Expression of PDGF-B, the gene encoding the platelet-derived growth factor B chain, has been implicated as a participant in an autocrine growth loop in the human osteosarcoma cell line U2-OS. In previous work, we identified a primary site in the PDGF-B promoter, the SIS proximal element (SPE), which is critical for transcription of the PDGF-B gene in U2-OS cells. We also identified Sp1 as one of the SPE-binding proteins in U2-OS nuclear extracts. In the present work, we have identified another SPE-binding protein to be Sp3. Gel mobility shift assays showed that both Sp1 and Sp3 require the CACCC motif within the SPE for binding. In vitro transcription assays showed that Sp1 or and Sp3 is necessary for transcription of the PDGF-B gene. Cotransfection experiments functionally demonstrated that Sp1 and Sp3 can independently or additively activate the PDGF-B promoter through the SPE as well as a synthetic promoter. However, the CACCC motif within the SPE is not the only site within the minimal PDGF-B promoter through which Sp1/Sp3 acts; additional nested deletion analyses showed that multiple cis-acting elements within the minimal promoter are required for full level transcription of the PDGF-B gene in U2-OS cells.

Platelet-derived growth factor (PDGF) is a potent mitogen and chemotactant for cells that express functional PDGF receptors, typically cells of mesenchymal origin (Antoniades, 1991; Deuel et al., 1982). It is a homo- or heterodimeric protein consisting of two related polypeptide chains, A and B (Johnsson et al., 1981). The three isoforms of PDGF (AA, AB, and BB) stimulate biological responses by binding to two cell surface PDGF receptors, α and β (Claesson-Welsh, 1994).

A functional, contributory role for PDGF-B in the development and maintenance of cancer cells is supported by several observations: (i) the B chain gene of PDGF was identified as the cellular homolog of the simian sarcoma virus oncogene, v-sis (Dooblittle et al., 1983; Waterfield et al., 1983), (ii) when under the control of a strong promoter, both v-sis and its cellular homolog PDGF-B caused the transformation of mouse 3T3 cells or human fibroblasts (Robbins et al., 1982; Clarke et al., 1984; Gazit et al., 1984; Stevens et al., 1988), (iii) the cellular PDGF-B gene has been shown to be constitutively expressed in a large percentage of human tumor cells, cells of both mesenchymal and epithelial origin, whereas it was not detectably expressed in the normal counterparts of these cells (Eva et al., 1982; Peres et al., 1987; Maxwell et al., 1990), and (iv) expression of a recombinant, dominant-negative mutant of PDGF-B in PDGF-B-expressing astrocytoma cells resulted in a substantial reduction in the tumorigenic growth of the cells (Shamah, 1993). The above evidence, especially the fact that high level expression of the cellular form of the sis oncogene is sufficient for oncogenesis, implies that loss of its transcriptional regulation provides a contributory step in neoplastic transformation.

In U2-OS, a human osteosarcoma cell line, coexpression of the PDGF-B gene as well as the PDGF receptor genes is associated with the constitutive activation of the PDGF receptors (Betzoltz et al., 1984). Studies of both v-sis and the PDGF-B gene in U2-OS and other cell types have implicated the presence of an intracellular, autocrine growth circuit in which PDGF-BB binds and activates the PDGF receptors within a cellular processing compartment prior to secretion (Graves et al., 1984; Betsholtz et al., 1984; Bejcek et al., 1989, 1992). Because these interactions may be inaccessible to therapeutic strategies such as extracellularly added PDGF antibodies or PDGF receptor antagonists, activation of the endogenous PDGF-B gene remains an important step within the PDGF-B autocrine loop, a step that could potentially be targeted to achieve suppression of the growth-promoting circuit.

With regard to the regulatory mechanisms that underlie PDGF-B gene activation, earlier work from our laboratory (Jin et al., 1993, 1994) and others (Khachigian et al., 1994) identified a dominant regulatory element within the PDGF-B promoter (the SPE or SIS proximal element, at −64 to −45) within which lies a CACCC motif. Ablation of the CACCC motif results in a loss of promoter activity roughly equal to the loss associated with ablation of the TATA box. Such CACCC motifs (also called CA boxes or GT boxes) have been shown to be binding sites for Sp1 as well as other newly identified members of the Expanding Sp family of transcription factors (Hagen et al., 1992, 1994). Studies of both v-sis and the PDGF-B gene in U2-OS and other cell types have implicated the presence of an intracellular, autocrine growth circuit in which PDGF-BB binds and activates the PDGF receptors within a cellular processing compartment prior to secretion (Graves et al., 1984; Betsholtz et al., 1984; Bejcek et al., 1989, 1992). Because these interactions may be inaccessible to therapeutic strategies such as extracellularly added PDGF antibodies or PDGF receptor antagonists, activation of the endogenous PDGF-B gene remains an important step within the PDGF-B autocrine loop, a step that could potentially be targeted to achieve suppression of the growth-promoting circuit.
phoretic mobilities. Both Sp1 and Sp3 are shown to require the intact CACCC motif for binding to the SPE, and their binding is necessary for efficient transcription of the PDGF-B gene. Using in vitro transcription assays, depletion of Sp1 and Sp3 binding activity from U2-OS nuclear extracts is associated with a ~80% decrease in transcription. Production of recombinant Sp1 or-and Sp3 in U2-OS cells is associated with a substantial increase in PDGF-B transcription. Lastly, we show by nested deletion mutations that the full level of PDGF-B transcription requires multiple cis-acting elements within the 250-bp promoter region.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The human osteosarcoma cell line, U2-OS, was purchased from the American Type Culture Collection. The cells were propagated in Dulbecco’s minimal essential medium supplemented with 10% fetal calf serum.

**Plasmids**—The PDGF-B luciferase reporter plasmid, RA1uc, the linker substitution mutant, J13, and the TIluc-derived reporter plasmids have been previously described (Jin et al., 1994). pGL3-RA1uc and pGL3-J13 were created by cloning the 0.4-kilobase pair PDGF-B promoter fragments from RA1uc and J13, respectively, into the pGL3basic vector (Promega). pGL3basic is a modified luciferase reporter plasmid with reduced background activity and enhanced reporter sensitivity for use in both in vitro and in vivo applications (Sherf and Wood, 1994). Vectors enabling expression of recombinant Sp1 or Sp3 in mammalian cells (CMV-Sp1 and CMV-Sp3) have been previously described (Hagen et al., 1994). NAluc was constructed by cloning an NcoI-HindIII fragment from the 0.4-kilobase pair PDGF-B promoter fragment into the luciferase reporter plasmid pSV0AL5. SDA1uc, SPA1uc, and SCP1uc were constructed by cloning polymerase chain reaction-generated fragments from −103 to +75, −64 to +75, and −64 to +6 of the PDGF-B promoter into the HindIII site of pSV0AL5.

**Identification of Sp3 as an SPE Binding Protein**—In an initial gel shift study of nuclear proteins that bound to the SPE element in the PDGF-B promoter, we identified Sp1 as one of the protein components of the top (t) SPE binding complex (Jin et al., 1994). In this study, approximately half of the t as well as the entire bottom (b) SPE-binding complex remained after treatment of the nuclear extract with the anti-Sp1 antiserum. Yet, the addition of competitor oligonucleotides that contained either a consensus Sp1 binding site or a CACCC box could still completely abolish the remaining t and b complexes. Because two additional Sp family members, Sp3 and Sp4, have been shown to recognize the Sp1 consensus binding site and CACCC-box with affinities similar to Sp1 (Hagen et al., 1992; Fraley and Wimbro, 1992), we sought to determine whether Sp3 and/or Sp4 were the other component(s) of the t and b SPE-binding complexes.

U2-OS nuclear extracts were first depleted of Sp1 by treatment with adenovirus major late promoter was performed with 200 ng of Hael I-linearized template (Farhan and Cornell, 1991), 6 μM MgCl2, 200 nM ATP, 600 nM CTP and UTP, 25 nM GTP, 10 μCi of [α-32P]-GTP (3000Ci/mmol) and otherwisethesameasdescribedaboveat24°C. The run-off transcript from the adenovirus major late promoter was analyzed on a 6% polyacrylamide gel. The proteins were transferred onto nitrocellulose filters (Schleicher & Schuell) using a semi-dry transfer apparatus. The filters were incubated with blocking buffer (3% bovine serum albumin, 50 μM Tris-HCl, pH 7.5, 20 mM NaCl, 10 mM Na2HPO4) overnight at room temperature. The filters were then incubated at 4°C overnight with rabbit polyclonal anti-Sp1 or anti-Sp3 antiserum (Santa Cruz) or anti-Sp1 antiserum. Stripped blots were then washed, blocked, and rehybridized with antiserum.

**RESULTS**

Identification of Sp3 as an SPE Binding Protein—In an initial gel shift study of nuclear proteins that bound to the SPE element in the PDGF-B promoter, we identified Sp1 as one of the protein components of the top (t) SPE binding complex (Jin et al., 1994). In this study, approximately half of the t as well as the entire bottom (b) SPE-binding complex remained after treatment of the nuclear extract with the anti-Sp1 antiserum. Yet, the addition of competitor oligonucleotides that contained either a consensus Sp1 binding site or a CACCC box could still completely abolish the remaining t and b complexes. Because two additional Sp family members, Sp3 and Sp4, have been shown to recognize the Sp1 consensus binding site and CACCC-box with affinities similar to Sp1 (Hagen et al., 1992; Fraley and Wimbro, 1992), we sought to determine whether Sp3 and/or Sp4 were the other component(s) of the t and b SPE-binding complexes.
from binding of the amino-terminal truncated form of Sp3.3. However, the faster migrating Sp3-containing complex (about 60 and 58 kDa), whose identities are unknown at this stage. However, the faster migrating Sp3-containing complex (about 60 and 58 kDa), whose identities are unknown at this stage. However, the faster migrating Sp3-containing complex (about 60 and 58 kDa), whose identities are unknown at this stage.

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Functional Analyses of Sp1 and Sp3 Contributions to PDGF-B Transcription—The contributions of Sp1 and Sp3 to transcription of the PDGF-B gene was evaluated using an in vitro transcription assay supported by U2-OS nuclear extracts (Fig. 3). The wild-type PDGF-B luciferase reporter plasmid (RAluc) gave a 112-nucleotide major in vitro transcript detected by primer extension analysis in this assay (Fig. 3, lane 1). The addition of an excess of either an Sp1 consensus or a wild-type SPE oligonucleotide to the incubation at concentrations known to abrogate Sp1 and Sp3 binding to the SPE produced an 85% decrease in transcript formation (Fig. 3, lanes 3 and 4). No effect was seen when an equivalent amount of a nonspecific heterologous oligonucleotide was added (Fig. 3, lane 2). Likewise, the addition of the Sp1 and SPE oligonucleotides had no nonspecific inhibitory effect upon transcription initiated from the adenovirus major late promoter (Fig. 3, lanes 6-9). These results indicated that binding of one or both of these Sp factors was necessary for PDGF-B transcription to occur in U2-OS cells.

To test the individual abilities of Sp1 and Sp3 to activate or repress PDGF-B transcription, we conducted cotransfection experiments in U2-OS cells using expression plasmids for human Sp1 and Sp3. Complexes with different electrophoretic mobilities (i.e., t and b) coincides with our observation of multiple Sp3 bands in Western blots of both U2-OS nuclear extracts (Fig. 1B) and HeLa nuclear extracts (Hagen et al., 1994). In U2-OS cells, the 97-kDa species of Sp3 is much more prominent than the other two smaller species (about 60 and 58 kDa), whose identities are unknown at this stage. However, the faster migrating Sp3-containing complex (i.e., b) observed in gel mobility shift assays seems to result from binding of the amino-terminal truncated form of Sp3.3. There was still a trace amount of the t complex remaining following the double supershift with both anti-Sp1 and anti-Sp3 antisera (Fig. 1A, lane 5). It did not disappear upon further addition of either antisera or addition of an anti-Sp4 antisera (data not shown), leaving the identity of this residual 5% of the t complex unknown.

Binding of Sp1/Sp3 Correlates with PDGF-B Promoter Activity—Our previous study indicated that directed mutations of the CACCC motif within the SPE dramatically reduced the activity of the PDGF-B promoter, whereas sequences immediately flanking the CACCC motif were less sensitive to mutation (Jin et al., 1994). To determine the effect of these mutations upon binding of Sp1 and Sp3, we performed gel mobility shift assays using double-stranded oligonucleotides that contained the same base substitutions within the SPE as those in the luciferase reporter plasmids (Jin et al., 1994). Base substitutions within the CACCC motif abolished both the t and b SPE-binding complexes, whereas oligonucleotides containing mutations of nucleotides flanking the CACCC motif still formed both SPE-binding complexes but at reduced levels (Fig. 2, A and B). In summary, mutations that functionally impaired transcription from the PDGF-B promoter also eliminated or diminished binding of Sp1 and/or Sp3; this clearly suggested that Sp1 and/or Sp3 regulated transcription of PDGF-B through binding to the CACCC motif within the SPE.

Gel mobility shift assays were performed using a wild-type SPE probe or YL0, YL3, YL5, or YL7 mutant SPE probes. The sequence of the Sp1 competitor oligonucleotide is: 5'-ATTCCGATCGGGCCGGCGGCGA (consensus Sp1 binding site is underlined). Concentrations of competitor oligonucleotides are shown as molar excesses relative to the probe concentration. Positions of the t and b complexes and the free probe are indicated. €

**Fig. 1. Gel mobility shift and Western blot assays of U2-OS nuclear extracts with anti-Sp1 and anti-Sp3 antisera.** A, gel mobility shift assays were performed using a wild-type SPE probe (5'-GGAAAGGCTGTCTCCACCCACCTCTCGCACTCTCCCTTCCTCTCCTT- T, SPE sequence underlined). The nuclear extract used in lane 2 was depleted of Sp1. Positions of the two complexes (t and b), the anti-Sp1 antiserum supershifted band and the anti-Sp3 antiserum supershifted band are indicated. B, Western blot assays were performed using U2-OS nuclear extracts as described under "Experimental Procedures." 100 µg of protein was loaded onto the gel. The filter was first probed with an anti-Sp1 antiserum (upper) and then was stripped of the probe and reprobed with an anti-Sp3 antiserum (lower). Thick arrows indicate the positions of the two species of Sp1 protein (105 and 95 kDa). Thin arrows indicate the positions of the three species of Sp3 protein (97, 60, and 58 kDa).

3 G. Suske, unpublished results.
Result also shows that both Sp1 and Sp3 can activate through sp1 binding site underlined); SPE (lanes 4 and 9), 5'-GGAAAGGGTCGTGCCTACACCCACCCACCTCTCGCACTCTCCCTTCTCCCT (SPE sequence underlined). Concentrations of competitor oligonucleotides were at a 150-fold molar excess of the template concentration. The arrows indicate the position of the in vitro transcription products initiated from the RA1uc template detected by primer extension analysis (left panel) or the run-off transcripts initiated from the adenosine major late promoter (AdML, right panel).

Sp1 or Sp3 (Fig. 4A). Cotransfection with either CMV-Sp1 or CMV-Sp3 significantly augmented the expression of a luciferase reporter gene driven by the wild-type PDGF-B promoter (pGL3-RA1uc) 10- and 7-fold, respectively. An additive effect of 16-fold increase in the expression of the luciferase reporter gene was seen when both CMV-Sp1 and CMV-Sp3 were cotransfected, suggesting that Sp1 and Sp3 can simultaneously bind and activate the PDGF-B promoter.

To determine what portion of this activation resulted from the CACCC motif within the SPE, we then cotransfected the Sp1 or/and Sp3 expression vectors with a luciferase reporter gene that contained a PDGF-B promoter with the CACCC motif within the SPE ablated (pGL3-13luc). Expression from this reporter gene was also enhanced by CMV-Sp1 or CMV-Sp3 about 7- and 4-fold, respectively, and enhanced by cotransfection of both CMV-Sp1 and CMV-Sp3 about 13-fold (Fig. 4A). This result suggests that the SPE CACCC motif was not the only site within the PDGF-B promoter through which Sp1 and Sp3 exert their effects.

Although the pGLbasic vector was designed to minimize or eliminate spurious Sp-like consensus sites from the vector, it was still possible that a significant portion of the Sp1/Sp3-induced activation might be arising from Sp1/Sp3 interactions with vector sequences. To rule out this possibility, we constructed a minimal, synthetic promoter comprised of a 20-bp oligonucleotide containing either the wild-type SPE sequence or a J13 mutant SPE sequence. This 20-mer was inserted upstream of the luciferase gene in the reporter plasmid, TIluc, which consists of the adenosine major late promoter TATA box and the murine terminal deoxynucleotidyl transferase RNA initiation sequence (Jin et al., 1994). Overexpression of Sp1 enhanced SPE-TIluc expression approximately 5.0-fold, and overexpression of Sp3 enhanced SPE-TIluc expression approximately 3.7-fold. Overexpression of both Sp1 and Sp3 enhanced SPE-TIluc expression approximately 7.4-fold (Fig. 4B), consistent with the additive effect also seen on the PDGF-B promoter (Fig. 4A). That the observed additive effect was smaller than simple summation of the individual effects may reflect saturation of the binding sites by overexpressed Sp factors. Overexpression of Sp1 or Sp3 alone or together had no significant effect upon either TIluc or J13-TIluc (Fig. 4B).

That was through GC or CACCC motifs outside of the SPE. This result also shows that both Sp1 and Sp3 can activate through the CACCC motif within the 20-bp SPE context alone as well as in a synthetic promoter, confirming that both Sp1 and Sp3 are activating transcription factors in U2-OS cells.

Multiple cis-acting Elements Are Necessary for the Full Level of PDGF-B Transcription—Our previous studies indicated that the SPE and the TATA box were the most critical cis-acting elements in the PDGF-B promoter in U2-OS cells, whereas other sequences in the 5′-flanking region of the PDGF-B promoter individually had much less of a contribution, if at all, to PDGF-B promoter activity (Jin et al., 1994). However, these sequences might be required to cooperate with the SPE and the TATA box to produce the full level of PDGF-B promoter activity. To test this hypothesis, a series of nested deletion mutations were constructed from the RA1uc reporter construct and used in transient transfection analysis (Fig. 5). Deletion of the most 5′ portion of the RA1uc promoter, to −250 bp (construct RA1uc, −250 to +75), had no effect upon promoter activity consistent with the notion that the −250 to +75 region of the PDGF-B gene contains all of the elements necessary to support...
transcription. Further 5' deletion, to −103 bp (construct SDA-luc, −103 to +75) and to −64 bp (construct SPAluc, −64 to +75), showed 56 and 94% reductions, respectively, in luciferase activities. To test whether sequences 3' of the transcription start site might also contribute to PDGF-B transcription, the SPAluc construct was further deleted at the 3' end to +6 bp (construct SPCluc, −64 to +6). The luciferase activity of SPCluc was 4-fold lower than that of SPAluc, and showed only 1.6% of the activity of the full-length promoter (RA luc). Therefore, the SPE is necessary but not sufficient for the full length transcription of the PDGF-B gene in U2-OS cells, which requires multiple elements both upstream and downstream of the SPE.

DISCUSSION

In this work, we have characterized the proteins that bind to the SPE and determined their functional contributions in regulating transcription of the PDGF-B gene in human U2-OS osteosarcoma cells. We have shown by gel mobility supershift assays that a second protein component of the t SPE-binding complex and the only protein component of the b SPE-binding complex are two species of Sp3 with different electrophoretic mobilities. We have also demonstrated that the CACCC motif within the SPE, which is essential for PDGF-B promoter activity, is essential for binding both Sp1 and Sp3. Using an in vitro transcription assay with U2-OS nuclear extracts, we have shown the complete dependence upon Sp1 and/or Sp3 for transcription of the PDGF-B gene. By cotransfection assays in U2-OS cells, we have demonstrated that both Sp1 and Sp3 can activate the PDGF-B promoter as well as a synthetic promoter through the SPE with Sp1 having a stronger activation potential than Sp3. Although the CACCC motif within the SPE is the dominant site through which Sp1 and Sp3 act, other GC and CACCC motifs in the PDGF-B promoter are likely to be responsible for part of the transcriptional activation by Sp1 or Sp3. Finally, our nested deletion analyses of the PDGF-B promoter have shown that multiple cis-acting elements are necessary for full level transcription of the PDGF-B gene in U2-OS cells.

Four members of the Sp family of transcription factors have been identified so far: Sp1, Sp2, Sp3, and Sp4. Except Sp2, all three of the other Sp factors bind to GC and CACCC box elements with comparable affinities. All three proteins contain a highly conserved DNA binding domain consisting of three zinc finger motifs at the carboxyl terminus. Sp3 and Sp4 also contain several putative functional domains homologous to those of Sp1. These include: (i) the amino-terminal glutamine and serine/threonine-rich regions similar to the A and B transactivation domains of Sp1; (ii) the highly charged domain immediately 5' of the zinc fingers, similar to the C transactivation domain of Sp1; (iii) the carboxyl-terminal domain similar to the D transactivation domain of Sp1 (Sp3). Although Sp4 is only expressed in limited cell types, Sp3, like Sp1, is ubiquitously expressed (Hagen et al., 1994). However, the function generally ascribed to Sp3 differs significantly from that of Sp1. Unlike Sp1, which is a potent activator for a wide variety of promoters, Sp3 has to date mainly been described as an inhibitory transcription factor (Hagen et al., 1994; Majello et al., 1994, 1995; Dennig et al., 1995). Sp3 can repress Sp1-mediated activation of responsive promoters, presumably by competition with Sp1 for their common binding sites. Our data indicate, however, that Sp3 can also act as an activator on the PDGF-B promoter as well as a synthetic promoter. Sp1 and Sp3 can both independently and additively activate the PDGF-B promoter through binding to the CACCC motifs within the SPE and to other GC and CACCC motifs within the PDGF-B promoter. However, functional interactions between the SPE and Sp1 or Sp3 do not appear to result in equivalent levels of activation; Sp1 appears to be a more potent activator than Sp3. This might reflect the intrinsic difference in the activation domains of Sp1 and Sp3 (Hagen et al., 1994) and/or differential regulation of Sp1 and Sp3 activity in U2-OS cells.

Besides the CACCC motif, the 10-bp sequence substituted in the J14 mutant reporter plasmid is also important for the activity of the SPE, because any of the two nucleotide transversion mutations within the J14 domain reduced the formation of SPE-binding complexes (Fig. 2B) as well as the promoter activity, and an aggregate mutation of this sequence results in promoter activity comparable with that of the CACCC knockout mutant J13 (Jin et al., 1994). This sequence does not contain any discernible transcription factor binding sites. One possible function of this sequence is that it may be required for the stabilization or correct conformation of Sp-SPE complexes formed on the adjacent CACCC site.

Our previous linker-scanning mutagenesis across the entire 250-bp 5' flanking sequence of the PDGF-B promoter identified the SPE and TATA box as the most critical cis-acting elements along with several minor ones (Jin et al., 1994). Our present nested deletion analyses demonstrated that the promoter sequence between the SPE (i.e., −64) and the +6 site of the PDGF-B promoter confers only 1.6% of the activity of which the full-length (i.e., −250 to +75) PDGF-B promoter is capable. Therefore, other cis-acting elements both upstream and downstream of the SPE, although individually not as critical as the SPE and TATA box, are required to cooperate with the SPE and TATA box to achieve full level PDGF-B promoter activity. This is consistent with the notion that synergy among multiple activators may enhance preinitiation complex formation by promoting stabilized binding of TFIIID to the promoter and subsequent recruitment of other basal transcription factors, whereas a single molecule of activator is unable to do so.
In U2-OS osteosarcoma cells, we are now able to partially explain the activated expression of the PDGF-B gene by demonstrating the functional interaction of Sp1 and Sp3 with CACC motifs within the PDGF-B promoter. More detailed understanding awaits the characterization of other cooperating elements and factors. Furthermore, other regulatory events such as DNA methylation of the endogenous PDGF-B promoter and nucleosome phasing at specific activation sites may confer higher level specificity and complexity of regulation of PDGF-B expression in vivo. Nevertheless, characterization of the SPE and its interactions with Sp1 and Sp3, which play essential roles in regulation of PDGF-B transcription, will help us to define the molecular interplay among multiple cis-acting elements and trans-acting factors in the regulation of PDGF-B transcription under various conditions. It is through a better understanding of the mechanism of activated PDGF-B transcription in U2-OS cells that we hope to further elucidate the contribution of aberrant growth factor synthesis to the neoplastic state of these human osteosarcoma cells.

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al., 1995; Buratowski, 1995; Sauer et al., 1995a, 1995b). Among the minor elements within the PDGF-B promoter are multiple GC and CACC boxes that are potential Sp1/Sp3 binding sites. Our cotransfection experiments demonstrated that both Sp1 and Sp3 could still augment luciferase expression from reporter constructs in which the CACC motif within the SPE had been mutated, although to a lesser degree than with the wild-type reporter construct. This suggests that Sp1 and Sp3 also activate through these other GC or CACC boxes and that this activation can partially compensate for the reduced expression resulting from mutation of the SPE. The loss of multiple GC and CACC boxes in the SDAluc construct possibly accounts for the 56% reduction in its promoter activity. The 7-fold reduction in promoter activity of the SPAluc construct compared with that of the SDAluc construct might result from the combined effect of losing the SIS distal element, AP-1 and ETS sites. Finally, sequences 3′ to the transcription initiation site also seem to be important because deletion of this region further reduced promoter activity 4-fold. Careful analysis of this region by systematic linker-scanning mutagenesis should identify the putative cis-acting elements.

In U2-OS osteosarcoma cells, we are now able to partially explain the activated expression of the PDGF-B gene by demonstrating the functional interaction of Sp1 and Sp3 with CACC motifs within the PDGF-B promoter. More detailed understanding awaits the characterization of other cooperating elements and factors. Furthermore, other regulatory events such as DNA methylation of the endogenous PDGF-B promoter and nucleosome phasing at specific activation sites may confer higher level specificity and complexity of regulation of PDGF-B expression in vivo. Nevertheless, characterization of the SPE and its interactions with Sp1 and Sp3, which play essential roles in regulation of PDGF-B transcription, will help us to define the molecular interplay among multiple cis-acting elements and trans-acting factors in the regulation of PDGF-B transcription under various conditions. It is through a better understanding of the mechanism of activated PDGF-B transcription in U2-OS cells that we hope to further elucidate the contribution of aberrant growth factor synthesis to the neoplastic state of these human osteosarcoma cells.

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