Smad2 and Smad3 are downstream transforming growth factor-β (TGF-β) signaling molecules. Upon phosphorylation by its type I receptor, Smad2 or Smad3 forms a complex with Smad4 and translocates to the nucleus where the complex activates target gene transcription. In the present study, we report that Smad3 binds directly to the osteopontin (OPN) promoter and that Smad4 interacts with the Hox protein and displaces it from its cognate DNA binding site in response to TGF-β stimulation. In gel shift assays, the glutathione S-transferase-Smad3 fusion protein was found to bind to a 50-base pair DNA element (−179 to −229) from the OPN promoter. Also, we found that both Hoxc-8 and Hoxa-9 bound to a Hox binding site adjacent to Smad3 binding sequence. Interestingly, Smad4, the common partner for both bone morphogenic protein and TGF-β signaling pathways, inhibited the binding of Hox protein to DNA. FLAG-tagged Smad4 coimmunoprecipitated with HA-tagged Hoxa-9 from cotransfected COS-1 cells, demonstrating an interaction between Smad4 and Hoxa-9. Transfection studies showed that Hoxa-9 is a strong transcriptional repressor; it suppresses the transcription of the luciferase reporter gene driven by a 124-base pair OPN promoter fragment containing both Smad3 and Hox binding sites. Taken together, these data demonstrate a unique TGF-β-induced transcription mechanism. Smad3 and Smad4 exhibit different functions in activation of OPN transcription. Smad3 binds directly to the OPN promoter as a sequence-specific activator, and Smad4 displaces the transcription repressor, Hoxa-9, by formation of Smad4/Hox complex as part of the transcription mechanism in response to TGF-β stimulation.

Transforming growth factor-β (TGF-β) superfamily growth factors regulate a diverse range of cellular functions, including proliferation, differentiation, extracellular matrix secretion, and cell adhesion. TGF-β is also one of the most abundant of the known growth factors stored within the bone matrix. When bone is resorbed during remodeling, TGF-β is released and stimulates the proliferation of precursor cells of osteoblast lineage, which induces new bone formation (1, 2). TGF-β decreases bone resorption by inhibiting both proliferation and differentiation of osteoclast precursors (3, 4) and by inducing apoptosis of osteoclasts (5). Osteopontin (OPN), the major non-collagenous bone matrix protein, is a secreted, arginine-glycine-aspartate (RGD)-containing phosphorylated glycoprotein (6, 7). OPN expression is rapidly induced by both bone morphogenic protein (BMP) and TGF-β (8, 9). It is produced by osteoblasts (10), as well as osteoclasts (10, 11). OPN regulates the adhesion, attachment, and spreading of osteoclasts to the bone surface during bone resorption (12). Most recently, OPN has been demonstrated to regulate the bone remodeling in response to mechanical stress (13), a novel mechanism by which osteoclast function is related to mechanical loading of the bone tissue.

Signal transduction by members of TGF-β superfamily is mediated by two types of transmembrane receptors (14). Smads are the direct substrates for kinase receptors. Upon phosphorylation, Smads translocate to the nucleus and recruit DNA-binding protein(s) to regulate gene expression. Smads are functionally classified into three groups: receptor-regulated Smads (R-Smads) including Smad1, -5, and -8 for BMP (15–19) and Smad2 and -3 for TGF-β/activin signaling pathway (20–23); co-Smad Smad4, which hetero-oligomerizes with R-Smads (24–29); and anti-Smads, Smad6 and -7, which block signals from being transduced into the nucleus via a mechanism of associating with type I kinase receptors (30–33) or competing with Smad4 for pathway-specific Smads (34). The activated type I receptors phosphorylate specific R-Smads. Upon dissociation of the phosphorylated R-Smad from type I receptor, it interacts with Smad4 and moves to the nucleus as a heteromeric complex. Once in the nucleus, Smads can be recruited to many DNA-binding proteins such as c-Jun/c-Fos, Fast-1, Fast-2, vitamin D receptor, glucocorticoid receptor, and Hoxc-8 to regulate transcriptional responses (24, 35, 37). It is not clear, however, whether the Smad4-R-Smads complex still remains associated after translocating to the nucleus (38, 39).

In a previous study (40), we have demonstrated an interaction between Smad1 and Hoxc-8 in the BMP signaling pathway. The interaction between Smad1 and Hoxc-8 breaks the equilibrium of Hoxc-8 binding to its DNA binding site and results in the transcriptional activation of OPN in response to BMP2 stimulation (40). Here we show evidence that the DNA-binding protein Hoxa-9 interacts with Smad4, but not with Smad3 (which binds to OPN promoter), and the interaction between Smad4 and Hoxa-9 results in the transcriptional activation of OPN in response to TGF-β stimulation. Also, unlike most DNA-binding proteins interacting with Smads that are transcriptional activators, Hoxa-9 functions as a strong transcriptional repressor, similar to Hoxc-8.

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‡ The abbreviations used are: TGF-β, transforming growth factor-β; OPN, osteopontin; SBE, Smad binding element; bp, base pair; GST, glutathione S-transferase; HA, hemagglutinin; BMP, bone morphogenic protein.
Fig. 1. TGF-β activates OPN gene transcription. A, diagram of OPN promoter-reporter constructs showing wild-type (Hex-pGL3) and mutated Smad binding site (mSBE-pGL3). B and C, Hex-pGL3, mSBE-pGL3 (B), or mHex-pGL3 (C) was transfected into Mv1Lu cells alone or cotransfected with a Hoxa-9 expression vector (HA-Hoxa-9). The cells were then treated with or without 5 ng/ml TGF-β for 48 h, and the luciferase activities were measured. Luciferase activities are normalized to pRL-SV40 and are presented as relative light units of mean ± S.D. of triplicates.

EXPERIMENTAL PROCEDURES

Cell Lines—Mink lung epithelial cell line Mv1Lu (ATCC, Manassas, VA) was maintained in minimal essential medium containing 10% fetal bovine serum and nonessential amino acids. COS-1 (ATCC) cells were maintained in Dulbecco’s modified Eagle’s medium supplied with 10% fetal bovine serum.

Plasmid Constructs—OPN promoter luciferase reporter constructs (Hox-pGL3 and mHex-pGL3), bacteria expression vectors for GST-Smad2, GST-Smad3, GST-Smad4, GST-Hoxa-9, as well as mammalian expression vectors Smad3-FLAG and Smad4-FLAG were described previously (40). mSBE-pGL3, which bears four nucleotide alterations at the Smad binding site in the OPN promoter −168 to −229 region, was generated by standard polymerase chain reaction cloning technique using primers 5′-TATAACCGGCTCTAACATGGCATAATGAAAGG-3′ (upstream) and 5′-TATACCTGAGTCACAAAGAGATCGTA-3′ (downstream) and inserted in between MluI and XhoI sites of the pGL3-control vector (Promega). An MluI and an XhoI site were added to the up- and downstream primers (bold), respectively. Mutated nucleotides are shown underlined. HA-Hoxa-9 expression vector was constructed in a similar strategy using a full-length Hoxa-9 cDNA vector (40). These DNA fragments were end-labeled with [γ-32P]ATP using T4 polynucleotide kinase. The subsequent experiment was performed as described previously (41). For supershift assays, anti-FLAG M2 antibody (Eastman Kodak Co.) was added to the binding reaction and incubated for an additional 10 min at room temperature.

Immunoprecipitation and Western Blotting—COS-1 cells were transfected with expression vectors as indicated in Fig. 6 using Tfx-50 according to manufacturer’s description (Promega). Twenty-four hours post-transfection, the cells were switched to Dulbecco’s modified Eagle’s medium containing 0.2% fetal bovine serum for 12 h and then treated with 10 ng/ml TGF-β1 (R & D Systems, Minneapolis, MN) for 30 min where appropriate. The cell lysates were immunoprecipitated with anti-FLAG M2 antibody (Eastman Kodak Co.) and immunoblotted with anti-TATA-box binding protein (TBP) monoclonal antibody (Babco, Berkeley, CA) and anti-GAPDH monoclonal antibody (Promega) as described previously (41). For the gel shift assay (see Fig. 4), the cells were treated with 10 ng/ml TGF-β1 for 12 h before cells were lysed.

Transfection and Luciferase Assays—Mv1Lu cells were transiently transfected with the constructs indicated in Fig. 1 using a mixture of cationic and neutral lipids (Tfx-50, Promega) as described previously (41). When increasing amounts of expression vectors were transfected, total DNA was kept constant by addition of pcDNA3 (Invitrogen, Carlsbad, CA). An internal control plasmid pRL-SV40 was cotransfected to monitor the transfection efficiency. Luciferase activities were assayed 48 h post-transfection with separate substrates to detect the luciferase (firefly) in the promoter-luciferase reporter plasmid and to the second luciferase (Renilla), encoded by the pRL-SV40 vector (dual luciferase assay kit, Promega) according to the manufacturer’s directions. Values were normalized to the renilla luciferase activity expressed from the pRL-SV40 plasmid. Luciferase values shown in the figures are representative of transfection experiments performed in triplicate in at least three independent experiments.

Electrophoretic Mobility Shift Assays—A 50-bp SBE (Smad binding element) corresponding to nucleotides −179 to −229, and a 25-bp Smad binding element (used in Fig. 4) corresponding to nucleotides −203 to −228 of the OPN promoter region were generated by annealing pairs of oligonucleotides (sense strands are shown). 5′-CTAAATGCAGTC-TATAATGAAGGTTGATCTAATGACATGCATTACACCT-3′ for SBE (underlined sequences were changed to ACCCTT and GCGC, respectively, in mutant probe m-SBE), and 5′-CTAAATGCAGTCCTAATAATGAAAGGTTGATCTAATGACATGCATTACACCT-3′ for Smad binding element. The probe OPN5 used in Fig. 5 was described previously (40). These DNA fragments were end-labeled with [γ-32P]ATP using T4 polynucleotide kinase. The subsequent experiment was performed as described previously (41). For supershift assays, anti-FLAG M2 antibody (Eastman Kodak Co.) was added to the binding reaction and incubated for an additional 10 min at room temperature.

Immunoprecipitation and Western Blotting—COS-1 cells were transfected with expression vectors and cotransfected with anti-TATA-box binding protein (TBP) monoclonal antibody (Babco, Berkeley, CA) and anti-GAPDH monoclonal antibody (Promega) according to the manufacturer’s description (Promega). Twenty-four hours post-transfection, the cells were switched to Dulbecco’s modified Eagle’s medium containing 0.2% fetal bovine serum for 12 h and then treated with 10 ng/ml TGF-β1 (R & D Systems, Minneapolis, MN) for 30 min where appropriate. The cell lysates were immunoprecipitated with anti-TATA-box binding protein (TBP) monoclonal antibody (Babco, Berkeley, CA) and immunoblotted with anti-TATA-box binding protein (TBP) monoclonal antibody (Promega) as described previously (41). For the gel shift assay (see Fig. 4), the cells were treated with 10 ng/ml TGF-β1 for 12 h before cells were lysed.

Transfection and Luciferase Assays—Mv1Lu cells were transiently transfected with the constructs indicated in Fig. 1 using a mixture of cationic and neutral lipids (Tfx-50, Promega) as described previously (41). When increasing amounts of expression vectors were transfected, total DNA was kept constant by addition of pcDNA3 (Invitrogen, Carlsbad, CA). An internal control plasmid pRL-SV40 was cotransfected to monitor the transfection efficiency. Luciferase activities were assayed 48 h post-transfection with separate substrates to detect the luciferase (firefly) in the promoter-luciferase reporter plasmid and to the second luciferase (Renilla), encoded by the pRL-SV40 vector (dual luciferase assay kit, Promega) according to the manufacturer’s directions. Values were normalized to the renilla luciferase activity expressed from the pRL-SV40 reporter plasmid. Luciferase values shown in the figures are representative of transfection experiments performed in triplicate in at least three independent experiments.
RESULTS AND DISCUSSION

**TGF-β Activates OPN Gene Transcription**—To investigate the molecular basis of TGF-β-induced OPN expression, the effect of TGF-β on OPN promoter activity was tested. Hox-pGL3 (Fig. 1A), a previously characterized OPN promoter-luciferase reporter (40), was used. Hox-pGL3 contains a 124-bp OPN promoter fragment (nucleotides −166 to −290) inserted upstream of SV40 promoter in the pGL3-control vector. This 124-bp fragment contains a SBE (nucleotides −217 to −222) and a well characterized Hox binding site (TAAT, nucleotides −195 to −198) separated by 20 base pairs. Mv1Lu cells, a TGF-β-responsive cell line, were transiently transfected with Hox-pGL3 reporter construct. The transfected cells were then incubated in the presence or absence of 5 ng/ml TGF-β for 48 h before the luciferase activity was assayed. As shown in Fig. 1B, treatment of transfected cells with TGF-β stimulated OPN promoter-reporter activity more than 5-fold. To verify whether SBE is involved in the TGF-β-induced transcriptional activity, a shorter, mutant version of OPN promoter-luciferase reporter was constructed (nucleotides −168 to −229) in pGL3-control vector (Fig. 1A). This 61-bp OPN promoter reporter construct (mSBE-pGL3) bears four nucleotide alterations in the Smad binding sequence (CAGTCT to CCAATGG). Transfection study showed that mutation of the SBE greatly reduced the basal and TGF-β-induced reporter activity. These results suggested that the TGF-β-induced OPN gene transcription is mediated via the SBE and that the direct binding of Smad3 to OPN promoter might be required for its gene activation.

**Hoxa-9 Inhibits OPN Gene Transcription**—Previous studies showed that Hox-8 inhibits OPN gene transcription in BMP signaling pathway through a functional Hox binding site adjacent to the SBE (40). Therefore, we examined whether this Hox binding element is also involved in TGF-β signaling. A Hoxa-9 expression vector (HA-Hoxa-9) was cotransfected with Hox-pGL3, mSBE-pGL3, or mHox-pGL3 reporter construct. As shown in Fig. 1B, coexpression of a small amount of Hoxa-9 (50 ng of HA-Hoxa-9 plasmid DNA) significantly inhibited the promoter reporter activities of both wild-type (Hox-pGL3) and SBE mutated (mSBE-pGL3) constructs in the presence or absence of TGF-β treatment. However, Hoxa-9 was unable to inhibit TGF-β-induced reporter activity when hox binding site was mutated (mHox-pGL3, Fig. 1C). These results indicate that Hoxa-9 negatively regulates TGF-β-induced OPN gene transcription via this hox site. Binding of Hoxa-9 to this Hox
binding element was examined in gel shift assays. As shown in Fig. 2, GST-Hoxa-9 bound to this DNA element (lane 3). The specificity of the binding was demonstrated by competition shift assays using unlabeled specific probe (lanes 4–6), as well as nonspecific probe (lanes 7–9). The GST itself did not bind to this probe (lane 2). Lane 1 is the probe with no added protein. These results suggest that the inhibitory effect of Hoxa-9 on the OPN promoter is mediated via the Hox binding site. 

**Smad3 Binds to the OPN Promoter**—We then investigated if the direct binding of Smad2 or Smad3 to OPN promoter is required. Gel shift assays were performed using affinity-purified GST-Smad2 or GST-Smad3 fusion protein and a 50-bp DNA fragment containing SBE (−179 to −229). Fig. 3A shows that GST-Smad3 effectively bound to SBE (lane 3), while GST-Smad2 bound weakly (data not shown). Thus, we focused attention on Smad3. The specificity of Smad3 binding was shown in a competition shift assay. Unlabeled SBE dose-dependently competed for Smad3 binding (lanes 4–6), and a 100-fold excess of it completely competed off Smad3 binding (lane 6). In contrast, an equal molar amount of a nonspecific probe did not show such an effect (lanes 7–9). GST-Smad4, the common partner for all pathway-specific Smads, did not bind to SBE (lane 10), nor did it affect Smad3 binding activity (lanes 11–13). To validate the DNA sequence in the SBE that confers the binding of Smad3, the core sequences CAGTCT for SBE and TAAT for Hox protein binding sites were mutated to ACCCTT and GCCG, respectively. As shown in Fig. 3B, mutations of SBE abolished binding of Smad3 (Fig. 3B, compare lane 7 to wild-type probe, lane 3).

Studies have shown that the MH1 domain, but not the full length of Smad3, is able to bind DNA (41, 42). Under our experimental conditions, however, full-length GST-Smad3 was found to bind to OPN promoter effectively (Fig. 3). To confirm that the full length of mammalian cell-expressed Smad3 also bound to the SBE, COS-1 cells were transfected with Smad3-FLAG expression vector. The transfected cells were treated with or without TGF-β (10 ng/ml) for 12 h before lyses were prepared, then gel shift assay was performed using the SBE probe. Western blot demonstrated that Smad3 was only expressed in the cells transfected with the Smad expression plasmid (Fig. 4B). As shown in Fig. 4A, a shifted band is seen from both TGF-β-treated and untreated lysates of Smad3-FLAG transfected cells (lanes 4 and 5). In contrast, the lyses from control cells did not yield such a band (lanes 2 and 3). To verify the presence of FLAG-tagged Smad3 in this new complex, anti-FLAG M2 antibody was added to the binding reaction. The addition of antibodies disrupted the newly formed DNA-protein complex (lanes 8 and 9), indicating that the complex contained FLAG-tagged Smad3. Lane 1 contained no protein, demonstrating the quality of the probe.

**Smad4 Interacts with Hoxa-9 in Cells**—Previously we have shown that Smad1 interacts with Hoxc-8 in response to BMP stimulation (40). To test whether TGF-β regulatory Smads interact with Hoxa-9 in a way similar to the interaction between Smad1 and Hoxc-8 in regulation of gene expression, GST-Smad2, -Smad3, or -Smad4 was coincubated with GST-Hoxa-9 in the binding reaction for gel shift assay. Neither GST-Smad2 nor GST-Smad3 interacted with GST-Hoxa-9 (Fig. 5, lanes 4 and 5). GST-Smad4, which did not bind to SBE, inhibited Hoxa-9 binding (Fig. 5, lane 6; Fig. 2, lanes 10–13). Addition of Smad2 or Smad3 did not enhance the inhibitory effect of Smad4 on Hoxa-9 binding (Fig. 5, lanes 7 and 8). Furthermore, the interaction of TGF-β regulatory Smads with Hoxa-9 was examined in mammalian cells. The carboxyl-terminally FLAG-tagged Smad3/Smad4 (Smad3/Smad4-FLAG) and amino-terminally HA-tagged Hoxa-9 (HA-Hoxa-9) were coexpressed in COS-1 cells in the presence or absence of TGF-β.
A

|                  | pcDNA3 | Smad3-FLAG | Smad4-FLAG | Hoxa-9 | TA-FG-beta |
|------------------|--------|-------------|-------------|--------|------------|
| IP: α-HA         | -      | +           | +           | +      | +          |
| IP: α-FLAG       | +      | -           | -           | -      | -          |

B

Western - α - FLAG

![Western blot diagram](image)

**FIG. 6.** Smad4 interacts with Hoxa-9 in cells. Coimmunoprecipitation assay showing an in vivo interaction between Smad4 and Hoxa-9. FLAG-tagged Smad3/Smad4 (Smad/Smad-FLAG) and HA-tagged Hoxa-9 (HA-Hoxa-9) were coexpressed in COS-1 cells with or without TGF-β stimulation. Whole cell extracts immunoprecipitated were performed using anti-HA antibodies followed by Western blotting utilizing antibodies against Smad4 and Hoxa-9.

(10 ng/ml) stimulation. Whole cell extract immunoprecipitations were performed using anti-HA antibodies followed by Western blot utilizing anti-FLAG M2 antibodies (top panel). Lanes 1 and 2, lysates from pcDNA3 transfected cells; lanes 3 and 4, Smad3-FLAG coexpressed with HA-Hoxa-9; lanes 5 and 6, Smad4-FLAG coexpressed with HA-Hoxa-9; lanes 7 and 8, Smad3/4-FLAG coexpressed with HA-Hoxa-9. In lanes 2, 4, 6, and 8, cells were treated with 10 ng/ml of TGF-β for 30 min before lysates were prepared. Lanes 9 and 10 (Western), whole cell lysates from Smad3-FLAG and Smad4-FLAG, respectively, transfected cells showing the molecular sizes of both proteins. The bottom two panels are Western blots showing the expression levels of Smad3/4-FLAG and HA-Hoxa-9.

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