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IDENTIFICATION OF NOVEL PROMOTER AND REPRESSOR ELEMENTS IN THE 5'-FLANKING REGIONS OF THE RAT INSULIN-LIKE GROWTH FACTOR-I GENE

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Summary: Although the major endocrine source of insulin-like growth factor-I (IGF-I) is the liver, little is known about IGF-I transcriptional mechanisms in this tissue. To evaluate the role of *cis*-regulatory elements, rat hepatocytes in primary culture were transfected with DNA constructs containing IGF-I 5' flanking sequences, fused to a luciferase reporter. We demonstrate the presence of a novel promoter ~0.5 kb upstream from exon 1 transcription initiation sites, together with a repressor element in this region, and a downstream repressor element which can modulate the activity of both endogenous and heterologous promoters.

Insulin-like growth factor I (IGF-I) is a 70-residue polypeptide with structure and actions similar to those of proinsulin (1). IGF-I modulates metabolism and growth, and is under control of growth hormone, insulin, and nutritional status. IGF-I also contributes locally to tissue function through autocrine/paracrine actions (2).

The rat IGF-I gene spans more than 90 Kb, and contains 6 exons encoding four IGF-I mRNA species (3). Multiple transcription initiation sites are present in both exons 1 and 2, similar to other genes lacking a TATA box in the promoter region (4). To date, rat IGF-I gene promoter activity has been studied in human neuroblastoma SK-N-MC cells (5) and rat dermal fibroblast and rat C6 glioma cells (6), revealing the presence of a proximal promoter region of exon 1. However, at present there is no knowledge of IGF-I regulatory elements active in the liver, the major source of circulating IGF-I.

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ABBREVIATIONS USED:

IGF-I, insulin-like growth factor 1; **DMEM**, Dulbecco's Modified Eagle's Medium; **AMV**, avian myeloblastosis virus; **BSA**, bovine serum albumin; **LUC**, luciferase; **SV40**, simian virus 40; **CMV**, cytomegalovirus; and **PBS**, phosphate buffered saline.

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To evaluate different 5' flanking regions, we have focused on DNA including exon 1, where most transcripts are initiated *in vivo* (4). Transcriptional activity was studied in hepatocyte primary culture, a model shown to reflect differentiated liver status (7). Our studies indicate promoter activity in constructs including previously identified transcription initiation sites, both promoter and repressor elements ~0.5 Kb upstream and a repressor element ~0.2 kb downstream from those sites.

MATERIALS AND METHODS: Male Sprague-Dawley rats were obtained from Harlan, Indianapolis, IN; rat tail collagen from Sigma, St. Louis, MO; collagenase from Worthington, Freehold, NJ; pGL2-Plasmids, GLprimer2, AMV reverse transcriptase and a luciferase assay kit from Promega, Madison, WI; a DNA sequencing kit from US Biochemicals, Cleveland, OH. Lumi-Gal 530 from Lumigen, Inc., Southfield, MI; restriction enzymes from New England BioLabs, Beverly, MA; and isotopes from Amersham, Arlington Heights, IL.

Development of constructs is shown in Figure 1. A 2.1 Kb *Pst*I fragment of IGF-I genomic DNA was kindly provided by Dr. Peter Rotwein (8). 0.6 Kb (*Sma* I-*Sac* I), 0.9 Kb (*Sac* I-*Bgl* II) and 1.5 Kb (*Sma* I-*Bgl* II) IGF-I DNA fragments were inserted into appropriate sites of the pGL2-Basic plasmid to generate B(-1124/-512) IGF-I, B(-512/+355) IGF-I and B(-1124/+355) IGF-I, respectively. To clone the 0.6 Kb fragment in the reverse direction, it was first inserted into pUC18 at *Xma* I-*Sac* I sites, then cut out with *Sac* I and *Hind* III, gel purified, and ligated into pGL2-Basic at the same sites to generate B(-512/-1124) IGF-I. To obtain 3'-deletion mutants, the 1.5 Kb fragment was first cloned into pUC18. The plasmid was then opened with *Pst* I and *Bgl* II, treated with exonuclease III, filled in, and re-ligated. Deletion mutants were sequenced, and inserts cut out with *Sma* I and *Hind* III, and subcloned into pGL2-Basic to create B(-1124/+267) IGF-I. B(-512/+267) IGF-I was obtained by digestion of

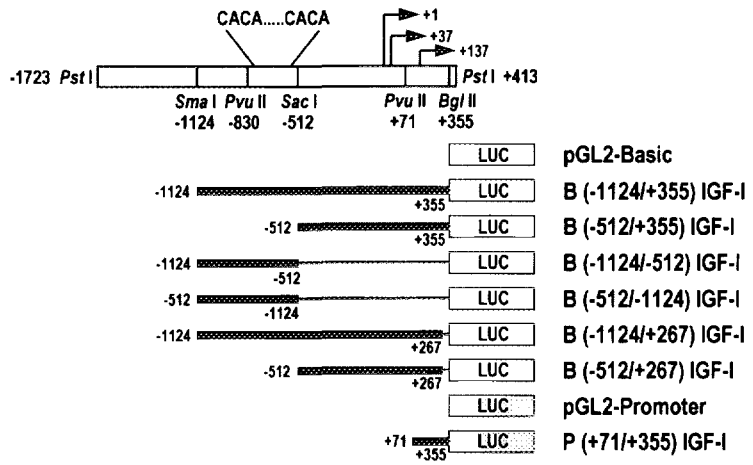

Figure 1. Development of constructs containing rat IGF-I flanking sequences, with a luciferase reporter. Restriction sites are shown for a 2.1 Kb IGF-I fragment. Three major transcription initiation sites in exon 1 are marked with arrows; sequence nomenclature is based on transcription initiation sites described by Adamo, et al (4). The "CA" rich region is indicated. In fusion plasmids, the rIGF-I sequence is shown as a solid bar, and deleted regions are shown as a line. The pGL2-Basic promoterless vector is shown as an open box, and pGL2-Promoter vector as a shaded box.

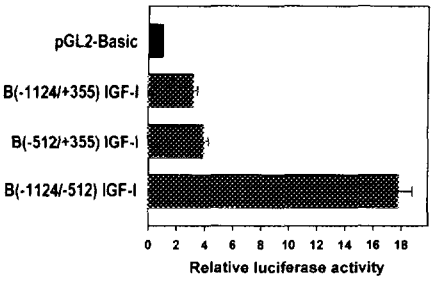

Figure 2. Promoter activity of 5'-flanking sequences of the rat IGF-I gene. Values reflect luciferase activity in hepatocytes transfected with pGL2-Basic chimeric constructs, normalized to cotransfected β-galactosidase activity; activity of pGL2-Basic was defined as 1. Mean ± SEM for at least three separate experiments with each condition, performed in triplicate. Statistical difference was assessed by analysis of variance.

B(-1124/+267) IGF-I with *Sma* I and *Sac* I, followed by fill-in and reclosure. To develop P(+71/+355) IGF-I, P(-1124/+355) IGF-I was first created by cloning the 1.5 Kb IGF-I fragment into pGL2-Promoter; the remaining upstream sequence was then removed by digestion with *Sma* I and *Pvu* II, followed by religation.

Hepatocytes were isolated as reported previously (9), followed by centrifugation through a Percoll cushion; final viability was close to 100%. Cells were plated at $3x10^6$ per 60-mm collagen coated plastic dish. After cells attached, medium was changed to DMEM/F12 supplemented with 3% serum, 10^{-7} M insulin and 10^{-7} M dexamethasone. Cells were transfected by the calcium phosphate method (9), using 5 μg of plasmid and 0.1 μg of CMV β-galactosidase DNA (provided by Dr. Grant MacGregor) (10). Cells were washed with PBS after exposure to DNA overnight, and medium was changed at 24 hr intervals for 48 hr, with serum free DMEM/F12 containing 1 mg/ml BSA, 0.1 mg/ml human transferrin, 10^{-7} M insulin and 10^{-7} M dexamethasone. Luciferase activity was determined according to the Promega protocol, and β-galactosidase activity was assayed with Lumi-Gal 530 according to the manufacturer's protocol. Promoter activity was normalized by β-galactosidase activity to adjust for transfection efficiency.

RESULTS: To determine which IGF-I 5' flanking regions modulate transcription, hepatocytes in primary culture were transfected with luciferase reporter gene constructs in which IGF-I sequences had been placed upstream from the luciferase expression region. As shown in Figure 2, luciferase activity was elevated ~3.2-fold with B (-1124/+355) IGF-I compared to promoterless pGL2-Basic ($p < 0.05$). Deletion of 0.6 Kb of upstream sequence [(B (-512/+355) IGF-I] had a further modest effect on luciferase activity, increasing luciferase activity to ~3.9-fold above pGL2-Basic ($p < 0.05$). These findings indicate the presence of a proximal promoter between -512 and +355 bp. However, deletion of this region resulted in increased luciferase expression [B(-1124/-512) IGF-I], approximately 18-fold greater than pGL2-Basic ($p < 0.05$). Furthermore, since luciferase expression produced by B(-512/-1124) IGF-I was below that of pGL2-Basic (not shown), the transcriptional activity of B(-1124/-512) IGF-I

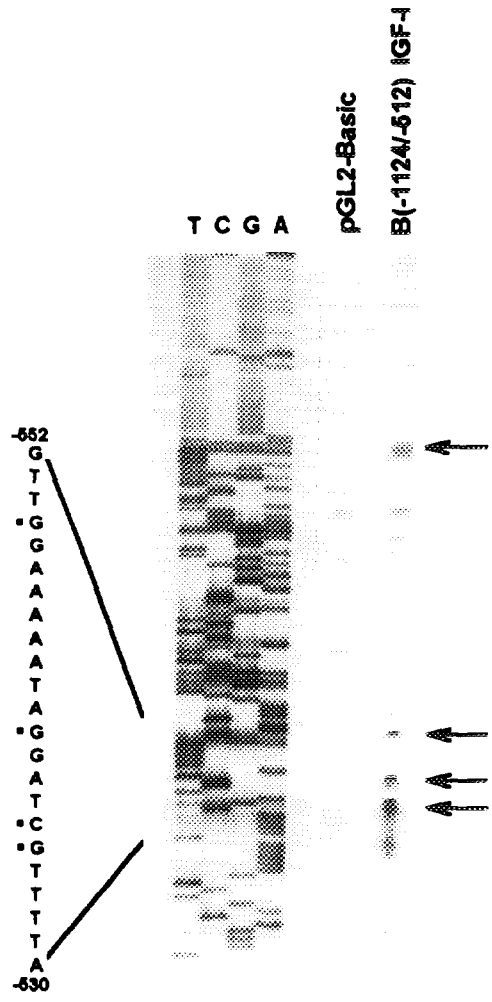

Figure 3. Primer extension analysis of transcriptional sites. Total RNA was isolated by the guanidinium isothiocyanate method (19). 20 µg of RNA from hepatocytes transfected with pGL2-Basic or B(-1124/-512) IGF-I were hybridized with end-labeled GLprimer2, according to the Promega protocol. The DNA sequencing ladder was generated using B(-1124/-512) IGF-I plasmid DNA with GLprimer2. IGF-I-specific transcription initiation sites are marked with arrows. The primer extension products were analyzed on a 6% polyacrylamide DNA sequencing gel.

appears to be due to a unidirectional distal promoter, rather than an enhancer which would be expected to be orientation-independent. Thus, at least two promoters appear to be located within the ~1.5 Kb IGF-I flanking region, one located upstream between -1124 and -512 bp, and the other between -512 and +355 bp, closer to transcription initiation sites as shown in Figure I. Since the promoter activity of the distal sequence [B(-1124/-512) IGF-I] was stronger than that of the combined proximal and distal sequences [B(-1124/+355) IGF-I], these results also suggest the presence of repressor elements in the region from -512 to +355 bp (see below).

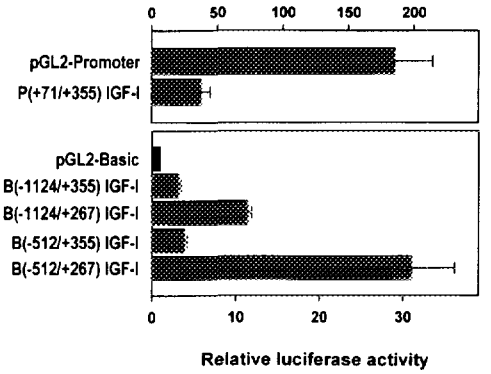

Figure 4. Evaluation of repressor activities in 5'-flanking sequences of the rat IGF-I gene. Experiments and data analysis were performed as in Figure 2. (Top) Comparison of luciferase activity in hepatocytes transfected with pGL2-Promoter alone, or P(+71/+355) IGF-I. (Bottom) Luciferase activity in hepatocytes transfected with either pGL2-Basic or constructs containing additional IGF-I 5'-flanking sequences. Mean ± SEM for at least three separate experiments.

Primer extension analysis was employed to examine transgene transcription initiation sites. To eliminate the effect of endogenous IGF-I mRNA, we utilized a primer specific to luciferase (GLprimer2). As shown in Figure 3, specific extension products were identified in the IGF-I sequence at positions -535/-536, -540, -549, and ~-634 nt, eliminating the possibility that upstream promoter activity could result from anomalous initiation in the vector. Separate primer extension studies with liver RNA also revealed both the recognized proximal initiation sites (4) and bands near the upstream promoter (not shown), but attempts at precise localization have been unsuccessful.

Since our initial experiments suggested the presence of repressor elements between -512 and +355 bp, we examined the effect of +71/+355 bp of IGF-I sequence when placed upstream from the SV40 promoter in pGL2-Promoter. As shown in Figure 4 (top), the ~0.3 Kb IGF-I fragment decreased promoter activity by ~70% ($p < 0.05$) -- also eliminating the possibility of reduced activity from competition between open reading frames. To dissect the region further, the -1124 to +355 bp fragment was digested with exonuclease III, resulting in B (-1124/+267) IGF-I; as shown in Figure 4 (bottom), 3' deletion increased expression 3-fold compared to B(-1124/+355) IGF-I ($p < 0.05$), suggesting that elements in the 88 bp region may mediate repressor activity.

Additional digestion of B(-1124/+267) IGF-I produced B(-512/+267) IGF-I, which provided a further increase in luciferase activity, 31-fold above pGL2-Basic and 3-fold above B(-1124/+267) IGF-I ($p < 0.05$), indicating that the 0.6 Kb region of the IGF-I gene may contain repressor elements as well as promoter regions.

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rIGF-I -763 CACACTCACA CACAATCACA CACACACACA CACACACTCA CACTCACTCA CACAC...AC ACACACTCAC ACACACAACT
hIGF-I  899 AATCAGAGAA AGAAAACACA CTCTGGCACA CAGACTCCCT CTGTCATACA CACAC...AC ACACACACAC ACACACACAC
P450    188 GGAAAAGCAT ATAGAACACG CACACACACA CACACACACA CACACACACA CACAC...AC AATCCCATGC CCTAGTAAGT
C4     -345 CACATGTATT CGTACACACA CACACACACA CACACACGCA CACGCACATG CAGAG...GG ACACGCACAC GCACACAAAC
Keratin 467 AGGACGCAAC TGAGCACGCG CACACACACA CACACACACA CACACACACA CACAGTATGT ATATGTTATT TCATTCAATT

Consensus seq. a           aCaC  CaCacaCACA CAcACaC Ca Cac CAcaca CAcA      A ac c   c  Ca  cAa
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Figure 5. Homology of rat and human IGF-I flanking regions with other eukaryotic promoter sequences. The sequence of the -1124/-512 bp rIGF-I fragment was compared to sequences in a eukaryotic promoter database, using the GCG Wisconsin package, with the FASTA program. Homologous sequences were aligned using the PILEUP program with GapWeight=5 and GapLengthWeight=0.3. The consensus sequence is indicated below the block of aligned sequences; nucleotides identical in all sequences are shown by capital letters, whereas nucleotides homologous in 4 out of 5 sequences are shown by lower case.

DISCUSSION: Our results indicate that the rat IGF-I gene contains a distal promoter between -1124 and -512 bp, a proximal promoter between -512 and +355 bp, repressor sequences within the former region, and a downstream repressor element located between +267 and +355 bp. The downstream repressor was active in both wild-type and heterologous promoters, while the upstream repressor region (-1124 to -512 bp) was most active in the absence of the downstream repressor, suggesting that these two regions may interact. To our knowledge, these findings constitute the first analysis of IGF-I elements active in hepatocytes, reflecting the major source of circulating IGF-I.

Our findings in hepatocytes indicating upstream repressor activity may be compared with previous studies in neuroblastoma SK-N-MC cells (5), and fibroblasts and C6 glioma cells (6). The data of Hall, et al (5) reveal a slight increase in promoter activity upon deletion of upstream sequences from -1114 to -823 bp, consistent with the increase in promoter activity we observed after deleting the -1124 to -512 bp region, and Lowe, et al (6) also found repressor activity between -1120 and -412 bp. Thus, several independent observations in combination suggest that the common region (-1114 to -823 bp) might be the locus of the upstream repressor elements.

Our results localize downstream repressor activity between +267 and +355 bp, and are consistent with analysis of the findings of Lowe, et al (6,11), which imply that reduction of the 3' terminus from +362 to +301 bp increased promoter activity ~2-fold in C6 glioma cells, although promoter activity did not change in fibroblasts. Conceivably, variation in activity could reflect cell type specific regulation of IGF-I expression.

We also observed promoter activity ~0.5 Kb upstream from previously identified exon 1 transcription initiation sites (4). Using SK-N-MC cells, Hall, et al (5) reported the greatest promoter activity with a construct with the 5'-terminus at -823 bp, supporting the hypothesis that critical elements may be located between -823 and -512

bp. The rat IGF-I gene contains two clusters of "CA" rich sequences (between -770 and -642 bp), which may be involved in mediating enhanced transcriptional activity. The -763 to -685 bp cluster is strongly conserved in both rat (8) and human (12) IGF-I genes, and is homologous to promoter regions in mouse complement C4 (13), sheep wool keratin (14), and a rat liver cytochrome P450 (15) gene (see Figure 5). Our finding is also consistent with the observation by Pao, et al (16) that an immobilized DNA fragment containing only sequences upstream of the major exon 1 transcription initiation sites (-5.4 kb to +2 bp) provided a strong signal in hepatic nuclear run-on assays. While Thomas, et al (17) have reported several footprints in the upstream regions of the IGF-I gene, further studies will be required to determine whether specific DNA-protein interactions may mediate the activator and repressor functions we have identified.

It has been difficult to study transcriptional regulation of IGF-I expression, both because of the complexity of the gene, and because of limited expression in hepatoma cell lines (18). While hepatocytes in primary culture maintain features of differentiated function which resemble that of the liver *in vivo* (7), examination of IGF-I regulation in hepatocytes has also been difficult, due to limited expression in this model as well; the B(-1124/+355) IGF-I construct provided luciferase activity only 3-4 fold above pGL2-Basic. However, the present identification of repressor regions has allowed the development of constructs with a 25- to 40-fold increase in promoter activity, which should be sufficient to permit studies of both hormonal regulation and the effects of transcription factor binding to contained regions.

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