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Three Clustered Sp1 Sites Are Required for Efficient Transcription of the TATA-less Promoter of the Gene for Insulin-like Growth Factor-binding Protein-2 from the Rat*

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Insulin-like growth factor-binding protein-2 (IGFBP-2) transcription in rat liver varies with developmental age and fasting. To define the DNA elements required for efficient expression of the TATA-less rat IGFBP-2 gene, the native or mutated promoter was fused to a promoterless luciferase reporter gene and transfected into BRL-3A rat liver and 293 human embryonic kidney cell lines. Luciferase activity decreased ~25-fold with progressive 5′ promoter deletions from nucleotide (nt) -581 to nt -189 (relative to ATG, +1). The smallest construct, however, still had >21-fold greater luciferase activity than the promoterless construct. In DNase I footprinting assays using native nt -276 to -36 promoter fragments or fragments containing block substitution mutations, BRL-3A nuclear extract and purified human transcription factor Sp1 protected a region from nt -234 to -215 containing one GC box and a broad region from nt -189 to -125 that contained three clustered but independent GC boxes. In gel retardation assays using an Sp1 oligonucleotide probe, BRL-3A extract formed two closely migrating complexes that were immunologically related to Sp1; Sp1 gave a single complex that co-migrated with the more retarded BRL-3A complex. Binding was competitively inhibited by oligonucleotides corresponding to each of the four GC boxes. The proximal three GC boxes were sufficient to allow trans-activation of the IGFBP-2 promoter by Sp1 in *Drosophila* SL2 cells. Independent block mutations indicated that all three of the GC boxes are required for promoter activity and are equally important. Thus, binding of Sp1 or Sp1-related proteins to three clustered GC boxes in the proximal IGFBP-2 promoter is essential for promoter activity. Multiple upstream regions also contribute to the full expression of the IGFBP-2 gene.

Insulin-like growth factor-binding protein-2 (IGFBP-2)[1] is a member of a family of six IGFBPs that bind IGF-I and IGF-II with high affinity, and have many structural and biological similarities (1, 2). Most cells secrete one or more IGFBPs (1), which determine the bioavailability and metabolic activity of the IGFs at the cellular level (1, 3).

Rat IGFBP-2 is a nonglycosylated protein of 270 amino acids (4). It is the predominant IGFBP in human and rat plasma during fetal life (5, 6). In the rat, IGFBP-2 levels decline after birth and are undetectable by three weeks of age (6). *In vitro*, IGFBP-2 has been shown to potentiate (7, 8) or inhibit (1, 9) the mitogenic activity of IGF-I in different experimental systems. It is expressed near cell layers undergoing rapid proliferation in the embryo (10) and in the brain at sites of ischemic injury (11, 12), suggesting that IGFBP-2 may target IGFs in the microenvironment to growing cells.

We have studied the *in vivo* regulation of IGFBP-2 gene expression with the overall goal of understanding how IGFBPs modulate the actions of IGFs. IGFBP-2 mRNA is expressed at higher levels in most fetal tissues than in the corresponding adult tissues, with the developmental decrease being most marked in liver (4, 13). In normal adult rats, IGFBP-2 expression persists at high levels only in the choroid plexus of the brain (14). IGFBP-2 mRNA levels, however, are increased in adult rat liver in catabolic states such as diabetes and fasting (13, 15). Changes in the rate of transcription of the IGFBP-2 gene in rat liver during development and fasting account at least in part for the changes in IGFBP-2 mRNA abundance (16).

To better understand the basis for this transcriptional regulation, we have cloned the 5′ region of the rat IGFBP-2 gene and demonstrated that it contains promoter activity when inserted in the sense orientation with respect to a luciferase reporter gene (17). The promoter lacks basal elements such as TATA and CCAAT in the vicinity of the transcription start sites,[2] but is very GC-rich. We now show that multiple regions of the 5′-flanking sequence contribute to basal promoter activity. In particular, we demonstrate that the integrity of each of three clustered GC boxes in the proximal promoter is critical for efficient transcription of the gene. Finally, we provide evidence that Sp1 or an Sp1-like protein present in BRL-3A cells trans-activates the IGFBP-2 gene by binding to these GC boxes.

MATERIALS AND METHODS

Reagents—Enzymes were purchased from the following commercial suppliers: Promega Corp. (Madison, WI), Boehringer Mannheim, New England Biolabs (Beverly, MA), Amersham Corp., Pharmacia LKB Biotechnology Inc., United States Biochemical Corp., and Perkin-Elmer Cetus. Polymerase chain reactions were performed on a DNA thermal cycler (Perkin-Elmer Cetus). Radionucleotides were purchased from Amersham Corp. Oligonucleotides were synthesized using a 394 DNA/RNA synthesizer from Applied Biosystems (Foster City, CA). Fetal calf serum (lot no. 11111074) was obtained from HyClone (Logan, UT). The BCA (bicinchoninic acid) protein assay kit was from Pierce Chemical

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[1] The abbreviations used are: IGFBP-2, insulin-like growth factor-binding protein-2; IGF, insulin-like growth factor; nt, nucleotide(s).

[2] Several sites of transcription initiation have been identified between nt -151 and nt -88 by primer-extended reverse transcription and ribonuclease protection (17, 23).

Co. Calcium phosphate transfection kits were obtained either from Pharmacia LKB Biotechnology Inc. or from Specialty Media (Lavallette, NJ). Protease inhibitors were purchased from Sigma. DNA alternating copolymers, poly(dI-dC)·poly(dI-dC) and poly(dA-dT)·poly(dA-dT), were from Pharmacia LKB Biotechnology Inc. The transcription factor Sp1, purified from HeLa cells infected with recombinant vaccinia virus containing a cDNA encoding the 696 carboxyl-terminal amino acids of human Sp1, was purchased from Promega Corp. Rabbit antisera directed against residues 520–538 of human Sp1 (18) and against human fibronectin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and Promega Corp., respectively.

Preparation of Plasmids for Transfection—Promoter deletion mutants were prepared by digestion of rat IGFBP-2 genomic clone H12-9 (17) with restriction enzyme *Nhe*I (restriction site at nucleotide −36 with respect to the translation start site, +1) and the restriction enzymes shown in Fig. 1 to give variable 5′ ends from nt −581 to nt −189. The resulting restriction fragments were made blunt-ended using either the Klenow fragment of DNA polymerase I or T4 DNA polymerase and cloned into plasmid pA3LUC (provided by Dr. W. M. Wood, University of Colorado) using *Hin*dIII linkers (17). Block substitution mutations in the four proximal GC boxes were generated in the context of nt −581 to −36 promoter fragments by replacing 10 base pairs of native sequence with a *Bgl*II linker (5′-CTAGATCTAT-3′) between nt −139 and −130 (ΔBox 1), nt −163 to −154 (ΔBox 2), nt −183 to −174 (ΔBox 3), or nt −226 to −217 (ΔBox 4). The mutant promoter fragments were prepared by *Bgl*II digestion and ligation of appropriate pairs of 5′ and 3′ fragments generated by polymerase chain reaction that had overlapping *Bgl*II sequences and cloned into the *Hin*dIII site of plasmid pA3LUC. Luciferase construct plasmids used in the transfection studies were prepared by the alkaline lysis method followed by double banding on cesium chloride gradients. They were verified by DNA sequencing using the chain termination method (19). Expression plasmids containing cDNA encoding the 696 carboxyl-terminal amino acids of human Sp1 in-frame with the translation initiation ATG of the *Drosophila* alcohol dehydrogenase (adh) gene (pP_{adh}Sp1-in) and an out-of-frame control (pP_{adh}Sp1-out) were provided by Dr. Robert Tjian (University of California at Berkeley) (20) and prepared using a DNA purification kit (Qiagen Inc., Chatsworth, CA).

Transfection of BRL-3A, 293, and SL2 Cells—The BRL-3A cell line (ATCC CRL 1442), established from Buffalo rat liver, was grown in modified Ham's F-12 medium (Biofluids, Rockville, MD) supplemented with 5% fetal calf serum and 2 mM glutamine. Cells were detached weekly by incubation with 2% chicken serum, 0.075% trypsin, and 7 units/ml collagenase and passaged at 1:6 dilution. The cells were replated at approximately 2×10^6 cells/100-mm dish 72 h prior to transfection. Luciferase reporter plasmids (30 μg) were transfected into BRL-3A cells by electroporation as described previously (17). Plasmid pTKGH (50 μg) containing the human growth hormone reporter gene under the control of the thymidine kinase promoter was cotransfected to normalize for the efficiency of transfection. Forty-eight hours after transfection, media were collected and frozen, to be assayed later for growth hormone using a solid phase immunoradiometric kit (Nichols Institute Diagnostics, San Juan Capistrano, CA). Cells were disrupted with 1 ml of lysis buffer (1% Triton X-100, 25 mM glycylglycine, pH 7.8, 15 mM $MgSO_4$, 4 mM EGTA, 1 mM dithiothreitol) and luciferase activity determined immediately using an automated luminometer (Berthold Clini-Lumat, London Diagnostics, Eden Prairie, MN) as described previously (17).

The human embryonic kidney cell line 293 (ATCC CRL 1573 (21)) was grown in minimal Eagle's medium (Life Technologies Inc.) supplemented with 10% fetal calf serum and 2 mM glutamine and passaged 1:3 weekly using 0.05% trypsin, 0.02% EDTA. Twenty-four hours before transfection, cells were trypsinized and plated at a density of 6×10^6 cells/100-mm dish. They were cotransfected with luciferase reporter plasmids (10 μg) and plasmid pTKGH (10 μg) using the calcium phosphate method (19). Cells and conditioned media were harvested and assayed as before 48 h after transfection.

The *Drosophila* SL2 cell line (20) was grown in M-3 media (Quality Biological Inc, Gaithersburg, MD) supplemented with 10% heat-inactivated (56 °C, 30 min) fetal calf serum, 10 mM glutamine, and 20 μg/ml gentamycin (Life Technologies Inc.). The cells were kept in tightly closed flasks at room temperature. Every 3–4 days, cells were suspended by shaking and passaged by diluting the suspended cells with 3 volumes of fresh media. Cells were plated at a density of 15×10^6/100-mm dish 24 h prior to transfection. Luciferase reporter plasmids, and the indicated Sp1 expression vector (pP_{adh}Sp1-in or pP_{adh}Sp1-out) or plasmid pGEM-7Zf(−) (Promega Corp.), 10 μg each, were cotransfected into SL2 cells using the calcium phosphate method. Cells were harvested 48 h after transfection, washed with phosphate-buffered saline, and resuspended in 0.5 ml of buffer (0.25 M Tris, pH 8.0, 1 mM dithiothreitol). Cells were lysed by freezing and thawing followed by sonication and cell debris removed by centrifuging twice in a microcentrifuge (10 min, 4 °C). Luciferase activity was assayed as before and corrected for protein measured by the BCA method using bovine serum albumin as a standard.

Preparation of Nuclear Extracts—BRL-3A cells were grown to confluence as described above. Nuclear extracts were prepared as described by Hennighausen and Lubon (22) with minor modification of the buffers. Briefly, cells were washed twice with phosphate-buffered saline, scraped into fresh phosphate-buffered saline, and collected by centrifugation. Nuclei were sedimented by centrifugation after lysis of the cells with Nonidet P-40. Pelleted nuclei were then stirred gently with 3 volumes of extraction buffer (20 mM Hepes, pH 7.9, at 4 °C, 25% glycerol, 400 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 0.1 mM benzamidine, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 2 μg/ml pepstatin) for 30 min at 4 °C. Chromatin was pelleted by centrifugation (60 min, 33,800 rpm (110,000 × *g*), 4 °C) in a Ti70 rotor (Beckman Instruments Inc.) and the extract dialyzed twice for 2 h against 50 volumes of dialysis buffer (identical to the extraction buffer except that it contained 20% glycerol and 100 mM NaCl but no $MgCl_2$). The dialyzed extract was cleared of precipitated material by centrifugation (30 min, 17,500 rpm (36,600 × *g*), 4 °C) in an SS-34 rotor (Du Pont) and stored as 100-μl aliquots in liquid nitrogen. Protein concentration, assayed by the BCA method, was 2 μg/μl.

DNase I Footprinting—Plasmid H12-9 was digested with *Bst*EII (nt −727) and *Ear*I (nt −276), and the *Bst*EII/*Ear*I promoter fragment electroeluted by standard methods (19). The *Bst*EII/*Ear*I fragment was cut with *Bsm*AI (nt −392) and the coding strand labeled with [α-^{32}P]dCTP (3000 Ci/mmol; Amersham Corp.) using the Klenow fragment of DNA polymerase I. After digestion with *Sfa*NI (nt −676), the nt −676 to −392 fragment was purified by polyacrylamide gel electrophoresis and reverse phase chromatography (Elutip-D, Schleicher & Schuell). The nt −453 to −189 promoter fragment labeled on the noncoding strand was prepared similarly by digesting plasmid H12-9 with *Cfr*10I (nt −453) and *Sty*I (nt −189). Probes covering the region between nt −276 and nt −36, labeled on the noncoding strand, were obtained similarly by digesting plasmid H12-9, and pGEM-7Zf(−) plasmids containing mutations ΔBox 1, ΔBox 2, ΔBox 3, and ΔBox 4, with *Ear*I (nt −276) and *Nhe*I (nt −36).

Nuclear extracts were preincubated for 10 min at room temperature in a 50-μl volume containing 1 μg of poly(dI-dC)·poly(dI-dC), 10 mM Hepes, pH 7.9, 10% glycerol, 50 mM NaCl, 2% polyvinyl alcohol, 0.1 mM EDTA, 0.25 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 0.05 mM benzamidine, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin. Preliminary experiments established the optimal amount of DNase I (Stratagene, La Jolla, CA) to use in reactions without extract (0.003–0.0125 unit), with purified Sp1 (0.006 unit), and with BRL-3A extract (0.1- 0.3 unit). Probes (3–6 fmol, 15,000 cpm) were added and the reactions incubated on ice for 15 min. Tubes were placed at room temperature for 2 min and 50 μl of a 10 mM $MgCl_2$, 5 mM $CaCl_2$ solution was added followed by the addition of DNase I. After 2 min, the reaction was terminated by adding 100 μl of a stop solution (0.2 M NaCl, 20 mM EDTA, 1% sodium dodecyl sulfate, 250 μg/ml yeast tRNA), the reaction mixtures extracted sequentially with phenol:chloroform (1:1) and chloroform, and the DNA precipitated with ethanol. Samples were dissolved in formamide buffer and resolved on a 6% polyacrylamide, 7 M urea gel alongside Maxam-Gilbert sequencing reactions (19). Gels were dried and autoradiographed at −70 °C in cassettes with intensifying screens.

Gel Shift Assay—Complementary oligonucleotides corresponding to regions of the rat IGFBP-2 promoter, and oligonucleotides containing a consensus binding site for the transcription factor Sp1 or the *Bgl*II linker, were synthesized. Random sequences were used to flank the Sp1 site and the *Bgl*II linker. The oligonucleotides were purified by polyacrylamide gel electrophoresis, and equimolar quantities of complementary strands were annealed in buffer (10 mM Tris, pH 8.0, 1 mM EDTA, 10 mM NaCl) by heating at 100 °C for 5 min and cooling to room temperature. The oligonucleotide pairs were designed to provide a 5′-recessed end for labeling with [α-^{32}P]dATP (3000 Ci/mmol) using the Klenow fragment of DNA polymerase I. Sequences of the oligonucleotides (with the coordinates of the 5′ and 3′ nucleotides of the IGFBP-2 promoter coding strand (17)) are written below. The GC box within each oligonucleotide of the rat IGFBP-2 promoter as well as the consensus recognition sites for Sp1 and the *Bgl*II linker are underlined.

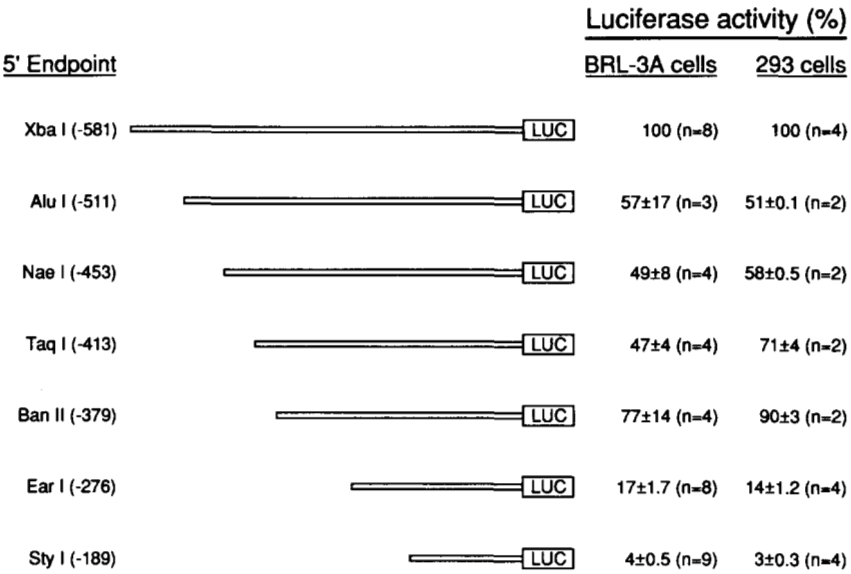

FIG. 1. **Promoter activity of rat IGFBP-2 promoter deletion constructs.** Restriction enzymes used to generate the 5′ ends of the deletion constructs are shown at the *left*. Restriction sites are shown in *parentheses*, numbered relative to ATG +1. The 3′ end of each promoter fragment is the *Nhe*I restriction site at nt −36. Deletion constructs are shown schematically by *open bars* and the promoterless luciferase reporter gene (LUC) by *open boxes*. Reporter plasmids (30 μg) and plasmid pTKGH (50 μg) were cotransfected into BRL-3A cells by electroporation; 293 cells were cotransfected with 10 μg of reporter plasmid and 10 μg of pTKGH by calcium phosphate precipitation. After 48 h, the media were collected and the cells lysed. Luciferase activity in the lysates was measured immediately by luminometry and growth hormone in the medium determined by radioimmunoassay. Transfections were performed at least in duplicate within each experiment. Luciferase activity (normalized for growth hormone content) is expressed as the percent of activity in the nt −581 to nt −36 construct assayed in the same experiment. The mean ± S.E. of n separate experiments is shown on the right. The construct with its 5′ end at nt −189 exhibited >21-fold and 26-fold greater activity than the promoterless pA3LUC plasmid in BRL-3A cells and 293 cells, respectively.[3]

Box 1 −147/−120	5′AAGGCAGGGGGGGCGGGGAGAAGCAGGC3′
Box 2 −171/−144	5′TGGTCTCCAAAAGGGGGAGGGGAGAAGG3′
Box 3 −187/−160	5′TTGGGGGGGAAGGGAGTGGTCTCCAAAA3′
Box 4 −237/−210	5′GGTGGGCAAGTGGGCGTGTGCGCAAGCA3′
Sp1	5′CGATCGATCGGGGCGGGGCGATCGATCA3′
*Bgl*II	5′GATCGATCCTAGATCTATGATC3′

Oligonucleotides 1–6

BRL-3A nuclear extracts were preincubated for 10 min in a buffer containing 6 μg of poly(dA-dT)·poly(dA-dT), 12.5 mM Hepes, pH 7.5, 10% glycerol, 50 mM KCl, 6.25 mM $MgCl_2$, 5 μM $ZnSO_4$, 0.05% Nonidet P-40. Probes (4–9 fmol, 20,000 cpm) were then added and the incubation continued on ice for 15 min. In competition studies, unlabeled double-stranded oligonucleotides that had been made blunt-ended using the Klenow fragment of DNA polymerase I, were added immediately before the probe. Reactions (15 μl) were then loaded on a 5% nondenaturing polyacrylamide gel (38:1, acrylamide:bisacrylamide) that had been pre-run at 100 V for 1 h at 4 °C. The gel and electrophoresis buffer contained 0.5 × TBE (45 mM Tris borate, 1 mM EDTA, pH 8.3) and 0.05% Nonidet P-40. Electrophoresis was carried out at 15 mA for 2 h. Gels were dried and autoradiographed at −70 °C using intensifying screens.

In studies designed to immunologically identify the protein component of the protein-DNA complexes, antibodies were added at the same time as the nuclear extract and allowed to react for 15 min at room temperature in a final volume of 20 μl. Bovine serum albumin was added to bring the final protein concentration (including antibody) to 10.25 μg/tube. As the antibodies were provided in different vehicles, buffer composition was equalized by addition of 10 mM Tris, pH 8.0, 14 mM NaCl and 25 mM sodium phosphate, pH 7.5 (final concentrations).

RESULTS

Identification of Functional Regions in the Promoter of the Rat IGFBP-2 Gene—To map the regions that confer basal activity on the rat IGFBP-2 promoter, we constructed a series of promoter deletion mutants having variable 5′ ends (from nt −581 to nt −189) and a common 3′ end at nt −36 (relative to the translation initiation site at nt +1) (Fig. 1). The promoter fragments were inserted into the promoterless reporter plasmid pA3LUC in the sense orientation and transfected by electroporation into BRL-3A cells. These constructs contained all of the transcription start sites described previously (17, 23).[2]

Removal of the regions located between nt −581 and nt −511 decreased promoter activity in BRL-3A cells by 43% (Fig. 1). The activity of the promoter remained unchanged by progressive deletions until nt −413, but increased by 30% when the deletion was extended to nt −379. Deletion of the sequence between nt −379 and nt −276 reduced the activity by 4.5-fold relative to the construct with a 5′ end at nt −379. A further 4.3-fold drop in activity was obtained after removing sequences extending from nt −276 to −189. The shortest promoter fragment tested (5′ end at nt −189), although having only 4% of the activity of the construct with its 5′ end at nt −581, exhibited >21-fold greater activity than the promoterless pA3LUC plasmid (Fig. 1).[3] Thus, deletion mapping suggests the existence of at least three broad regions that stimulate promoter activity (nt −581 to −511, nt −379 to −276, and nt −276 to −189), and one region that inhibits promoter activity (nt −413 to −379).

The deletion constructs also were transfected into the human embryonic kidney cell line 293, known to express IGFBP-2 (24). The same three regions with positive promoter activity in BRL-3A cells and a region with inhibitory activity were identified in 293 cell transfections. The 5′ end of the region displaying inhibitory activity appeared to extend further upstream than in BRL-3A cells, to nt −453. Luciferase activity using the shortest promoter fragment (nt −189 to −36), although only ~3% that of the nt −581 to −36 construct, was 26 ± 6 (S.E., n = 4) times greater than that of the promoterless construct.[3] These results indicate that the functional importance of these regu-

[3] The -fold increase in promoter activity compared with the corresponding promoterless construct could not be evaluated precisely in BRL-3A cells because activity in the promoterless construct was not significantly different from background activity. Taking the minimum significant increase in activity that we could have detected ($p < 0.05$, one-tailed t test) as the activity in the promoterless construct, we calculated the activity of the nt −189 to nt −36 construct as >21-fold that of the promoterless construct. In 293 cells, luciferase activity of the promoterless construct was significantly different from background activity. After subtraction of background activity, the -fold increase in promoter activity was calculated as 26 ± 6-fold (S.E.).

latory sequences is conserved in both cell lines.

BRL-3A Nuclear Extract Protects Sequences in the Distal Regions of the Rat IGFBP-2 Promoter—DNase I footprinting analysis was used to identify the specific sites in the distal rat IGFBP-2 promoter shown by deletion analysis to be functionally important (Fig. 2). Using a nt −676 to −392 promoter fragment labeled on the coding strand as probe, 20 or 40 μg of BRL-3A nuclear extract strongly protected the region between nt −552 and −534 from DNase I digestion (Fig. 2, *lanes 1–4*). This protected site might account for the decrease in promoter activity that was observed when the region between nt −581 and −511 was deleted.

Using a nt −453 to nt −189 promoter fragment labeled on the noncoding strand, two regions were protected from DNase I digestion by incubation with BRL-3A nuclear extract (Fig. 2, *lanes 5–8*). The most 5′ footprint extended from nt −347 to nt −323 and contained a strong DNase I hypersensitive site at nt −332. The protected sequence is contained within the nt −379 to nt −276 region of the rat IGFBP-2 promoter whose deletion is responsible for a 4.5-fold drop in activity in transfection experiments (Fig. 1). The second region within this fragment that was protected by BRL-3A extract was located between nt −276 and nt −189, a region whose deletion led to an additional 4.3-fold decrease in promoter activity (Fig. 1). The nucleotide coordinates of this protected region were mapped in experiments to be described below.

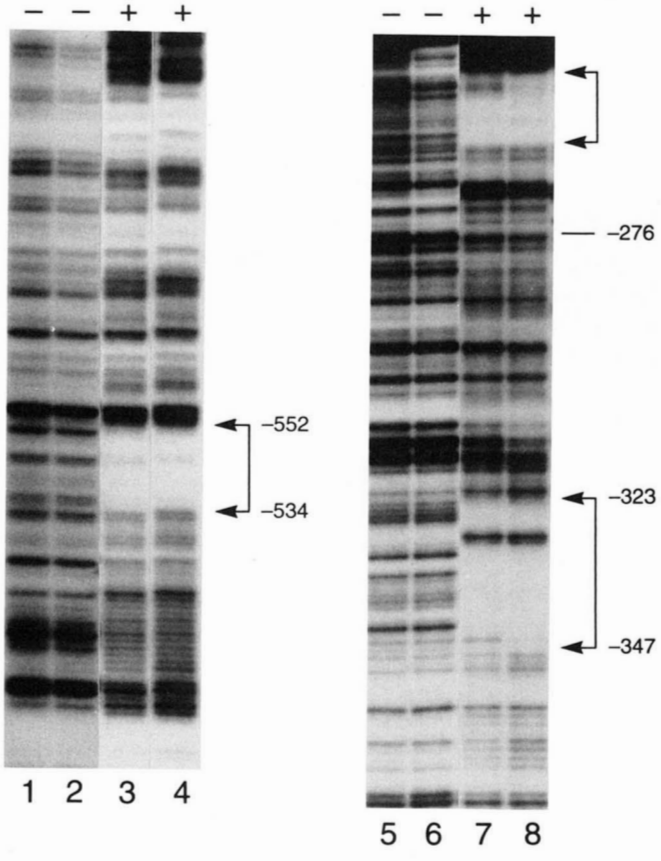

FIG. 2. **DNase I protection assay of the distal regions of the rat IGFBP-2 promoter.** Promoter fragment nt −676 to −392 labeled on the coding strand (*lanes 1–4*), and fragment nt −453 to −189 labeled on the noncoding strand (*lanes 5–8*), were digested with DNase I in the absence (*lanes 1, 2, 5,* and *6*) or presence of 20 μg (*lanes 3* and *7*) or 40 μg (*lanes 4* and *8*) of BRL-3A nuclear extract. The sites protected from DNase I digestion are bracketed; their positions are given relative to the translation initiation site. The nucleotide sequence protected between nt −552 and −534 is 5′ACTCAAGGTCATTGTTCCT3′ (coding strand); the sequence protected between nt −347 and −323 is 5′GAGAGTAAGAATAACTTGCCAGCGT3′ (noncoding strand). A DNase I hypersensitive site is visible at nt −332 in *lanes 7* and *8*. The nucleotide coordinates of the protected region located upstream of nt −276 were mapped in Fig. 3.

BRL-3A Nuclear Extract Protects a Cluster of GC Boxes Near the Transcription Start Sites of the Rat IGFBP-2 Promoter—The preceding results indicate that multiple sequences in the distal 5′-flanking region (nt −581 to −189) increase IGFBP-2 transcription ~ 25-fold and that sequences downstream from nt −189 increase IGFBP-2 promoter activity at least 21-fold compared to the promoterless pA3LUC construct. As the IGFBP-2 promoter lacks a TATA box to direct the efficient initiation of transcription, we have devoted the remainder of this study to defining the proximal elements that are required for basal promoter activity.

The rat IGFBP-2 promoter is GC-rich in the region of transcription initiation, providing many potential binding sites for nuclear proteins that recognize GC-rich sequences. To determine which of these sites are recognized by nuclear proteins in BRL-3A cells, we incubated a nt −276 to −36 promoter fragment labeled on the noncoding strand with 10 μg of nuclear extract. Two regions of the fragment were strongly protected from DNase I digestion by the extract, one extending from nt −189 to −125 and a second located between nt −234 and −215 (Fig. 3, *lane 1 versus 3*). This latter footprint most likely corresponds to the poorly resolved protected region seen between nt −276 and −189 in Fig. 2.

The sequences protected between nt −189 and −125 and between nt −234 and −215 include three GC boxes and one GC box, respectively, and resemble sequences that bind the transcription factor Sp1 (25–27). Incubation of the nt −276 to −36 fragment with 30 ng of purified human Sp1 protected both the nt −189 to −125 and the nt −234 to −215 regions from DNase I digestion, reproducing almost exactly the pattern of protection observed with BRL-3A extract (Fig. 3, *lane 2 versus 3*).

The size of the broad footprint covering nt −189 to −125 suggested that it might contain three tandem Sp1 binding sites, since a single Sp1 molecule typically protects 18–20 nt (25). To determine the exact number of binding sites in this region, we created independent block mutations of the potential Sp1 binding sites by replacing nt −139 to −130 (ΔBox 1), nt −163 to −154 (ΔBox 2), and nt −183 to −174 (ΔBox 3) with a 10-nt *Bgl*II linker.[4] A similar block mutation, ΔBox 4, was created at nt −226 to −217. DNA fragments (nt −276 to −36) containing these individual block mutations or the native sequence were compared in DNase I protection assays. The fragment containing the ΔBox 2 mutations no longer bound Sp1 and was not protected by either BRL-3A extract or Sp1 over the internal region nt −163 to nt −146 (Fig. 3, *lanes 7–12*). Binding to the 5′ and 3′ sites was not affected by the ΔBox 2 mutations, demonstrating that the nt −189 to −125 footprint is composed of three distinct Sp1 binding sites. Similarly, block mutations in Box 1 and Box 4 abolished protection (over nt −143 to −125 and nt −234 to −215, respectively) by purified Sp1 or BRL-3A extract (Fig. 3, *lanes 1–6* and *lanes 19–24*). Although protection over Box 3 (nt −189 to −170) was lost when the corresponding block mutant was incubated with purified Sp1, this region was still partially protected following incubation with BRL-3A nuclear extract (Fig. 3, *lanes 13–18*).

These results indicate that purified Sp1 binds to four sites between nt −234 and −125. Binding to each site appears to be

[4] The *Bgl*II mutations were centered over GC boxes 1 and 4, but not over Box 3, because sequence analysis suggested two potential overlapping sites (nt −184 to −173 and nt −177 to −168). Therefore, we replaced a region common to both of these potential sites with the *Bgl*II linker. The *Bgl*II substitution was targeted to the 5′ end of Box 2 so as not to change sequences in the immediate vicinity of nt −151, one of the transcription start sites described previously (17).[2]

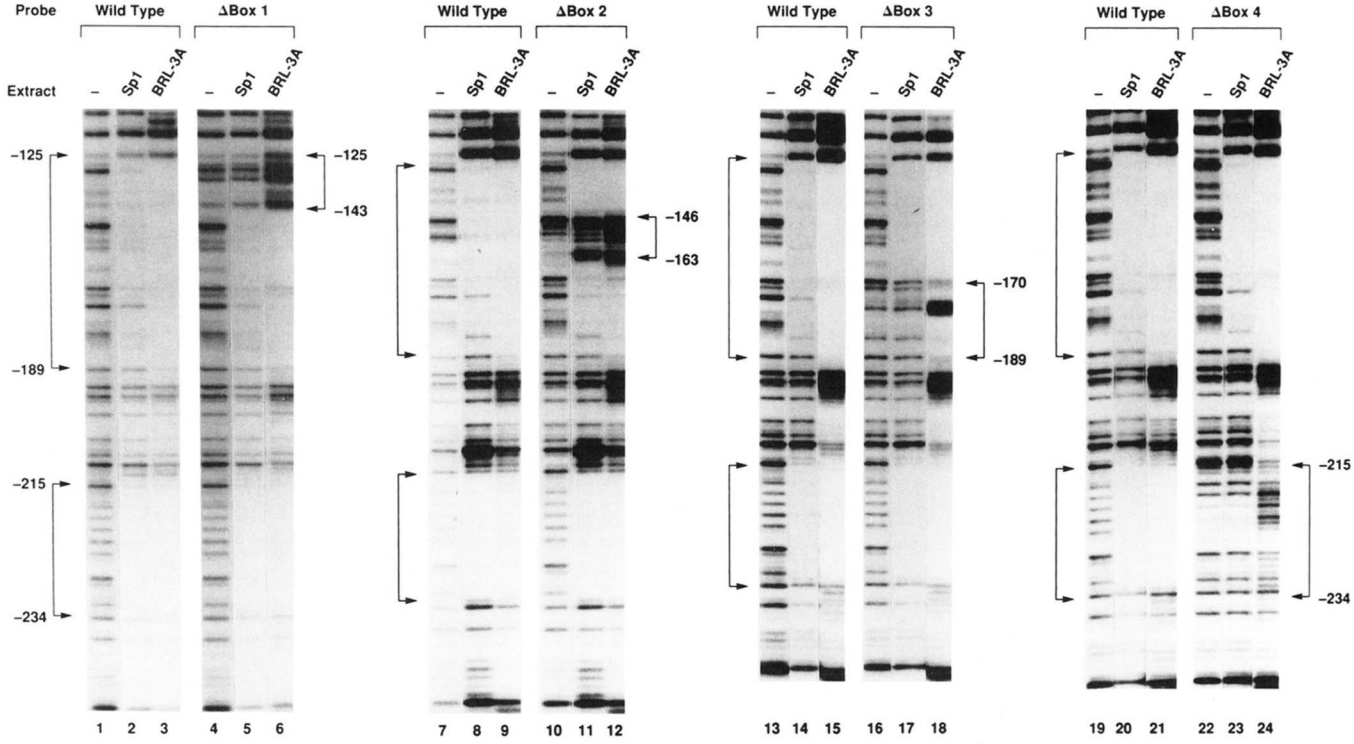

FIG. 3. **DNase I footprinting analysis of the proximal region of the rat IGFBP-2 promoter.** DNA fragments corresponding to nt −276 to −36 of the wild-type rat IGFBP-2 promoter or the same region containing *Bgl*II block mutations in one of the four GC boxes (ΔBox 1, ΔBox 2, ΔBox 3, and ΔBox 4) were labeled on the noncoding strand and digested with DNase I in the absence (–) or in the presence of purified Sp1 (30 ng) or BRL-3A nuclear extract (10 μg). Digestion of the wild-type fragment analyzed on the same gel as each mutant fragment is shown in each panel; the sites protected (nt −189 to −125 and nt −234 to −215) are *bracketed* on the *left* side. The region whose protection was eliminated by the individual block mutation is *bracketed* on the *right* side of each panel and its nucleotide boundaries given. Sp1 did not protect the region closest to nt −189 nearly as well as BRL-3A extract even when added at double concentration (results not shown).

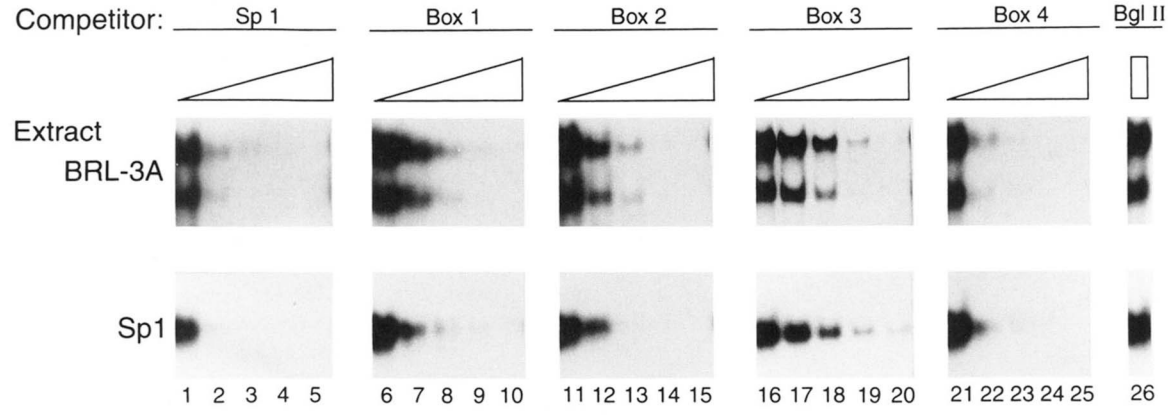

FIG. 4. **Gel shift analysis showing the relative ability of the GC boxes from the rat IGFBP-2 promoter to competitively inhibit binding of an oligonucleotide probe containing a consensus Sp1 binding site.** Labeled oligonucleotide probe (20,000 cpm) was incubated with either 1 μg of BRL-3A extract (*top panel*) or 1 ng of purified Sp1 (*bottom panel*) in the presence of increasing molar excess (0, 30, 100, 300, 1000 ×) of oligonucleotides corresponding to the Sp1 consensus sequence (*lanes 1–5*) or to sequences of the rat IGFBP-2 promoter containing GC box 1 (*Box 1, lanes 6–10*), GC box 2 (*Box 2, lanes 11–15*), GC box 3 (*Box 3, lanes 16–20*), or GC box 4 (*Box 4, lanes 21–25*). Box 1, Box 2, Box 3, and Box 4 oligonucleotides correspond to nt −147 to −120, nt −171 to −144, nt −187 to −160, and nt −237 to −210 of the rat IGFBP-2 promoter, respectively. Only the relevant portion of the autoradiograms is shown. The Sp1 probe formed two specific protein-DNA complexes with BRL-3A extract and one complex with purified Sp1 (see also Fig. 6). The autoradiograms used to construct the composite figure were exposed for 6 h. The specificity of complex formation is demonstrated by the inability of the *Bgl*II linker oligonucleotide to competitively inhibit binding (1000-fold molar excess, *lane 26*).

independent, since it was not affected by single block mutations in any of the other sites. BRL-3A nuclear proteins bind indistinguishably from Sp1 to Boxes 1, 2, and 4. The partial protection by BRL-3A extract with the ΔBox 3 mutant may indicate that other nuclear proteins bind to adjacent sequences when Sp1 is unable to bind to Box 3 or that insertion of a *Bgl*II linker creates an unexpected binding site in this region.

The Trans-acting Factor Binding the Cluster of GC Boxes in BRL-3A Extract Is Sp1 or an Sp1-like Protein—Gel shift assays were used to compare the binding specificity of Sp1, and of the nuclear factors in BRL-3A extract, to each of the four GC boxes. BRL-3A extract formed two protein-DNA complexes with a radiolabeled double-stranded oligonucleotide containing a 10 base pairs consensus binding site (26, 27) for the transcription factor Sp1 (Fig. 4, *top*). Oligonucleotide probes corresponding to the four GC boxes also formed two complexes with BRL-3A extract having the same mobility as those observed using the Sp1 probe; no significant binding was observed to other pro-

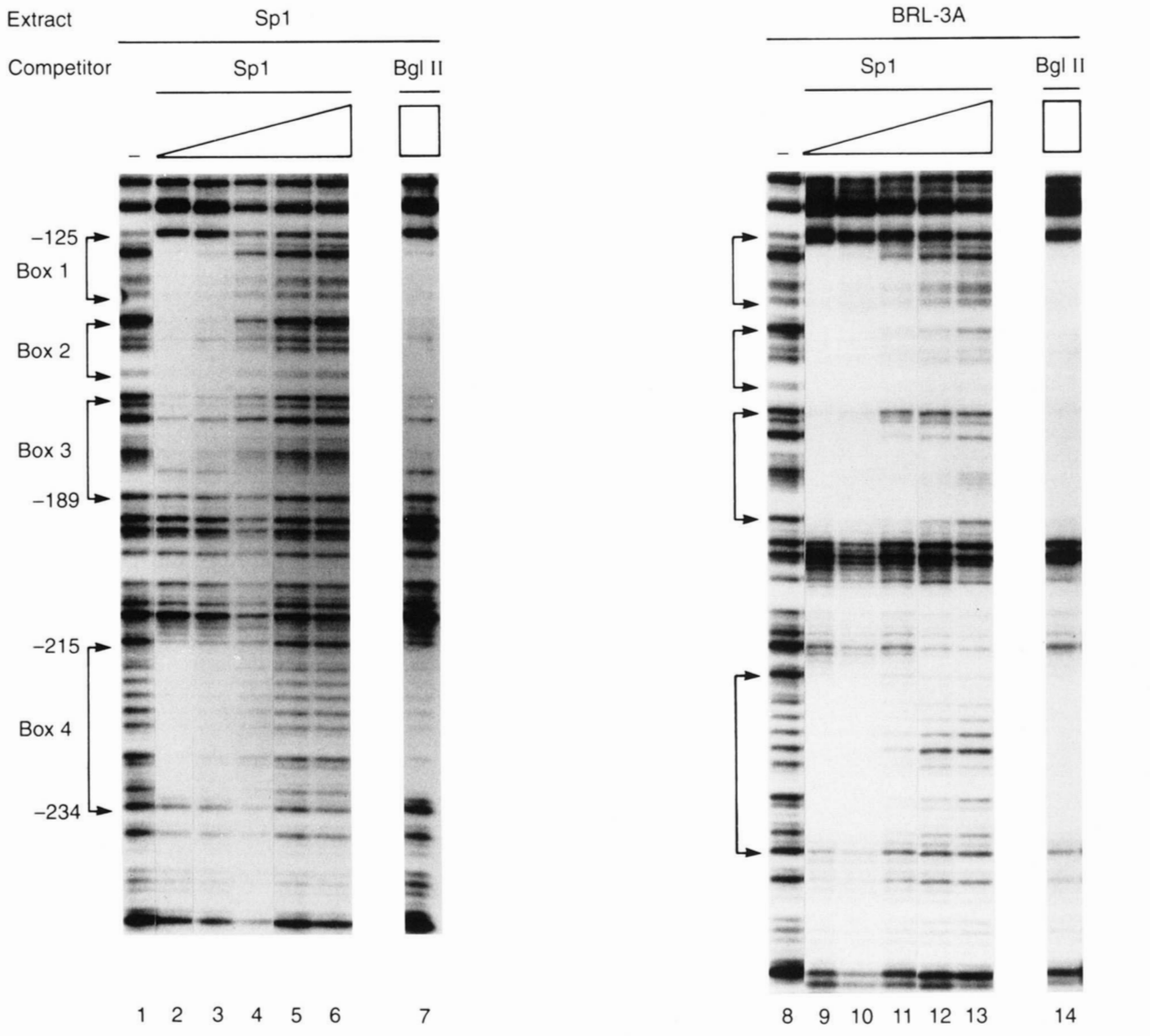

FIG. 5. **Sp1 oligonucleotide inhibits protection of GC boxes 1–4 of the rat IGFBP-2 promoter by Sp1 and BRL-3A extract in DNase I footprinting assays.** Promoter fragment nt –276 to –36 was labeled on the noncoding strand. Increasing molar excess (0, 50, 200, 1000, 5000 ×) of the oligonucleotide corresponding to the Sp1 consensus sequence was added immediately before the probe to DNase I digestions performed in the presence of 30 ng of Sp1 (*lanes 2–6*) or 10 μg of BRL-3A extract (*lanes 9–13*). The pattern of digestion by DNase I obtained in the absence of DNA binding protein is shown in *lanes 1* and *8*. A 5000-fold excess of *Bgl*II linker oligonucleotide did not eliminate the protection observed with Sp1 (*lane 7*) or BRL-3A extract (*lane 14*). The boundaries of the two regions protected with Sp1 and BRL-3A extract containing Boxes 1, 2, and 3 (nt –189 to –125) and containing Box 4 (nt –234 to –215) are given. The region protected by each individual box is indicated.

teins in the BRL-3A extract (results not shown). Purified Sp1 formed a single complex (Fig. 4, *bottom*) that migrated similarly to that of the upper BRL-3A complex (*cf.* Fig. 6). The Sp1 oligonucleotide probe also formed two complexes with similar migration when incubated with nuclear extracts from 293 embryonic kidney cells (not shown).

Formation of two specific protein-DNA complexes also has been reported with other nuclear extracts and probes corresponding to GC boxes from various genes or consensus Sp1 sequence oligonucleotides (28–31). In some cases this has been attributed to differences in post-translational modification, such as the presence of dephosphorylated and phosphorylated forms of Sp1, with apparent molecular masses 95 and 105 kDa, respectively (32). It is unlikely that the two bands in our experiments represent Sp1 multimers, as we have not used a large excess of protein relative to the probe, the condition in which multimerization occurs (33), and the doublet was not seen with Sp1 itself. Alternatively, one of these bands might represent an Sp1-like protein rather than authentic Sp1 (Ref. 29 and see below).

Formation of complexes of BRL-3A nuclear proteins and Sp1 with the consensus Sp1 oligonucleotide probe was completely inhibited by incubation with 100-fold molar excess of unlabeled Sp1 oligonucleotide (Fig. 4, *lanes 1–5*), but not by 1000-fold excess of an oligonucleotide containing the *Bgl*II linker (Fig. 4, *lane 26*).[5] In addition, oligonucleotides corresponding to the regions of the rat IGFBP-2 promoter that contain GC boxes 1, 2, and 4 competitively inhibited the formation of the Sp1 and BRL-3A complexes, although they were slightly less potent than the Sp1 oligonucleotide. The GC box 3 oligonucleotide was a less effective competitor; 300-fold molar excess did not completely inhibit formation of the protein-DNA complexes ob-

[5] Complexes formed with oligonucleotide probes corresponding to the four GC boxes showed a similar specificity of inhibition (results not shown).

tained with BRL-3A extract and Sp1 (Fig. 4, *lanes 16–20*). These results suggested that GC Box 3 had a lower affinity for Sp1 and BRL-3A nuclear proteins than did Boxes 1, 2, and 4.

To determine whether this apparent difference in affinity also was observed in the context of the natural promoter, competitive DNase I footprinting assays were performed using the nt −276 to −36 promoter fragment labeled on the noncoding strand (Fig. 5). Protection of each of the four GC boxes by Sp1 and BRL-3A extract was decreased when Sp1 oligonucleotide was added at 200-fold excess or higher concentration. Binding of Sp1 and BRL-3A extract to Box 3 and the other three GC boxes was inhibited at the same concentrations of added oligonucleotide. These results indicate that in the natural promoter, the affinity of Box 3 for Sp1 and BRL-3A nuclear proteins is similar to that of the other three GC boxes.

The two major proteins in BRL-3A extract that bind the Sp1 oligonucleotide were identified immunologically by gel shift analysis performed in the presence of antibodies raised against human Sp1 (Fig. 6). Extract incubated with increasing concentrations of antibodies to Sp1 prior to incubation with the Sp1 oligonucleotide probe inhibited formation of the two Sp1-DNA complexes in dose-dependent fashion. In the reactions that received the Sp1 antibodies, some protein-DNA complexes did not enter the gel and presumably represent aggregated material (Fig. 6, *lanes 2–4*). In experiments using larger amounts of BRL-3A extract (not shown), a supershifted band (*i.e.* a complex with reduced mobility) also was detected above the more slowly migrating protein-DNA complex. Binding of antibodies to purified Sp1 gave both a supershift and some aggregated material that did not enter the gel (Fig. 6, *lane 9*). In contrast, antibodies directed against human fibronectin did not inhibit the formation of the protein-DNA complexes, nor did it lead to the formation of supershifted or higher order complexes with either BRL-3A extract or purified Sp1 (Fig. 6, *lane 5–7* and *lane 10*). These results suggest that the proteins in BRL-3A extract responsible for forming the two protein-DNA complexes with Sp1 oligonucleotide and oligonucleotides corresponding to the four GC boxes are immunologically related to human Sp1.

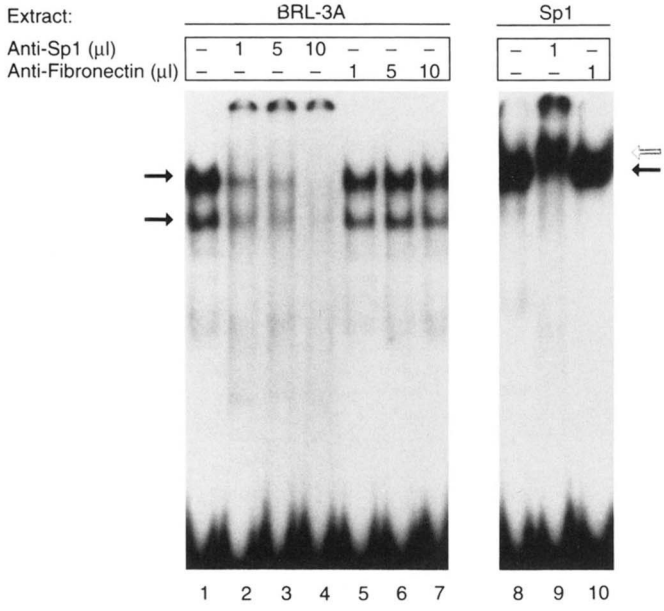

FIG. 6. **Immunologic identification of the BRL-3A nuclear proteins binding the Sp1 consensus recognition site.** Increasing volumes of rabbit antibodies (1 μg of IgG/μl) to the human transcription factor Sp1 (*lanes 2, 3, 4,* and *9*) or human fibronectin (*lanes 5, 6, 7,* and *10*) were incubated with 0.25 μg of BRL-3A extract (*lanes 1–7*) or 1 ng of purified Sp1 (*lanes 8–10*) for 15 min, after which a labeled Sp1 oligonucleotide (20,000 cpm) was added and gel shift analysis performed. The *solid arrows* on the *left* indicate the positions of the two specific protein-DNA complexes observed with BRL-3A extract. The supershifted (*lane 9*) and normally migrating (*lanes 8* and *10*) Sp1-DNA complexes are indicated on the *right* by the *open* and *closed arrows*, respectively. In the presence of Sp1 antibody, aggregated Sp1-DNA complexes were observed that did not enter the gel (*lanes 2, 3, 4,* and *9*). Both panels are from the same autoradiogram. In the absence of antibody, purified Sp1 formed a complex that migrated nearly identically with the upper complex observed with BRL-3A extract.

TABLE I

Sp1 stimulates rat IGFBP-2 promoter activity in Drosophila SL2 cells

Plasmids in which IGFBP-2 promoter fragments containing the first three GC boxes (*Sty*I-*Nhe*I, nt −189 to −36) or all four GC boxes (*Ear*I-*Nhe*I, nt −276 to −36) fused to a promotorless luciferase reporter gene were transfected into the *Drosophila* SL2 cell line together with an in-frame Sp1 expression vector (pP_{adh}Sp1-in (Sp1-in)), an out-of-frame Sp1 expression vector (pP_{adh}Sp1-out (Sp1-out)), or an unrelated vector (pGEM-7Zf(−). Plasmids (10 μg each) were cotransfected into SL2 cells by calcium phosphate precipitation. Cells were lysed and analyzed for luciferase activity 48 h later. Luciferase activity (arbitrary units) was normalized for the amount of protein in each cell lysate, and is shown for each of two experiments. Transfections were performed in duplicate within each experiment and the mean ± S.E. is shown. The ratio of luciferase activity obtained with cotransfection of the in-frame Sp1 expression vector to the activity obtained with cotransfection of the out-of-frame Sp1 expression vector (Sp1-in/Sp1-out) is calculated.

	Luciferase activity			Relative activity Sp1-in/Sp1-out
	Unrelated	Sp1-out	Sp1-in	
*Sty*I-*Nhe*I construct				
Experiment 1	NT[a]	38 ± 1	420 ± 124	10.8 ± 3.1
Experiment 2	19 ± 8	40 ± 1	271 ± 62	6.8 ± 1.6
*Ear*I-*Nhe*I construct				
Experiment 1	NT[a]	138 ± 8	769[b]	5.6 ± 0.4
Experiment 2	36 ± 11	77[c]	1395 ± 9	18.1 ± 0.1

[a] Not tested.

[b] Single sample. The relative activity of the duplicate sample was 24.5 ± 0.4-fold. Because of the large variation, only the sample with lower relative activity is tabulated.

[c] Single sample. The luciferase activity of the duplicate sample using the out-of-frame Sp1 expression vector was 264, considerably higher than the pGEM-7Zf(−) plasmid control. Even using this value for the out-of-frame Sp1 plasmid, the in-frame Sp1 expression vector would have stimulated luciferase activity 5.0 ± 0.1-fold.

Sp1 Can Trans-activate the Rat IGFBP-2 Promoter—To formally demonstrate that Sp1 can trans-activate the rat IGFBP-2 promoter, we performed transfection experiments in the *Drosophila* cell line SL2 (Table I). The factors comprising the basal transcription machinery are conserved between *Drosophila* and mammals, yet SL2 cells do not contain any endogenous Sp1 (20). Reporter plasmids containing rat IGFBP-2 promoter fragments with either three (nt −189 to −36) or four (nt −276 to −36) GC boxes were cotransfected with in-frame or out-of-frame Sp1 expression plasmids. With both promoter fragments, cotransfection with in-frame Sp1 increased luciferase activity 5–18-fold relative to the out-of-frame Sp1 construct. These results indicate that the proximal three GC boxes are sufficient for Sp1 to trans-activate the IGFBP-2 promoter in SL2 cells.

Full Activity of the IGFBP-2 Promoter Requires the Integrity of the Three Proximal GC Boxes—We next studied the functional consequence of independent block mutations in each of the four GC boxes (ΔBox 1, ΔBox 2, ΔBox 3, and ΔBox 4). Plasmids containing the mutations in the context of a reporter plasmid exhibiting full activity (nt −581 to −36) were transfected into BRL-3A or 293 cells (Fig. 7). Independent mutations in Box 1, 2, or 3 reduced promoter activity by ~ 80% in BRL-3A cells and by ~ 60% in 293 cells. The block mutation in Box 4 decreased promoter activity to a lesser extent than mutations in the other three GC boxes in BRL-3A cells (60%) and did not decrease activity in the 293 cell line (Fig. 7). Thus, the integrity of all four GC boxes is required for full activity of the rat IGFBP-2 promoter in BRL-3A cells, but the first three GC boxes alone are sufficient in the 293 cell line.

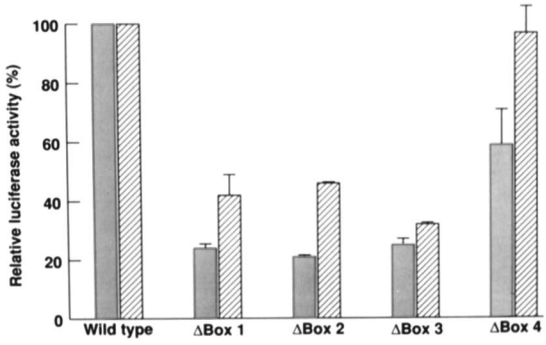

FIG. 7. **Activity of the rat IGFBP-2 promoter containing individual block mutations in the four GC boxes.** Plasmids containing either the nt −581 to −36 (*Xba*I-*Nhe*I) promoter fragment (wild type) or the same fragment harboring individual block mutations of the GC boxes (ΔBox1, -2, -3, -4) were cotransfected with plasmid pTKGH into BRL-3A cells (*solid bars*) and 293 cells (*hatched bars*). For BRL-3A transfection, *Xba*I-*Nhe*I and pTKGH plasmids were added at 30 and 50 μg, respectively; 10 μg of both plasmids were transfected into 293 cells. Each transfection was performed in duplicate within an experiment. For BRL-3A cells, the results represent the mean ± S.E. of five (ΔBox 1, -2, -3) or three (ΔBox 4) independent experiments. For 293 cells, the results are the mean ± S.E. of three (ΔBox 1, ΔBox 4) or 2 (ΔBox 2, ΔBox 3) experiments. Luciferase activity, normalized for growth hormone content, is expressed as a percentage of the activity of the wild-type construct transfected in the same experiment. In these experiments, the nt −189 to −36 construct had 5.0 ± 0.6% and 2.7 ± 0.2% luciferase activity of the nt −581 to −36 construct in BRL-3A and 293 cells, respectively.[6]

TABLE II

Comparison of the nucleotide sequences of the four rat IGFBP-2 GC boxes and the Sp1 consensus sequence

The position of the four GC boxes of the rat IGFBP-2 promoter are given relative to ATG, +1. The Sp1 consensus sequence is from Briggs *et al.* (27) and Kadonaga *et al.* (26). Alternative nucleotides in the consensus sequence are indicated at positions 1, 2, and 7–10. Nucleotides differing from the consensus are underlined and in bold.

	Nucleotide sequence	Position
Box 1	G G G G C G G G G **A**	−138 to −129
Box 2	G G G G **A** G G G G **A**	−158 to −149
Box 3	**A** G G G **A** G **T** G G **T**	−177 to −168
Box 4	**T** G G G **C** G T G **T** G	−227 to −218
Sp1 consensus	G G G G C G G G G C T A T A A T	

DISCUSSION

Regulation of IGFBP-2 gene transcription accounts at least in part for the temporal and metabolic regulation of IGFBP-2 mRNA abundance in rat liver (16). In the present study, we have undertaken a characterization of the functional regions of the IGFBP-2 promoter. By deletion mapping, three broad regions, nt −581 to −511, nt −379 to −276, and nt −276 to −189, were identified that contributed positively to promoter activity in BRL-3A cells. Overall, deletion of nt −581 to −189 reduced promoter activity by more than 95%. DNase I footprinting experiments identified sequences protected by BRL-3A nuclear extracts within these stimulatory regions. Deletion analysis also suggested the presence of negative cis-elements located between nt −413 and −379, although preliminary footprinting experiments have not revealed protected sequences in this region (results not shown). The same regions are important for promoter activity in the human embryonic kidney cell line 293.

Although elements upstream of nt -189 contribute significantly to IGFBP-2 promoter activity, the deletion construct with its 5′ end at nt −189 retains more than 21-fold greater promoter activity than the promoterless plasmid, suggesting that important cis-elements also are located downstream of nt −189. As the IGFBP-2 promoter lacks TATA and CCAAT boxes that commonly specify the start site and determine the efficiency of transcription (34), in this study we have focused on identifying the elements in the proximal region of the promoter that are required for the efficient activation of transcription. This region is GC-rich (17) and contains four GC boxes that are potential binding sites for the transcription factor Sp1. DNase I footprinting analysis of native and mutated nt −276 to −36 DNA fragments revealed that BRL-3A nuclear extract protects a broad region between nt −189 and −125 that contains three possible Sp1 binding sites, and a fourth region between nt −234 and −215 that contains a single Sp1 binding element. The same four sites were protected by purified Sp1. Transcription of many TATA-less promoters is activated by Sp1 (31, 35–38), suggesting that Sp1 may act at these proximal Sp1 binding sites to increase IGFBP-2 gene transcription.

Although GC-rich regions are recognized by many nuclear proteins other than Sp1 (39), we have demonstrated that the protein in BRL-3A extracts that binds to the four GC boxes between nt −234 and −125 is Sp1 or a closely related protein. In gel retardation assays, BRL-3A extract formed two specific protein-DNA complexes with an oligonucleotide probe containing the consensus Sp1 recognition sequence. The upper complex migrated similarly to the complex formed between the Sp1 oligonucleotide and purified human Sp1. Formation of both complexes was competitively inhibited by unlabeled oligonucleotides corresponding to the four GC boxes of the IGFBP-2 promoter and by a polyclonal antiserum raised against amino acid residues 520–538 of human Sp1 which are conserved in rat Sp1 (40). When used as probes in mobility shift assays, each of the four GC boxes formed the same two protein-DNA complexes as the Sp1 consensus oligonucleotide and gave no other major retarded band (results not shown). In addition, Sp1 trans-activated rat IGFBP-2 promoter fragments that contain three or four GC boxes when cotransfected into the *Drosophila* SL2 cell line that lacks endogenous Sp1. Four Sp1-like proteins (BTEB, Sp2, Sp3, and Sp4) recently have been cloned (40–42), some of which bind the Sp1 consensus sequence with comparable affinity to authentic Sp1, are indistinguishable from Sp1 in gel shift assays, or are homologous to Sp1 in the region against which the Sp1 antiserum was raised. It remains to be determined, therefore, whether Sp1 itself or a closely related protein activates the rat IGFBP-2 promoter in BRL-3A cells.

Despite the fact that the nucleotide sequences of the four GC boxes of the rat IGFBP-2 promoter differ somewhat from the Sp1 consensus recognition sequence (26, 27) (Table II), purified Sp1 or Sp1-like proteins in BRL-3A extract bind with similar specificity to these sites. In competitive gel shift experiments, the binding of labeled Sp1 consensus oligonucleotide to these proteins was inhibited with similar potency by unlabeled Sp1 consensus oligonucleotide and by unlabeled oligonucleotides containing Box 1, 2, or 4. In contrast, the Box 3 oligonucleotide appeared less potent as a competitor (Fig. 4) and as a probe (not shown). The apparent difference in potency of Box 3, however, appears to be a function of the relative position of the GC box within the oligonucleotide used in the gel shift assays and of the length of the flanking sequence, rather than a reflection of

[6] Promoter activity in constructs containing block mutations in Box 1, 2, or 3 are higher than the activity in a nt −189 to nt −36 deletion construct. This may reflect the fact that the block mutations were examined in a nt −581 to nt −36 promoter fragment, which contains at least two upstream regions that stimulate gene transcription. Unless these positive effects require interactions with Sp1 that are abolished in the block mutations in the individual boxes, the residual activity represents the net effect of decreased basal activity because of the block mutation and enhanced activity because of the two upstream regions.

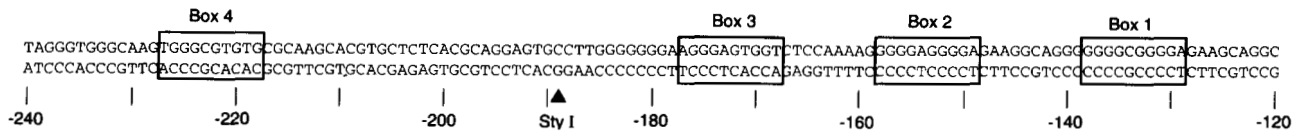

FIG. 8. **Nucleotide sequence of the proximal rat IGFBP-2 promoter.** The nucleotide sequence is taken from Brown and Rechler (17). The location of the four GC boxes is indicated. Initial assignment was based on sequences most closely matching the Sp1 binding site and verified by gel shift assays. The position of Box 3 was assigned after observing that the 10-base pair sequence corresponding to nt −177 to −168 (but not to nt −184 to −173 as predicted by Binkert *et al.* (23)) flanked by random sequence formed the expected protein-DNA complexes with Sp1 and BRL-3A extract. The position of the *Sty*I restriction site at nt −189 is indicated by a *closed triangle*.

the affinity of Box 3 in the context of the natural promoter.[7] Indeed, in DNase I footprinting assays, unlabeled oligonucleotide containing the consensus Sp1 sequence competitively inhibited the binding of purified Sp1 or BRL-3A extract to each of the four GC boxes contained in the labeled nt −276 to −36 DNA fragment at the same -fold excess.

The nucleotide sequence of the four GC boxes differs from that of the Sp1 consensus sequence (Table II) in positions previously shown to have little effect on Sp1 binding. Based on the crystal structure of the protein-DNA complex of the closely related zinc finger protein Zif268 (or EGR-1), Kriwacki *et al.* (43) proposed a model in which high affinity binding of Sp1 required zinc finger 3 to contact G's at positions 2 and 3, and zinc finger 2 to contact G's at positions 4 and 6. These four G's are conserved in each of the four GC boxes of the rat IGFBP-2 promoter (Table II). Binding of Sp1 to the GC boxes of the IGFBP-2 promoter was not affected by differences at positions 1 (Box 3), 9 (Box 4), and 10 (Boxes 1, 2, and 4). These results are consistent with the views that positions 1 and 10 in the Sp1 consensus sequence are least important for binding affinity (25) and that the six residues at the 5′ end of the Sp1 consensus sequence (GGGGCG) contribute more to total binding energy than the 4 residues at the 3′ end (GGGC) (43). Similarly, a C to A transversion at position 5 of Boxes 2 and 3 of the IGFBP-2 promoter had no major effect on binding affinity. Although the same central A transversion reduced the binding affinity 3-fold in oligonucleotide binding studies (44), sites containing this mutation in the context of several natural promoters (25, 35, 45, 46) were functional and bound Sp1 with high affinity.

Sp1 binding to the four GC boxes of the IGFBP-2 promoter appears to be independent and noncooperative. Block mutation in any one box does not affect binding to the other three boxes in DNase I footprinting assays (Fig. 3). As purified Sp1 and BRL-3A extract were used at only two to three times saturating concentrations in these experiments, the fact that the other sites are still fully protected implies that they bind Sp1-like proteins with unchanged affinity and that major positive cooperativity does not take place between the sites. In fact, cooperative binding of Sp1 to a cluster of GC boxes has never been demonstrated (33, 36, 47, 48).

The proximal three GC boxes in the IGFBP-2 promoter are separated by only 9 and 10 nucleotides (Fig. 8). Clustering of GC boxes in close proximity to the transcription start site is typical of TATA-less promoters (35–37, 49, 50), in contrast to TATA-containing promoters in which a single GC box is usually found adjacent to elements that bind other basal transcription factors (28, 29, 51–53). Constructs containing the three clustered GC boxes were sufficient to allow trans-activation of the IGFBP-2 promoter by Sp1 in SL2 cells. Independent block mutations in any of the three proximal GC boxes reduced promoter activity to the same extent in BRL-3A (~80%) and 293 (~60%) cells,[6] implying that each box is required and equally important for promoter function. This functional equivalence differs from the situation in another TATA-less promoter that has three clustered GC boxes, the rat transforming growth factor α promoter, in which the GC box closest to the transcription start site is functionally the most important (37).

In the TATA-containing promoter of the human argininosuccinate synthetase gene, the three clustered GC boxes are synergistically activated by Sp1; promoter activity was eight times greater in constructs with three functional sites than predicted from the separate activities of the sites (47). Synergistic interactions of the proximal three GC boxes in the IGFBP-2 gene was suggested by the fact that promoter activity in BRL-3A cells decreased disproportionately when a single member of the GC box cluster was inactivated.[8] Tjian and co-workers (33) have proposed that synergistic activation results from the association of Sp1 monomers by protein-protein interactions to form tetramers, with one monomer of each tetramer binding to a single GC box. Tetramers binding to adjacent GC boxes then can stack to form multimeric Sp1 complexes, creating a DNA loop juncture and producing a more effective activation surface for the transcription machinery (54, 55).

In natural TATA-less promoters, mutations in Sp1 binding sites located near transcription initiation sites decrease the extent of transcription initiation and alter the relative usage of alternative initiation sites (36–38), suggesting that Sp1 may be involved both in the activation of transcription and in positioning the initiation complex. Transcription initiation at TATA-less promoters utilizes the same general initiation factors as transcription in TATA-containing promoters, including participation of the TATA binding protein as part of the TFIID complex (56).[9] Based on studies with artificial promoters containing the six GC boxes of the SV40 early promoter fused to the initiator element of the adenovirus major late promoter, Tjian and co-workers (56, 57) proposed that Sp1 bound to a GC box would recruit the TFIID complex through protein-protein interactions with a heat-labile tethering factor that presumably is part of the TFIID complex. In this model, the TATA binding protein does not bind directly to the promoter but is anchored to the DNA by the tethering protein bound to Sp1. Alternatively, the TATA binding protein has been shown to bind with lower affinity to the nt −30 region of many TATA-less promoters (58) and may be stabilized by transcription factors such as Sp1. Whatever the mechanism by which Sp1 attracts the TFIID complex to the DNA, this interaction may not be sufficient to properly position the transcription initiation complex. In artificial promoters, the transcription start site appears to be determined by the initiator element rather than by Sp1 (59). Studies are in progress to evaluate the role of Sp1 in determining the specificity and usage of the transcription start sites in the IGFBP-2 promoter.

The fourth GC box of the IGFBP-2 promoter is less critical for promoter activity than the other three GC boxes, particularly

[7] Y. R. Boisclair, A. L. Brown, S. Casola, and M. M. Rechler, unpublished results.

[8] The luciferase activity of constructs in which GC boxes 1, 2, and 3 were intact was 6–9 times greater than the sum of the activities observed in constructs in which only a single GC box was intact and the other two boxes were inactivated by point mutations (see Footnote 7).

[9] In TATA-containing promoters and presumably also in TATA-less promoters, transcription activation by Sp1 also depends on the presence of a coactivator protein that is part of the TFIID complex (56, 57).

in 293 cells where its inactivation by mutation did not significantly alter expression of the luciferase reporter gene. Box 4, the most upstream site, is separated from Box 3 by 40 nt (Fig. 8), a spatial arrangement that suggests that its function may differ from that of the three clustered boxes. The presence of GC box 4 was not necessary for trans-activation of the IGFBP-2 promoter by Sp1 in *Drosophila* SL2 cells. Box 4 might contain a binding domain for a factor that is more abundant in 293 than in BRL-3A cells and competitively inhibits activation by Sp1. Antagonism between Sp1 and nuclear factors Zif268, NF-1, and the Sp1-like protein BTEB has been described (40, 51, 60).

In summary, we have demonstrated that multiple regions of the rat IGFBP-2 promoter are important for full activation. We have localized four functional GC boxes in the proximal promoter region and demonstrated that they are activated by Sp1 or Sp1-like proteins. The factors binding to elements in upstream regions of the promoter, not yet defined but required for full expression of the gene, might interact with Sp1-like proteins bound to proximal GC boxes (61, 62), thereby increasing the concentration of activator proteins near the transcription initiation machinery.

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REFERENCES

1. Rechler, M. M. (1993) *Vitam. Horm.* **47,** 1–114
2. Shimasaki, S., and Ling, N. (1991) *Prog. Growth Factor Res.* **3,** 243–266
3. Clemmons, D. R. (1992) *Growth Regul.* **2,** 80–87
4. Brown, A. L., Chiariotti, L., Orlowski, C. C., Mehlman, T., Burgess, W. H., Ackerman, E. J., Bruni, C. B., and Rechler, M. M. (1989) *J. Biol. Chem.* **264,** 5148–5154
5. Zapf, J., Schmid, C., Guler, H. P., Waldvogel, M., Hauri, C., Futo, E., Hossenlopp, P., Binoux, M., and Froesch, E. R. (1990) *J. Clin. Invest.* **86,** 952–961
6. Donovan, S. M., Oh, Y., Pham, H., and Rosenfeld, R. G. (1989) *Endocrinology* **125,** 2621–2627
7. Bourner, M. J., Busby, W. H., Jr., Siegel, N. R., Krivi, G. G., McCusker, R. H., and Clemmons, D. R. (1992) *J. Cell. Biochem.* **48,** 215–226
8. Hill, D. J., and Hogg, J. (1992) *Adv. Exp. Med. Biol.* **321,** 113–122
9. Feyen, J. H. M., Evans, D. B., Binkert, C., Heinrich, G. F., Geisse, S., and Kocher, H. P. (1991) *J. Biol. Chem.* **266,** 19469–19474
10. Wood, T. L., Streck, R. D., and Pintar, J. E. (1992) *Development (Camb.)* **114,** 59–66
11. Klempt, M., Hutchins, A.-M., Gluckman, P. D., and Skinner, S. J. M. (1992) *Development (Camb.)* **115,** 765–772
12. Lee, W.-H., Clemens, J. A., and Bondy, C. A. (1992) *Mol. Cell. Neurosci.* **3,** 36–43
13. Ooi, G. T., Orlowski, C. C., Brown, A. L., Becker, R. E., Unterman, T. G., and Rechler, M. M. (1990) *Mol. Endocrinol.* **4,** 321–328
14. Tseng, L. Y.-H., Brown, A. L., Yang, Y. W.-H., Romanus, J. A., Orlowski, C. C., Taylor, T., and Rechler, M. M. (1989) *Mol. Endocrinol.* **3,** 1559–1568
15. Orlowski, C. C., Brown, A. L., Ooi, G. T., Yang, Y. W.-H., Tseng, L. Y.-H., and Rechler, M. M. (1990) *Endocrinology* **126,** 644–652
16. Tseng, L. Y.-H., Ooi, G. T., Brown, A. L., Straus, D. S., and Rechler, M. M. (1992) *Mol. Endocrinol.* **6,** 1195–1201
17. Brown, A. L., and Rechler, M. M. (1990) *Mol. Endocrinol.* **4,** 2039–2051
18. Jackson, S. P., and Tjian, R. (1988) *Cell* **55,** 125–133
19. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
20. Courey, A. J., and Tjian, R. (1988) *Cell* **55,** 887–898
21. Graham, F. L., Smiley, J., Russell, W. C., and Nairn, R. (1977) *J. Gen. Virol.* **36,** 59–72
22. Hennighausen, L., and Lubon, H. (1987) in *Methods in Enzymology. Guide to Molecular Cloning Techniques* (Berger, S. L., and Kimmel, A. R., eds) pp. 721–735, Academic Press, San Diego
23. Binkert, C., Margot, J. B., Landwehr, J., Heinrich, G., and Schwander, J. (1992) *Mol. Endocrinol.* **6,** 826–836
24. Binkert, C., Landwehr, J., Mary, J.-L., Schwander, J., and Heinrich, G. (1989) *EMBO J.* **8,** 2497–2502
25. Jones, K. A., Kadonaga, J. T., Luciw, P. A., and Tjian, R. (1986) *Science* **232,** 755–759
26. Kadonaga, J. T., Jones, K. A., and Tjian, R. (1986) *Trends Biochem. Sci.* **11,** 20–23
27. Briggs, M. R., Kadonaga, J. T., Bell, S. P., and Tjian, R. (1986) *Science* **234,** 47–52
28. Robidoux, S., Gosselin, P., Harvey, M., Leclerc, S., and Guérin, S. L. (1992) *Mol. Cell. Biol.* **12,** 3796–3806
29. Descheemaeker, K. A., Wyns, S., Nelles, L., Auwerx, J., Ny, T., and Collen, D. (1992) *J. Biol. Chem.* **267,** 15086–15091
30. Johnson, M. R., Bruzdzinski, C. J., Winograd, S. S., and Gelehrter, T. D. (1992) *J. Biol. Chem.* **267,** 12202–12210
31. Ma, L., Weeda, G., Jochemsen, A. G., Bootsma, D., Hoeijmakers, J. H. J., and van der Eb, A. J. (1992) *Nucleic Acids Res.* **20,** 217–224
32. Jackson, S. P., MacDonald, J. J., Lees-Miller, S., and Tjian, R. (1990) *Cell* **63,** 155–165
33. Pascal, E., and Tjian, R. (1991) *Genes & Dev.* **5,** 1646–1656
34. Mitchell, P. J., and Tjian, R. (1989) *Science* **245,** 371–378
35. Lee, J.-K., Tam, J. W. O., Tsai, M.-J., and Tsai, S. Y. (1992) *J. Biol. Chem.* **267,** 4638–4645
36. Blake, M. C., Jambou, R. C., Swick, A. G., Kahn, J. W., and Azizkhan, J. C. (1990) *Mol. Cell. Biol.* **10,** 6632–6641
37. Chen, X., Azizkhan, J. C., and Lee, D. C. (1992) *Oncogene* **7,** 1805–1815
38. Jolliff, K., Li, Y., and Johnson, L. F. (1991) *Nucleic Acids Res.* **19,** 2267–2274
39. Faisst, S., and Meyer, S. (1992) *Nucleic Acids Res.* **20,** 3–26
40. Imataka, H., Sogawa, K., Yasumoto, K., Kikuchi, Y., Sasano, K., Kobayashi, A., Hayami, M., and Fujii-Kuriyama, Y. (1992) *EMBO J.* **11,** 3663–3671
41. Hagen, G., Müller, S., Beato, M., and Suske, G. (1992) *Nucleic Acids Res.* **20,** 5519–5525
42. Kingsley, C., and Winoto, A. (1992) *Mol. Cell. Biol.* **12,** 4251–4261
43. Kriwacki, R. W., Schultz, S. C., Steitz, T. A., and Caradonna, J. P. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 9759–9763
44. Letovsky, J., and Dynan, W. S. (1989) *Nucleic Acids Res.* **17,** 2639–2653
45. Yu, C.-Y., Motamed, K., Chen, J., Bailey, A. D., and Shen, C.-K. J. (1991) *J. Biol. Chem.* **266,** 8907–8915
46. Dawson, P. A., Hofmann, S. L., van der Westhuyzen, D. R., Südhof, T. C., Brown, M. S., and Goldstein, J. L. (1988) *J. Biol. Chem.* **263,** 3372–3379
47. Anderson, G. M., and Freytag, S. O. (1991) *Mol. Cell. Biol.* **11,** 1935–1943
48. Barrera-Saldana, H., Takahashi, K., Vigneron, M., Wildeman, A., Davidson, I., and Chambon, P. (1985) *EMBO J.* **4,** 3839–3849
49. Zong, J., Ashraf, J., and Thompson, E. B. (1990) *Mol. Cell. Biol.* **10,** 5580–5585
50. Farnham, P. J., and Kollmar, R. (1990) *Cell Growth & Diff.* **1,** 179–189
51. Nehls, M. C., Grapilon, M. L., and Brenner, D. A. (1992) *DNA Cell Biol.* **11,** 443–452
52. Sakai, T., Ohtani, N., McGee, T. L., Robbins, P. D., and Dryja, T. P. (1991) *Nature* **353,** 83–86
53. Sartorelli, V., Webster, K. A., and Kedes, L. (1990) *Genes & Dev.* **4,** 1811–1822
54. Mastrangelo, I. A., Courey, A. J., Wall, J. S., Jackson, S. P., and Hough, P. V. C. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 5670–5674
55. Su, W., Jackson, S., Tjian, R., and Echols, H. (1991) *Genes & Dev.* **5,** 820–826
56. Pugh, B. F., and Tjian, R. (1990) *Cell* **61,** 1187–1197
57. Pugh, B. F., and Tjian, R. (1991) *Genes & Dev.* **5,** 1935–1945
58. Wiley, S. R., Kraus, R. J., and Mertz, J. E. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 5814–5818
59. O'Shea-Greenfield, A., and Smale, S. T. (1992) *J. Biol. Chem.* **267,** 1391–1402
60. Ackerman, S. L., Minden, A. G., Williams, G. T., Bobonis, C., and Yeung, C.-Y. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 7523–7527
61. Li, R., Knight, J. D., Jackson, S. P., Tjian, R., and Botchan, M. R. (1991) *Cell* **65,** 493–505
62. Sun, J.-M., Penner, C. G., and Davie, J. R. (1992) *Nucleic Acids Res.* **20,** 6385–6392