent activity of pSaBLUC was decreased by 68% ($p < 0.05$) when fetal bovine serum was omitted (posttransfection) from the culture, whereas activity of the pGL2-P vector was unaffected ($p > 0.05$) by presence or absence of serum (Fig. 3), suggesting that a serum factor(s) affects transcriptional function(s) of the 110-bp fragment in HepG2 cells.

Identification of bHLH Core Motifs That Bind HepG2 Nuclear Protein(s)

Putative *cis*-acting elements within the 110-bp fragment were identified by electrophoretic mobility shift assay as well as comparison to consensus recognition sequences reported for other transcription factors. Two bHLH transcription factor binding motifs (E-boxes) were identified at nt –843 (5'-CACCTG-3') and nt –776 (5'-CAAATG-3'), respectively. Complementary oligonucleotides spanning these two motifs (designated S1 and S2) (Fig. 4A) were synthesized, annealed, and used in electrophoretic mobility shift assays to examine for binding to HepG2 nuclear proteins. Radiolabeled S1 probe formed two closely migrating, specific DNA–protein complexes (C1, C2) with HepG2 nuclear protein(s) (Fig. 4B). Radiolabeled S2 probe yielded an identical electrophoretic mobility shift pattern (C1 and C2) with HepG2 nuclear proteins (data not shown). Formation of both complexes was completely abolished by addition of 10-fold molar excess of unlabeled double-stranded S1 or S2 to the binding reaction (Fig. 4B). By contrast, binding to S1 was not inhibited by addition of an unrelated double-stranded oligonucleotide from the porcine uteroferrin (UF) gene promoter (Fig. 4B).

To demonstrate that the bHLH motif is responsible for the binding interaction with HepG2 nuclear protein(s), three block mutations, two of which overlap the bHLH recognition sequence, were separately introduced within the S1 oligonucleotide (Fig. 5A). These mutant oligonucleotides were subsequently used as unlabeled DNA competitors in electrophoretic mobility shift assays with radiolabeled S1 probe. Mutl, which had an intact bHLH motif (i.e., two intact E-box half sites), completely inhibited the binding of the wild-type (wt; S1) probe to HepG2 nuclear protein(s) (Fig. 5B). Mut2, in which the two (CA) nucleotides defining one half-site of the bHLH motif were altered, competed with the wild-type (wt) probe, but only at higher molar concentrations (Fig. 5B). Binding of the wt probe was unaffected (i.e., no dose-dependent inhibition) by coincubation with excess mut3, which had four out of six bases altered from the native bHLH motif (CA→CG, TG→TT) and which altered both E-box half-sites, or the unrelated UF oligonucleotide (Fig. 5B).

The bHLH Motifs Bind a 48-kDa Nuclear Protein

To characterize the protein(s) that bind these bHLH motifs, HepG2 nuclear extracts were used in Southwestern blot analysis with ^{32}P-labeled S1 probe. Two proteins with molecular masses of 48 kDa (major band) and 118 kDa (minor band) were observed to bind labeled S1 (Fig. 6); however, significant reduction in binding intensity with addition of a 20-fold molar excess of unlabeled S1 oligonucleotide was observed only with the smaller protein (Fig. 6).

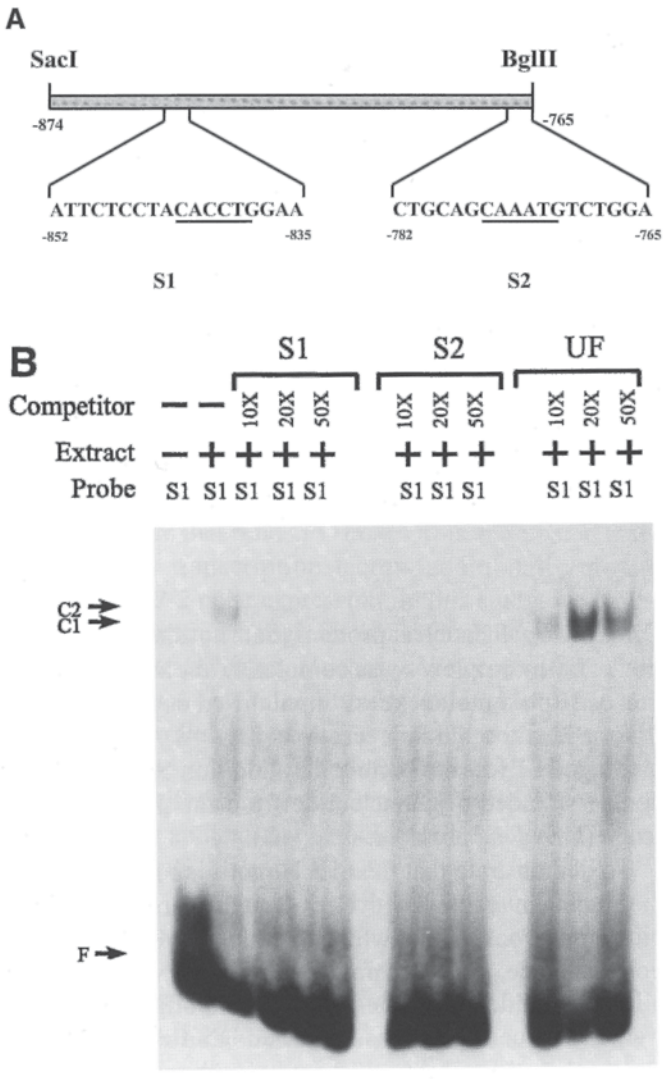

Fig. 4. HepG2 nuclear proteins interact with oligonucleotides (S1 and S2) containing core HLH transcription factor binding motifs. **(A)** Location and nucleotide sequence of S1 and S2. Core HLH motifs were identified at nt –843 and nt –776 within the 110-bp region of the IGFBP-2 gene, and oligonucleotides (S1 and S2) containing these and adjacent nucleotides were synthesized, annealed, and used in gel mobility shift assays. **(B)** Binding of S1 probe to HepG2 nuclear extract. Radiolabeled S1 was incubated with 10 μg of HepG2 nuclear extract in the absence or presence of increasing concentrations of unlabeled double-stranded oligonucleotide. An unrelated oligonucleotide (UF) was also used in competition studies as a control.

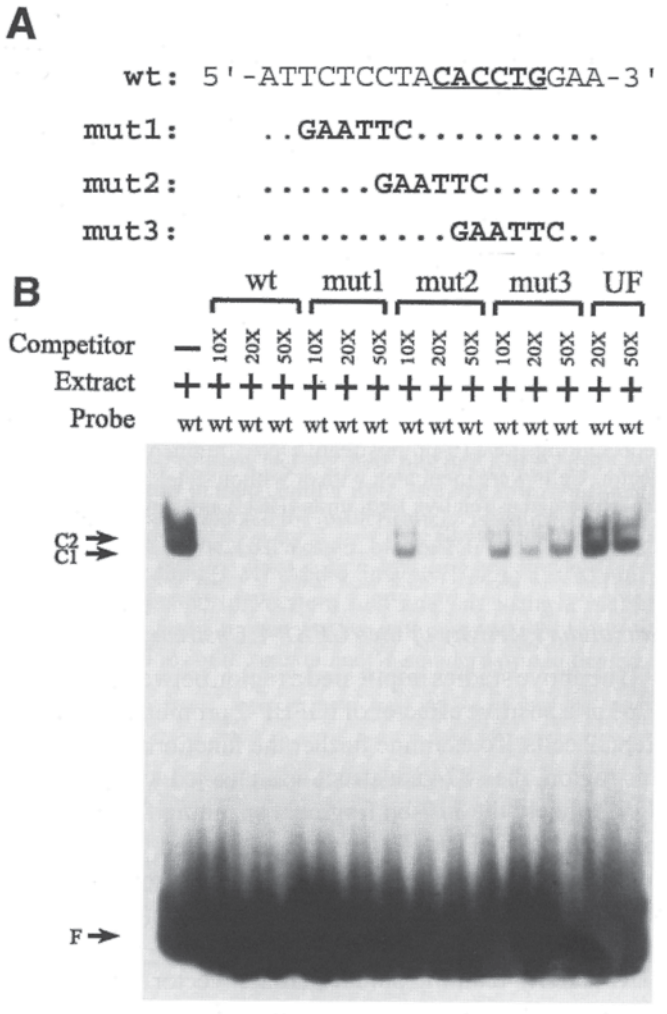

Fig. 5. S1 oligonucleotide binding to HepG2 nuclear proteins is abolished by mutation of the E-box. **(A)** Nucleotide sequence of the wild type (wt) and mutant oligonucleotides (upper strands shown) used in gel mobility shift assays. **(B)** Competition among the wt and mutant oligonucleotides in gel mobility shift assays using HepG2 nuclear extract. An unrelated oligonucleotide (UF) was used as negative control.

The HepG2 nuclear protein that specifically bound the basic helix–loop–helix (bHLH) core motif exhibits a molecular mass consistent with that of AP-4, a bHLH transcription factor whose expression has not been reported for hepatic cells. To evaluate further the possibility that AP-4 is the nuclear factor binding this putative regulatory sequence, nuclear extracts were prepared from HepG2 cells previously transfected with an expression vector encoding human (full-length) AP-4, and used in Southwestern blot analysis as described above. Expression of transfected AP-4 vector in HepG2 cells increased the abundance of the 48 kDa protein as monitored via increased binding of the labeled S1 probe, relative to nuclear extracts prepared from nontransfected cells (Fig. 6).

Regulation of the IGFBP-2 Gene 110-bp Region by AP-4

To address the functional relevance of the interactions between AP-4 and upstream bHLH core motifs, each half-site of both E-boxes was mutated (CA→GA, TG→TC) within the context of the chimeric pSaBLUC construct (Fig. 7A), and the wt and mutant (MUT) constructs used in transient cotransfection assays with an AP-4 expression vector or the corresponding empty expression vector. In empty expression vector cotransfected cells, the promoter activity of the wt reporter construct was threefold higher than that for the MUT reporter construct and pGL2-P plas-

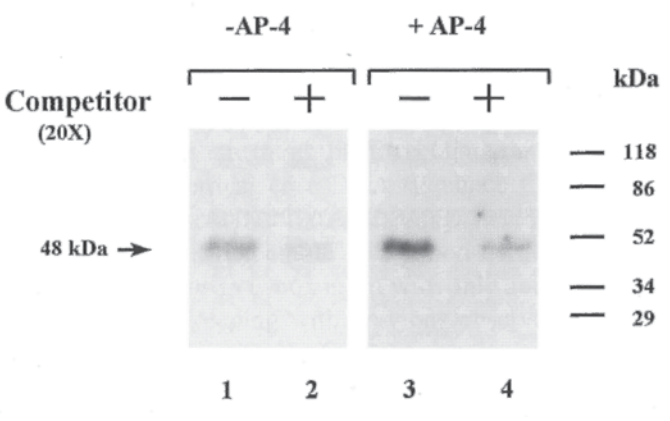

Fig. 6. Southwestern blot of S1 oligonucleotide-binding protein(s). Equivalent amounts of nuclear proteins from nontransfected (lanes 1 and 2) or AP-4 expression-vector transfected (lanes 3 and 4) HepG2 cells were resolved by 10% SDS-PAGE and subjected to Southwestern analysis with ^{32}P-labeled double-stranded S1 oligonucleotide. The specificity of the S1 DNA–protein interaction was examined using a 20-fold molar excess of unlabeled double-stranded oligonucleotide in each panel.

mid (Fig. 7B). The latter two DNAs did not differ in ability to direct LUC expression. Cotransfection of the human AP-4 expression plasmid increased the promoter activity of the wt construct up to fivefold relative to that for the pGL2-P plasmid (Fig. 7B). However, this increase in promoter activity in AP-4 cotransfected cells was not apparent for the mutant reporter construct (Fig. 7B); in this case, no difference was observed for mutant pSaBLUC and pGL2-P. This result indicates that binding of AP-4 to the E-box(es) stimulates the *trans*-activation by the 110-bp fragment of promoter activity. Activity of the pGL2-P vector was induced on cotransfection with AP-4 vector, although not to the same extent as for the wt construct. This result is in line with the known transcriptional activity of AP-4 for SV40 gene promoters *(27)*.

Discussion

The present study characterized the functional activity of the 5'-flank of the porcine IGFBP-2 gene and identified positively acting DNA sequence elements within this region that are *trans*-activated by AP-4, a member of the bHLH family of transcription factors. Although previous studies *(22,23,25,26)* documented the presence, within the proximal promoter region, of specific protein–DNA interactions that may underlie transcriptional activation of the IGFBP-2 gene, the present study specifically searched for contributions of the more distal sequences and, as a result, implicated a bHLH protein in the transcriptional regulation of this gene. Additionally, in contrast to the same earlier studies that suggested the functional importance of multiple Sp1 transcription factor binding G/C-boxes located proximal to the transcription start site and highly conserved among mammalian IGFBP-2 genes *(16,25,26)*, our studies indicated a relative lack of positive activity for these sequences in the present system. Indeed, >95% of this promoter's activity was dependent on the presence of the region extending between –1397 and –765 bp, suggesting that the proximal region is inactive or contains sequences that bind *trans*-repressor(s) that mask the positive effects of Sp1 binding to G/C-boxes.

The identity of AP-4 as a *trans*-activator of the IGFBP-2 gene was demonstrated by a combination of approaches including:

1. Southwestern blotting, in which binding of consensus E-box motifs to a 48-kDa nuclear protein, similar to AP-4, was demonstrated.
2. Cotransfection of AP-4 expression plasmid and pSaBLUC construct containing the bHLH motifs, which showed increased SV40 promoter activity in the presence of AP-4, but not with corresponding empty expression vector.
3. Mutation of both half-sites of each of the two E-boxes within the context of the pSaBLUC construct, and subsequent evaluation of SV40 promoter activity in the presence of AP-4 expression plasmid, which resulted in loss of *trans*-activator function of the overexpressed AP-4.

The lack of available AP-4 antiserum precluded confirmation of AP-4 as a component of the complexes C1 and C2 identified by electrophoretic mobility shift assays; however, the functional assays described above, as well as competitive gel-shift assays using wt and mut oligonucleotides, are consistent with C1 and/or C2 as specific complexes of AP-4 and DNA. In this regard, although Southwestern blot analysis indicated that two distinct HepG2 nuclear proteins bind the CACCTG motif, the increased abundance of the 48-kDa protein in cells transfected with an AP-4 expression vector and the lack of significant reduction in binding activity of the 118-kDa protein with the labeled probe in the presence of excess unlabeled homologous oligonucleotide, suggest that the 48-kDa nuclear protein represents the functional E-box binding component in HepG2 cells. Similarly, HeLa cell AP-4 protein preparations purified by DNA sequence affinity chromatography contain a functionally unrelated protein of molecular mass 116 kDa *(27)*.

The molecular mechanism by which IGFBP-2 gene expression is regulated in hepatic as well as other cell types is undefined. Previous studies have demonstrated the binding of Sp1 to multiple G/C boxes located proximal to the transcription start site of the rat IGFBP-2 gene, which regulate basal activity of this promoter *(25,26)*. However, no functional studies have been reported that document whether these G/C boxes act within the context of more distal elements. The current studies demonstrated a lack of activity for the smallest IGFBP-2 promoter construct, which contains all of these G/C boxes, consistent with cell context influencing IGFBP-2 promoter activity. Several reasons might explain the apparent cell context-dependent *trans*-activator function of the Sp1 binding sites; these

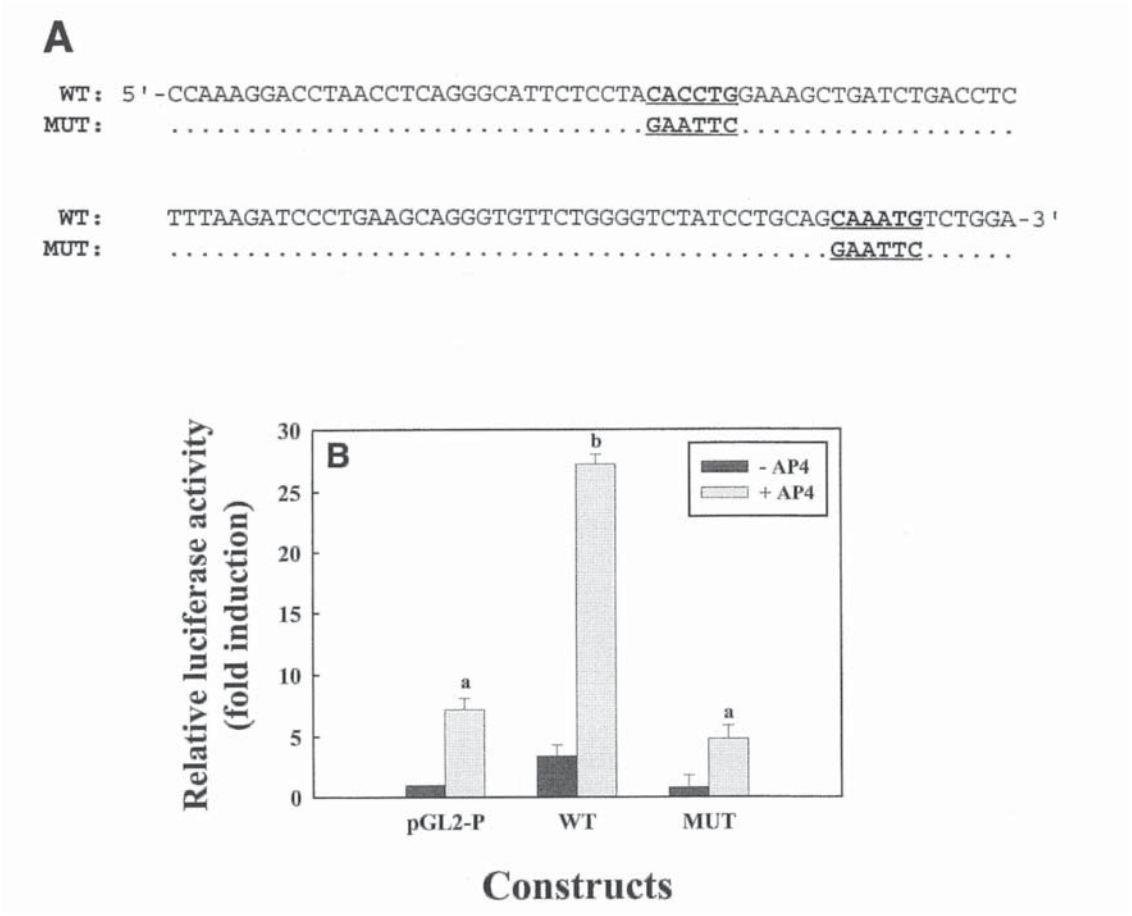

Fig. 7. AP-4 *trans*-activates a functional promoter via the 110-bp fragment. **(A)** Nucleotide sequence showing block mutations introduced in place of both bHLH motifs within the 110-bp fragment. **(B)** Stimulation of the activity of the 110-bp fragment by AP-4. Block mutations were introduced in place of the 6-bp core bHLH binding motifs (A) and the resultant double-stranded 110-bp oligonucleotide tested for ability to enhance SV40 promoter-driven LUC gene expression. The wild type (wt) and mutant (MUT) pSaBLUC constructs and the parent (pGL2-P) vector were separately cotransfected with AP-4 expression vector or the corresponding empty expression vector (pCMV). Results are expressed as fold-induction over the empty vector + cotransfected pGL2-P plasmid and represent means ± SEM of three independent cotransfection experiments (n = 3 replicates/reporter construct/experiment).

include cell-specific *trans*-repressor(s) that binds to sequences proximal or overlapping to the G/C boxes and opposes the positive effects of Sp1, lack of Sp1 in HepG2 cells, and differential cell-type expression of distinct Sp-related family members, some of which are known to interact directly with Sp1 and to inhibit its activity *(28)*. Although we did not evaluate any of these possibilities, the finding that IGFBP-2 promoter activity is markedly influenced by sequences upstream of nt –764 that are not G/C-rich suggests that the contribution of Sp1 or other Sp family proteins to IGFBP-2 gene expression is likely a minor component in HepG2 cells. It remains to be determined whether this is true for other tissues and/or cell types that are known to coexpress the IGFBP-2 and Sp1 genes, such as the porcine endometrium of pregnancy *(16,29)*.

It is interesting that the size of the IGFBP-2 mRNA in HepG2 cells is ~4.2 kb, similar to that for IGFBP-2 transcripts in human fetal liver *(7)*, the human embryonic liver cell line WRL 68 *(7)*, and lung tumor cell lines *(30)*, but distinct from the 1.6- to 2.0-kb IGFBP-2 transcripts found for adult rat *(6,19)*, human *(5)*, and pig *(12)* liver tissues. Indeed, it is unclear if this larger transcript represents incompletely processed IGFBP-2 pre-mRNA or a longer mature mRNA owing to heterogeneity in the length of 5'- and/or 3'-UTR. The former possibility seems unlikely, since HepG2 cells synthesize and secrete the corresponding protein of correct molecular mass, as detected on Western blots. In human hepatic cells, the presence of the 4.2-kb transcript appears to be inversely related to the degree of differentiation, with adult liver exhibiting the 1.6 kb mRNA and fetal liver manifesting both the 1.6- and the 4.2-kb IGFBP-2 transcripts *(7)*.

AP-4 was first identified as a nuclear protein that binds to the SV40 enhancer and, as a consequence, activates viral late gene transcription *(27)*. In several cellular as well as viral gene enhancers, AP-4 binding sites overlap the recognition sequences of other transcription factors, such as AP-1, suggesting their possible interactions *(27,31)*. The bHLH motif is present in a variety of enhancers that bind other bHLH transcription factors, which are implicated in

lineage determination and in the control of cell proliferation and differentiation *(32–36)*. Unlike AP-4 *(31)*, however, these proteins have the ability to form heterodimeric complexes with different members of this large family, but like AP-4 dimers, share the ability to recognize and bind the conserved symmetrical core DNA sequence CANNTG. Results of the present mobility shift assays demonstrated a lower affinity, but detectable interaction of the HepG2 48-kDa protein and an E-box motif with only one half-site intact. This is in keeping with previous observations for other bHLH proteins *(37,38)*. Computer analysis identified additional canonical E-boxes both upstream and downstream of the 110-bp fragment, which raises the interesting possibility of AP-4 interactions across the entire flanking region of the gene studied here. Experiments to measure the respective promoter-enhancing activity of this entire region after systematic deletion of all potential AP-4 binding sites are required to examine this scenario. In addition, we have noted the presence of E-box motifs in the published immediate 5'-flanking sequence of the human IGFBP-2 gene, although the human sequence corresponding to the more upstream 110-bp region of the porcine gene is unavailable, thereby precluding their direct comparison.

In summary, these results demonstrate the functional importance of upstream DNA sequences in the control of IGFBP-2 gene transcription. We have shown that IGFBP-2 promoter activity is increased by a distal 633-bp region, which is not G/C-rich. This region encompasses a number of noncontiguous core bHLH transcription factor binding motifs, at least two of which interact with a high degree of specificity with an HepG2 nuclear protein that likely is AP-4 or another bHLH protein of identical size. Whether this protein and/or its corresponding DNA sequence motif interacts with other transcription factors and nuclear enhancer binding proteins to direct IGFBP-2 gene transcription in vivo remains to be determined. Stimulation of IGFBP-2 synthesis may constitute a novel mechanism by which E-box binding proteins potentially may affect cell proliferation, cell differentiation, and apoptosis.

Materials and Methods

Materials

Antibiotic-antimycotic solution (ABAM), fetal bovine serum (FBS), and calf thymus DNA were obtained from Sigma Chemical Co. (St. Louis, MO). Restriction enzymes and poly (dI:dC) were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Minimal Essential Medium (MEM), Hank's Balanced Salt Solution (HBSS), and TRIzol were purchased from Gibco BRL (Gaithersburg, MD). The luciferase assay system and five-strength reporter lysis buffer were from Promega Corp. (Madison, WI). Radionucleotides and BioTrans nylon membranes were obtained from ICN Pharmaceuticals (Irvine, CA). Nitrocellulose membranes were from Schleicher and Schuell (Keene, NH). Oligonucleotides were synthesized by the DNA Synthesis Core Facility of the Interdisciplinary Center for Biotechnology Research (ICBR) at the University of Florida (Gainesville, FL).

RNA Isolation and Analysis

Total cellular RNA was isolated from confluent HepG2 cell cultures using TRIzol reagent following the manufacturer's instructions. Thirty micrograms of RNA were fractionated in a 1.5% agarose-formaldehyde gel, blotted to a BioTrans nylon membrane, and hybridized to nick-translated porcine IGFBP-2 cDNA (gel-purified, full-length insert) as previously described *(12,16)*. The IGFBP-2 transcript was identified by autoradiography at –80°C. The size of the mRNA was calculated based on lengths of 5 and 2 kb, respectively for 28S and 18S rRNAs *(12)*.

Western Blot Analysis

HepG2 cells were grown to near confluence in MEM (containing 10% FBS) and were then incubated in serum-free MEM for 24 h. Medium was removed, and the cells rinsed and incubated in fresh serum-free MEM for an additional 24 h. Conditioned medium (CM) was collected after the second incubation and centrifuged briefly to remove cells and insoluble material. CM proteins were lyophilized, reconstituted in SDS-PAGE sample buffer, and subjected to SDS-PAGE under nonreducing conditions. Following gel electrophoresis, proteins were transferred to a nitrocellulose membrane *(39)*. The filter was blocked in Tris-buffered saline (TBS, pH 7.4) containing 1% Carnation nonfat dry milk and then incubated with rabbit antiserum to full-length recombinant porcine IGFBP-2 (generated and characterized in this laboratory) for 24 h at 4°C. The filter was rinsed in five changes of TBS containing 0.2% Tween-20 and incubated with [^{125}I]-protein A (1×10^6 cpm/mL) for 2 h at room temperature. The blot was washed in five changes of TBS/Tween-20 at room temperature and the IGFBP-2 antibody–protein A complexes identified by autoradiography at –80°C.

Plasmid Constructions and Reporter Enzyme Assay

Restriction fragments of the porcine IGFBP-2 gene (–1397, –874, –764, and –305 to +73 bp, relative to first codon) were subcloned in the sense orientation into the multiple cloning site region of the promoter-less pGL2 enhancer vector (Promega Corp). The open reading frame specified by the IGFBP-2 translation initiation codon (+1) was shifted relative to that for LUC to eliminate secretion of LUC protein via the IGFBP-2 leader peptide. The resultant chimeric constructs and, in parallel, the parent vector (20 µg each) were transiently transfected into subconfluent HepG2 cells using the polybrene method as previously described *(40)*. Forty-eight hours after transfection, cells were washed, lysed, and assayed for LUC enzyme activity using a luminometer (EG&G Berthold, Bad Wilbad, Germany). LUC activity was normalized for total protein con-

tent of the cellular extract and expressed as fold induction relative to the longest DNA construct (–1397 IGFBP-2LUC).

To map further the distal regulatory sequence(s) of the porcine IGFBP-2 gene, the –874 IGFBP-2LUC construct was cleaved with *Sac*I and *Bgl*II to yield the 110-bp fragment spanning from nt –874 to nt –765. This DNA fragment was inserted in the sense orientation into the pGL2-Promoter (pGL2-P) vector (Promega Corp), which contains the SV40 promoter linked to the downstream LUC coding sequence. This IGFBP-2 construct, designated pSaBLUC, and in parallel, the parent pGL2-P vector (20 μg) were transiently transfected into subconfluent HepG2 cells by the polybrene method and LUC activity determined as above. To examine the functional role of bHLH recognition sequences in IGFBP-2 gene activity, the two bHLH binding motifs located within the 110-bp region were altered by block mutation, and oligonucleotides containing these mutated sequences were subcloned upstream of the SV 40 promoter fused to the LUC reporter gene in pGL2-P. Identity of all constructs was verified by DNA sequencing. All transfection experiments were repeated three or four times, and within each experiment, triplicate transfections were performed with each individual DNA construct. In many cases, results were confirmed with multiple independent preparations of plasmid DNA prepared using CsCl density gradient centrifugation.

Preparation of Nuclear Extracts

Nuclear extracts from confluent HepG2 cells were prepared following procedures described by Gonzalez et al. *(41)*. Cells were washed twice in PBS, scraped into fresh PBS, and collected by low-speed centrifugation (1000*g*) for 4 min. Cells were resuspended in five times the pellet volume of TEG buffer (10 m*M* Tris-HCl, pH 7.4, 1.5 m*M* EDTA, 10% glycerol, 0.15 m*M* spermine, 0.5 m*M* spermidine, 0.1 m*M* phenylmethylsulfonylfluoride [PMSF], and 4 μg/mL aprotinin) and sedimented by centrifugation (1000*g*) for 5 min at 4°C. Pelleted cells were resuspended in 3 vol of fresh TEG buffer, homogenized with 30 strokes in a Dounce homogenizer with B pestle, and centrifuged at 800*g* for 10 min at 4°C. The resultant pellet was resuspended in extraction buffer (20 m*M* HEPES, pH 7.9, 0.1 m*M* EDTA, 5 m*M* $MgCl_2$, 2 m*M* dithiothreitol [DTT], 60 m*M* NaCl, 20% glycerol, 0.15 m*M* spermine, 0.5 m*M* spermidine, 0.1 m*M* PMSF, and 4 μg/mL aprotinin) and incubated on ice for 1 h with gentle shaking every 15 min. The nuclear suspension was centrifuged at 180,000*g* for 45 min at 4°C, and the extract dialyzed overnight against 200 vol of dialysis buffer (identical to extraction buffer except for omission of spermine, spermidine, PMSF, and aprotinin). The dialyzed extract was cleared of precipitated material by centrifugation (12,000*g*, 15 min, 4°C) and stored in 50-μL aliquots at –80°C. Protein concentrations of nuclear extracts were determined by the Bradford assay, with bovine serum albumin used as the standard.

Electrophoretic Mobility Shift Assay

Complementary oligodeoxyribonucleotides corresponding to sequences of the upstream IGFBP-2 gene region with homology to the bHLH transcription factor binding motif (CANNTG) were synthesized by the DNA Synthesis Core Facility at the University of Florida. The oligonucleotides (upper strand shown) used for binding and competition assays were as follows (putative bHLH transcription factor binding sites are underlined and mismatches with wt sequence are indicated as lower-case letters): S1, 5'-ATT CTCCTACACCTGGAA-3'; S2, 5'-CTGCAGCAAATG TCTGGA-3'; mut1, 5'-ATgaattcACACCTGGAA-3'; mut2, 5'-ATTCTCgaattcCTGGAA-3'; mut3, 5'-ATTCTCCTAC gaattcAA-3'; UF, 5'-GGGGCGGGAGGGCCGCCCCG-3'. Equimolar quantities of complementary strands were annealed by incubation at 65°C for 20 min and subsequent cooling to room temperature. Double-stranded oligonucleotides were end-labeled using T4 polynucleotide kinase (PNK). HepG2 nuclear extracts (10 μg protein) were preincubated on ice for 15 min in a buffer containing 10 m*M* Tris-HCl, pH 7.4, 60 m*M* KCl, 1 m*M* $MgCl_2$, 0.5 m*M* EDTA, 0.5 m*M* DTT, and 10% glycerol. ^{32}P-labeled DNA probe (30,000 cpm) was then added, and the incubation continued on ice for 30 min. In competition studies, excess unlabeled double-stranded oligonucleotide was added 15 min prior to addition of the radioactive probe. Reactions (45 μL) were loaded onto 5% nondenaturing polyacrylamide gels that were pre-electrophoresed at constant 33 mA/gel for 1 h at room temperature. Electrophoresis was carried out at constant 33 mA/gel for 4 h. Gels were dried and subjected to autoradiography to detect the presence of radioactive complexes.

Southwestern Blot Analysis

Approximately 150 μg of HepG2 nuclear proteins were subjected to 10% SDS-PAGE and electroblotted to a nitrocellulose membrane. After transfer, the membrane was blocked with 5% Carnation nonfat dry milk in TEN buffer (10 m*M* Tris-HCL, pH 7.4, 1 m*M* EDTA, 60 m*M* NaCl) for 20 min at room temperature. The membrane was rinsed briefly with TEN buffer and then incubated in the same buffer, supplemented with sonicated calf thymus DNA (5 μg/mL), for 15 min at room temperature. Double-stranded ^{32}P-labeled probe (1×10^5 cpm/mL) was then added, and the membrane incubated overnight at 4°C. For competition studies, the filter was preincubated with 10- to 50-fold molar excess of unlabeled double-stranded oligonucleotide for 15 min prior to addition of the labeled probe. Unbound probe was removed by two 15-min washes in TEN buffer, and the binding protein(s) was visualized by autoradiography at –80°C.

Statistical Analysis

Differences in LUC activity (adjusted for protein content of cell extract) between DNA constructs were evalu-

ated by least-squares analysis of variance. When significant differences were detected ($P < 0.05$), means were separated by orthogonal contrasts. For each construct, the data are presented as mean ± SEM.

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