Transforming growth factor-β (TGF-β) family members, which include bone morphogenetic proteins (BMPs) and TGF-βs, elicit their cellular effects by activating specific Smad proteins, which control the transcription of target genes. BMPs and TGF-βs have overlapping as well as specific effects on mesenchymal cell differentiation for which the mechanisms are incompletely understood. Here we report that Id1, a dominant negative inhibitor of basic helix-loop-helix proteins, is a direct target gene for BMP. BMP, but not TGF-β, strongly activates the Id1 promoter in an Smad-dependent manner. We identified two BMP-responsive regions in the mouse Id1 promoter, which contain three distinct sequence elements; one region contains two Smad binding elements (SBEs), and the other region contains a GGCGCC palindromic sequence flanked by two CAGC and two CGCC motifs. Whereas SBEs and GGCGCC sequence are critically important, the CAGC and CGCC motifs are needed for efficient BMP-induced Id1 promoter activation. Smads are part of nuclear transcription factor complexes that specifically bind to SBEs and GGCGCC sequence in response to BMP but not TGF-β. Multimerization of the all three distinct sequence motifs is needed to generate a highly sensitive and BMP/Smad-dependent specific enhancer. Our results provide important new insights into how the BMP/Smad pathway can specifically activate target genes.

Depending on their extracellular stimuli, mesenchymal precursor cells can differentiate into muscle, fat, bone, or cartilage. Members of the transforming growth factor-β superfamily, which includes TGF-βs, activins, and bone morphogenetic proteins (BMPs), have important roles in directing cell fate choices of mesenchymal cells (1–3). BMPs stimulate osteoblast differentiation and inhibit myogenic differentiation (4–6). TGF-βs and activins inhibit myogenic and late osteoblast differentiation (2, 7–9). The mechanisms that govern the ability of different TGF-β family members to induce similar but also specific differentiation effects are unclear.

Like other members of the TGF-β superfamily, BMPs exert their effects through distinct combinations of two different types of serine/threonine kinase receptors, i.e. type I receptors (also termed activin receptor-like kinases, or ALKs) and type II receptors (10, 11). For BMPs, three distinct type II receptors for BMP, i.e. BMP type II receptor (BMPR-II) and activin type II receptors (ActR-II and ActR-IIIB), and three distinct type I receptor, i.e. BMPR-IA, BMPR-IB, and ALK-2, have been identified. The mechanism of receptor activation has been best characterized for TGF-β but is very likely to occur in an analogous fashion for BMPs (10, 11). Receptor activation involves BMP-induced hetero-oligomerization of two sequentially acting kinases, with the type I receptor acting as a substrate for the type II receptor kinase. This is consistent with the notion that the type I receptor has been shown to confer signaling specificity to the type I-type II heteromeric complex (10, 11). Activated type I receptors initiate intracellular signaling through phosphorylating specific Smad proteins (12–14). Whereas Smad2 and Smad3 are phosphorylated by TGF-β and activin receptors, Smad1, Smad5, and Smad8 are phosphorylated by BMP type I receptors.

Based upon their functional properties, Smads can be divided into three distinct subclasses: they are (i) receptor-regulated Smads (R-Smads), which transiently interact with activated type I receptors and become phosphorylated on two serine residues in their carboxy-terminal SSXS motif, (ii) common partner Smads (Co-Smads), which assemble into heteromeric complexes with R-Smads that accumulate into the nucleus, where these complexes regulate the transcription of target genes, and (iii) inhibitory Smads (i.e. Smad6 and Smad7), which potently interfere with TGF-β/Smad signaling by competing with R-Smads for interaction with type I receptors or by inducing receptor degradation (12–16).

R- and Co-Smads are transcription factors (12–14). Transcriptional activation of R- and Co-Smads is mediated via their conserved carboxy-terminal regions, known as MH2 domains, which can recruit transcriptional co-activators CBP/p300. Smad3 and Smad4 were found to preferentially recognize, via their conserved amino-terminal regions known as MH1 domains, the 5′-GTCT-3′ sequence (also termed the Smad binding element (SBE)) (17–19). Multimers of SBE, when placed in front of a minimal promoter, can provide a strong enhancer function for TGF-β. SBE-like sequences are critically important for TGF-β-induced activation of multiple TGF-β-responsive genes (12–14). BMP R-Smads have been shown to weakly bind GCAT motifs or GCCG containing sequences found in promoters of the BMP target genes Xvent2B and Smad6, respectively (20, 21). Mutation of these sequences decreased BMP-induced promoter activation. However, BMP inducibility of reporter constructs containing multimerized GC-rich sequences is very low and requires R-Smad overexpression (22,
23. Therefore, the mechanism on how the BMP/Smad pathway specifically activates target genes is still incompletely understood.

The affinity of Smads for DNA is weak (18). Smads need thus to co-operate with other DNA binding factors to bind efficiently to promoters of target genes. The 30-zinc finger nuclear protein OAZ associates with BMP R-Smads in response to BMP (24). Expression of OAZ is cell type-specific and cannot be detected in mesenchymal cells. A member of the core binding factor (CBF) family of transcription factors, Cbfal, also termed Runx-related gene 2 (RUNX2) was shown to interact directly with Smad1 and Smad5 (25, 26). RUNX2 precedes the appearance of osteoblasts, and mice deficient in RUNX2 lack osteoblasts, and bone ossification is completely blocked (27, 28). RUNX2 and Smads have been shown to co-operate in transcriptional responses and BMP-induced osteoblast differentiation (29, 30). Ectopic expression of RUNX2 in C2C12 cells was found to induce many of the extracellular matrix proteins induced by BMPs (29). However, RUNX2 alone is not sufficient to induce the whole onset of osteoblast differentiation without co-operation with Smad5 (29). Thus, other BMP targets genes with critical roles in BMP-induced osteogenesis remain to be identified.

Inhibitors of differentiation (Id) proteins act as dominant inhibitors of basic helix-loop-helix transcription factors, including members of the MyoD family of myogenic transcription factors, and also pocket proteins, including the retinoblastoma protein (31). Id and basic helix-loop-helix proteins dictate in an opposite manner cellular programs of differentiation and proliferation in various cell types. Because BMPs can inhibit Id proteins can mimic certain BMP-induced responses, e.g. inhibiting myoblast differentiation (34, 35), Id proteins may serve an important effector function for BMPs. In the present paper we show that BMP specifically transcriptionally activates Id1 expression in C2C12 myoblasts and map two distinct BMP-specific responsive regions containing three pivotal distinct sequence motifs in the Id1 promoter.

**EXPERIMENTAL PROCEDURES**

**Expression and Reporter Plasmids—**Expression plasmids for Smads and type I receptors have been previously described (36). Mouse Id1 promoter fragment (−1575/+88) was used as a template for precise mapping of the BMP-responsive element, Id1 promoter fragments were PCR-amplified using mouse Id1 (−1575/+88) fragment as a template. For precise mapping of the BMP-responsive element, Id1 promoter fragments were PCR-amplified using mouse Id1 (−1231/+88) fragment as a template and subcloned between KpnI and XbaI sites upstream of minimal adenoviral major late promoter (MLP) reporter construct (17). Site-directed mutagenesis was performed using QuikChange site-directed mutagenesis kit (Stratagene) protocol. The wild-type GAGT sites were replaced by the mutated GaAGTGAG, the wild-type CAGC sites were changed into the mutated CAGTC sequence, the wild-type GCCG elements were replaced by GaCG-mutated sequence, and the wild type GCCGCC palindrome, GC′-1, and GC′-2 sequence elements were replaced with GattaC, IγCa, tCaC, respectively. All constructs were DNA sequence-verified.

**Cell Culture and Ligands—**C2C12, HepG2, and MDA-MB468 cells were cultured in Dulbecco’s modified Eagle’s medium (Sigma) containing 10% fetal calf serum (Sigma). Cells were grown in a 5% CO2-containing atmosphere at 37 °C. Recombinant BMPs were a gift from Dr. K. Sampath (Curis, Inc.), activin A was from Dr. Y. Eto (Ajinomoto Pharmaceutical), and recombinant TGF-β3 was obtained from Dr. K. Iwata (OSI Pharmaceuticals). Transient Transfections and Transcriptional Reporter Assays—Cells were seeded at a density of 1.5 x 10⁴ cells/cm² in 12-well (C2C12 cells) or 6-well (MDA-MB468 cells) plates. The next day, cells were transiently cotransfected with the reporters in the absence or presence of expression plasmids using pcDNA plasmid to keep total amount of transfected DNA constant. Transfection was carried out using the calcium phosphate co-precipitation method (4 µg of total plasmid DNA/well in the case of MDA-MB468 and HepG2 cells or FuGENE 6 (Roche Molecular Biochemicals) transfection reagent (500 µg of total plasmid DNA/well) in the case of C2C12 cells, following the manufacturer’s protocol. β-Galactosidase co-transfection was used as an internal control for normalizing transfection efficiency. Luciferase and β-galactosidase activity were quantified using the luciferase assay (Promega) with Victor luminometer (Wallace) as described previously (39).

**RNA Isolation and Northern Blot Analysis—**RNA was isolated with using Trizol reagent (Invitrogen) in accordance with manufacturer protocol. 20 µg of total RNA/lane was loaded on denaturing (6% formaldehyde) 1% agarose gels. Blotting and hybridization using 32P-labeled (Amer sham Biosciences, Inc.) probes were performed as described previously (36).

**Construction of BMP Reporter—**Synthetic oligonucleotides 5'-CGCGGCGGCGCGCTGGAGGCTGGCCG-3' (sense) and 5'-AGGACGGGGCCGGCGGCTGGAGGCTGGCCG-3' (antisense), containing two CAGC sites (underlined), GCCGCC palindrome (overlined), and MluI 5'-overhang (double-underlined), on the sense strand, and correspondingly, 5'-TCTCTGCGCTCTAAACGCTTGCAGG-3' (sense) and 5'-CTTAGCTCAAGCCGGCTTAGGAGGCTGGCCG-3' (antisense) containing two SBE sites (underlined), one GCCGCC palindrome, two CAGC sites, and an NheI 3' site. The annealed oligonucleotides containing palindromic and two CAGC sites or two SBE sites were also used as specific competitors in electrophoretic mobility shift assays (EMSA).

To determine the functional importance of different elements that are present in BRE-Luc, the following four derivative constructs were made. (i) The CAGC-1, 2- and GC′-3,4-containing oligonucleotides were multimerized twice and subcloned into the Smad1 site of MLP-Luc, (ii) SBE-2, 3- and GC′-5-containing oligonucleotides were multimerized twice and inserted into the NheI site of BRE-Luc, and (iii) GC′-3,4-containing oligonucleotides and (iv) CAGC-1, 2-containing oligonucleotides were multimerized twice, and both were subcloned into BglII site of 2x (SBE-2, 3 and GC′-5) reporter construct.

**Western Blot Analysis—**Western blotting was performed as described previously (36). Polyclonal rabbit PS1 antibody that specifically recognizes phosphorylated Smad1 and/or Smad5 (used in a dilution 1:1000) has been previously described (40). Antibody against Id1 diluted in assay 1:1000 (was from Santa Cruz Biotechnology). Secondary horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Amer sham Biosciences, Inc.) was used in a 10³-fold dilution. Detection was performed by enhanced chemiluminescence (ECL, Amer sham Biosciences, Inc.).

**Electrophoretic Mobility Shift Assays—**Nuclear extracts from untransfected C2C12 cells and C2C12 cells stimulated with BMP-6 (100 ng/ml) or TGF-β3 (5 ng/ml) were prepared according to Schreiber et al. (41). GST-Smad fusion proteins were prepared as described before (17). The binding reaction containing 20 µl 32P-end-labeled oligonucleotides and appropriate nuclear extracts (15 µg of protein/sample) or GST-Smad proteins (500 µg of purified protein/sample) were performed as described in Denny et al. (17). A × 100, × 250, or × 500 molar excess of unlabelled oligonucleotides was used as a competitor where indicated. One µg of purified anti-Smad5 (42) and anti-Smada2 and three µg of anti-Smad4 (43) rabbit polyclonal antibodies were used for supershift experiments.

**RESULTS**

**Id1 Is a Direct BMP Target Gene—**Previous studies show that BMPs, but not TGF-β, potently induce the expression of Id proteins (32, 33, 44), which are inhibitors of basic helix-loop-helix proteins (31). Because we wanted to obtain new insights into the molecular mechanism of how certain genes can be selectively induced by BMP, but not by TGF-β, the Id genes were molecularly targeted for further study. We found that total Smad4 and BMP7 potently induce mRNA levels of Id1, Id2, and Id3 in C2C12 cells (Fig. 1A). Induction of Id expression by BMP peaks around 1–2 h. Among the three genes analyzed, Id3 was found to be most stably induced within the 1–24 h time period, and Id1 was found to be most rapidly induced upon BMP stimulation.
challenge (Fig. 1A); Id1, likely to be an immediate early BMP response gene, was therefore chosen for further analysis. Induction of Id1 by BMP6 was indeed observed in the presence of cycloheximide (CHI), indicating that de novo protein synthesis is not required for this response (Fig. 1B). Id1 is thus a direct BMP target gene. Id1 mRNA was actually more induced in the presence of BMP6 and CHI (Fig. 1B), probably as a result of an increase in mRNA stability or loss of transcriptional repressors by CHI. The addition of actinomycin D (ActD), an inhibitor of transcription, inhibited the BMP-induced effect on Id1 expression (Fig. 1B), indicating that the up-regulation of the Id1 protein is transcriptionally dependent. BMP6 stimulation also resulted in an up-regulation of the Id1 protein in C2C12 cells. This effect was blocked by ActD and CHI (Fig. 1C), indicating that in these cells BMP does not increase the Id1 expression by increasing the stability of the Id1 protein.

BMP/Smad-induced Activation of the Id1 Promoter—When we transfected the mouse or human Id1-Luc reporter constructs in C2C12 cells and stimulated the cells with BMP or TGF-β, we found that only BMPs activated these Id1 reporters (Fig. 2, A and B). On another transcriptional reporter, TGF-β activated a transcriptional response, indicating that C2C12 cells are TGF-β-responsive, as previously reported (5). Consistent with these findings, BMP-induced activation of reporter is potently inhibited by dominant negative BMP type I receptors and stimulated by constitutively active (ca) BMP type I receptors, i.e., caALK2, caALK3, and caALK6 but not caALK4 and caALK5 (which are type I receptors for TGF-β and activin, respectively) (Fig. 2C and data not shown). To examine whether Smad proteins are involved in the BMP6-induced transcriptional activation of Id1-Luc reporter, we co-transfected C2C12 cells with the mouse Id1-Luc reporter and expression constructs for R-Smads, i.e., Smad1 or Smad5 or inhibitory Smads, i.e., Smad6 or Smad7. We found that Smad1 or Smad5 strongly promoted both basal and BMP-induced activation of this reporter (Fig. 2D) and that both I-Smads strongly inhibited both basal and BMP-induced luciferase levels (Fig. 2E). These findings suggest an involvement of Smad proteins in BMP-induced activation of Id1-Luc reporter. The observation that basal levels are reduced by I-Smads suggests that there is...
autocrine BMP signaling in C2C12 cells.

To show that Smad4 is required for the BMP6-induced activation of Id1-Luc reporter, we used MDA-MB468 cells. MDA-MB468 cells are human epithelial cells deficient in Smad4. In these cells, BMP6 has no effect on Id1-Luc reporter activity (Fig. 2F). However, co-transfection of an expression construct encoding Smad4 restored the BMP6 activation of Id1-Luc reporter, demonstrating that Smad4 is necessary for the BMP6 transcriptional effect. This BMP6-induced activation of Id1-Luc reporter can be further enhanced by co-expressing Smad4 with Smad1 or Smad5. These results suggest that the Co-Smad4 and R-Smads (i.e. Smad1 and Smad5) co-operate in BMP6-induced activation of Id1-Luc reporter.

Identification of BMP-responsive Region in Mouse Id1 Promoter

By performing sequential deletion analysis of mouse Id1 promoter (Fig. 3A), we found that Id1 promoter fragment −1231 to +88 (Id(−1231/+88)) had similar activity as Id(−1575/+88). The activity was completely lost in Id(−1070/+88) reporter construct. Subsequently, we sub-cloned Id(−1231/+996) fragment into a minimal promoter reporter construct and showed that it was highly sensitive to BMP when transfected into C2C12 cells (Fig. 3B). Upon subdividing this fragment into two smaller fragments Id(−1231/+1071) and Id(−1070/+996), we (nearly) lost completely the BMP responsiveness. In addition, Id(−1231 to −1055) reporter construct shows only little BMP responsiveness, which was regained when we extended this fragment to −1025. The minimal region, which remained BMP responsive, was Id1(−1133/−1025) fragment (Fig. 3B).

Identification of Two Distinct BMP-response Elements in Id1 Promoter

Interestingly, when we compared the mouse Id1 with the human Id1 promoter sequences, which are both strongly activated by BMPs (Fig. 2, A and B), we noted that these two promoters are most similar in the identified BMP-responsive region (Fig. 4). Importantly, we noticed within the Id1(−1133/−1025) fragment the presence of five GTCT motifs, also termed SBEs (17, 19), three GCCG sequences, which have previously shown to be important for activation of BMP-responsive promoters (22, 23), five conserved CGCC, and four conserved CAGC motifs. The latter two sequence motifs have not been previously implicated as BMP response elements.

To examine the importance of SBE, GCCG, CAGC, and CGCC sequence elements in BMP-induced activation of mouse Id1 promoter, we performed site-directed mutagenesis on several of these motifs and analyzed the effect on BMP-induced transcriptional activation of the Id1-Luc reporter constructs.

Fig. 2. BMPs, but not TGF-β, activate in a Smad-dependent manner the mouse Id1 promoter in C2C12 cells. C2C12 cells were transfected with mouse Id1-Luc (A) and human Id1-Luc (B) followed by treatment with or without BMPs (100 ng/ml) or TGF-β3 (5 ng/ml). C, C2C12 cells were co-transfected with the mouse Id1-Luc reporter construct and expression constructs for dominant negative ALK2 or ALK6 followed by treatment with or without BMP6 (100 ng/ml). In addition, C2C12 cells were co-transfected with the mouse Id1-Luc reporter construct and expression constructs encoding for ca versions of ALK2, ALK4, ALK5, and ALK6. Luciferase values normalized for transfection efficiency are shown. C2C12 cells (D and E) or MDA-MB468 cells (F) were co-transfected with mouse Id1-luc reporter construct in the absence or presence of indicated Smad expression plasmids. BMP R-Smad, Smad1 and Smad5; Co-Smad, Smad4; I-Smads, Smad6 and Smad7. Basal and BMP6-induced luciferase levels with S.D. normalized for transfection efficiency are shown.
**BMP-induced Activation of the Id1 Promoter**

A. Id1-luc constructs were transfected into C2C12 cells followed by treatment with or without BMP6 (100 ng/ml). The fragments of Id1 promoter cloned upstream of the MLP luciferase reporter were transfected into C2C12 cells and subsequently treated with or without BMP6 (100 ng/ml). Fold-induction values in the presence of BMP6 compared with basal levels are indicated. Luciferase values normalized for transfection efficiency are shown.

Comparing the effect of BMP on Id1-(−1133/−996) (construct 1; Fig. 5) with Id1-(−1133/−1025) (construct 9; Fig. 5A), we found that both fragments were potently activated by BMP6, but the Id1-(−1133/−996) was consistently more so than the Id1-(−1133/−1025) (Fig. 5). This indicates that SBE-4, SBE-5, CAGC-3, and CAGC-4 (Fig. 5A) may contribute to response but are not critically important. When we mutated the GCCG elements (GC-1 and GC-3; constructs 8 and 12), we found little change on the fold of BMP activation and even an increase in the level of BMP-induced activation compared with wild-type constructs (constructs 1 and 9) (Fig. 5). Mutation of CAGC-1 and CAGC-2 showed that both sites, but in particular CAGC-1, are important in mediating BMP response to this promoter. The maximal level of BMP-induced activation is severely affected by mutation of CAGC-1, and BMP fold of activation is greatly diminished by mutation of CAGC-2. Mutation of SBE-2 and SBE-3 led to a drastic reduction in BMP6 induction compared with the wild-type Id1 promoter, indicating that these sites are critically important. Upon mutation of both sites, the reduction was even greater, and this mutated reporter construct only minimally responded to BMP6 (Fig. 5). Also, the basal activity of the reporter construct was affected by mutation of SBE-2 and SBE-3. The reduction in basal transcriptional activity is likely caused by interference with autocrine BMP signaling (Fig. 2E).

Upon mutating the GC-3,4 elements (construct 13), which are arranged as a palindrome GCCGCC, we observed a pronounced inhibitory effect on both basal and fold activation by BMP6. The inhibitory effect upon mutating the palindrome was intermediate to the effect we found upon mutating both CAGC or both SBE elements (Fig. 5). When we mutated GC-1 and GC-2 (construct 14), we affected BMP-induced activation but much less as mutating the GC-3,4 sequence motifs. GC-1 and GC-2 are needed for most efficient activation of the Id1 promoter by BMP. Because GC-1,2 sequence elements overlap with putative binding sites for Egr-1, Sp-1, and YY1, it is possible that these transcription factors participate in BMP-induced activation of Id1 promoter. However, we were unable to promote BMP-induced Id1 promoter activation by ectopic expression of YY1 and Egr-1 (data not shown).

**Binding of Smad5 and Smad4 to BMP Response Elements**—To test whether transcription factor complexes can bind to the BMP-responsive Id1 promoter fragments, we performed EMSA using nuclear extracts from C2C12 cells that were not treated or treated with BMP6 or TGF-β. We could identify binding complexes to the Id1 promoter fragment (−1070/−1025) containing SBE-2 and SBE-3 with nuclear extracts from cells treated with BMP6 but not with TGF-β. The BMP6-induced binding was specific since it could be competed with excess SBE containing oligonucleotides (Fig. 6A). In addition, no BMP6-induced binding was observed to Id1-(−1070/−1025), in which two SBEs were mutated (data not shown). Based upon previous studies (17), Smad4 is likely to mediate the direct interaction of the transcription factor complex with SBE-2 and SBE-3 containing Id1-(−1070/−1025) fragment. Indeed, we observed direct and specific binding of *Escherichia coli*-expressed GST-Smad4, but not GST-Smad5ΔMH2, to the Id1-(−1070/−1025) fragment (Fig. 6B).

To our surprise, we also found specific BMP-inducible (but not TGF-β-inducible) binding of transcription factor complexes on the Id1-(−1133/−1071) fragment containing GC-1 to GC-4 flanked by two CAGC sites using nuclear extracts from C2C12 cells (Fig. 6E). The BMP6-induced binding to Id1-(−1133/−1071) was competed with excess unlabeled wild-type fragment Id1-(−1105/−1080) containing GGGGCC palindrome flanked by two CAGC sites or the same unlabeled fragment in which the two CAGC motifs were mutated (Fig. 6C). However, BMP6-induced binding to Id1-(−1133/−1071) was not competed with the same unlabeled fragment in which the palindrome was mutated or with excess SBE oligonucleotides (Fig. 6C). These results indicate that GC motifs, but not CAGC elements, contribute mainly to the BMP6-induced binding. To determine which GC motifs are important, we compared the BMP6-induced binding to wild-type Id1-(−1133/−1071) or to the same fragment in which either GC-1,2 or GGGGCC palindrome were mutated. Whereas mutation of palindrome completely abrogated binding, BMP6-induced binding to the fragment with mutation of GC-1,-2 was slightly less efficient than to the wild type fragment (Fig. 6D). Taken together, these results indicate that GGGGCC palindrome is the main DNA binding determinant in Id1-(−1133/−1071). Furthermore, we could supershift the BMP-induced binding of the transcription factor complexes to the Id1-(−1133/−1071) fragment with a specific antibody to Smad5 and Smad4 but not with an antibody specific to Smad2 (used as a negative control in the experiment) (Fig. 6E). Thus, the BMP6-induced transcription factor complex that binds to Id1-(−1133/−1071) contains both Smad5 and Smad4. To determine whether the binding of Smads to Id1-(−1133/−1071) is direct, we used *E. coli*-expressed GST-Smad5ΔMH2 and GST-Smad4 proteins in EMSA. The Smad5ΔMH2 domain and Smad4 were found to specifically interact with Id1-(−1133/−1071) (Fig. 6E). The Smad DNA binding results are in full agreement with the activation of the Id1-luc reporter by overexpression of Smad4 and Smad5 (Fig. 2).
BMP-induced Activation of the Id1 Promoter

### Discussion

The present investigation shows that BMPs, but not TGF-β, rapidly activate the mouse Id1 promoter via two regions, one of which contains GCCG sequence elements flanked by CAGC motifs. The other region harbors two SBEs. Although SBE-2 and SBE-3 and GCCGCC palindromes are most important for DNA binding of Smads and conferring BMP responsiveness to the Id1 promoter, CAGC-1, -2, and GC'-5 motifs are needed for efficient activation.

A strict dose-dependent response is observed when cells transfected with BRE-Luc reporter are stimulated with increasing concentrations of BMP6 (Fig. 7C). The BRE-Luc reporter is also specifically activated by BMP6 but not TGF-β or activin in other BMP-responsive cell types, including HepG2 cells (Fig. 7D and data not shown). Whereas wild-type Id1 promoter can be activated by serum (37), we found that the BRE-Luc reporter is not activated by serum (Fig. 7D).
We observed that BMPs induce the Id1 expression by directly activating its promoter (Fig. 2). The effect of BMP on Id1 mRNA expression was found to be transcriptionally dependent and occurs in the absence of de novo protein synthesis (Fig. 1). Whereas a first report on BMP-enhanced Id1 expression had shown that BMP-induced effect on Id1 expression was (contrary to our findings) not blocked by ActD (33), our results are in agreement with subsequent studies in which BMP exerts effects on Id1 expression at the transcriptional level (32, 45). The level of activation of the mouse and human Id1 promoters

**Fig. 6. BMP-inducible binding of nuclear transcription factors and direct binding of Smads to Id1 promoter fragments.** A, an EMSA was performed with nuclear extracts from C2C12 cells non-treated or treated with TGF-β3 (5 ng/ml) and BMP6 (100 ng/ml) using Id1(−1070/−1025) containing SBE-2 and SBE-3 as a 32P-labeled probe. Comp., competitor. B, An EMSA of E. coli-expressed GST-Smad4 or GST-Smad5ΔMH2 using Id1(−1070/−1025) as a 32P-labeled probe. Unlabeled (SBE), was used as competitor in A and B. C, an EMSA was performed with nuclear extracts from C2C12 cells from non-treated or treated with BMP6 (100 ng/ml) and Id1(−1133/−1071) containing GC1–4 repeats flanked by CAGC motifs as a 32P-labeled probe. Wild-type Id1(−1105/−1080) (designated Pwt/CAGCwt), Id1(−1105/−1080) with either mutated GGCGCC palindrome (designated Pmut/CAGCwt), Smad4, or Smad5ΔMH2 containing SBE-2 and SBE-3 (designated (SBE)2wt), was used as competitors. D, an EMSA was performed with nuclear extracts from C2C12 cells from non-treated or treated with BMP6 (100 ng/ml) using wild-type Id1(−1133/−1071) (designated Wt) or the same fragment with GC1–4 mutated (designated GC1–4mut) or GC3–4 mutated (designated GC3–4mut) as a 32P-labeled probe. E, an EMSA was performed with nuclear extracts from C2C12 cells from non-treated or treated with BMP6 (100 ng/ml) or TGF-β3 (5 ng/ml) and Id1(−1133/−1071) as a labeled probe. Specific anti-Smad5, anti-Smad2, and anti-Smad4 antibodies (Ab) were added where indicated. F, an EMSA of E. coli-expressed GST-Smad5ΔMH2 or GST-Smad4 using Id1(−1133/1071) as a 32P-labeled probe, Id1(−1052/−1032) (designated (SBE)2) or Id1(−1105/−1080) (designated P/CAGC) were used as competitors. Where indicated, a 100-fold molar excess (in A and B) or a 250-fold molar excess (in C and F) of non-radiolabeled oligonucleotide was added as competitor. The retarded probe-protein complexes are indicated with an arrow or arrowheads, in the case of supershifted complexes.
that is achieved by BMP6 is high (17- and 15-fold for mouse and human promoters, respectively) compared with other promoters that are activated by specific extracellular stimuli. For example, we could only observe 5.5-fold induction by TGF-$\beta$ of the PAI-1 promoter, a gene that is strongly and directly induced by TGF-$\beta$ (17).

The observation that BMPs, but not TGF-$\beta$, strongly activate the Id1 promoter can be explained by the presence of Smad4 and Smad5 binding elements that are present in multiple copies in the Id1 promoter (Fig. 5). Smad5 and Smad4 are pivotal components in BMP pathway; their ectopic expression is sufficient to activate the Id1 promoter, and blocking of their activation or removing Smad4 abrogates the BMP-induced Id1-Luc activation (Fig. 2). The Id1 promoter is not activated by TGF-$\beta$, which is consistent with the finding that TGF-$\beta$ (unlike BMP) did not induce Smad binding to SBE or GGCGCC/CAGC-containing sequences in Id1 promoter fragments (Fig. 6). The flanking sequences of the SBEs previously were shown to be an important determinant for Smad binding (46). Probably, flanking sequences of the SBEs in Id1 promoter may not favor the in vivo binding of the Smad3/Smad4 complex. Of note, among the putative Drosophila Mad binding sites in vestigial quadrant enhancer and Ubx midgut enhancer, GC-rich sequences flanked by a CAGC motif were found to bind Drosophila Mad (which is highly similar to mammalian BMP R-Smads) with highest affinity (47). We are currently analyzing whether clusters of SBE and GGCGCC palindrome with flanking CAGC sequences in the promoters of other direct BMP target genes such as JunB and connective tissue growth factor are conferring BMP responsiveness to these genes.

SBE, CGCC, and to a lesser extent CAGC sequence motifs were identified as most important for BMP-induced Smad binding and activation of the Id1 promoter. SBE, but not CGCC or CAGC sequence elements, have previously been implicated in BMP R-Smad binding. The three sequence motifs are distinct but also have similarity to each other. The 5'-CAGACA-3' sequence is often found as SBE (17) in which the nucleotides indicated in bold are involved in making direct contact with Smad (18). Change of these three nucleotides indicated in bold to other nucleotides were also found to have a profound effect on Smad4 binding, whereas other nucleotides had less effect (19). The CAGC sequence motif is often followed by an extra C in the Id1 promoter and is thus similar to CAGACA sequence and may bind Smads. The CGCC element differs from the SBE sequence and has higher similarity to CAGC motif. If we presume that Smaad5 and Smad4 contact GGCGCC palindrome via their $\beta$-hairpin loops in an analogous fashion as Smad3 with SBE, then the GC-rich sequences are not expected to bind
Smad5 or Smad4 with high affinity (18). However, binding of BMP6 activated the Smad5-Smad4 complex to GGCGGC palindrome or SBE2 and SBE3 containing Id1 fragments appears equally strong. It is possible, therefore, that Smad5 and Smad4 make contact with GGCGGC palindrome via a domain distinct from a β-hairpin loop or that another cellular factor that is part of a common complex with Smad5-Smad4 facilitates the interaction of Smad5-Smad4 with GGCGGC palindrome.

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BMP induces myogenic differentiation (6). Id1 is strongly induced by BMP and is likely to be important mediator of the inhibitory effect of BMP on myogenic differentiation. Like BMPs, TGF-β also inhibits myogenic differentiation (9). However, TGF-β has been reported to have no effect on Id1 expression (44), and we found that TGF-β does not activate Id1 promoter (Fig. 2). Consistent with this notion, TGF-β has been shown to inhibit the activity of myogenin through a mechanism that is independent of interference with myogenin DNA binding (44) and is, thus, independent of Id proteins. Therefore, the mechanism by which BMP and TGF-β inhibit myogenic differentiation of mesenchymal precursor cells is likely to be different.

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