

Direct Binding of Smad1 and Smad4 to Two Distinct Motifs Mediates Bone Morphogenetic Protein-specific Transcriptional Activation of *Id1* Gene*

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Teresa López-Rovira‡§, Elisabet Chalaux‡¶, Joan Massagué||, Jose Luis Rosa‡, and Francesc Ventura‡**

From the ‡Departament de Ciències Fisiològiques II, Campus de Bellvitge, Universitat de Barcelona, Feixa Llarga s/n, 08907 L'Hospitalet de Llobregat, Spain and ||Cell Biology Program and Howard Hughes Medical Institute, Memorial Sloan-Kettering Cancer Center, New York, New York 10021

Bone morphogenetic proteins (BMPs) are potent inhibitors of myoblast differentiation and inducers of bone formation both *in vivo* and *in vitro*. Expression of *Id1*, a negative regulator of basic helix-loop-helix transcription factors, is up-regulated by BMPs and contributes to the antimyogenic effects of this family of cytokines. In this report, we have identified a specific BMP-2 immediate early response enhancer in the human *Id1* gene. Transcriptional activation of the enhancer was increased by overexpression of BMP-responsive Smads, and Smad4 and was completely abrogated in Smad4-deficient cells. Deletion analysis demonstrates that the responsive region is composed of two separate DNA binding elements, a set of overlapping GC boxes, which bind BMP-regulated Smads upon BMP stimulation, and three repeats of CAGAC boxes. Gel shift and oligonucleotide pull-down assays demonstrated that these two types of motifs were capable of binding their corresponding Smads. However, deletion or mutation of either DNA binding element was nonadditive, since disruption of either GC or CAGAC boxes resulted in complete or severe loss of BMP-2 responsiveness. These data suggest the simultaneous requirement of two independent DNA binding elements to allow functional cooperativity of BMP-regulated Smads and Smad4 in BMP-activated gene promoters.

During the process of differentiation, progenitor cells develop into more specialized phenotypes by highly specific changes in gene expression. The developmental maturation of precursor cells can be divided at least in two stages, the commitment of undifferentiated cells to a particular lineage followed by the terminal differentiation into a specific phenotype. Thus, a common progenitor such as undifferentiated mesenchymal cells can differentiate into osteoblasts, chondrocytes, myocytes, or adipocytes depending on the signals derived from the cellular environment.

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¶ Supported by a doctoral fellowship from the Fundació Pi i Sunyer.

** To whom correspondence should be addressed: Unitat de Bioquímica, Campus de Bellvitge, Universitat de Barcelona, Feixa Llarga s/n, 08907 L'Hospitalet de Llobregat, Spain. Tel.: 34-93-4024281; Fax: 34-93-4024213; E-mail: fventura@bellvitge.bvg.ub.es.

The complex framework of the molecular mechanisms by which regulation in gene expression governs cell fate includes tissue-determining transcription factors such as peroxisome proliferator-activated receptor γ 2 and C/EBPs for adipocytes or Cbfa1 for osteoblasts (1–3) as well as signal transduction regulators. One of the best known regulators of cell differentiation is the family of basic helix-loop-helix (bHLH)¹ transcription factors. Most of the members of this family have been shown to be involved in the development of different mammalian cell lineages. For example, two members of the bHLH family, MyoD and myf-5, execute myogenic lineage determination, whereas myogenin and MRF4 appear to execute the differentiation program (4). Other examples include the bHLH factors Mash1, Math, or neurogenin, which control neurogenesis in the nervous system (5, 6). All the members of this family have the ability to homo- or, more commonly, hetero-dimerize through their HLH domain and to bind DNA through their basic domain. Only one subfamily of HLH factors, known as Id proteins, lacks this basic region. Heterodimerization of Id proteins with bHLH is sufficient to block both bHLH DNA binding and function (7–9). Thus, Id proteins are mainly known as negative regulators of the commitment or differentiation that the bHLH factors promote not only in muscle cells but also in lymphoid or neurogenic precursors (10–12). Four mammalian Id proteins have been identified, Id-1 to -4, which have partially overlapping expression patterns and certain levels of functional redundancy (7, 8). For instance, mammalian Id1, -2, and -3 proteins are able to interact with E and/or myogenic proteins inhibiting muscle differentiation, whereas Id4 fails in this inhibition (13, 14).

Particular cell lineage commitment decisions depend on a complex network of gene expression of bHLH¹ transcription factors and their Id antagonists. This network is ultimately controlled by cell-intrinsic programs as well as by mutually exclusive extracellular-signaling factors. For example, a number of signals that induce the osteoblast phenotype, such as bone morphogenetic proteins (BMPs), repress myogenic differentiation *in vitro* (15–17) and induce bone formation after implantation in intramuscular sites *in vivo* (18). This ability to switch myogenic or neurogenic developmental fates has been related to the ability of BMPs to up-regulate Id1 expression and

¹ The abbreviations used are: bHLH, basic helix-loop-helix; TGF- β , transforming growth factor β ; BMP, bone morphogenetic protein; CREB, cAMP-response element-binding protein; ATF, activating transcription factor HEK-293-T; DMEM, Dulbecco's modified Eagle's medium; HEK cells, human embryonic kidney cells; bp, base pair(s); GST, glutathione *S*-transferase.

repress tissue-specific bHLH transcriptional activities (16, 19, 20).

The BMPs belong to the TGF- β superfamily of cytokines (21). Each ligand of this family exerts its biological function by inducing the formation of heteromeric complexes of specific type I and type II serine/threonine kinase receptors. Then type II receptor phosphorylates the type I receptor, which in turn propagates the signal inside the cell. Although several BMP receptor substrates and signal transducers may exist, the best known substrates and mediators of TGF- β family receptors are the Smad family of transcription factors. Eight members have been described for mammals, which fall into three subfamilies. The R-Smads are directly phosphorylated by the activated receptors at serine residues in their carboxyl terminus. R-Smads include Smads1, -5, and -8, which primarily function in BMP signaling, and the TGF- β /activin/nodal-specific Smad2 and -3. After phosphorylation, receptor-specific Smads hetero-oligomerize with Smad4, so far the only co-Smad isolated from mammals, and translocate to the nucleus. The third subfamily includes Smad6 and -7, which are named I-Smads for their ability to inhibit receptor-mediated signaling (22, 23).

Upon entry to the nucleus, Smads usually form complexes containing sequence-specific DNA binding factors and transcriptional coactivators or corepressors to achieve stable binding and transcriptional activation (24–26). Smad proteins have DNA binding activity at their amino-terminal domain (or MH1 domain) and transactivation activity in their carboxyl-terminal domain (MH2 domain). R-Smads and Smad4 bind with preference to the sequence CAGAC (27, 28), which is found in diverse TGF- β and BMP response elements (for review, see Ref. 24). However, the MH1 domain of Mad, the *Drosophila* homologue of Smad1/5, has been shown to bind a GC-rich sequence in Dpp-responsive *vestigial*, *Ultrabithorax*, *tinman*, and *labial* promoters (29–31). Smad binding to target promoters usually involves additional factors that increase the affinity and specificity of the resulting complex for the target DNA. A growing list of examples includes the proteins OAZ and Cbfa1, which direct BMP-activated Smads to *Vent-2* (32) or *osteocalcin* (33), respectively, Fast1 that directs nodal activated Smads to *Mix.2* (34), and Cbfa3 that directs TGF- β -activated Smads to *IgCa* (35).

Compared with much recent work on a large number of genes regulated by TGF- β , relatively little is known about transcriptional regulation by BMP in mammals and the interaction of mammalian Smads with natural BMP-responsive promoters. In this study, we identified a BMP-2-responsive region in the *Id1* promoter, which is an immediate early gene induced by BMP-2. This element is sufficient to confer BMP-2 responsiveness and requires Smad1/5 and Smad4 DNA binding motifs. These data suggest that a combination of distinct DNA binding activities of Smads is able to specify the choice of BMP-specific target genes independent of other transcription factors.

MATERIALS AND METHODS

Cell Culture and Transfection

C2C12 cells were cultured in DMEM supplemented with 20% fetal bovine serum and HEK-293-T cells in DMEM supplemented with 10% fetal bovine serum and 1 mM sodium pyruvate. HCT-116 and 5-18 cells (provided by B. Vogelstein, Johns Hopkins Oncology Center, Baltimore, MD) were cultured in DMEM supplemented with 10% fetal bovine serum and, for 5-18 cells, with 0.4 mg/ml G-418 plus 0.1 mg/ml hygromycin. Cells were grown in a 10% CO₂ atmosphere with 95% humidity.

C2C12 and HEK-293-T cells were transiently transfected with the indicated vectors by the calcium phosphate method as described previously (36). HCT-116 and 5-18 cells were transiently transfected with the Lipofectin procedure according to the manufacturer's protocol (Invitrogen).

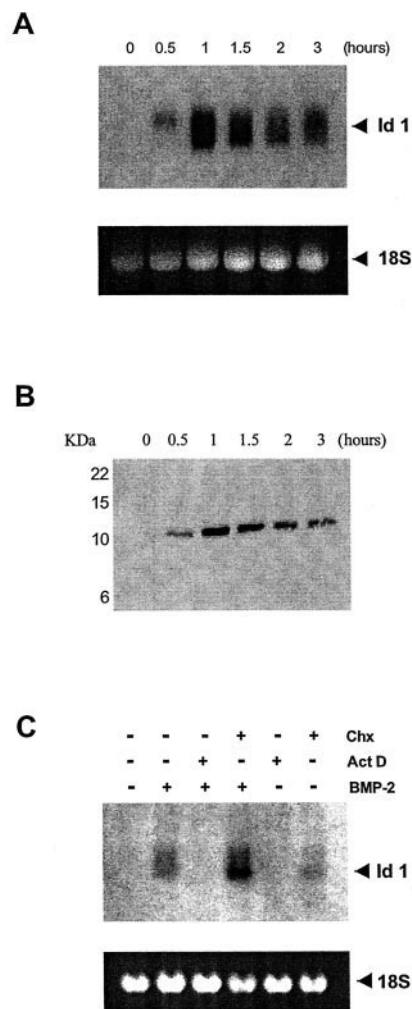


FIG. 1. *Id1* induction by BMP-2 correlates with an early pattern. C2C12 cells were treated with 1 nM BMP-2 for different times. Total RNA was extracted, analyzed by Northern blotting, and hybridized with the *Id1* probe (A), or total extracts were obtained and analyzed by Western blotting (B). C, C2C12 cells were treated with BMP-2 alone or with cycloheximide (CHX) or actinomycin-D (*Act D*) for 1 h. Total RNA was extracted, and *Id* expression was measured by Northern blot analysis.

Plasmid Constructs

The promoter region of the human *Id1* gene (–1370 to +86) was amplified by PCR and subcloned into pBluescript vector, sequenced, and further subcloned into the promoterless luciferase reporter vector pGL2-basic, pId1lux (Promega, Madison, WI). Sequences of the primers used for the PCR were 5'-GACAACTCTTCATCAGAGCTCGCT-3' (upstream) and 5'-CATGATTCCTTGTCGACTGCTGAAA-3' (downstream). 5' deletions were generated through partial and total *Sma*I digestions using the sites present in the vector and the promoter region. The pId 170 reporter construct (–170 to +86) corresponds to the minimal promoter and includes the endogenous TATA box. The 183-bp fragment was subcloned into *Sma*I sites of pId 170 or pGL2-fos, which contains the minimal *c-fos* promoter.

Constructs containing deletions within the 183-bp fragment were generated as follows. The 183-bp fragment was digested with *Hae*III, and fragments of 130 bp were subcloned into pId 170. The pId 183 reporter was digested with *Pst*I plus *Aat*II, *Nco*I plus *Aat*II, and *Pst*I plus *Nco*I, blunt-ended, and religated generating, respectively, pId 60, pId 183 Δ GC, and pId 120 reporters. pId GC was constructed by insertion of phosphorylated double-strand oligonucleotides into pId 170. Sequences of the oligonucleotides are 5'-CCATGGCGACCGCCGCGCGCGCCAGCCTGACAGTCCGTCCGGG-3' and its complementary sequence. Oligonucleotide 5'-GTCTCCATGGCTTTTATGAATGGGTGACGTCACAGG-3' and its complementary sequence was annealed and digested with *Nco*I plus *Aat*II and then subcloned into the pId 183. The resulting construct was digested with *Pst*I plus *Nco*I, blunt-ended, and

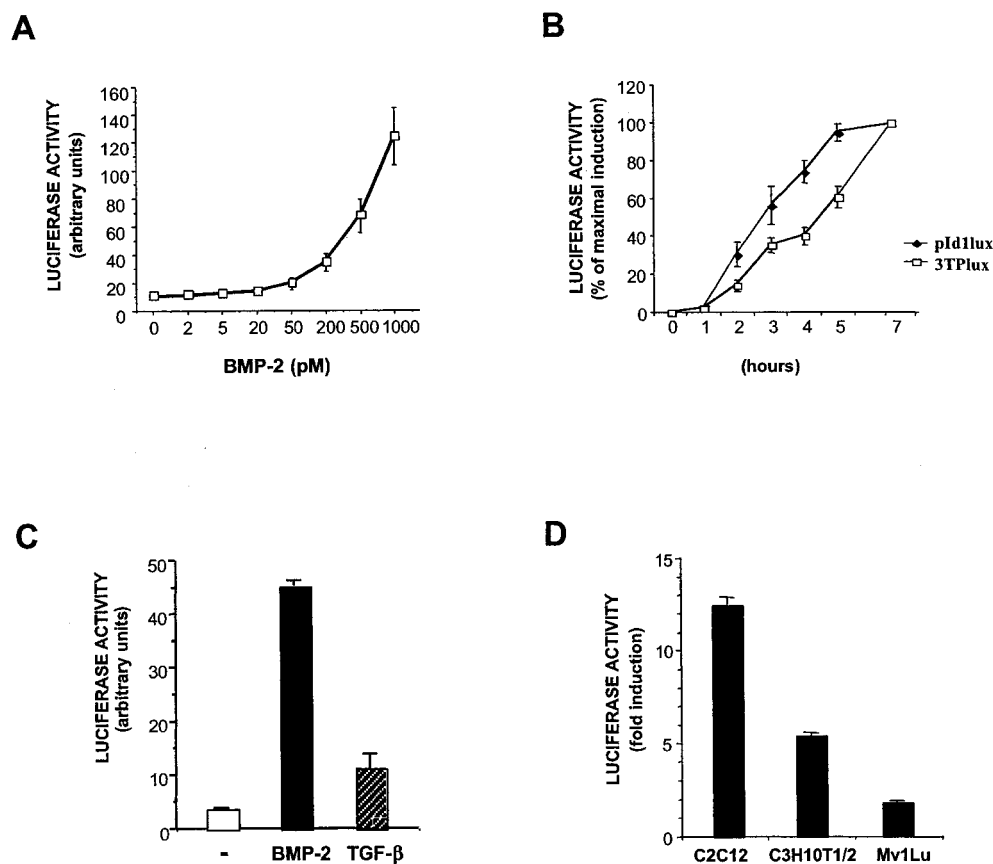


FIG. 2. Identification of a BMP-2-responsive region in the *Id1* promoter. A and C, C2C12 cells were transfected with the reporter pId1lux. 24 h later, cells were incubated with 0, 2, 5, 20, 50, 200, 500, or 1000 pM BMP-2 for 16 h (A) or 1 nM BMP-2 (B–D) or 200 pM TGF- β (B and C) in DMEM supplemented with 0.1% fetal calf serum. B, cells were transfected with pId1lux or 3TPlux and, the day after transfection, were treated with BMP-2 or TGF- β , respectively, for different times, and luciferase activity was assayed. D, C2C12, Mv1Lu, and C3H10T1/2 cells were transfected with the pId1lux reporter vector and treated for 16 h with BMP-2. The results are shown as the mean \pm S.E. of triplicates of three to five independent experiments.

religated to generate a reporter containing the minimal promoter with an 80-bp fragment originated from the 183-bp fragment. pId 120 plasmid was digested with *Aat*II, blunt-ended, and religated, generating the Δ ATF construct with a 4-bp deletion. Point mutations were performed using the QuikChangeTM site-directed mutagenesis kit (Stratagene, La Jolla, CA). Construct integrity was confirmed by restriction analysis and sequencing.

GST-Smad3 Δ MH2 (corresponding to Met¹ to Ala²²⁹) and GST-Smad4 Δ MH2 (from Met¹ to Glu³²¹) were gifts from P. ten Dijke. GST-Smad5 MH1 (from Met¹ to Phe¹⁴⁷) and GST-Smad1 MH1 (from Met¹ to Ser¹⁵¹) were generated by PCR and subcloned in the pGEX 4T-1 (Amersham Biosciences, Inc.) and verified by sequencing. Expression vector for FLAG-tagged Smad5 was provided by R. Nishimura.

Luciferase and β -Galactosidase Assays

C2C12, HCT-116, and 5-18 cells were split 24 h after transfection, cultured in DMEM supplemented with 0.1% fetal bovine serum and treated with 1 nM BMP-2 (Genetics Institute, Cambridge, MA) or 200 pM human recombinant TGF- β 1 (Sigma) for 16 h. Luciferase activities were quantified using the luciferase assay system (Promega). Luciferase values were normalized using β -galactosidase activity measured with the luminescent β -galactosidase detection kit II (CLONTECH Laboratories, Inc., Palo Alto, CA) and expressed as the mean \pm S.E. assayed in triplicate in three to five independent experiments.

RNA Analysis by Northern Blot Hybridization

RNA Isolation—C2C12 were treated with 1 nM BMP-2, 5 μ g/ml actinomycin D, or 10 μ g/ml cycloheximide for 1 h. Plates were washed twice with cold phosphate-buffered saline, and total RNA was prepared using the UltraspecTM RNA Isolation System (Biotecx Laboratories, Inc., Houston, TX).

Reverse Transcriptase-PCR—Two micrograms of total RNA was reverse-transcribed using a Ready-To-Go first-strand kit (Amersham Biosciences, Inc.) and oligo-dT as primer. PCR amplification was carried

out using 2 μ l of the reverse-transcribed RNA product and oligonucleotides Id1.1 5'-CGCTGAGGCGGCGCACTGAGG-3' and Id1.2 5'-TCAGCCAGTGATCATTGTAAT-3'. The resulting fragment was used as a probe and was directly labeled by incorporation of [α -³²P]dCTP using standard procedures.

Northern Blot Analysis—Samples of 20 μ g of RNA were separated by electrophoresis on a 1% agarose-formaldehyde gel and transferred to nylon membranes by capillary transfer. The RNA hybridization was carried out at 42 $^{\circ}$ C, and the membranes were then washed 3 times at 46 $^{\circ}$ C in 0.1% SDS and 0.1 \times SSC and subjected to autoradiography by exposing to Kodak MS film for 36 h at -80° C.

Western Blot Analysis

Total Extracts—After treatment with 1 nM BMP-2 for different times, C2C12 cells were washed with cold phosphate-buffered saline and lysed with sample buffer 1 \times (62.5 mM Tris, pH 6.8, 10% glycerol, 1% SDS, 100 mM dithiothreitol, and 0.25 mg/ml bromphenol blue).

Western Blot—Protein extracts were resolved on 15% SDS-polyacrylamide gels, transferred to nitrocellulose membranes (Millipore, Bedford, MA), and subjected to Western blot analysis using a 1:1000 dilution of antibody for Id1, sc-488 (Santa Cruz Biotechnology, Santa Cruz, CA). Immunocomplexes were visualized by developing membranes with a horseradish peroxidase-conjugated anti-rabbit IgG antibody (1:5000) followed by incubation with ECL Western blot reagent (Amersham Biosciences, Inc.).

Biotinylated Oligonucleotide Precipitation Assays

HEK-293-T cells, 48 h after transfection, or C2C12 cells, after 16 h of serum starvation, were treated with 1 nM BMP-2 for 1 h, washed twice with cold phosphate-buffered saline, and sonicated as described previously (32). Oligonucleotide 120 corresponds to the fragment included in the pId 120 reporter (the oligonucleotide 120mut is identical to 120 but contains the three CAGAC boxes mutated GTCTG to TAATG and CAGAC to CATTG); BRE oligonucleotide was described previously (32).

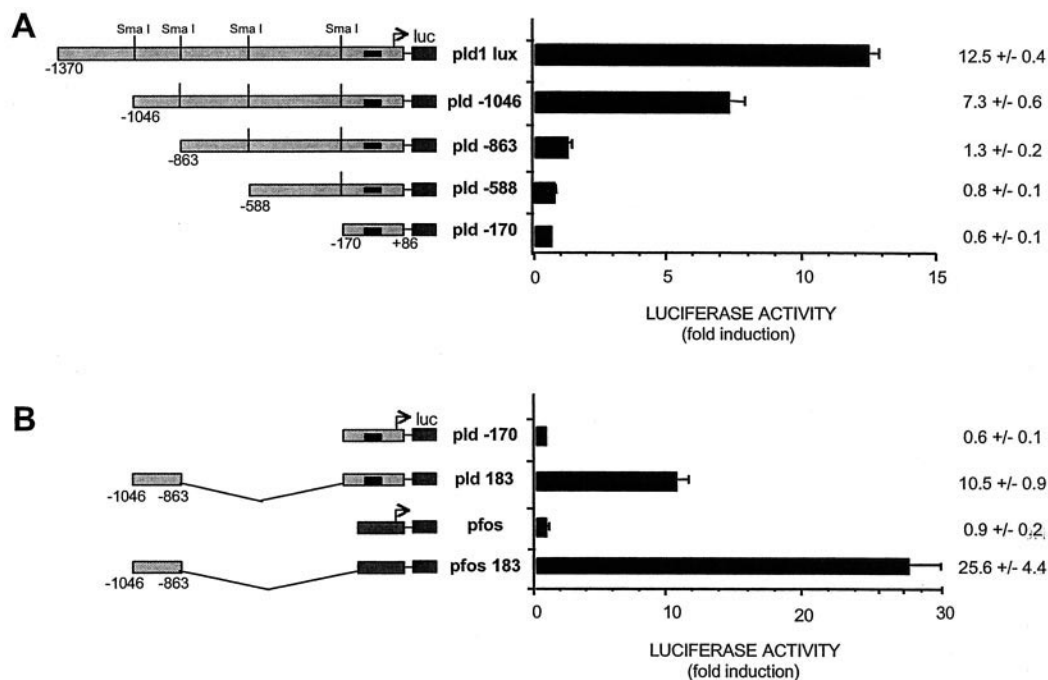


FIG. 3. **Characterization of Id promoter region required for BMP-2 responsiveness.** *A*, a series of 5' deletions of the 1.5-kilobase upstream region of the *Id* promoter were transfected in C2C12 cells. After treatment with BMP-2 1 nM for 16 h, luciferase activity was assayed. Nucleotides are numbered with respect to the transcription start site. *B*, the region comprised between -1046 and -863 was subcloned into the minimal non-responsive *Id1* promoter (pId 170) or an heterologous *c-fos* minimal promoter and transfected in C2C12 cells. 24 h later, cells were treated with BMP-2 for 16 h. The results are shown as the mean \pm S.E. of triplicates of three to five independent experiments.

After overnight incubation with biotinylated oligonucleotide, collection with streptavidin-agarose beads (Pierce), and washing with the lysis buffer, DNA-bound proteins were separated on 10% SDS-polyacrylamide gels and immunodetected as described above. Detection was performed using polyclonal anti-Smad4 or anti-Smad1 antibodies for assays with endogenous Smad4 and Smad1 or anti-FLAG (Sigma) and anti-Myc (Amersham Biosciences, Inc.) monoclonal antibodies for epitope-tagged Smads.

Electrophoretic Mobility Shift Assay

Nuclear Extracts—After 48 h of transfection, 293-T cells were washed twice with phosphate-buffered saline. Nuclear extracts were obtained as described previously (37). The protein content was determined using the Bradford protein concentration assay (Bio-Rad) with bovine serum albumin as standard.

Protein Purification—BL21 cells harboring a control plasmid or GST-Smad-encoding plasmids (GST-Smad1 MH1, GST-Smad3 Δ MH2, GST-Smad4 Δ MH2, and GST-Smad5 MH1) were grown and induced with 0.3 mM isopropyl-1-thio- β -D-galactopyranoside for 3 h. After sonication, fusion proteins were bound to with glutathione-Sepharose 4B (Amersham Biosciences, Inc.). After washing, MH1 domains were obtained by cleavage with thrombin protease (Amersham Biosciences, Inc.) at 22 °C for 16 h.

EMSA—The *Id1* promoter probes used in these assays were double-strand oligonucleotide corresponding to the complete 120-bp BMP-2-responsive region (see Fig. 5 for sequence), the 120-bp region containing the three CAGAC boxes mutated (120mut), GC oligonucleotide, and the CAGAC oligonucleotide. Probes were directly labeled by incorporation of [γ - 32 P]dATP using T4-polymerase kinase (MBI Fermentas, Vilnius, Lithuania). 10 μ g of nuclear proteins or 500 ng of purified MH1 Smad recombinant proteins were diluted to a final volume of 20 μ l in a reaction mixture containing 20 mM Tris, pH 7.9, 50 mM NaCl, 10% glycerol, 0.1 mM dithiothreitol, 1.25 μ g of poly(dI-dC). Samples were incubated at room temperature for 10 min before adding 0.1 pmol of labeled probe (5–10 \times 10 4 cpm). After a 20-min incubation, the reaction mixture was loaded onto a 5% polyacrylamide gel, 0.25 \times Tris-buffered EDTA, and 2.5% glycerol and resolved at 20 mA for 2.5 h. Gels were dried and autoradiographed. Where indicated, antibodies were added, and the reaction was incubated for an additional 15 min before loading on the gel.

RESULTS

Induction of *Id1* Expression Is an Immediate Early Response to BMP-2—Previous studies show that *Id1* mRNA levels increase after BMP-2 addition in various cell types, including mesenchymal and neuroepithelial cells (19, 20, 38, 39). To more accurately assess the temporal pattern of this response, we evaluated *Id1* mRNA and protein levels in C2C12 cells at different time points after BMP-2 addition. As shown in Fig. 1A, *Id1* mRNA levels were strongly increased upon BMP-2 stimulation, reached peak levels after 1 h, and decreased thereafter. *Id1* protein levels followed both the induction and the decay of mRNA levels, which is consistent with a rapid turnover of *Id1* protein in the cell, with a reported half-life of 20–30 min (8, 40) (Fig. 1B). This induction by BMP-2 was more evident if cells were serum-starved overnight before the addition of BMP-2, since *Id1* is also up-regulated by serum (41). To investigate whether the regulation of *Id1* by BMP-2 is mediated at the level of gene transcription and requires protein synthesis, we assessed the effect of the protein synthesis inhibitor cycloheximide and the inhibitor of transcription actinomycin D (Fig. 1C). BMP-2-mediated up-regulation of *Id1* mRNA was completely blocked by pretreatment of cells with actinomycin D (second versus third lanes from the left side), whereas the addition of cycloheximide even increased induction of *Id1* by BMP-2 (second versus fourth lanes). These data indicate that BMP-2 increases *Id1* mRNA likely through a transcriptional event that does not require protein synthesis.

To define the BMP-2-responsive elements in the *Id1* promoter, we isolated the upstream regulatory sequence of the human *Id1* gene (nucleotides -1370 to +86) by PCR and subcloned into the promoterless luciferase reporter vector pGL2-basic (referred thereafter as pId-lux). This region contains the TATA box as well as the transcription initiation site (at position +1) (41). Using this promoter-enhancer sequence to drive expression of a luciferase reporter gene, we observed strong

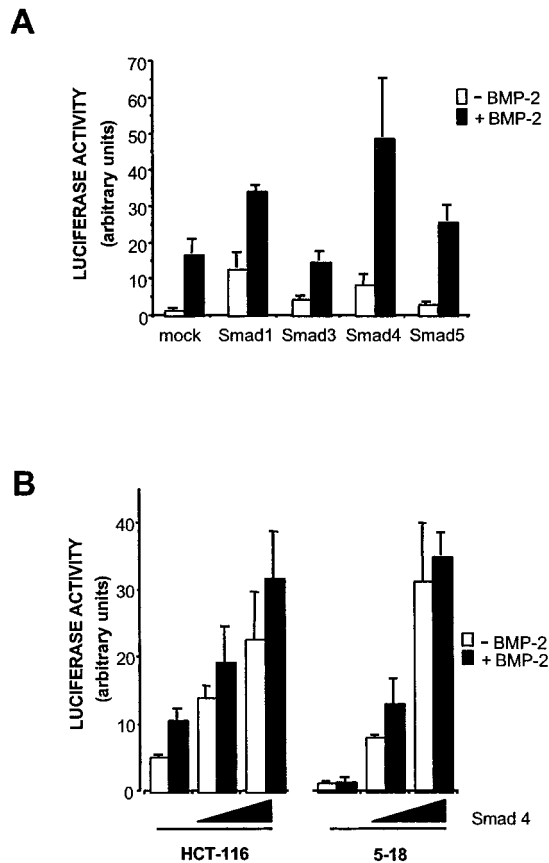


FIG. 4. Involvement of Smads in *Id1* induction by BMP-2. C2C12 cells were cotransfected with the pId 183 reporter in combination with Smads (A), HCT-116 cells and a Smad4-deficient clone, 5-18, were transfected with the pId 183 reporter, alone or with increasing amounts of Smad4 expression vector (B). Luciferase assays were performed after 16 h of treatment with 1 nM BMP-2. Total DNA was kept constant by the addition of empty vector. Results are shown as the mean \pm S.E. of triplicates of three to five independent experiments.

dose-response effects after BMP-2 addition (10–15-fold induction at 1 nM) when transfected in C2C12 cells (Fig. 2A). We then examined the time course of *Id1* reporter induction by BMP-2 in further detail. It has been shown that the reporter construct p3TP-lux binds Smad3 and -4 and confers immediate responses to TGF- β (42). As shown in Fig. 2B, C2C12 cells transfected with pId1 lux reporter construct and treated with BMP-2 gave an even faster pattern of induction than cells transfected with p3TP-lux and treated with TGF- β . It has been shown that TGF- β blocks myogenic differentiation of C2C12 cells by a mechanism independent of *Id1* and induces only a very modest up-regulation of *Id1* compared with the strong induction observed by BMP-2 (38). To determine whether pId-lux drives BMP-specific transcriptional responses, we transfected this construct into C2C12 cells. Incubation with 200 pM TGF- β in serum-depleted media for 16 h induced a marginal increase (2-fold) in reporter activity compared with the induction by BMP-2, suggesting the presence of a response element highly specific for BMP-2 (Fig. 2C). We also examined whether this promoter was BMP-2-inducible in different cell types. Mesenchymal C2C12 and C3H10T1/2 cells gave a BMP-2 responsiveness of 12- and 6-fold, respectively, whereas the epithelial Mv1Lu cell line also allowed induction by BMP-2 although to a much lesser extent (Fig. 2D). Altogether, these results suggest that this promoter sequence contains the necessary information to mediate BMP-2-dependent transcriptional activation of *Id1*, which is consistent with the transcriptional activation of the *Id1* gene in C2C12 cells.

Identification of *Id1* Promoter-Enhancer Sequences Required for BMP-2 Responsiveness—To characterize the elements within the 1.5-kilobase promoter-enhancer sequence responsible for the BMP-2-dependent transcriptional responses, we constructed a series of 5' deletions of the 1.5-kilobase upstream region included in pId-lux and transfected these constructs in C2C12 cells. Sequences from -1370 to -1046 can be deleted with a slight decrease in BMP-2-induced luciferase activity (Fig. 3A). The further removal of the region between -1046 to -863, however, resulted in a complete loss of BMP-2 responsiveness (Fig. 3A) without significant changes in basal reporter activity (data not shown). To test whether the 183-bp region (comprised between -1046 and -863) alone has the ability to render a minimal promoter responsive to BMP-2, we assayed the BMP-2 responses of two luciferase constructs containing this 183-bp region upstream of either a minimal *Id1* (from -170 to +86) or a heterologous *c-fos* minimal promoter. As shown in Fig. 3B, minimal promoters showed no response at all to BMP-2, whereas the cytokine activated both constructs bearing the enhancer. Moreover, this element behaves like a classical enhancer with similar activities when placed in both orientations (data not shown). Thus, these data suggest that the region from -1046 to -863 is necessary and sufficient for BMP-2 responsiveness of the *Id1* gene expression.

Smad1 and Smad4 Participate in Activation of the BMP-responsive Element—We next analyzed whether these transcriptional effects on *Id1* transcription were mediated through Smads. As shown in Fig. 4A, overexpression of Smad1, -5, and -4 increased both basal and BMP-2-induced activation of the minimal responsive reporter construct pId 183, whereas overexpression of Smad3 only induced a small increase in basal luciferase activity and did not modify BMP-2 responsiveness. We next assessed the functional role of Smads in transcriptional induction of the *Id1* promoter in cells lacking Smad4. Targeted deletion of Smad4 in the colorectal HCT116 cell line generated a clone (named 5-18) that lacks TGF- β and activin responses (27, 43). As shown in Fig. 4B, this Smad4-deficient clone exhibited a complete loss of BMP-2 responsiveness compared with the control HCT116 cells. In addition, co-transfection of increasing doses of Smad4 expression vector increased the basal luciferase activities in both cell lines and partially restored transcriptional responses to BMP-2 in the Smad4-deficient clone 5-18. Altogether, these data suggest that both Smad1 and -5, in conjunction with Smad4, can function as effectors of the BMP-2-induced transcription of the *Id1* gene.

Two Distinct DNA Elements Are Required for BMP-2 Responsiveness—Sequence analysis of the 183-bp enhancer reveals consensus sites for YY1, Sp1, Egr-1, ATF/CREB, and four CAGAC boxes (Fig. 5A). To investigate which part of the region was responsible of BMP-2 transcriptional induction, we performed further deletion analysis of pId120 using convenient restriction sites or oligonucleotide fusion to the minimal pId 170. 5' deletion of the region from -1046 to -985, which includes a CAGAC box, did not change BMP-2-dependent reporter inducibility when transfected into C2C12 cells (Fig. 5B). However, further deletion up to -945 or -925, which eliminates YY1, Sp1, and Egr1 binding sites in a GC-rich region, lead to almost a complete suppression of induction of reporter activity. Similarly, an internal deletion of this GC-rich region (pId 183 Δ GC) also abolished cytokine responses. In addition, constructs including 3' deletions including the three CAGAC boxes also showed loss of BMP-2 responsiveness (Fig. 5B). Taken together, the above results indicated that two separate elements, with no sequence homology between them, were the critical determinants in the *Id1* promoter-enhancer for the BMP-2 responsiveness.

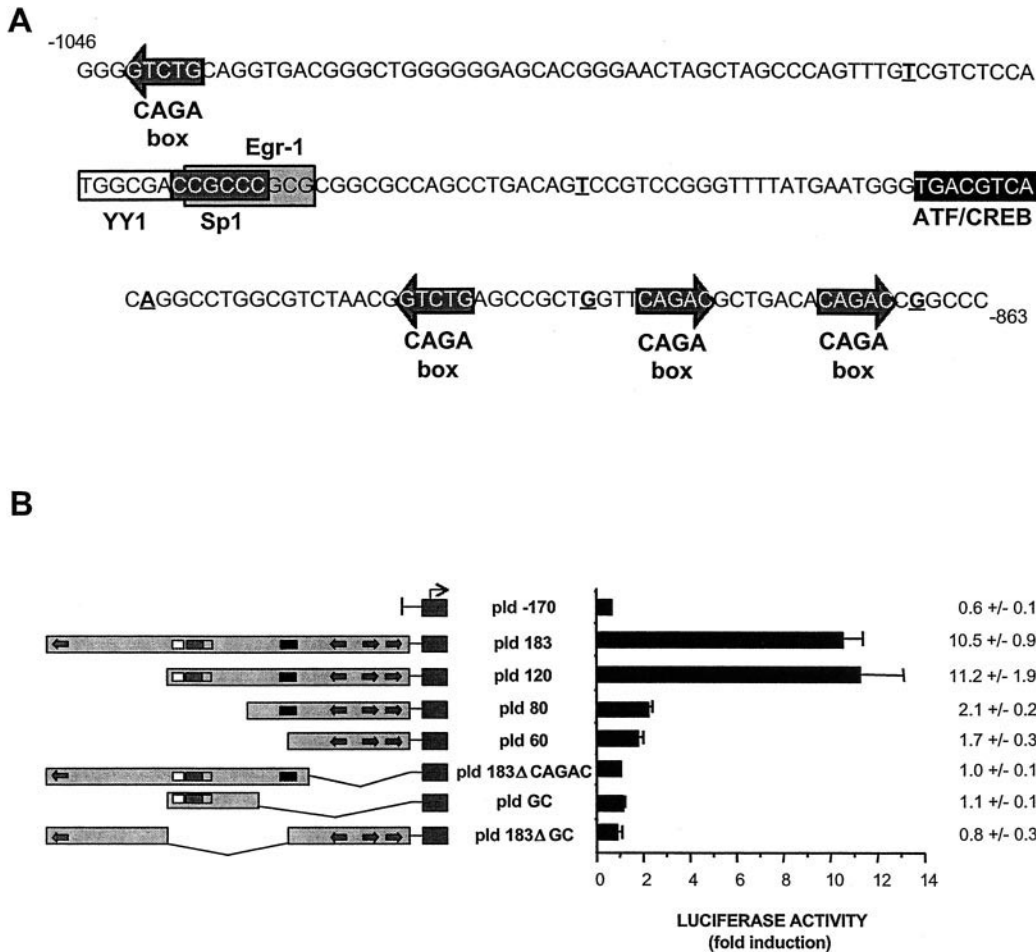


FIG. 5. **Two separate elements are required for BMP-2 responsiveness.** *A*, nucleotide sequence of the 183-bp responsive region. This 183-bp region includes different consensus sequences: 4 CAGAC boxes and YY1, Egr1, Sp1, ATF/CREB binding sites. The nucleotides *underlined* and in *bold* correspond to divergences between human and mouse or rat sequences in this region of the *Id1* promoter. *B*, deletion analysis of the 183-bp region, from -1046 to -863. As indicated, appropriate deleted constructs were transfected in C2C12 cells, and luciferase assay was performed as described. Results are shown as the mean ± S.E. of triplicates of three to five independent experiments.

Examination of these separate elements revealed that one of them contains CAGAC boxes, which have been shown to bind Smad3 and Smad4 (27, 42, 44). The other element contains a GC-rich region that includes five overlapping motifs identical or highly similar to the sequence GCCGNCGC (GC box) (Fig. 6A). This sequence has been identified as a binding site for Mad (the *Drosophila* ortholog of Smad1) in several Dpp-responsive genes (29, 30). To explore the biological significance of this motif, we generated a series of constructs containing the minimal responsive reporter construct pId 120 (from -985 to -863) with the corresponding mutations listed in Fig. 6A. As shown in Fig. 6B, point mutation of the Egr1 site (*mEgr1*) did not show significant effects on luciferase inducibility by BMP-2. However, point mutations that disrupt the two overlapping GC boxes located more 5' (GCmut1) showed about half the inducibility compared with the wild type promoter. Moreover, mutations of the three GC boxes located more 3' (GCmut2) or disruption of four GC boxes (GCmut3) strongly decreased BMP-2 responsiveness to a levels similar to those obtained with a full deletion of the GC region (previously shown in Fig. 5B). We also analyzed the effects of mutating an ATF/CREB site located between these CAGAC and GC essential regions. ATF/CREB sites and CREB or ATF-2 binding activity have been shown to cooperate with Smads in some TGF-β or BMP transcriptional responses (45–48). Fig. 6B shows that point mutation of the ATF/CREB site resulted in 20% inhibition of BMP-2 inducibility.

Direct Binding of BMP-regulated Smads to the GC-overlapping Motifs of Id1 Promoter—We next tested whether mammalian Smad1 and -4 bind to the BMP-responsive elements of *Id1* promoter using two complementary approaches. Oligonucleotide pull-down assays of HEK-293-T cells transfected with FLAG epitope-tagged Smads showed that Smad1 or Smad4 alone or in combination are able to bind to the BMP-2-responsive region (-985/-863) (Fig. 7A, left panel). On the contrary, Smad3 did not show significant binding either alone or in combination with Smad4 (Fig. 7A, right panel). To further characterize their binding specificities as well as the effects of receptor activation for Smad binding, we performed electrophoretic mobility shift assays. FLAG-tagged Smad1 was expressed in HEK-293-T cells in the presence or absence of a constitutively active form of BMPR-IB, BMPR-IB(QD). As shown in Fig. 7B, Smad1 bound to the GC-rich probe as a broad band plus other weaker bands of lower mobility, and this binding is enhanced by coexpression of BMPR-IB(QD) (third versus second lanes). These multiple bands could represent various forms of DNA binding complexes because of the multiple GC binding sites present in the enhancer, or alternatively, the Smad1-containing complexes could also incorporate endogenous Smads or coactivators in HEK-293-T cells. To further confirm that those bands correspond, in fact, Smad-containing complexes, the addition of anti-FLAG antibody supershifted the complexes (fourth and fifth lanes). We also used the complete BMP-responsive region as a probe to be shifted by the

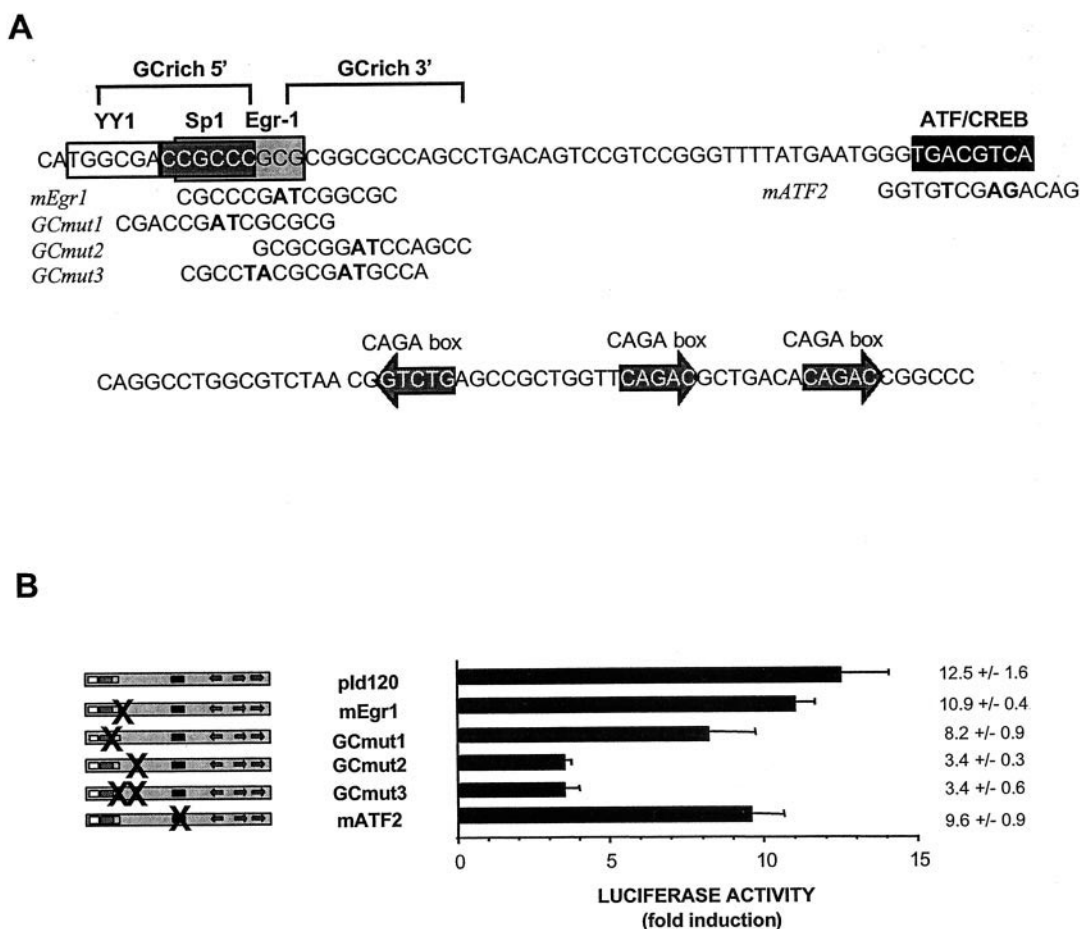


FIG. 6. **Mutational analysis of the GC-rich enhancer.** A, nucleotide sequence of the 120-bp region. Consensus sites are boxed, and substituted nucleotides are in bold. B, C2C12 cells were transfected with constructs containing the indicated mutations. After treatment with 1 nM BMP-2, cells were lysed, and luciferase activity was measured. The results are shown as the mean \pm S.E. of triplicates of three to five independent experiments.

same extracts, obtaining similar results to those of the GC region alone (data not shown).

To also define whether endogenous Smads also bind to the BMP-responsive region upon cytokine stimulation, we conducted oligo pull-down assays using C2C12 cells treated with BMP-2 for 1 h. We included as a control an oligonucleotide containing the BMP-2-responsive region of the *Xvent-2* gene (32). As shown in Fig. 7C, we could detect binding of Smad1 and Smad4 to both responsive regions. Binding of Smad1 to the Id-1-responsive region required BMP-2 stimulation, whereas Smad4 showed a slight binding in the basal state, which is strongly increased upon BMP-2 stimulation. Mutation of the three CAGAC boxes abrogates binding of Smad4 in the basal state and decreases the BMP-2-stimulated binding of both Smad1 and -4 to the mutated probe.

The previous experiments demonstrate the presence of Smad1 and 4 in the BMP-2-responsive binding complex but cannot determine whether or not binding of Smads to the distinct DNA motifs is direct. To address this issue, we used recombinant Smad-MH1 domains. As shown in Fig. 8A, Smad1, Smad3, Smad5, and Smad4 MH1 domains bound to the complete BMP-2-responsive probe. To determine their binding specificity to the distinct motifs, we performed similar assays using a probe containing the complete responsive region with mutated CAGAC motifs as well as probes exclusively containing the GC-overlapping motifs or a probe containing the three CAGAC motifs.

Whereas Smad1, -3, and -5 bound the CAGAC-mutated com-

plete probe as well as the CAGAC and the GC regions, Smad4 showed lower affinity binding to the CAGAC-mutated or GC probes compared with its binding to the CAGAC probe (Fig. 8, B, C, and D). Taken together, these results suggest that BMP-2 induces the association of BMP-restricted Smads and Smad4, which synergistically bind to their preferentially bound GC and CAGAC motifs and induce transcriptional activation of the *Id1* gene.

DISCUSSION

In this study we have identified a BMP-2 immediate early response region in the *Id1* promoter in C2C12 cells and shown the basis of selective BMP responsiveness of this region. The responsive region is composed of two distinct DNA motifs, a set of overlapping GC boxes, which differentially bind pathway-restricted Smads, and three repeats of CAGAC boxes. These two elements are capable of binding their corresponding Smads separately. However, specific transcriptional activation of *Id1* promoter requires the synergistic cooperation of both types of elements, since deletion or mutation of either element results in almost a complete loss of BMP-2 responsiveness. In conclusion, our present results suggest that cooperative direct binding of Smad proteins to distinct DNA motifs could mediate activation of BMP-specific target genes.

Id family members encode negative regulators of the bHLH transcription factors that have been found to play a central role in the control of mammalian development (6, 7, 49). Because *Id* proteins behave similarly biochemically and most tissues ap-

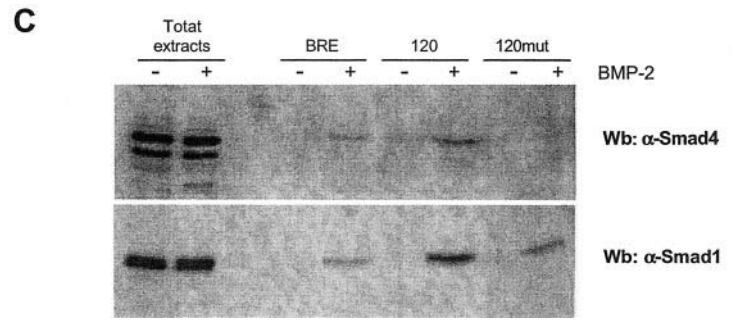
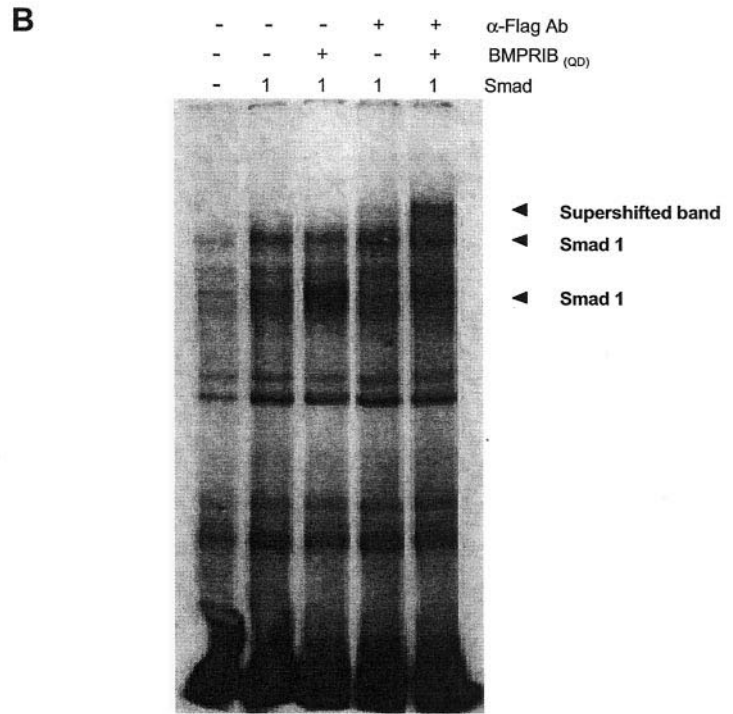
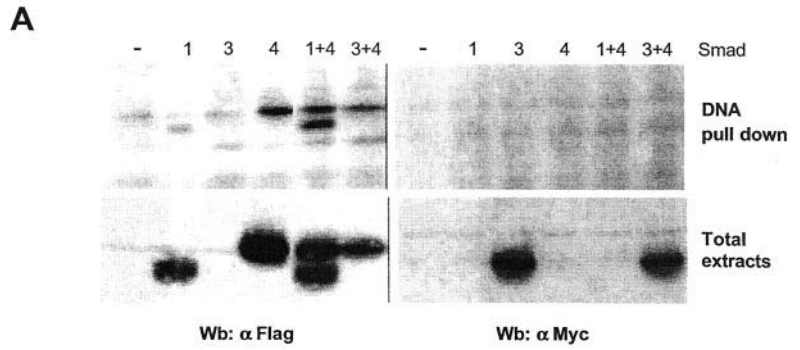


FIG. 7. Smads are capable of binding the BMP-2-responsive region. *A*, HEK-293-T cells were transfected with FLAG-epitope or Myc epitope-tagged Smads as indicated. Two days later total extracts were obtained, and oligonucleotide pull-down assay was performed using biotinylated double-stranded oligonucleotides corresponding to the 120-bp region (see “Materials and Methods” for sequence). Total extracts and the precipitated complexes were analyzed by immunoblotting using anti-FLAG and anti-Myc antibodies. *B*, nuclear extracts were obtained 2 days after transfection of HEK-293-T cells with FLAG epitope-tagged Smad1. After the addition of ³²P-labeled probe, extracts (10 μg of protein) were incubated with anti-FLAG antibody (*Ab*) where indicated. The probe corresponds to the GC-rich region (described under “Materials and Methods”). Smad overexpression was tested by Western blot (*Wb*) with anti-FLAG antibody (data not shown). *BMPR-IB*_(OD), constitutively active BMP type I receptor. *C*, C2C12 cells were treated with BMP-2 for 1 h, and the pull-down assay was performed using biotinylated double-stranded oligonucleotides corresponding to the BRE, the *Id1*-responsive 120-bp region as well as the 120-bp oligonucleotide containing the three CAGAC boxes mutated (120mut). Oligo-bound Smad1 and Smad4 were monitored by Western blot with specific anti-Smad1 and anti-Smad4 antibodies.

pear to express multiple *Id* genes, there are some levels of functional redundancy between different *Id* genes. For instance, during neurogenesis and angiogenesis, the expression patterns of *Id1* and *Id3* are highly overlapping with each other, which is supported by the fact that *Id1*^{-/-};*Id3*^{-/-} double knock-out is required for neurogenesis and angiogenesis *in vivo* (49). BMP has been shown to generate the same profile of induction for both *Id1* and -3 in myogenic and neurogenic cell lines (39). Interestingly, sequence analysis of the *Id3* promoter reveals a region with a pattern of GC motifs and CAGAC boxes spaced similarly to those present in the *Id1* promoter, raising the possibility that BMP-activated Smads may coordinately induce of *Id1* and *Id3*. BMP induction of *Id* family members

may function as a molecular switch, inhibiting developmental programs such as myogenesis or neurogenesis, which are regulated by bHLH transcription factors, thereby promoting the initiation of alternative programs such as osteogenesis or astrocytogenesis (6, 20, 38, 49). The role of Smads as nuclear effectors of TGF-β family signals is well established (for review, see Refs. 24 and 25). However, the molecular events that allow selective regulation of many important target genes of the TGF-β family remain to be defined. Only a few mammalian BMP-responsive promoters have been characterized (50–53). The relatively low DNA binding affinity and specificity of Smads has limited the progress in this area. Some of the BMP-responsive elements described to

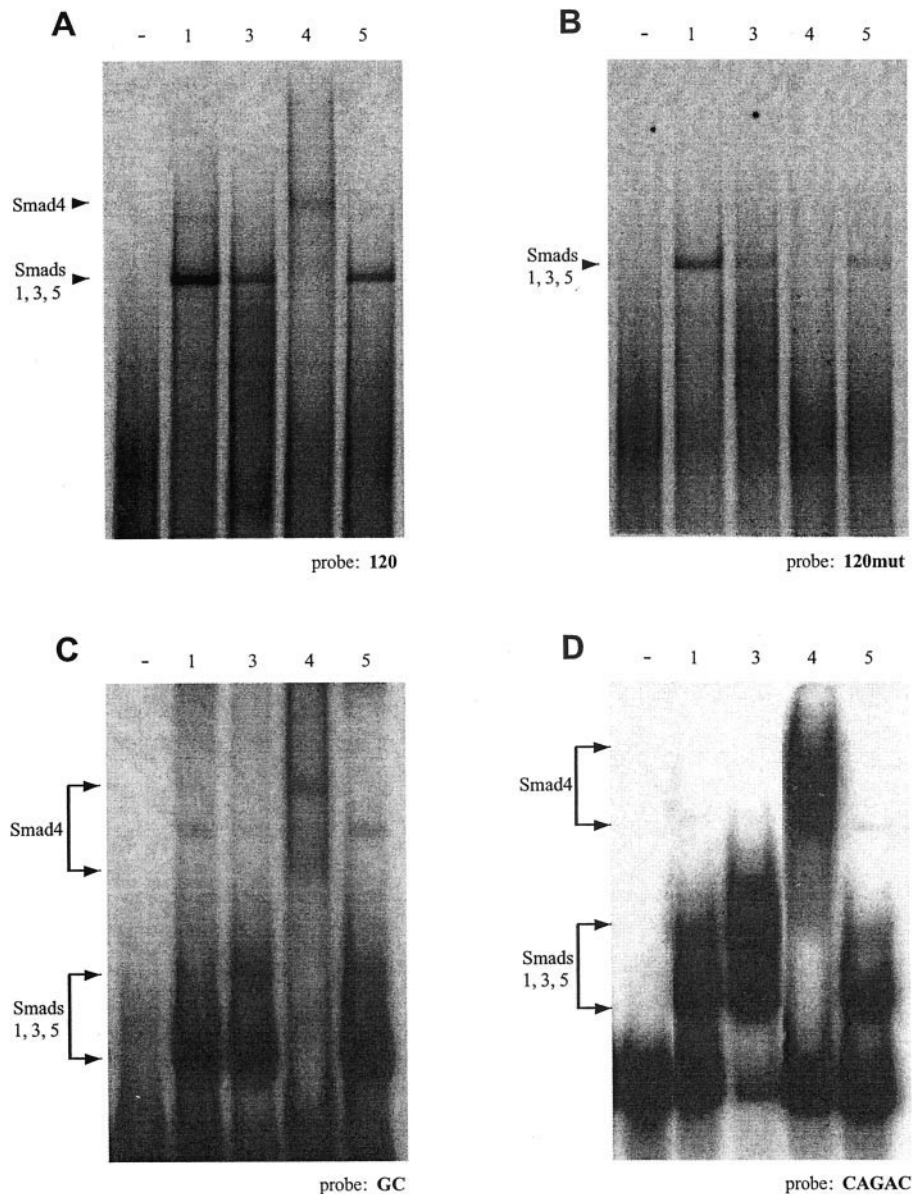


FIG. 8. Smads directly binds to the GC-rich and the CAGAC regions. Purified Smad MH1 domains were tested for DNA binding. Gel shift was performed using 32 P-labeled probes for the complete 120-bp BMP-2-responsive region (A), the 120-bp region containing the three CAGAC boxes mutated (120mut) (B), the overlapping GC motifs (C), or the three CAGAC boxes (D).

date do not discriminate between BMP, activin, or TGF- β signals (51), or their responsiveness in reporter constructs requires multimerization of BMP response elements or overexpression of BMP-signaling molecules (51–53). In contrast to these properties, the BMP-responsive region of *Id1* identified here specifically responds to BMP-2 without the need for further modification of the promoter/enhancer region or overexpression of BMP pathway components.

Our genetic evidence strongly suggests that Smads play an essential role in mediating this response. Cells with a targeted deletion of *Smad4* are unable to activate the *Id1* reporter in response to BMP-2, whereas complementation of these cells by expression of exogenous *Smad4* restores BMP-2 responsiveness. Furthermore, the ability to activate *Id1* is specific to BMP-activated Smads. Overexpression of BMP-activated Smads enhances both the basal and BMP-stimulated transcriptional activity of this promoter, whereas TGF- β -activated Smads do not.

We have identified a set of three CAGAC sequences in the *Id1* promoter as elements necessary for its responsiveness to BMP. The CAGAC motif was originally identified as a binding site for Smad3 and -4 based on random oligonucleotide selec-

tion experiments (27). The crystal structure of the MH1 domain of Smad3 bound to the CAGAC motif has been solved (44). Three amino acids in a β -hairpin that contact DNA are invariant among all Smads, suggesting that BMP and TGF- β /activin-specific Smads share this DNA contacting ability. Indeed, Smad1 has been shown to bind to the CAGAC sequence both *in vitro* (44) and in target promoters (32, 42, 53). Our results show that these CAGAC boxes in the *Id1* promoter are essential for its response to BMP.

Our results, however, also show that these CAGAC boxes are not sufficient for the BMP response. We have identified a nearby GC-rich sequence (the “GC box”) that is required, together with the CAGAC boxes, for the BMP response. Previous work on the transcriptional activation of Dpp-target genes such as *vestigial*, *tinman*, or *Ultrabithorax* in *Drosophila* indicated a direct interaction of the Smad1 orthologue, Mad, with a GC-rich motifs in the promoter region of these genes (29–31). We show that activation of the BMP-signaling pathway results in increased binding of Smad1 to the GC region from the *Id1* BMP response element. Furthermore, *in vitro* this region binds recombinant Smad MH1 domains, although Smad4 showed an

apparent preference for binding to the CAGAC region over the GC region (Fig. 8).

Pathway-restricted Smads have conserved differences in a few amino acids in the DNA binding region, which could confer different binding specificities. An interesting observation is that the helix H2 in the MH1 domain of Smads contains several lysine residues that are completely solvent exposed. BMP-activated Smads contain additional lysine residues in the H2 helix that are not present in TGF- β /activin Smads (44). Structural studies suggested that this H2 helix could be modeled into the major groove of DNA, and basic residues have been shown to bind preferentially GC rich sequences (44, 54). In agreement with this, it has been recently shown that point mutations in the H2 helix are important for specific DNA binding and transcriptional activation of Smad3 (55).

Several studies show that additional proteins may be required as DNA binding cofactors in order for BMP-activated Smads to recognize specific target genes (28, 32, 48, 56, 57). Although we cannot rule out the requirement of additional proteins for Smad recognition of the *Id1* promoter, it is possible that the cooperative binding of Smad1/5 and Smad4 to the GC box and the CAGAC boxes is sufficient for the specificity of this recognition process. Sequence comparison of the human, mouse, and rat *Id1* genes reveals that the CAGAC and GC boxes are highly conserved, whereas the sequences surrounding them are less well conserved (see Fig. 5 for details). Furthermore, mutation of Egr1 or Sp1 sites in the BMP-responsive region of *Id1* results in at most a slight decrease in inducibility (Fig. 6), and overexpression of Sp1 or Egr-1 does not augment the BMP response (data not shown). Mutation of the single ATF/CREB site only partially decreased the magnitude of the induction by BMP-2, which suggests some level of cooperativity between ATF binding factors and Smad complexes (45, 46, 48, 58). Thus, none of the other recognizable sites within the BMP-responsive region of the *Id1* promoter appears to be essential for this response.

The low binding affinity of Smads for isolated CAGAC or GC boxes would explain the need to multimerize Smad binding motifs to obtain strongly responsive reporter constructs (51, 53) and, more importantly, the requirement for clusters of multiple Smad binding motifs in natural promoter regions that may respond to Smads without the agency of other DNA binding cofactors. It would also explain our observation that either deletion of CAGAC or GC motifs abolishes the BMP responsiveness of *Id1*. The co-operative binding ability of the Smad subunits in the BMP-activated complex together with the geometry of this complex and of the matching GC and CAGAC boxes in the *Id1* promoter may provide a strong and highly selective interaction leading to transcriptional activation of this important regulator of cell differentiation.

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