Transcriptional Regulation of the Rat Insulin-like Growth Factor-I Gene Involves Metabolism-dependent Binding of Nuclear Proteins to a Downstream Region*

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Ching-I Pao, Juan-li Zhu, David G. Robertson, Kai-wei M. Lin, Paul K. Farmer, Svijetana Begovic, Guang-je Wu, and Lawrence S. Phillips§

From the Division of Endocrinology and Metabolism, Department of Medicine and the Department of Microbiology/Immunology, Emory University School of Medicine, Atlanta, Georgia 30322

Insulin-like growth factor-I (IGF-I) gene transcription is mediated largely via exon 1. In an initial search for regulatory regions, rat hepatocytes were transfected with IGF-I constructs. Since omission of downstream sequences led to reduced expression, we then used in vitro transcription to evaluate potential metabolic regulation via downstream regions. With templates including 219 base pairs of downstream sequence, transcriptional activity was reduced 70–90% with hepatic nuclear extracts from diabetic versus normal rats. However, activity was comparable with templates lacking downstream sequences. The downstream region contained six DNase I footprints, and templates with deletion of either region III or V no longer provided reduced transcriptional activity with nuclear extracts from diabetic rats.

Nuclear protein binding to regions III and V appeared to be metabolically regulated, as shown by reduced DNase I protection and activity in gel mobility shift assays with nuclear extracts from diabetic rats. Southwestern blotting probes corresponding to regions III and V recognized a 65-kDa nuclear factor present at reduced levels in diabetic rats. These findings indicate that a downstream region in exon 1 may be important for both IGF-I expression and metabolic regulation. Altered concentration or activity of a transcription factor(s) binding to this region may contribute to reduced IGF-I gene transcription associated with diabetes mellitus.

The insulin-like growth factors (IGFs) are polypeptides with sequence, structure, and biological actions similar to those of insulin (1). Since circulating levels of IGF-I are more responsive to changes in metabolic status than are levels of IGF-II (2, 3), IGF-I is thought to be a more important regulatory factor during postnatal life. While IGF-I is expressed in many organs and tissues, consistent with paracrine regulation and a role as a local growth factor, its expression is 50–100 times higher in the liver than in other tissues, consistent with hepatic origin of circulating IGF-I, and a role as an endocrine regulator of growth (1, 3, 4). In the liver, IGF-I expression appears to be regulated pretranslationally (5–7). Modulation at the level of gene transcription is indicated by findings such as decreased IGF-I gene transcription in streptozotocin-diabetic animals (7) and the ability of insulin to stimulate IGF-I gene transcription in hepatocyte primary culture (8). However, underlying mechanisms are poorly understood.

The single IGF-I gene gives rise to a complex family of mRNAs with both size and coding sequence heterogeneity (9–11) and polypeptides which are products of multiple translational initiation sites. Multiple in-frame initiator codons within 5’ sequences specify different amino-terminal signal peptides, and preproIGFs with signal peptides containing 22, 32, or 48 amino acids are synthesized depending on utilization of different AUGs (9, 12). Initiation of transcription is also complex, as several laboratories have identified multiple transcription initiation sites in exons 1 and 2 of the rat, sheep, and human genes (12–16). In the rat IGF-I gene, initiation sites extend over 140 bp in exon 1, and 770 bp in exon 2 (13). However, Adamo et al. (13) found that two initiation sites in exon 1 could account for 70–80% of IGF-I gene transcription in adult rat liver. Thus, although rat IGF-I gene transcription is regulated by two distinct promoters, the exon 1 promoter appears to be dominant.

Since relatively little is known about molecular regulation of IGF-I gene transcription, we have focused on the liver; while several laboratories have begun to study the basis of IGF-I gene transcription in different immortal cell lines (14, 17–19), there has been little evaluation of underlying mechanisms in the dominant source of IGF-I production (1, 3, 4). Analysis of findings from several laboratories suggests that downstream regions may play a role in IGF-I gene expression (17–20), but such an hypothesis has not been tested in the liver. In the present study, we demonstrate that sequences downstream from the exon 1 major transcription initiation site are important both for hepatic IGF-I expression and metabolic regulation, we characterize nuclear protein binding to downstream sequences, and we identify two regions that may be involved in the decreased IGF-I gene transcription associated with diabetes mellitus.

MATERIALS AND METHODS

Chemicals—Restriction endonucleases and DNA modifying enzymes were obtained from New England Biolabs (Beverly, MA); streptozotocin from Pfannstielh (Waukegan, IL); [γ-32P]ATP (6000 Ci/mmol), [α-32P]dATP, and [α-32P]JdGTP (800 Ci/mmol) were from Amersham Corp. Oligonucleotides were from Operon Technology (Alameda, CA);
Luciferase and lucifin were from Boehringer Mannheim. Superscript RNAse H reverse transcriptase was from Life Technologies, Inc., and DNase I was from Pharmacia Biotech Inc. All other chemicals (of molecular biology grade) were purchased from Sigma.

Animals—Male Sprague-Dawley rats (Charles River, Lexington, MA), weighing approximately 120–160 g, were fed ad libitum. Chronic diabetes was produced through tail vein injection of streptozotocin. Streptozotocin at 250 mg/kg was used to produce diabetic mice, with sacrifice of animals two days after injection.

Transfection— Constructs with IGF-1 sequences cloned into a luciferase reporter (p0Luc) were generously provided by Dr. Peter Rotwein from Washington University, as reported previously (14). Relative to the rIGF-I exon 1 major transcription initiation site (7, 13), was purified from a polyacrylamide gel and described previously (28). Pairs of oligonucleotides, corresponding to +42/+68 bp ( oligo III ), +79/+101 bp ( oligo IV ), and +129/+152 bp ( oligo V ) downstream from the exon 1 major transcription initiation site, were annealed, labeled, and gel purified.

Gal Mobility Shift Assay—End-labeled 272-bp Accl/BglII fragments were incubated with 0.5–15 μg of nuclear extract in 25 μl of binding buffer containing 10 μM Tris, pH 7.5, 50 μM KCl, 1 μM EDTA, 0.5 μM DTT, 0.2% Nonidet P-40, 20 μg of bovine serum albumin, 4 μg of poly(dI-dC), and 10% glycerol at 25°C for 25 min. Protein-DNA complexes were separated from free probe at 4°C on a 5% polyacrylamide gel (0.5 × TBE (45 μM Tris, pH 8.0, 45 mM boric acid, 1 mM EDTA) at 11 V/cm for 2–3 h, and visualized by autoradiography. For double-stranded oligonucleotides, the probe was incubated with 5–15 μg of extract in same buffer, except that 200 μg/ml of salmon sperm DNA and 24 μg/ml of pBR322 were included to reduce nonspecific binding.

DNA Sequence Determination—End-labeled 272 bp Accl/BglII fragments were incubated with 4–24 μg of nuclear extract in 25 μl of binding buffer containing 10 μM Hepes, pH 7.9, 50 μM KCl, 1 μM EDTA, 0.5 μM DTT, 1 μg of poly(dI-dC), and 10% glycerol at 25°C for 25 min. An equal volume of binding buffer containing 10 μg MgCl2, 2 μl CaCl2, and 10 units/ml of DNase I was added, and the sample was incubated at 25°C for 2 min. The reaction was stopped with buffer containing 40 μM DNase I treated with 5 μl of phenol and 1 μl of chloroform.

DNase I Protection Assay—End-labeled 272 bp Accl/BglII fragments were incubated with 4–24 μg of nuclear extract in 25 μl of binding buffer containing 10 μg Hepes, pH 7.6, 25 μM KCl, 0.15 μM spermine, 0.5 μM spermidine, 1 μl EDTA, 10% glycerol, and 2.0 μM sucrose. The homogenate was layered onto a 2 ml sucrose cushion and centrifuged at 27,000 rpm in an SW28 rotor at 4°C for 1 h. The nuclear pellet was resuspended in lysing buffer containing 10 μg Hepes, pH 7.6, 100 μM KCl, 3 μM MgCl2, 0.1 μM EDTA, and 10% glycerol at a concentration of 10 A260/ml. Nuclei were lysed by adding one-tenth of a volume of 4 × (NH4)2SO4, and chromatin was removed by centrifugation at 39,000 rpm in an SW40 rotor for 2 h. Nuclear proteins were concentrated by (NH4)2SO4 precipitation (0.33 g/ml) and dialyzed against buffer containing 25 μg Hepes, pH 7.6, 100 μM KCl, 0.1 μM EDTA, and 10% glycerol for 25 min. Protein-DNA complexes were separated from free probe at 4°C on a 5% polyacrylamide gel (0.5 × TBE (45 μM Tris, pH 8.0, 45 mM boric acid, 1 mM EDTA) at 11 V/cm for 2–3 h, and visualized by autoradiography. For double-stranded oligonucleotides, the probe was incubated with 5–15 μg of extract in same buffer, except that 200 μg/ml of salmon sperm DNA and 24 μg/ml of pBR322 were included to reduce nonspecific binding.

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EDTA and 10 μg/ml tRNA and deproteinized with phenol. DNA was precipitated with ethanol, electrophoresed on an 8 M urea, 6% polyacrylamide gel, and visualized by autoradiography.

Southwestern Blotting—20 μg of extract were mixed with an equal volume of loading buffer containing 5 mM Tris, pH 6.8, 200 mM DTT, 5% SDS, 20% glycerol, and 0.05% pyronin Y. Proteins were denatured at 100 °C for 3 min, separated on a 10% SDS-polyacrylamide gel, and then blotted onto nitrocellulose paper (29). The blots were incubated with buffer containing 10 mM HEPES and 5% nonfat dry milk at 25 °C for 1 h, and then incubated with binding buffer containing 10 mM HEPES, pH 8.0, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.25% nonfat dry milk, 5 μg/ml salmon sperm DNA, and 1 × 10⁶ cpm/ml probe at 25 °C for 1 h. After washing with 2 changes of binding buffer containing 300 mM NaCl at 25 °C for 2 h, the filter paper was air dried and subjected to autoradiography.

RESULTS

Sequences Downstream from Exon 1 Transcription Initiation Sites Are Important for IGF-I Gene Expression and Metabolic Regulation—Constructs with IGF-I 5′-flanking sequences were transfected into rat hepatocytes in primary culture, as summarized in Fig. 1. Relative to expression of a promoterless luciferase reporter vector (p0LUC), expression was decreased 30% by the presence of IGF-I sequence extending from −4 to −32 bp relative to the exon 1 major transcription initiation site (7, 13, 14). Expression was increased 40 and 113% with constructs containing 1.86 and 1.26 kilobase pairs of 5′ sequence, respectively, and the same 3′ terminus. Compared with expression of p(−1859−32)LUC (40% above that of p0LUC), increased expression was obtained with constructs containing additional downstream sequences. Expression was increased 126% above p0LUC with a construct containing 1.86 kilobase pairs of 5′ sequence and 3′ sequence terminating at −35 bp, and maximum expression, 230% above p0LUC, was obtained with a construct containing the same 5′ sequence and 3′ sequence extending to +180 bp; expression was significantly greater than that of the construct with similar 5′ sequence but lacking downstream sequence (p < 0.005). Thus, these findings suggested that downstream sequences enhanced IGF-I gene expression.

Because of relatively low expression in transient transfection studies (possibly due to the difficulty in maintaining IGF-I gene expression in hepatocytes) as well as the difficulty of transfecting cells in primary culture (31)), a different model was used to test the hypothesis that downstream sequences contribute to metabolic regulation of IGF-I gene transcription. A genomic IGF-I template containing 471 bp of upstream sequence and 219 bp of downstream sequence was incubated with nuclear extracts from the livers of normal and diabetic rats, and in vitro transcriptional activity was evaluated by primer extension. As shown in Fig. 2A, the dominant transcription initiation site in vitro was identical to that used in vivo. The signal originated from RNA polymerase II transcripts, since it was sensitive to α-amanitin (not shown). Moreover, no signal was detected when extracts were incubated in the absence of a DNA template, indicating that signals originated from tran...
Since in vitro transcriptional activity for the adenovirus major late promoter template driven by nuclear extracts from the livers of diabetic rats was comparable or greater to that with extracts from normal rats (Fig. 2), we concluded that nuclear extracts from the livers of diabetic rats contained adequate transcriptional machinery and that changes in IGF-I gene transcription were likely to be specific. Using templates containing downstream sequence, in vitro transcriptional activity of nuclear extracts from the livers of diabetic rats was reduced 90%, compared with nuclear extracts from the livers of normal rats, (p, 0.05), but transcriptional activity with an IGF-I template lacking downstream sequence was not significantly decreased with extracts from diabetic versus normal rats (p, 0.1, Fig. 2C). Since this observation is consistent with our previous finding (7), that IGF-I gene transcription rates were reduced ~97% in the livers of diabetic rats as compared with normal rats, downstream sequences may be important for both IGF-I expression and metabolic regulation.

Nuclear Protein(s) Binding to Downstream Sequences—To search for regions that might be involved in gene regulation, the binding of hepatic nuclear factors to the 272-bp AccI/BglII fragment was first studied by gel mobility shift analysis, as shown in Fig. 3. Densitometric scanning revealed that the intensity of shifted protein-DNA complexes was reduced 30–60% with extracts from streptozotocin-diabetic as compared with normal rats. Binding was specific, since formation of DNA-protein complexes could be competed with an unlabeled 272-bp fragment but not with pBR322 DNA (not shown). All protein-DNA binding studies were repeated with at least three different batches of extracts, and extract activity was monitored by in vitro transcription assays.

Protein Binding Sites Assessed by DNase I Footprinting—Protein binding sites were determined on both coding and noncoding strands, and results are shown in Fig. 4. Region I corresponded to the major transcription initiation site in exon 1. Five additional protected regions were observed consistently, with footprints at +17 to +25 (region II), +42/+68 (region III), +79/+101 (region IV), +129/+152 (region V), and +155/+169 (region VI). Binding of factors in nuclear extracts from the livers of diabetic rats as compared with normal rats, downstream sequences may be important for both IGF-I expression and metabolic regulation.

Region III and V Are Necessary for the Diabetes-associated Reduction in IGF-I Gene Transcription—The importance of downstream regulation of rIGF-I transcription by guest on July 24, 2018http://www.jbc.org/Downloaded from
Downstream regions in metabolic regulation was evaluated with in vitro transcription assays using deletion mutants as templates, as shown in Fig. 6. A template lacking regions IV and V (+42/+152) no longer provided reduced transcriptional activity with nuclear extracts from diabetic versus normal rats (panel B). Similar results were also obtained with templates lacking regions III (+42/+68) or V (+129/+152) (panel C). In contrast, a template with deletion of region IV continued to exhibit decreased transcriptional activity with nuclear extracts from diabetic versus normal rats (panel D). While the potential involvement of other regions is still being investigated in our laboratory, these data were reproducible with different batches of extracts, and the transcriptional activities of normal and diabetic extracts remained comparable as determined with the adenovirus major late promoter as template.

Nuclear Factors Associated with Regions III and V Are Reduced by Streptozotocin-induced Diabetes—Double-stranded oligonucleotides corresponding to regions III (+42/+68 bp) and V (+129/+152 bp) were used in gel mobility shift analyses to examine DNA-protein interactions, as shown in Fig. 7. Addition of pBR322 DNA was necessary to decrease nonspecific binding (lanes 2 and 10 versus lanes 4 and 12). The association of nuclear factors with region V was stronger than that with region III, especially in formation of complex II (lanes 3 and 4 versus lanes 11 and 12). While formation of both complexes I and II could be competed with unlabeled oligonucleotides (lane 3 versus lanes 5 and 6 and lane 11 versus lanes 13 and 14), cross-competition was incomplete (lane 3 versus lanes 7 and 8 and lane 11 versus lanes 15 and 16). Thus, binding of nuclear factors to regions III and V was relatively specific, particularly for complex II. Activities of nuclear extracts from the livers of normal and diabetic rats are shown in Fig. 8. Nuclear proteins associated with region IV showed similar affinity with normal and diabetic rat liver extracts. In contrast, nuclear protein binding to regions III and V was reduced with extracts from diabetic animals, typically 30–50% of that of extracts from normal rats.

Identification of Protein Factors Associated with Regions III and V—To characterize the size and relative abundance of proteins associated with regions III and V, hepatic nuclear extracts from normal and diabetic rats were subjected to polyacrylamide gel electrophoresis and then blotted to nitrocellulose and probed with corresponding oligonucleotides, as shown in Fig. 9. Proteins with apparent molecular weight of ~65 kDa were associated with both regions III and V and were present in extracts from both normal and diabetic animals. However, apparent abundance of the ~65-kDa protein in extracts from diabetic animals was ~75% of normal with region III and...
50% of normal with region V, as determined by densitometric scanning.

**DISCUSSION**

The IGF-I promoters analyzed to date have several common features, such as lack of a "TATA" box, presence of transcription "initiator" sequences (13, 32), and binding sites for well recognized transcription factors such as Sp1, C/EBP, and HNF-1 located upstream from the major transcription initiation sites (32). The present studies demonstrate that sequences downstream from the major transcription initiation site in exon 1 are important for both IGF-I gene expression and metabolic regulation. Within the −54/+219 bp region of exon 1, we found six loci of binding with hepatic nuclear factors; protected regions were similar to those described by Thomas et al. (33). With our model, DNase I footprinting and gel mobility shift assays revealed that nuclear factors in the livers of diabetic rats have reduced interactions with region III (−42/+68) and region V (+129/+152). Transfection studies revealed a 230% increase in expression with a construct containing 180 bp of downstream sequence (including both regions III and V). Our findings are consistent with those of Hall et al. (14), who observed that the presence of downstream sequence increased IGF-I gene expression when the same constructs were transfected into SK-N-MC cells and Lowe et al. (18) and Adamo and co-workers (19) who found that downstream sequence increased IGF expression when constructs were transfected into C6 glioma cells. Further evidence of biological significance was provided by in vitro studies; specific differences in IGF-I transcriptional activity between normal and diabetic rat liver extracts could be detected only in the presence of downstream sequences.

The two protein-DNA complexes observed in gel mobility shift assays with oligonucleotides III and V likely result from binding of multiple nuclear factors rather than formation of a dimer, since only complex I could be cross-competed with both oligonucleotides III and V. A putative common factor could interact with motifs such as CCTGC(G/C)CA found within both regions III and V. In both gel mobility shift assays and Southwestern blotting studies, the formation of protein-DNA complexes could be competed with unlabeled oligonucleotides but was not blocked with a great excess of pBR322 DNA, indicating that binding was specific.

The DNA-binding protein(s) identified by Southwestern blotting appears to be metabolically regulated, as reduced binding was provided by hepatic nuclear extracts from diabetic as compared with normal rats. While gel mobility shift assays point to the presence of at least two DNA-binding proteins, we do not yet know if other putative factors are metabolically regulated as well. Lack of identification of a second DNA-binding factor by Southwestern blotting may also be attributed to the denaturing conditions used in this procedure, which could interfere with protein-protein interactions that may be stabilized by the caging effect in gel mobility shift analysis.

A number of viral and cellular transcriptional units contain essential sequences which are downstream from transcription initiation sites (34–37). Such downstream elements may influence RNA elongation, processing, and translation, in addition to transcription initiation. Promoters commonly associated with housekeeping and growth control genes often require downstream elements to achieve full gene expression (38). Moreover, intragenic enhancers or activators have been described for numerous extrahepatic genes such as immunoglobulins (39), adenosine deaminase (40), and muscle creatine kinase (41). Thus, the requirement for both upstream and downstream elements to achieve full gene expression is not unique to the IGF-I gene.

There has been relatively little characterization of tissue-specific and hormone response elements of the IGF-I gene. Since this manuscript was submitted, Nolten et al. (42) recently found that C/EBP and HNF-1 can stimulate hIGF-I gene expression.

**FIG. 7. Binding of nuclear proteins to oligonucleotides III and V.** Each binding reaction contained 6 μg of nuclear protein from normal rat liver, 5000 cpm probe, pBR322 plasmid DNA or/and cold double stranded oligonucleotides as indicated. Protein/DNA complexes were separated on a 6% polyacrylamide gel.
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