THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 270, No. 42, Issue of October 20, pp. 24844–24851, 1995

Printed in U.S.A.

Transcription Factor AP-2 Regulates Human Insulin-like Growth Factor Binding Protein-5 Gene Expression*

(Received for publication, May 16, 1995, and in revised form, August 21, 1995)

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Insulin-like growth factor binding protein-5 (IGFBP-5) is an important modulator of IGF actions. IGFBP-5 mRNA is abundant in human fibroblasts and is regulated by cAMP. To understand the molecular mechanism underlying this cell type-specific expression and regulation, we isolated the 5′-flanking region of the human IGFBP-5 gene and fused it to a promoter-less reporter plasmid encoding luciferase. Transient transfection of the construct into fibroblasts displayed both constitutive and cAMP-induced promoter activity in an orientation-specific manner. Sequence analysis revealed the existence of distal and proximal consensus AP-2 recognition sites located 5′ from the TATA box. Both sequences bound specifically to human AP-2 *in vitro* by gel shift mobility assay. The possible role of AP-2 was examined by cotransfection of AP-2-deficient HepG2 cells with the IGFBP-5 promoter construct and a human AP-2 expression construct. Cotransfection with AP-2 significantly elevated IGFBP-5 promoter activity. This *trans*-activation was IGFBP-5 promoter and AP-2 specific. In AP-2 abundant fibroblasts, expression of AP-2B, a dominant-negative inhibitor of AP-2, suppressed IGFBP-5 promoter activity. In HepG2 cells, AP-2B alone had no significant effect, but the AP-2-induced activation of promoter activity was inhibited by AP-2B in a dose-dependent manner. The relative functional importance of the putative AP-2 binding sites was examined using a number of deletion mutants and point mutations. When the first two distal CCCCACCC-like putative AP-2 sites were deleted or mutated, there was no change in AP-2-induced *trans*-activation. Deletion or mutation of the proximal GCCNNNGGC-like sequences, however, abolished the AP-2-induced activation. These results suggest that AP-2 regulates the IGFBP-5 gene expression through the proximal GCCNNNGGC-like sequences. This AP-2-mediated *trans*-activation contributes at least in part to the constitutively high expression of IGFBP-5 in fibroblasts and to the cAMP responsiveness of this gene.

Insulin-like growth factor I and II (IGF-I and IGF-II)[1] are multifunctional growth factors required for normal development and growth in vertebrate animals. IGF-I mediates many of the growth-promoting effects of growth hormone during postnatal life (1) and both IGF-I and -II have been shown to be important for fetal growth in gene targeting experiments (2).

In extracellular fluids the IGFs are bound to one of the members of a family of soluble, high affinity binding proteins (3). These IGF binding proteins (IGFBPs) act as carrier proteins in plasma to control the efflux of IGFs from the vascular space and prolong their half-lives. More importantly, since they bind to IGFs with higher affinities than the IGF receptors, IGFBPs provide a means of localizing IGFs on target cells. Furthermore, they can alter the biological activity of IGFs by modulating their interaction with IGF receptors (1). Six distinct IGFBPs, designated as IGFBP-1 to 6, have been isolated and cloned and each represents an individual gene product. They share relatively high amino acid sequence similarity but each has distinct structural and biochemical properties and each is subject to differential tissue-specific expression, developmental and hormonal regulation (4). This suggests that each IGFBP may play a specific role in regulating the biological actions of IGFs in defined tissues.

IGFBP-5 is the most conserved of the six known IGFBPs. This 252-amino acid protein is more than 97% identical among human, rat, and mouse (5). IGFBP-5 has the unique property of associating with cell surfaces and adhering to extracellular matrix (1). When associated with the extracellular matrix, it has been shown to potentiate the effect of IGFs on fibroblast growth (6). In addition, IGFBP-5 may also be involved in cell differentiation. The expression of IGFBP-5 is greatly increased during terminal differentiation of the mouse C2 myoblast cell line *in vitro* and overexpression of IGFBP-5 alters the differentiation of these cells (5, 7). IGFBP-5 mRNA is abundant in several tissues in adult animals, including kidney, brain, gut, uterus, and cardiac and skeletal muscle but is minimal in liver (5, 8, 9). In the rat embryo, IGFBP-5 mRNA is detected as early as embryonic day 10.5 and is mainly distributed at the surface ectoderm, the notochord, the floor plate, limb buds, precursor cells for neuronal cells, and specific muscle cells (10). *In vitro*, the level of expression of IGFBP-5 varies between cell types and is regulated by specific factors such as RA and IGF-1 in a cell type-specific manner (11–14). Previously we reported that IGFBP-5 mRNA was significantly induced by forskolin in human fetal skin fibroblasts, suggesting that IGFBP-5 is regulated by intracellular cAMP levels (15). A similar increase was also reported in rat osteoblast-like cells by parathyroid hormone through a cAMP-mediated mechanism (16). The molecular mechanisms underlying the tissue-specific expression and hormonal regulation of the IGFBP-5 gene are undefined.

This study was undertaken to characterize *cis*-acting sequences and their corresponding *trans*-acting factors that are responsible for the constitutively high expression in connective tissues and for the cAMP responsiveness of the human IG-

* This work was supported by National Institutes of Health Grant AG02331 from the National Institute of Aging. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: IGF, insulin-like growth factor; IGFBP, insulin-like growth factor binding protein; SV40, Simian virus 40; RA, retinoic acid; CAT, chloramphenicol acetyltransferase; bp, base pair(s); kb, kilobase(s).

FBP-5 gene. We have cloned the 5′-flanking region of the human IGFBP-5 gene from a genomic DNA library and confirmed the structure published recently by Allander *et al.* (17). We report here that the developmentally regulated transcription factor AP-2 stimulates transcription of the IGFBP-5 gene and that this *trans*-activation is at least in part responsible for the constitutively high expression in fibroblasts and for the cAMP responsiveness of this gene. We found that AP-2 regulates IGFBP-5 gene expression through a 5′-GCCNNNGGC-3′-like sequence but not 5′-CCCCACCC-3′ sequences *in vivo*, although both sequences have been proposed as consensus AP-2 binding sites (18, 19) and both were capable of binding to AP-2 *in vitro*.

MATERIALS AND METHODS

Cloning of the IGFBP-5 Gene—A human genomic DNA library constructed in Lambda Fix II vector (Stratagene, La Jolla, CA) was screened using a 627-base pair (bp) human IGFBP-5 cDNA, which was labeled with [^{32}P]dCTP (Amersham) by a random priming kit (Boehringer Mannheim). DNA from plaque-purified positive clones was digested with *Not*I, and the resulting fragments were selected by size and hybridized to a 261-bp cDNA fragment spanning the first exon of IGFBP-5. The selected inserts were subcloned into Bluescript vector (Stratagene). The nucleotide sequence of the largest fragment (7 kb) was partially sequenced following the dideoxy nucleotide method using double-stranded DNA sequencing with Sequenase Version 2 (U. S. Biochemical Corp.). This fragment contains 1.3 kb of the 5′-flanking region of the gene.

Plasmid Construction—IGFBP-5 promoter/luciferase constructs (pBP5P/Luc) were made by fusing human IGFBP-5 DNA fragment to a promoter-less luciferase vector (pGL2-Basic, Promega, Madison, WI). A DNA fragment consisting of 1278 nucleotides 5′ to the ATG translation start codon was generated by polymerase chain reactions. The ATG codon was mutagenized into GTG to avoid potential frameshifts. The DNA fragment was cloned into pGL2-Basic vector in a sense or antisense orientation. The constructs were verified by DNA sequencing. The human AP-2 expression plasmid SPRSV-AP-2 and the control vector SPRSV-LTR were provided by Dr. Trevor Williams (19). The human AP-2B expression plasmid pSG5-AP-2B was provided by Dr. Michael A. Tainsky (20).

Mutagenesis—Two sets of mutants were constructed to analyze the function of the multiple putative AP-2 binding sites. Sequence position numbering in the human IGFBP-5 gene is relative to the cap site, which is 772 bp 5′ to the first nucleotide of ATG (17). In the first set, mutants with various lengths of the 5′-flanking region was generated by polymerase chain reaction or by site-directed mutagenesis. Construct pBP5P1 is identical to pBP5P except the deletion of the 5′ 122-bp sequence. This construct contains all the putative AP-2 sites. Further deletion of the region from −381 to −128 where the two CCCCACCC sites are located generated the construct pBP5P2. Oligonucleotide 5′-TACAAACTG-GCTGCTATTTAAAAGCGC-3′ was used to further delete 15 nucleotides from bp −52 to −37 and thus deleted all three putative AP-2 binding sites, generating the construct pBP5P3. All of the sequence 5′ of the TATA box was deleted in the construct pBP5P4. These DNA fragments were subcloned into pGL2-Basic vector and all plasmids were sequenced to verify proper orientation.

In the second set of mutants, points mutants of pBP5P/Luc were generated by site-directed mutagenesis as described previously (6). Plasmid DNA was transfected into *Escherichia coli* strain CJ236. A 60-ml culture was inoculated and cultured with a fresh colony of CJ236 and was then infected with helper phage R408. After 5 h the bacteria were pelleted. Phagemid particles were precipitated in 16% polyethylene glycol and ammonium acetate, and single strand phagemid DNA was isolated by binding to glassmilk following the manufacturer's protocol (Bio 101, La Jolla, CA). The following complementary oligonucleotides containing mutagenic mismatches were used. Oligonucleotide 5′-TCACACGGGGTGGGCTTTGGAGAGGCCTTCTA-3′ mutated the first distal putative AP-2 site (CCCCACCC, located at bp −147 to −140) to CCAAAGCC, generating mutant MU1. Oligonucleotide 5′-AAACTCA-CAGGTGTAGGCTATGGAGAGGCCTTC-3′ mutated the two overlapping distal putative AP-2 sites (CCCCACCCCCACCC, located at bp −147 to −134) to CCAAAGCCTCACAC, generating mutant MU2. Oligonucleotide 5′- TTAAATAGCCGGACAATGTCTGCCAGCCAG-3′ mutated the proximal putative AP-2 site (GCCAGGGGC, located at bp −47 to −39) to TACAGTGTC, generating mutant MU3. These oligonucleotides were phosphorylated with T4 polynucleotide kinase (Promega) for 1 h at 37 °C. An aliquot of the synthesis mixture was used to transform *E. coli* strain DH5 αF′ and the positive colonies were selected by ampicillin resistance. DNA from the resulting colonies was amplified and DNA sequencing was used to determine the clones containing the correct sequences.

Cell Culture and DNA Transfection—Cell cultures were grown in 6-well Falcon tissue culture plates. Human fetal dermal fibroblasts (GM10, Human Mutant Genetic Cell Repository, Camden, NJ), normal and SV40 T-antigen transformed human fetal lung fibroblasts (IMR-90, MRC-5, AG 2804, and AG 10076, NIA Aging Cell Culture Depository, Camden, NJ), and human glioblastoma tumor cells (T98G, American Type Culture Collection, Rockville, MD) were plated at 3–8 × 10^5 cells/well and maintained in 2 ml of Eagle's minimal essential medium supplemented with serine (21 μg/ml), pyruvate (110 μg/ml), asparagine (30 μg/ml), penicillin (100 units/ml), streptomycin (100 μg/ml), and 10% fetal calf serum. Human hepatoma and rhabdomyosarcoma cells (HepG2 and A673, American Type Culture Collection) were plated at 4–5 × 10^5 cells/well and were maintained in 2 ml of Dulbecco's minimal essential medium with low glucose (1 g/liter) supplemented with 4 mM glutamine, antibiotics, and 10% calf serum or fetal calf serum. After culture overnight, cells were exposed to calcium phosphate transfection buffer with 2 μg of luciferase test plasmid DNA for approximately 6–18 h followed by a 3-min glycerol shock. After transfection the cells were washed twice and maintained in growth medium. The transfected cells were harvested 2–3 days later unless otherwise specified. 0.5 μg of pSV-β-galactosidase control vector DNA was cotransfected to control for transfection efficiency.

Luciferase Activity Assay—The amount of cellular extract used in the luciferase assay was normalized relative to β-galactosidase activity. Luciferase activities were determined using the Luciferase Assay System (Promega). β-Galactosidase activity was assayed by monitoring the conversion of *o*-nitrophenyl-β-D-galactopyranoside to galactose and *o*-nitrophenyl at $A_{420\ nm}$. Each experiment was repeated 3–4 times with duplicate samples.

Gel Mobility Shift Assay—The assays were conducted with the following double-stranded oligonucleotides in which the nucleotides that differ from the wild type sequence are underlined: A1WT (−57) 5′-CTGGCTGGCAGCCAGGGGCCGGCTATT-3′ (−30); A2WT (−152) 5′-CCTCTCCCCACCCCCACCCCGTGTGA-3′ (−127); A1MU (−57) 5′-CTGGCTGGCATACAGTGTCCGGCTATT-3′ (−30); and A2MU (−152) 5′-CCTCTCCAAAGCCTCACACCGTGTGA-3′ (−127). The consensus AP-2 oligonucleotide 5′-GATCGAACTGACCGCCCGCGGCCC-3′ was purchased from Promega. Complementary single-stranded oligonucleotides were hybridized at room temperature in 100 mM NaCl after heating for 5 min at 95 °C. Oligonucleotides were end-labeled using T4 polynucleotide kinase (Promega) and [^{32}P]ATP (3,000 Ci/mmol, Amersham). The labeled probes were purified by chromatography (NAP column, Pharmacia Biotech Inc.). The radiolabeled probes (10,000 cpm) were incubated with or without competing oligonucleotides and bacterially expressed human AP-2 (Promega) in pH 7.5 buffer containing 4% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 50 mM NaCl, 0.5 mM dithiothreitol, 10 mM Tris-HCl, and 50 μg/ml poly(dI-dC)-poly(dI-dC) in a final volume of 20 μl. After 20 min incubation at 4 °C, the DNA-protein complexes were separated by electrophoresis on a 4% polyacrylamide gel in 0.5 × TBE buffer and visualized by autoradiography. For supershift assay experiments, samples were preincubated with 1 μl of antiserum against human AP-2 (Santa Cruz Biotechnology, Santa Cruz, CA) or 1 μl of anti-IGFBP-5 antibody for 1 h at room temperature before the addition of ^{32}P-labeled DNA.

RNA Isolation and Northern Blot Analysis—RNA was isolated from cell cultures by the TriReagent following the manufacturer's instructions (Molecular Research Center, Inc., Cincinnati, OH) and was quantified by spectrophotometry. RNA samples were size-fractionated on a 1.2% agarose formaldehyde gel, blotted, and fixed onto nylon membrane (Biotrans, ICN Biochemical, Inc.), and hybridized with the [^{32}P]dCTP-labeled human 627-bp IGFBP-5 cDNA. A cDNA probe for glyceraldehyde-3-phosphate dehydrogenase (Ambion, Austin, TX) was used to assess relative amounts of RNA in the Northern blots.

RESULTS

Cell-specific Expression and cAMP Regulation of the Human IGFBP-5 Gene—The levels of IGFBP-5 mRNA in several cultured human cell lines were compared by Northern blot analysis (Fig. 1*A*). IGFBP-5 mRNA was most abundant in fibroblastic cell lines such as IMR-90, GM-10, MRC-5, and the glioblastoma cell line T98G (*lanes 1, 2, 5*, and *3*, respectively). It was not appreciably detected in HepG2 cells and A673 cells

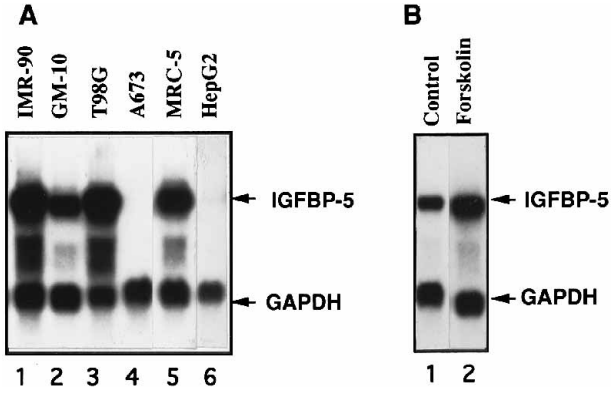

FIG. 1. **Northern blot analyses of IGFBP-5 mRNA levels.** Total cellular RNA was prepared as described under "Materials and Methods" from different cell lines. Ten-μg RNA aliquots were electrophoresed and transferred to a nylon membrane and then hybridized with a ^{32}P-labeled human IGFBP-5 cDNA probe. The amounts of RNA in each lane were verified with a glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) cDNA probe. The *arrows* denote the 6-kb IGFBP-5 message and 1.4-kb glyceraldehyde-3-phosphate dehydrogenase message. *A*, expression of IGFBP-5 mRNA in several human cell lines; *B*, effects of forskolin on IGFBP-5 mRNA expression in GM-10 fibroblasts. Confluent fibroblast monolayers were incubated in Eagle's minimal essential medium (*lane 1*) or Eagle's minimal essential medium plus forskolin (10 μM, *lane 2*) for 18 h.

(*lanes 6* and *4*). More sensitive reverse-transcriptase polymerase chain reaction, however, revealed the presence of minimal levels of mRNA in A673 and HepG2 cells (data not shown). When GM-10 fibroblasts were treated with forskolin, the IGFBP-5 mRNA abundance increased significantly (2.1-fold, Fig. 1*B*), as reported previously (15). These results suggest that IGFBP-5 is expressed in a cell type-specific manner and that forskolin induces IGFBP-5 mRNA expression in human fibroblasts.

Functional Characterization of the IGFBP-5 Gene Regulatory Region—To investigate the molecular mechanism underlying this cell type-specific expression and cAMP induction, the 5′-flanking region of the human IGFBP-5 gene was isolated. The 5′-flanking region was fused to a luciferase reporter vector and used to transfect GM-10, HepG2, and A673 cells. In all three cell lines, the IGFBP-5 regulatory/promoter construct had significantly greater activity in directing the reporter gene expression when inserted in the sense orientation compared with the antisense orientation (Fig. 2). The basal promoter activity was higher in transfected GM-10 cells than in A673 and HepG2 cells. Elevation of intracellular cAMP levels by forskolin treatment significantly increased the promoter activity in GM-10 cells (Fig. 2*C*), but this effect was not seen in HepG2 and A673 cells (Fig. 2, *A* and *B*). These results indicate that this promoter/regulatory region contains elements essential for cell-specific expression and cAMP responsiveness.

AP-2 Binds to the Putative AP-2 Elements in the IGFBP-5 Regulatory Region—Since cAMP induces IGFBP-5 expression, we searched the DNA sequence of the regulatory region for consensus cAMP responsive *cis* elements. Several putative AP-2 binding sites but no consensus sequence for cAMP responsive element or AP-1 binding site was found in this region. The DNA sequence 5′-CCCCACCCCCACCC-3′ at position −147 to −134 contains two overlapping AP-2 elements 5′-CCCCACCC-3′ found in the porcine plasminogen activator gene and the rat tyrosine aminotransferase gene (18). Another DNA sequence 5′-GCCAGGGGC-3′ adjacent to the TATA box at position −47 to −39 contains sequence identical to the consensus AP-2 element 5′-GCCNNNGGC-3′ described by Williams and Tjian (19). In this proximal region, there are two other sequences homologous to the consensus sequence, 5′-

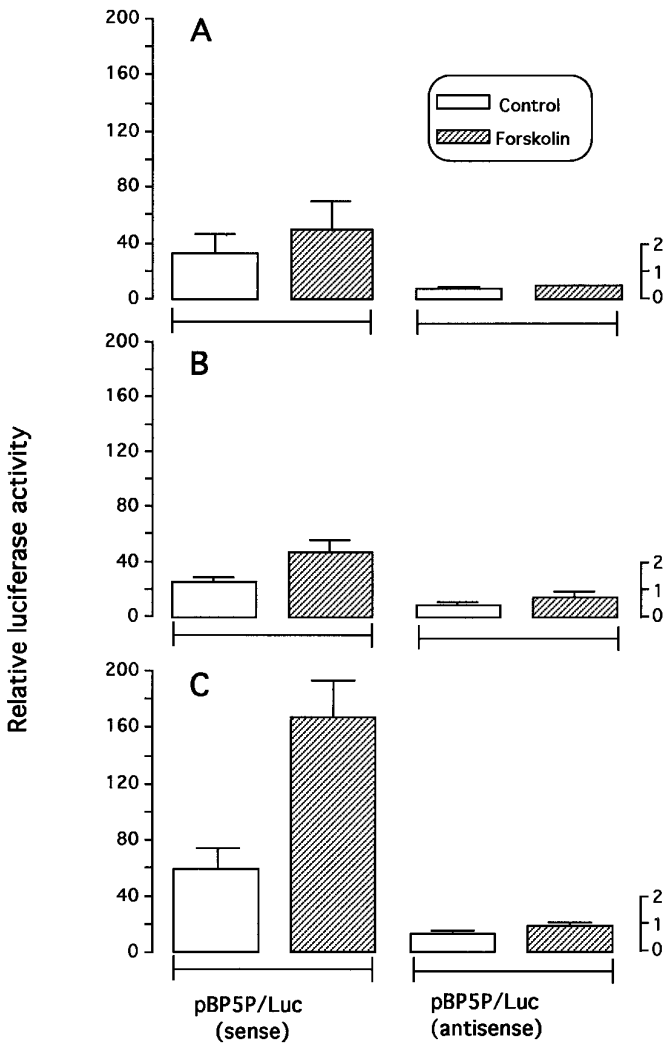

FIG. 2. **Effect of forskolin on human IGFBP-5 promoter activity in HepG2 (*A*), A673 (*B*), and GM-10 (*C*) cells.** A 1278-bp DNA fragment of the human IGFBP-5 gene 5′-flanking region was fused to a luciferase reporter gene (pGL2-Basic) in a sense or antisense orientation to generate pBP5P/Luc (*sense*) and pBP5P/Luc (*antisense*), respectively. These constructs and the promoter-less vector (pGL2-Basic) were transiently transfected into cells. After growing in complete medium for 48 h, cells were incubated in serum-free medium with or without forskolin (10 μM) for another 18 h. The cellular extracts were prepared and the luciferase activity was measured as described under "Materials and Methods." The relative luciferase activities represent the relative fold value *versus* the promoter-less pGL2-Basic plasmid. pSV-β-galactosidase was used as an internal control. For all transfection experiments the results reported were obtained from at least three independent experiments each carried out in duplicate.

GGCTGGCAG-3′ at position −55 to −47 and 5′-GGGGCCGGC-3′ at position −43 to −35. To examine whether AP-2 could bind to these putative sites, gel shift mobility assays were performed using ^{32}P-labeled oligonucleotides derived from bp −57 to −30 (probe A1) and bp −152 to −126 (probe A2). Bacterially expressed human AP-2 formed a protein-DNA complex with probe A1 (Fig. 3*A, lane 2*). The AP-2·DNA complex with probe A1 was competed effectively by 50-fold excess of unlabeled A1WT oligonucleotide as well as 50-fold excess of AP-2 consensus oligonucleotide (Fig. 3*A*, *lanes 3* and *4*), but was not inhibited by 100-fold excess of unlabeled A1MU oligonucleotide in which the putative AP-2 sites were mutated (Fig. 3*A, lane 5*). Similarly, probe A2 also formed specific protein-DNA complex with AP-2 (Fig. 3*B*). This complex was inhibited by unlabeled A2WT oligonucleotide and AP-2 consensus oligonucleotide but not A2MU oligonucleotide. To determine that

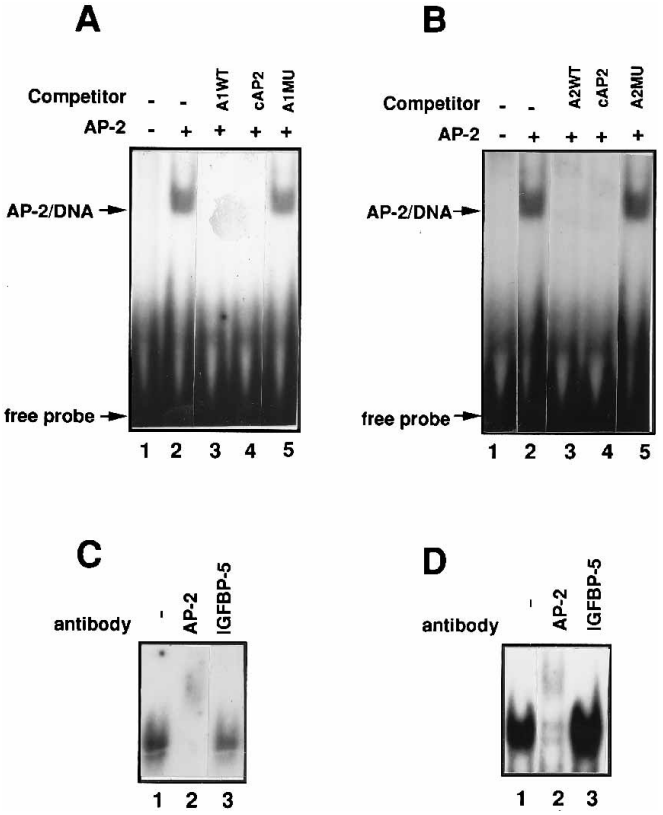

FIG. 3. **Binding of human AP-2 to the putative AP-2 binding sites of the human IGFBP-5 promoter.** Gel-shift analysis of bacterially expressed human AP-2 was performed with (*A*) ^{32}P-labeled A1 or (*B*) ^{32}P-labeled A2 double-stranded oligonucleotides as probes. The probes were incubated with (*lane 1*) or without AP-2 (*lanes 2–5*). Competitive binding was shown by inclusion of 50-fold excess of unlabeled wild type oligonucleotide (*lane 3*, A1WT or A2WT), mutated oligonucleotide (*lane 5*, A1MU or A2MU), or consensus AP-2 oligonucleotide (*lane 4*, cAP-2). Supershift assays for AP-2·A1 (*C*) or AP-2·A2 complexes (*D*) were performed without (*lane 1*) or with 1 μl of anti-AP-2 antibody (*lane 2*) or 1 μl of anti-IGFBP-5 antibody (*lane 3*).

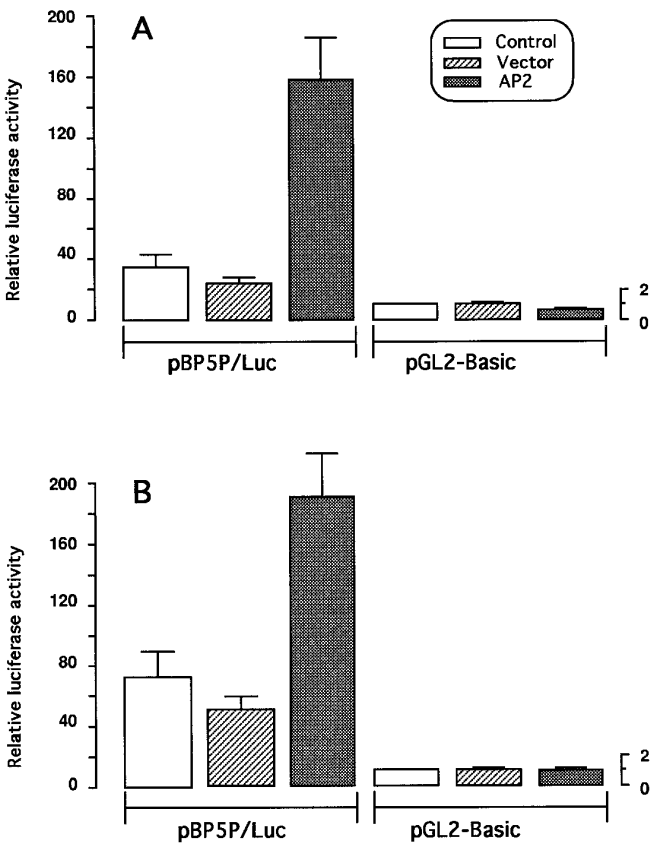

FIG. 4. **Effects of AP-2 on human IGFBP-5 promoter activity in HepG2 (*A*) and GM-10 (*B*) cells.** Human hepatoma cells (HepG2) or fibroblasts (GM-10) were transiently transfected with either pGL2-Basic or pBP5P/Luc and cotransfected with either a human AP-2 expression plasmid pSV-AP-2 (AP-2), the pSV vector without AP-2 sequence (vector) or pBluescript DNA control (control). Cellular extract preparation and the luciferase assays were performed as described for Fig. 2.

AP-2 is actually the protein binding to these sites, supershift experiments were performed using an antiserum against AP-2. As shown in Fig. 3, *C* and *D*, the AP-2·A1 and AP-2·A2 complex was supershifted by addition of an anti-AP-2 antibody (*lane 2*). In contrast, the complexes were not affected by addition of anti-IGFBP-5 antibody (*lanes 3,* Fig. 3, *C* and *D*, respectively). The results indicate that AP-2 can bind specifically to these two locations of the IGFBP-5 gene *in vitro*.

AP-2 Regulates IGFBP-5 Expression in Vivo—To examine whether AP-2 is functionally involved in regulating IGFBP-5 expression, we performed a series of cotransfection studies using the IGFBP-5 promoter construct. If the promoter activity of the IGFBP-5 gene is regulated by AP-2, then expression of exogenous AP-2 in cells that lack endogenous AP-2 should increase IGFBP-5 promoter activity. Conversely, interference of the *trans*-activating activity of AP-2 in AP-2 abundant fibroblasts should reduce the promoter activity. Fig. 4 shows the results obtained when the human AP-2 expression construct SPRSV-AP-2 (19) was cotransfected with the pBP5P/Luc construct in the AP-2 deficient HepG2 cells. pBP5P/Luc plasmid had a low basal promoter activity that was induced up to 5-fold by the cotransfection of AP-2 (Fig. 4*A*). In contrast, AP-2 had no effect on the luciferase activity in cells transfected with vector pGL2-Basic. The fibroblasts, which have abundant endogenous AP-2 (28), had a significant level of promoter activity (Fig. 4*B*). Cotransfection of the SPRSV-AP-2 construct increased the promoter activity but the magnitude was smaller. These results suggest that AP-2 is capable of activating IGFBP-5 gene expression *in vivo* when expressed in human cells.

AP-2 binds DNA as a homodimer with the binding and dimerization domain located at the C-terminal region of the protein (19). AP-2B, an alternatively spliced product from the AP-2 gene, has the activation domain of AP-2 but lacks the dimerization and DNA binding domain and can specifically interfere with endogenous AP-2 activity in a dominant-negative manner (20). We therefore cotransfected construct pBP5P/Luc with a AP-2B expression construct (pSG5-AP-2B) (20) in GM-10 fibroblasts. Expression of AP-2B significantly suppressed the basal IGFBP-5 promoter activity in these cells (Fig. 5*A*). Similar inhibition of IGFBP-5 promoter activity by AP-2B expression was also obtained with T98G cells (data not shown). In HepG2 cells, expression of AP-2B had no significant effect on the basal promoter activity, but it inhibited the AP-2 induced activation in a dose-dependent manner (Fig. 5*B*). Since it is known that SV40 T-antigen inhibits the *trans*-activation activity of AP-2 (21), we tested whether SV40 T-antigen-induced cellular transformation could suppress the constitutively high expression of endogenous IGFBP-5 mRNA in fibroblasts. As shown in Fig. 6, two untransformed human fetal lung fibroblast cell lines, MRC-5 and IMR-90, both had high levels of IGFBP-5 mRNA (*lanes 1*, *2*, *5*, and *6*) similar to GM-10 fibroblasts (*lane 9*). When these cells were transformed by SV40 T-antigen, the IGFBP-5 mRNA expression was almost completely abolished (*lanes 3, 4, 7,* and *8*). This inhibition by SV40 T-antigen-induced transformation was specific for IGFBP-5,

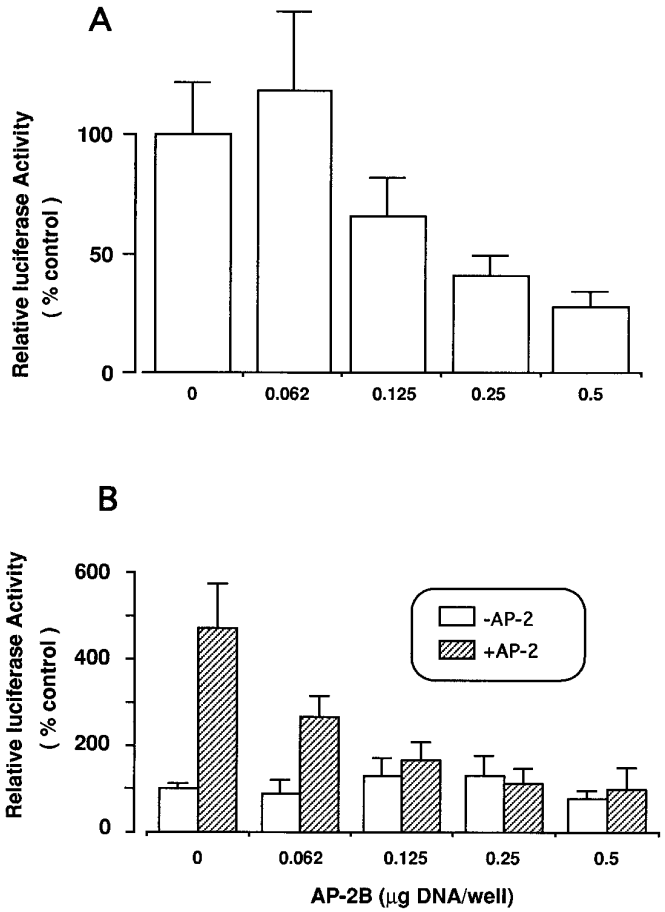

FIG. 5. *A*, inhibition of AP-2 activity by AP-2B on IGFBP-5 promoter activities in GM-10 fibroblasts. Human fibroblasts (GM-10) were transiently transfected with the pBP5P/Luc construct (2 μg) and an AP-2B expression plasmid. *B*, effect of AP-2B on AP-2-induced *trans*-activation in HepG2 cells. Human hepatoma cells (HepG2) were transiently transfected with pBP5P/Luc and cotransfected with a human AP-2 expression plasmid pSV-AP-2 (+AP-2) or pBluescript plasmid DNA (−AP-2). The cells were cotransfected with various amounts of AP-2B. The micrograms of transfected AP-2 B expression plasmid DNA are shown at the *bottom*. The differences in AP-2B DNA amount were compensated for by using pBluescript DNA.

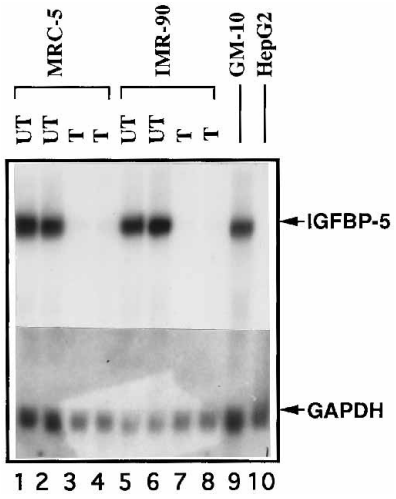

FIG. 6. **Northern blot analyses of IGFBP-5 mRNA levels in normal (*UT*) and SV40 T-transformed (*T*) human fibroblasts.** Ten μg RNA aliquots were electrophoresed and transferred to a nylon membrane and then hybridized with a ^{32}P-labeled IGFBP-5 cDNA probe and a glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) probe. The *arrows* denote the 6-kb IGFBP-5 message and 1.4-kb glyceraldehyde-3-phosphate dehydrogenase message. The RNA samples loaded in *lanes 1* and *2, 5* and *6* are from normal human fetal lung fibroblast lines MRC-5 and IMR-90, respectively. *Lanes 3* and *4*, *7* and *8* are RNA samples isolated from their corresponding transformed cell lines, AG 2804 and AG10076. RNA samples isolated from GM-10 or HepG2 cells were used as positive and negative controls.

since no significant difference was seen in glyceraldehyde-3-phosphate dehydrogenase expression. These results indicate that AP-2 regulates transcription of the human IGFBP-5 gene and contributes at least in part to its constitutively high expression in human fibroblasts.

The trans-Activation of IGFBP-5 Gene by AP-2 Is Biphasic—In the cotransfection studies with GM-10 cells, we noticed that transfection of high amounts of AP-2 DNA often failed to stimulate the promoter activity and was even inhibitory. High-level expression of AP-2 has been shown to cause transcriptional "self-interference" and result in inhibition of the AP-2 mediated *trans*-activation in an "artificial" reporter construct 3XAP2RE$_{hMt}$-tK chloramphenicol acetyltransferase (a construct made by fusing three AP-2 responsive elements of human MtIIA gene to the herpes simplex virus thymidine kinase promoter in the vector pBLCAT2 (22). We therefore conducted studies to examine whether high-level expression of AP-2 could result in reduced *trans*-activation ability of AP-2 using the IGFBP-5 promoter. Transfection of various amounts of SPRSV-AP-2 DNA in AP-2-deficient HepG2 and A673 cells significantly elevated the promoter activity of the IGFBP-5 gene dose dependently (Fig. 7, *A* and *B*). This *trans*-activation was most appreciable at a dose of 0.5 μg of DNA/well. In AP-2 abundant GM-10 fibroblasts, however, AP-2-induced *trans*-activation was only obtained at low doses (Fig. 7*C*). At high doses, the *trans*-activation ability of AP-2 was diminished. These results are unlikely to be attributable to differences in transfection efficiency because the luciferase activity was normalized by cotransfection of a β-galactosidase control vector. These results suggest that the *trans*-activation of human IGFBP-5 gene transcription by AP-2 is biphasic.

AP-2 Regulates IGFBP-5 Gene Expression through a Proximal GCCNNNGGC Site—Since multiple putative AP-2 binding sites (CCCCACCC or GCCNNNGGC-like sequences) are present on the promoter/regulatory region of the human IGFBP-5 gene and they were all capable of binding to recombinant human AP-2 *in vitro*, we conducted studies to investigate the relative functional importance of these sites *in vivo* using HepG2 cells. A number of deletion mutants, in which these AP-2 binding sites had been deleted separately, were generated and transfected with or without SPRSV-AP-2 vector in HepG2 cells. Deletion of the first 5′ 122 bp slightly increased the basal promoter activity but had little effect on AP-2-induced activation (pBP5P1/Luc, Fig. 8). Further deletion of the region from −381 to −128 where the two CCCCACCC sites are located significantly reduced the basal promoter activity (pBP5P2/Luc). AP-2 expression, however, still increased the promoter activity up to 4.5-fold. When the proximal region (−52 to −37) was deleted, both the basal activity and AP-2-induced activation was abolished (pBP5P3/Luc and pBP5P4/Luc), suggesting that this proximal region is necessary for AP-2-induced activation. The functional importance of these sites was further examined using site-directed mutagenesis. When the first distant putative AP-2 site CCCCACCC (located at bp −147 to −138) was mutated to CCAAAGCC, there was no change in AP-2-induced activation (*MU1*, Fig. 9). Mutation of the two distant putative AP-2 sites CCCCACCCCCACCC (located at bp −147 to −134) to CCAAAGCCTCACAC appeared to slightly increase the AP-2 induced activation (*MU2*, Fig. 9), but these changes were not significant. When the proximal putative AP-2 site

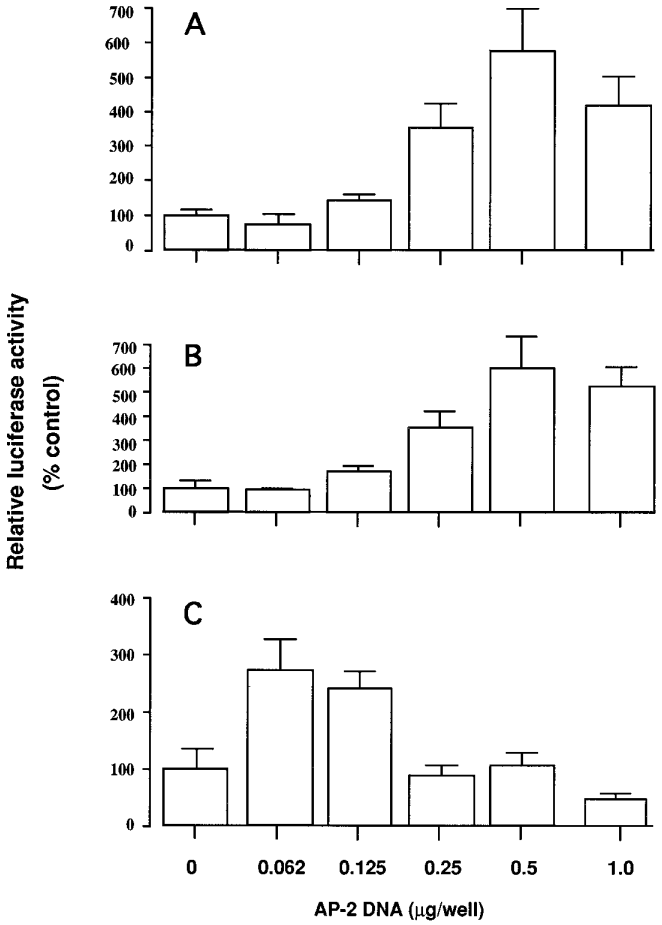

FIG. 7. **Effect of varying amount of pSV-AP-2 plasmid DNA on human IGFBP-5 promoter activity in HepG2 (*A*), A673 (*B*), and GM-10 (*C*) cells.** Cells were transiently transfected with pBP5P/Luc (2 μg) and cotransfected with pSV-AP-2 (*AP-2*) or pBluescript DNA control (*control*). The cellular extracts preparation and the luciferase assay were performed as described in the legend to Fig. 2. The differences in AP-2 DNA amount were compensated for by using pBluescript DNA.

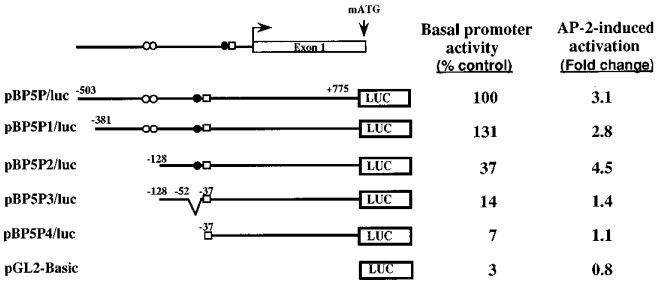

FIG. 8. **Mapping of the functional AP-2 binding sites in the human IGFBP-5 promoter.** The schematic diagram shows the pBP5P/Luc construct and a series of 5′ and internal deletion mutants fused to pGL2-Basic reporter gene. The putative AP-2 binding sites are indicated with *open circles* (CCCCACCC) or *solid circles* (GCCNNNGGC), respectively. The TATA box is indicated as an *open square*. These constructs were cotransfected into HepG2 cells with a human AP-2 expression plasmid (pSV-AP-2) or a control plasmid (pSV). The luciferase activities induced by AP-2 represent averages of three independent experiments of the relative fold increase as compared to the control lacking AP-2.

GCCAGGGGC (located at bp −47 to −39) was mutated to GACAGTGTC (*MU3*, Fig. 9), the AP-2-induced activation was diminished. These results suggest that the proximal GCCNNNGGC sequence is the primary site for AP-2 to bind and trans-activate the human IGFBP-5 gene *in vivo*.

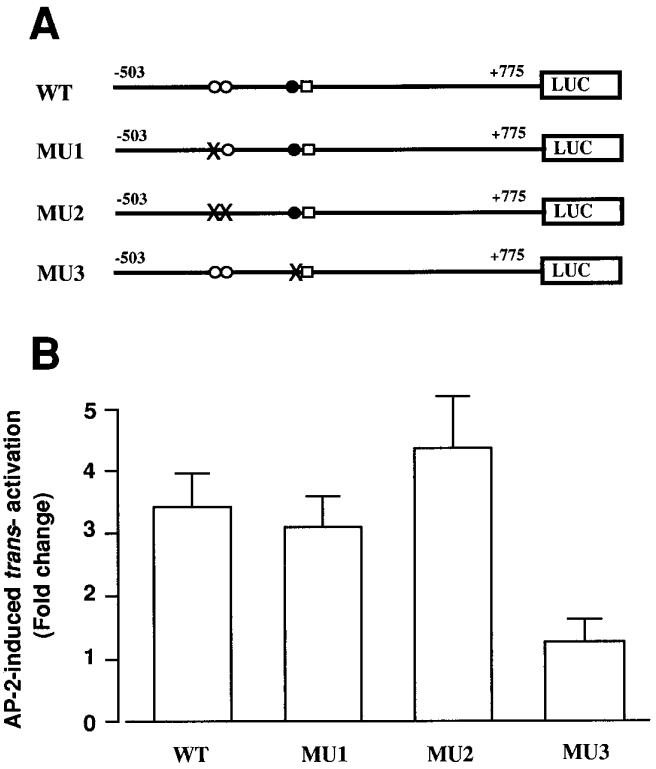

FIG. 9. *A*, schematic diagram showing the pBP5P/Luc construct and a series of point mutant constructs. The putative AP-2 binding sites are indicated with *open circles* (CCCCACCC) or *solid circles* (GCCNNNGGC), respectively. The TATA box is indicated as an *open square*. The mutated site is indicated as a *cross*. *B*, effects of point mutations in the putative AP-2 binding sites of human IGFBP-5 promoter. The wild type and mutant constructs were cotransfected into HepG2 cells with a human AP-2 expression plasmid (pSV-AP-2) or a control plasmid (pSV). The luciferase activities stimulated by AP-2 represent averages of the relative fold increases compared to the control lacking AP-2 in four independent experiments.

DISCUSSION

The results from this study have, for the first time, examined the role of specific *cis*-acting sequences and a transcription factor in the regulation of IGFBP-5 gene expression. We have identified the promoter/regulatory region of this gene that showed constitutive promoter activity and cAMP responsiveness when transfected in human fibroblasts. We were able to demonstrate that transcription factor AP-2 regulates IGFBP-5 gene expression through a proximal 5′-GCCNNNGGC-3′-like sequence of this promoter region. This AP-2-induced *trans*-activation contributes at least in part to the constitutively high expression in fibroblasts and to the cAMP responsiveness of this gene.

The IGFBP-5 gene has been recently cloned and characterized from rat, mouse, and human (23, 9, 17). A DNA fragment containing the 5′-flanking region of the human gene has been shown to be able to direct expression of a reporter gene in human breast cancer cells (17). A more in-depth analysis using the mouse promoter in human hepatoma HepG2 cells suggests that the IGFBP-5 promoter has a simple structure, requiring the TATA box and no more than 125 nucleotides of additional 5′ DNA to generate primary promoter activity (24). The *cis*-acting sequences and their corresponding *trans*-acting factors responsible for the developmental- and cell type-specific regulation of IGFBP-5 expression have not been reported.

Several lines of evidence point to a role for AP-2 in regulating human IGFBP-5 gene transcription. Elevation of intracellular cAMP levels increases IGFBP-5 mRNA levels in human skin fibroblasts (Ref. 15 and this study). Since the IGFBP-5 pro-

moter activity was induced to a similar extent by forskolin treatment (Fig. 2*C*), this cAMP-induced increase in mRNA is a transcriptionaly regulated event. Sequence analysis had indicated the presence of several consensus AP-2 binding sites in the promoter region of the human IGFBP-5 gene. AP-2, originally purified from HeLa cells, is a 52-kDa nuclear protein that functions as dimer (21). AP-2 exerts its function in mediating regulation of gene expression in response to a number of diverse signal transduction pathways. Elevation of AP-2 transactivity can be elicited by RA (25). In addition to RA, AP-2 mediates transcriptional activation in response to two other signal pathways, the cAMP-dependent protein kinase A pathway and the phorbol ester/diacylglycerol-inducible protein kinase C pathway (26, 27). This induction of AP-2 activity is mediated by a mechanism independent of increased AP-2 mRNA and independent of protein synthesis. A similar effect by forskolin was observed with IGFBP-5 expression in this study and IGFBP-5 expression has been reported to be regulated by RA (11, 12, 14). The spatial and temporal expression pattern of IGFBP-5 is similar to that of AP-2, the latter is known to be expressed primarily in neural crest cells and their major derivatives (28). IGFBP-5 mRNA expression like AP-2 is restricted to a subset of ectodermal and mesodermal tissues during rodent embryogenesis (10). Neither AP-2 nor IGFBP-5 is significantly expressed in endodermally derived tissues such as liver. This overlapping expression is also reflected *in vitro*. As shown in this study, IGFBP-5 mRNA is abundant in fibroblasts and glioblastoma cells, which are known to have high levels of endogenous AP-2 (28). In the AP-2 deficient HepG2 cells, IGFBP-5 mRNA levels are very low. This similarity in regulation and pattern of expression is suggestive of a role for AP-2 in regulating IGFBP-5 expression.

The potential role of AP-2 in regulating IGFBP-5 expression was addressed by DNA binding studies and transfection studies. Gel shift mobility assay data suggest that the regions from −152 to −127 and from −57 to −30 in the IGFBP-5 promoter were capable of binding to human AP-2 and mutation of these regions reduced binding, suggesting that AP-2 may be involved in binding to these regions and regulating this gene. Evidence supporting this conclusion is that cotransfection of HepG2 cells or fibroblasts with a human AP-2 expression plasmid increased IGFBP-5 transcription level. Northern analysis showed that the levels of IGFBP-5 mRNA were extremely suppressed in the SV40 T-transformed human fibroblasts (Fig. 6), suggesting SV40 T-induced cellular transformation inhibits the expression of IGFBP-5. This could occur through the inhibition of AP-2, since SV40 large T-antigen has been shown to inhibit the *trans*-activating activity of AP-2 by directly binding to AP-2 and thereby preventing the formation of an AP-2·DNA complex (21). Furthermore, specific interference of the *trans*-activation activity of AP-2 in human fibroblasts by expression of AP-2B, a dominant-negative inhibitor of AP-2, resulted in reduced IGFBP-5 promoter activity. These results suggest that AP-2 is involved in regulating human IGFBP-5 gene expression. The extent to which AP-2 might be central in controlling IGFBP-5 expression is still not clear. Our transient transfection studies with HepG2 cells, however, have shown that expression of exogenous AP-2 can impart to hepatoma cells the ability to express IGFBP-5 promoter driven transgenes, and AP-2B counteracts this activation. These data strongly argue that AP-2 plays an important role in regulating human IGFBP-5 gene expression.

DNase I footprinting experiments have shown that AP-2 binds to GC-rich sequences that are present in a variety of cellular and viral promoters and sequences of individual AP-2 binding sites can vary substantially (21, 26). Based on the sequence comparison of AP-2 binding sites identified by DNase I footprinting in a number of cellular and viral promoters, sequences such as 5′-CCCCA(G/C)(G/C)C-3′ have been proposed as consensus sites (18). DNase I footprinting, however, does not generate information regarding the contribution of individual nucleotides within the binding sites. Using methylation interference assays and missing contact probing assays, Williams and Tjian (19) identified a palindromic sequence, 5′GCCNNNGGC-3′, as the core recognition sequence for AP-2 binding. In the promoter region of the human IGFBP-5 gene, both consensus sequences for AP-2 binding are present. The DNA sequence 5′-CCCCACCCCCACCC-3′ at position −147 to −134 contains two overlapping AP-2 elements 5′-CCCCACCC-3′ found in the porcine plasminogen activator gene and the rat tyrosine aminotransferase gene (18). The DNA sequence at position −55 to −35 contains sequences identical or close to the consensus AP-2 element 5′-GCCNNNGGC-3′ (19). Although both sequences were capable of binding to AP-2 *in vitro*, the promoter activity data of serially 5′-deleted IGFBP-5 promoter constructs showed that the region between positions −52 and −37 contains determinants important for the AP-2-induced activation of transcription. Using a number of point mutants, we were able to assign the AP-2-induced activation of the promoter to the proximal sequences which contain sequences identical or close to the 5′-GCCNNNGGC-3′ consensus AP-2 binding sequences. The finding that the proximal 5′-GCCNNNGGC-3′ sequence but not the distal 5′-CCCCACCC-3′ sequences is the functional site that mediates the *trans*-activation activity of AP-2 *in vivo* is interesting in the context of AP-2 binding sites. It also suggests that being able to bind to a DNA sequence *in vitro* does not necessarily impart functional involvement *in vivo*. In the human immunodeficiency virus type I gene, AP-2 and NF-κB have been shown to be able to bind to the same region in a mutually exclusive manner (29). It should be noted that the sequence 5′-CCCCACCC-3′ contains sequences identical to the retinoblastoma control element, 5′-CCACCC-3′. The retinoblastoma control element motif has been identified as being important for retinoblastoma-induced *trans*-activation (30).

AP-2 has been implicated to play a crucial role in the control of gene expression in response to cell differentiation signals within neural crest and epidermal cell lineages (28). In the human tetracarcinoma cell line PA-1, constitutive suppression of AP-2 transactivator function by stably expressing AP-2B resulted in a RA-resistant phenotype and these cells became tumorigenic, suggesting that normal function of AP-2 is required for these cells to respond to this differentiation signal (20). Similarly, the neuronal differentiation of human SH-SY5Y neuroblastoma cells, induced by activation of the protein kinase C signal pathway, is accompanied by increased AP-2 *trans*-activator activity (31). Intriguingly, this protein kinase C-induced SH-SY5Y neuronal differentiation is dependent on IGF-I. Pahlman *et al.* (32) reported that while IGF-I is mitogenic for proliferative SH-SY5Y cells, it loses its mitogenic response and strongly enhances the neuronal differentiation when added with phorbol 12-myristate 13-acetate. This "switch" from a mitogenic effect to a differentiation role for IGF-I is puzzling since both mitogenic and differentiation effects are mediated through the IGF-I receptor and this receptor is expressed and functional in both proliferative and differentiated SH-SY5Y cells. We speculate that this switch may be related to the potential changes in IGFBPs, since our finding that AP-2 regulates IGFBP-5 expression raises the possibility that the production of IGFBP-5 may be activated during the protein kinase A-induced differentiation in SH-SY5Y cells. Neuroblastoma cells have been shown to synthesize a number

of IGFBPs (33, 34), and an increase in IGFBP-5 production is observed during differentiation of myoblasts (7).

The mechanism underlying the biphasic effect of *trans*-activation of human IGFBP-5 gene transcription by AP-2 remains to be understood, but it is probably related to the transcriptional self-interference of AP-2 reported previously. Kannan *et al.* (22) found that overexpression of AP-2 causes inhibition of AP-2 *trans*-activation activity mediated by its activation domain using an artificial promoter construct. Our data with a functional promoter are similar in that overexpression of AP-2 results in reduced AP-2 *trans*-activation activity. It has been speculated that the AP-2 activity results from a complex of proteins and that there are cofactors necessary for the AP-2 *trans*-activation activity, some of which are present in limiting amounts. Excess AP-2 molecules may interact with one or more of these putative cofactor(s), making them unavailable for the AP-2 molecules that are bound to the target site and therefore causing autointerference (22). Such autointerference has been reported with GAL4-VP16 (35). In support of the concept of transcriptional self-interference of AP-2, our studies with cell lines expressing different levels of endogenous AP-2 showed that greater amounts of AP-2 plasmid are required for the autoinhibitory effect in AP-2-deficient cells such as HepG2 cells.

In summary, we have demonstrated that AP-2 regulates transcription of the human IGFBP-5 gene. We have identified the proximal 5′-GCCNNNGGC-3′-like sequences in the human IGFBP-5 gene where AP-2 is able to bind and *trans*-activate the gene. It should be noted that the structure of the IGFBP-5 promoter is highly conserved, since proximal 200 bp of human mouse and rat IGFBP-5 gene promoters are more than 90% identical (9, 17, 23). In particular, the proximal AP-2 binding sequence found in human gene is identical to the analogous part of the rat and mouse genes. In addition, cAMP induces IGFBP-5 mRNA in other mammalian species (16). Therefore, the regulation of IGFBP-5 gene expression by the transcriptional factor AP-2 is likely to be a general mechanism for mammals. The identification of the role of AP-2 and of a functional binding motif in the IGFBP-5 gene has provided necessary information to warrant future interest. Normal AP-2 function is involved in the control of a number of cellular events including cell growth and differentiation (36). The involvement in these events is an interesting characteristic AP-2 has in common with the IGF system. Further studies will focus on these specific cellular events and how they work together to control IGFBP-5 levels and cell growth and differentiation.

Acknowledgments—We are indebted to Dr. Trevor Williams, Yale University, for the AP-2 expression construct and Dr. Michael A. Tainsky, the University of Texas M. D. Anderson Cancer Center, for the AP-2B expression construct. We thank Drs. Wendie Cohick and Jane Clarke, University of North Carolina at Chapel Hill, for their helpful discussion and comments.

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