In Vitro Transcription of the Rat Insulin-like Growth Factor-I Gene*

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Although the liver is the major source of circulating insulin-like growth factor-I (IGF-I), relatively little is known about the regulation of IGF-I gene transcription in this tissue. Since transcripts are initiated largely in exon 1, we established an in vitro transcription system to evaluate activation of transcription via the major exon 1 initiation site. Transcription of a G-free cassette reporter was directed by rat IGF-I genomic fragments, and the adenovirus major late promoter was used as an internal control. Tissue specificity was demonstrated by a 60–90% decrease in transcripts with spleen extracts as compared with liver. 54 base pairs (bp) of upstream sequence were sufficient to direct IGF-I gene transcription, and activity increased 5-fold with 300 bp of upstream sequence. DNase I footprinting revealed four protected regions between –300 and –60 bp; binding was confirmed by gel shift analysis, and tissue specificity was demonstrated by reduced shifts with spleen extracts. The necessity of transcription factor binding to such sites was established by competition analysis, which revealed a specific decrease in IGF-I transcription in the presence of a competing fragment. Use of this in vitro transcription system should permit analysis of the function of individual transcription factors involved in regulation of IGF-I gene expression.

Insulin-like growth factor-I (IGF-I) is a 70-amino acid peptide that is similar to proinsulin in structure (1) and has major anabolic effects on growth, development, and metabolism during both fetal and postnatal life (2, 3). The liver is the major origin of IGF-I acting in an endocrine mode (4), but IGF-I is also synthesized in many other tissues, with local autocrine/paracrine actions (2–4). Although IGF-I was first thought to be regulated mainly by growth hormone, it is now recognized that nutrition, local cellular factors, and other hormones also modulate IGF-I production (2–4).

The rat IGF-I gene contains at least six exons with total length over 80 kb (5). A single copy of the IGF-I gene gives rise to four major mRNA species, with size differences due primarily to multiple polyadenylation sites (6). Transcription is initiated at multiple loci in exons 1 and 2, but exon 1 transcripts predominate in all tissues (7).

While IGF-I production appears to be regulated mainly at the level of gene transcription, underlying mechanisms are not well understood. The 5'-flanking sequences for IGF-I genes from several species exhibit common features, including lack of a TATA box, the presence of "initiator" elements, and binding sites for recognized transcription factors such as Sp1, C/EBP, HNF-1, and AP-1. However, although studies in neuroblastoma SK-N-MC cells (8), rat fibroblasts, and rat C6 glioma cells (9, 10) indicate the presence of functioning promoter regions in exon 1, it has been more difficult to characterize IGF-I transcription in the liver, the dominant source of IGF-I in vivo (4). While the regulation of many genes has been examined by transient transfection in cultured cell models, this approach is less well suited to the liver, because IGF-I expression in immortal cell lines tends to be low (11) and cultured primary hepatocytes are difficult to transfect (12). Accordingly, we have utilized nuclear extracts of normal rat liver in an in vitro transcription system to examine the effect of 5'-flanking sequences on IGF-I gene expression. We demonstrate that maximal promoter activity requires –300 bp upstream from the major exon 1 transcription initiation site and that binding of nuclear factors to this region is essential for IGF-I gene expression.

EXPERIMENTAL PROCEDURES

Chemicals—Restriction endonucleases, T4 polynucleotide kinase, Klenow fragment of DNA polymerase I, and mung bean nuclease were obtained from New England Biolabs (Beverly, MA); a 24-bp oligonucleotide containing consensus Sp1 binding sites was from Stratagene (La Jolla, CA); nucleotides and DNase I were ordered from Pharmacia (Piscataway, NJ); [a-32P]ATP, [a-32P]dATP, [a-32P]CTP, and [a-32P]GTP were from DuPont/NEN (Boston, MA); a 24-bp oligonucleotide containing consensus Sp1 binding sites was from Stratagene; consensus Sp1 binding sites were from Stratagene; [a-32P]cAMP, [a-32P]cGMP, [a-32P]cAMP, and [a-32P]cGMP were from Amersham Corp.; oligonucleotides were from Operon Technology (Alameda, CA); and other molecular biology grade reagents were from Sigma.

Animals—Male Sprague-Dawley rats (Charles River, Lexington, MA), weighing 120–160 g, were fed ad libitum. Animals were sacrificed by cervical dislocation, and livers or spleens were used for nuclear extract preparation immediately.

Liver/Spleen Nuclear Extract Preparation—Nuclear extracts were prepared according to modifications of the methods of Gorski et al. (13) and Triezenberg et al. (14). Briefly, 10–12 g of tissue were homogenized in 85 ml of buffer containing 10 mm Hepes, pH 7.6, 25 mm KCl, 0.15 mm spermine, 0.5 mm spermidine, 1 mm EDTA, 10% glycerol, and 2.0 mm sucrose. The protease inhibitors leupeptin, aprotonin, and pepstatin A (all at 1 (g/ml), 0.5 mm PMSF, and 1 mm benzamidine were added just before use. The homogenate was layered onto a 2 × sucrose cushion and centrifuged at 27,000 rpm in an SW 28 rotor for 1 h at 2 °C. Nuclei were resuspended in lysing buffer containing 10 mm Hepes, pH 7.6, 100 mm KCl, 0.1 mm EDTA, 1 mm DTT, 3 mm MgCl2, 10% glycerol, leupeptin, aprotonin, pepstatin A (all at 1 (g/ml), and 0.1 mm PMSF. Nuclei were lysed by adding 0.1 volume of 4 (g ammonium sulfate, and lystate was left on ice for 30 min with gentle mixing. Chromatin was removed by
centrifugation at 39,000 rpm in an SW 40 rotor for 1.5 hr at 2°C. Nuclear protein was concentrated by (NH₄)₂SO₄ precipitation (0.33 g/ml) and dialyzed against buffer containing 25 mM Hepes, pH 7.6, 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, and 10% glycerol for 4 hr at 4°C. Nuclear protein was frozen and stored at -70°C.

Template Construction—The plasmids pUC13(C₂AT)₃⁷³, pML(C₂AT)₁⁹⁰, and pML(C₂AT)₂⁷⁰ were kindly provided by Dr. Lee E. Babiss. Although there are four transcription initiation sites in exon 1, the G-free cassette was placed downstream from site 3 because this is the major initiation site in vivo (15), and there are no G residues immediately downstream from this site. Two oligonucleotides 5'-GT-TATAGAATTCACCATGGTCATTTCAGGG-3' (2471/2454) and 5'-GCTGCATACGTAAGAAGAGGGATTTAGAG-3' (213/14) were used as primers for PCR amplification (16). After digestion with EcoRI and SnaBI, the gel-purified fragment was subcloned into pUC13(C₂AT), and the construct, pIGF471(C₂AT), was sequenced. The 5'-upstream sequence was extended to 1 kb by insertion of a 581-bp NcoI IGF-I genomic fragment, and the recombinant was designated as pIGF1050(C₂AT). A series of 5'-deletion mutants were then prepared by conventional subcloning, as summarized in Fig. 1. Plasmid DNAs were purified by standard CsCl gradient methods, linearized, deproteinized, and used as templates in transcription assays.

In Vitro Transcription Assay—The optimal assay conditions were defined in preliminary studies. In general, reactions (30 μl) contained 1.0 μg of IGF-I template DNA, 50 ng of pML(C₂AT)₁⁹⁰ or pML(C₂AT)₂⁷₀, 60 μg of liver nuclear extract (L, lane 1, 3, and 4) or spleen nuclear extract (S, lane 2), and 1 μg of pUC13(C₂AT) (lane 1) or pGF₄₇₁(C₂AT) (lanes 2-4). In lane 4, the reaction contained 3 μg/ml α-amanitin. The RNA was electrophoresed on an 8 M urea, 6% polyacrylamide gel. A pBR322 plasmid DNA digested by Hpal I was used as a size marker.

![Fig. 1. Construction of DNA templates. The template pGF₄₇₁(C₂AT) was constructed first (see “Materials and Methods”). The 5'-upstream region was extended by adding a 580-bp NcoI genomic fragment to produce pGF₁₀₅₀(C₂AT). The templates pGF₇₀₀(C₂AT) and pGF₅₀₀(C₂AT) were constructed by deleting NarI/NarI and Ban/l/Ndel fragments from pGF₁₀₅₀(C₂AT) and pGF₄₇₁(C₂AT), respectively. The BanI and AccI fragments, containing 136 and 54 bp of 5'-upstream sequence together with the 373-bp G-free cassette, were excised from pGF₅₀₀(C₂AT), subcloned on the SmaI site on pUC19, and designated as pIGF₁₃₆(C₂AT) and pIGF₅₄(C₂AT), respectively. The exon 1 transcription initiation site designated as +1 is indicated above the constructs; for convenience, the initiation sites identified by Adamo et al. (15) are shown below.](image1)

![Fig. 2. Expression of the IGF-I gene in vitro. Each reaction contained 50 ng of pML(C₂AT)₁⁹⁰, 60 μg of liver nuclear extract (L, lanes 1, 3, and 4) or spleen nuclear extract (S, lane 2), and 1 μg of pUC13(C₂AT) (lane 1) or pGF₄₇₁(C₂AT) (lanes 2-4). In lane 4, the reaction contained 3 μg/ml α-amanitin. The RNA was electrophoresed on an 8 M urea, 6% polyacrylamide gel. A pBR322 plasmid DNA digested by Hpal I was used as a size marker.](image2)
taining 5 \( \mu \)g of tRNA and 60 \( \mu \)g of proteinase K and incubating for 30 min at 37°C. The RNA was purified by phenol/chloroform extraction, ethanol-precipitated, and then applied onto an 8M urea, 6% polyacrylamide gel, and visualized by autoradiography. For competition studies, the oligonucleotides 5'-AACGTCTGCTAACCCTGTCA-3' and 5'-AAA-CAGCTGGGGGAACATTCG-3' were used as primers to synthesize a 183-bp (6240/258) fragment by polymerase chain reaction (16). The 183-bp fragment including footprint sites I–IV (partial) was used as a competitor in transcription assays in order to ensure that competition was specific.

DNase I Protection Assay—The end-labeled DNA fragment was incubated with 4–24 \( \mu \)g of nuclear extract in 20 \( \mu \)l of binding buffer containing 25 mM Hepes, pH 7.6, 0.1 mM EDTA, 50 mM KCl, 1 mM DTT, 1 \( \mu \)g of poly(dI-dC), and 5% glycerol at 25°C. After incubation for 2 min at 25°C, reactions were carried out for 2 min at 25°C. Reactions were stopped by buffer containing 10 mM MgCl\(_2\), 2 mM CaCl\(_2\), and 0.25 mM NaCl, 200 \( \mu \)g/ml proteinase K, and 20 \( \mu \)g/ml tRNA. After incubation for 30 min at 37°C, the purified DNA was resolved on an 8% urea, 6% polyacrylamide gel and visualized by autoradiography.

Gel Mobility Shift Assay—An end-labeled 247-bp DNA (~300/54) was incubated with 1–4 \( \mu \)g of nuclear extract in 25 \( \mu \)l of binding buffer containing 10 mM Tris, pH 7.5, 50 mM KCl, 1 mM EDTA, 0.5 mM DTT, 1% Nonidet P-40, 20 \( \mu \)g of bovine serum albumin, 4 \( \mu \)g of poly(dI-dC), and 10% glycerol for 25 min on ice. Protein-DNA complexes were separated at 4°C on a 5% polyacrylamide gel in 1 X TBE (89 mM Tris, 89 mM borate, 2 mM EDTA) at 11 volts/cm for 2–3 h and visualized by autoradiography.

RESULTS

In Vitro Transcription of the Rat IGF-I Gene—In initial experiments, the pIGF471(C2AT) construct was used to examine rIGF-I gene expression in vitro, as shown in Fig. 2. With the IGF-I template, the G-free cassette (C2AT) provided a 373-bp signal. A second template from the adenovirus major late gene promoter (AdMLP) was included in all reactions as an internal control, with a shorter C2AT cassette providing a 190- or 270-bp signal. In the presence of a nuclear extract from normal rat liver, a strong IGF-I signal was observed (lane 3). The IGF-I signal was abolished by use of vector (pUC13(C2AT)) as template (lane 1), and both the IGF-I and AdMLP signals were abolished by addition of \( \alpha \)-amanitin (lane 4). These findings indicate that the IGF-I transcripts were directed by RNA polymerase II and originated from an authentic promoter on the rIGF-I gene and that 471 bp of 5'-flanking sequence contain cis-elements sufficient to activate the IGF-I promoter in vitro. A nuclear extract from rat spleen was transcriptionally active as shown by a strong AdMLP signal but provided an IGF-I
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Fig. 5. The effect of 5'-deletions on IGF-I gene expression. Panel A, assays here and below were performed in the presence of 60 mM KCl, 6 mM MgCl₂, 60 μg of extract at 30 °C for 45 min, as described under "Materials and Methods." The DNA templates were pIGF₁₀⁵₀(C₂AT), pIGF₁₀⁴₇₁(C₂AT), pIGF₁₀₃₀(C₂AT), pIGF₁₀₅⁴(C₂AT), pIGF₁₀₃₃(C₂AT), and pIGF₁₀₁₃₆(C₂AT). The signal obtained from pIGF₁₀⁵⁴(C₂AT) was designated as 1. Mean ± S.E. for four different nuclear extracts is shown.

The tissue specificity of DNA-protein interactions within the −300-bp promoter was evaluated by gel mobility shift analysis (Fig. 8). Using a 247-bp DNA fragment (−300/−54) as a probe, DNA-protein complexes were readily apparent with a liver nuclear extract but barely detectable with a spleen nuclear extract (panel A). The formation of DNA-protein complexes was specific, because binding with liver extracts could be competed with a 100 × excess of unlabeled probe (lane 6) and with a 50 × molar excess with spleen extracts (lane 10); binding was not competed with a 300 × excess (0.35 μg) of Sp1- and PvuII-digested pBR322 plasmid DNA (not shown). The reduced binding observed with spleen nuclear extracts was due primarily to the absence of tissue-specific IGF-I-related factors, because the spleen nuclear extract provided stronger binding activity than the liver nuclear extract when an Sp1 probe was used (panel B, lanes 2 and 3 versus 4 and 5). The formation of DNA-protein complexes with Sp1 was also found to be specific via competition studies (data not shown). Taken together, our findings suggest that the majority of transcription factor binding within the −300-bp IGF-I promoter region is likely to be tissue-specific.

To determine whether DNA-protein interactions within the −300-bp region might be essential for IGF-I gene expression, in vitro transcription was performed with and without addition of a 183-bp DNA fragment (−240/−58 bp), which included DNase I-protected regions I–III and IV (partial). Although a 5 × molar excess of the competing fragment decreased IGF-I transcription 15%, AdMLP transcription was unaffected. With a 25 × molar excess, IGF-I transcription was decreased by 70% (Fig. 9, lane 3 versus lane 1), but AdMLP transcription was minimally affected, showing that competition was specific under these conditions. With a 100 × molar excess of the competing fragment, IGF-I transcription was decreased by 90% (lane 4 versus lane 1), while 45% of AdMLP activity was retained. In contrast, a 35 × excess of a 190-bp (−178/+10) AdMLP fragment decreased AdMLP expression by 45% but did not interfere with transcription.

5'-Flanking Sequences Essential for rIGF-I Gene Expression—To identify promoter regions, we examined in vitro transcription supported by IGF-I templates with different lengths of 5'-flanking sequence: −1050, −701, −471, −300, −136, and −54bp with respect to the major exon 1 transcription initiation site, as shown in Fig. 5. In four separate experiments, core promoter activity was consistently detected with the pIGF₁₀⁵⁴(C₂AT) construct. Transcriptional activity was increased 2.2-fold with −136 bp of 5'-flanking sequence and was 5-fold with −300 bp of 5'-flanking sequence (both p < 0.05 versus −54 bp). Transcriptional activity was decreased −50% with another 171 bp of 5'-flanking sequence, and there was no significant change in promoter activity with further addition of upstream sequence to −1050 bp. Similar results were obtained when circular plasmid DNA was used a template, indicating that changes in promoter activity were not due to restraint effects (data not shown).

DNA-Protein Interactions in rIGF-I 5'-Flanking Regions—Potential transcription factor binding within the critical region from −300 to −54 bp was examined with DNase I protection analysis, as shown in Fig. 6. Three major protected regions were identified on the coding strand at −119/−100 (I), −149/−126 (II), and −213/−193 bp (III), with weaker binding at −248/−238 bp (IV). Protection on the noncoding strand was generally comparable, except that incomplete protection was observed above region III from −202/−186 bp, and protection remained weak with region IV. Comparisons with transcription factor consensus sequences indicate homology with binding sites for both ubiquitous and liver-specific factors, as summarized in Fig. 7 and Table I.
IGF-I gene expression (not shown). With a 70 × molar excess of the AdMLP competing fragment, expression of both IGF-I and AdMLP was decreased (not shown), consistent with competition for general transcription factors. In combination, these findings suggest that binding of transcription factors to the -240/-58-bp region is necessary to direct IGF-I gene transcription from the major exon 1 initiation site.

## DISCUSSION

The present studies demonstrate that the molecular regulation of rIGF-I gene expression can be evaluated by in vitro transcription with nuclear extracts and genomic templates. Specific IGF-I transcripts from the major exon 1 transcription initiation site were detected by expression of a 373-bp G-free cassette (C2AT), while shorter 190- or 270-bp G-free cassettes reflected transcription of the adenovirus major late promoter, as an internal control. Using nuclear extracts shown to be transcriptionally competent on the basis of AdMLP activity, we found that IGF-I transcriptional activation was liver-specific, because transcription was decreased ~90% with spleen extracts. Mixing experiments indicated that reduced transcriptional activation with spleen extracts may be attributable to the lack of putative tissue-specific activators, a finding similar to previous observations with the L-type pyruvate kinase and albumin genes (13, 17). Transcription was polymerase II-dependent, as shown by inhibition with α-amanitin. While basal promoter activity could be detected with 54 bp of IGF-I 5′-flanking sequence, a 5-fold stronger signal was detected with 300 bp of 5′-flanking sequence, with a decrease in signal strength with addition of further 5′-sequence. Within the -300/-54-bp region, four DNase I footprints were identified, and competition studies indicated that binding of putative transcription factors to such regions is necessary for IGF-I gene expression in vitro.

The present finding of maximal promoter activity with -300 bp of sequence 5′ to the major exon 1 transcription initiation site, in an in vitro transcription system driven by normal liver extracts, may be compared with observations by other workers.

The amount of protein used was 4, 8, 16, and 24 μg in lanes 2-5, respectively. Panel B, a DdeI/PvuII (-350/-66) fragment was labeled on the noncoding strand. The amount of protein used was 8, 16, 24, and 32 μg in lanes 2-5, respectively.

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**FIG. 6.** DNase I protection assay. The probe was incubated with liver nuclear extract for 20 min at 25 °C and then digested with DNase I at 20 units/ml for 2 min as described under “Materials and Methods.” The DNA was electrophoresed on an 8 M urea, 6% polyacrylamide gel. The DNA size marker (M) was pBR322-digested by Hpal I. Protected regions were determined with Maxam-Gilbert sequencing (A = G) using naked DNA digested with DNase I as a negative control (lane 1). Panel A, an NcoI/AccI (-471/-54) fragment was labeled on the coding strand.
who evaluated promoter activity by transient transfection in extrahepatic immortal cell lines. Hall et al. (8) examined rIGF-I gene expression in SK-N-MC neuroblastoma cells and found very limited promoter activity with a construct extending from 2533 to 1190 bp (see Fig. 1), and maximal promoter activity with a construct with the 5' terminus at 21 kb. Similar findings in SK-N-MC cells transfected with human IGF-I constructs were reported by Kim et al. (18), who found greatest activity with a construct extending from 21.8 kb to 1181 bp. However, Jansen et al. (19) also reported greatest activity in SK-N-MC cells with a human IGF-I construct extending from 2690 to 155 bp. In contrast, Lowe and Teasdale (9) found maximal promoter activity in rat dermal fibroblasts and C6 glioma cells with a rat IGF-I construct extending from 2550 to 1224 bp and observed that addition of 700 bp of further 5'-flanking sequence led to reduced expression. More recently, Lowe (10) used a similar model to examine rIGF-I gene expression with constructs having the 3' terminus at +40 bp and reported little fall off in expression when the 5'-flanking sequence was reduced to 2156 bp.

Variation among these findings may have several causes. Our studies utilized extracts from normal liver, while other workers (8–10, 18) utilized fibroblasts or immortal cell lines, in which the concentration and/or activity of transcription factors are recognized to be different from that of liver (20–22) and could contribute to the observed discrepancies in promoter activity. Because of the constraints of the G-free cassette system, our constructs contained little downstream sequence as compared with the chimeric rIGF-I genes used in transfection studies. However, both our laboratory (23) and other workers (8–10) have noted that downstream sequences may be important for expression and conceivably could also modify the interactions of transcription factors with upstream sequences. In addition, our system provided evaluation of transcripts initiated only at a defined site, whereas transient transfection studies included transcripts potentially initiated at multiple sites. Finally, formation of the transcription machinery to assemble initiation complexes in vitro also differs from that in vivo since chromatin structure is disrupted with the use of purified DNA as a template (24). Taken together, these possibilities may underlie our finding of maximal promoter activity with a −300-bp construct using normal liver extracts, as opposed to a −1-kb construct in SK-N-MC neuroblastoma cells (8), and −156-bp constructs in rat fibroblasts and C6 glioma cells (9, 10).

For many genes, the dominant control of liver-specific expression is at the level of transcription (25, 26) and depends on binding of trans-acting factors to cis-regulatory DNA sequences, often located in the 5'-flanking region. For the rat IGF-I gene, developmental activation is associated with the progressive appearance of DNase I-hypersensitive sites, consistent with the impact of trans-acting factors (27). In the present study, the four protected regions identified by DNase I footprinting are compatible with the location of DNase I cleavage sites described by Kikuchi et al. (27). While regions I and II are similar to locations HS3A and HS3B described by Thomas et al. (28), we are not aware of binding of nuclear factors to regions III and IV in previous reports on the rat IGF-I gene. The combined functional importance of these sites is illustrated by our competition study, in which expression was decreased 90% by the presence of a fragment containing sites I, II, III, and IV (partial).

The regulation of hepatic gene transcription depends in part on the coordinated contributions of basal transcription factors, tissue-specific transcription factors, and transcription factors that are modulated by hormonal and metabolic status (26). Thus, relatively liver-specific genes such as albumin contain binding sites for basal transcription factors such as TATA box binding protein (TBP), AP1, and NF-Y (29–31), as well as more liver-specific factors such as hepatic nuclear factors I, II, III,

| Region | Transcription Factor | Consensus Sequence | IGF-I Sequence |
|--------|---------------------|--------------------|----------------|
| I (-119/-110) | C/EBP | TTACGGTTAAGCTTTGGCAAG | gTgTAAGTTGGC |
| | AP1 | TGA/GT/GA | TgTCTCA |
| | NF1 | T'/GG'/AC/GCA | CgAATGTTGGCA |
| | HNF-1 | GTTTATTAAC | gTToTcAT |
| | DBP | T'/TA/TAGA | AtAcAT |
| II (-149/-126) | HNF-3 | T/TTTGA/G | TaTgAT |
| | HNF-5 | T'/TTTGA/G | TaTTG |
| | AP1 | TGA/GT/GA | TgC |
| | AF3 | TGGG/GT/GA | TGTT |
| III (-213/-193) | Spl | G/GGC/GGC/GGC/G | AC/TCC/TTAAGGGGGGGA |
| | Insulin | GGGGGAAT | GGGGAA |
| | E2F | TTTTG/GG/G | TTTT |
| IV (-248/-238) | TBP | TATAAA | TCAGTATT |
| | | | TATA |

TABLE I
Comparison of rIGF-I genomic DNA sequence within DNase I protection regions I-IV with the consensus sequences of the known transcription factors

Only the sequences of the message strand are shown. The nucleotides that are different from the consensus sequences are indicated by lowercase letters. The rIGF-I sequence is in bold.
and V (HNF-1, -2, -3, and -5), CCAAT/enhancer binding protein (C/EBP), and D-site binding protein (DBP) (30–34). Based on comparisons with consensus sequences for binding sites of such factors (35), it seems likely that both liver-specific and general transcription factors will be found to interact with the proximal IGF-I promoter region (36, 37).

In summary, the present studies constitute the first use of an in vitro transcription system to examine the regulation of IGF-I gene expression in normal liver, the major source of circulating IGF-I (4). In the future, this system should permit functional assays of promoter activity assessment to be combined with transcription factor binding in order to identify critical regula-

**Fig. 8. Gel mobility shift analysis.** Panel A, a 247-bp (−300/−54) DNA fragment was incubated with 1, 2, and 4 µg of nuclear protein from liver (lanes 2–4) or spleen (lanes 7–9) as described under “Materials and Methods.” In competition assays, 4 µg of protein was incubated with a 50 × (lanes 5 and 10) or 100 × (lanes 6 and 11) excess of unlabeled fragment on ice for 20 min before addition of probe. Panel B, a 24-bp double-stranded oligonucleotide containing Sp1 binding sites was incubated with nuclear extracts from liver (lanes 2 and 3) and spleen (lanes 4 and 5) for 20 min on ice as described. DNA-protein complexes were resolved on a 5% polyacrylamide gel and visualized by autoradiography.

**Fig. 9. Competition analysis.** A 183-bp fragment (−240/−58) was incubated with liver nuclear extract on ice for 20 min before pIGF300(C2AT) template DNA was added. The in vitro transcription assay was performed as described under “Materials and Methods.” φ174 DNA digested by HindIII was used as a size marker (M); lane 1, control; lane 2, 5 × excess; lane 3, 25 × excess; lane 4, 100 × excess.
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