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Bone morphogenetic protein-2 (BMP-2) transactivates Dlx3 through Smad1 and Smad4: alternative mode for Dlx3 induction in mouse keratinocytes

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ABSTRACT

Expression of the Dlx3 homeodomain gene is induced in terminally differentiated epidermal cells. Dlx3 regulates gene expression in skin and plays important roles in patterning of the embryonic ectoderm through differential sensitivity to bone morphogenetic protein (BMP) signaling. We analyzed the expression of BMP family members in murine keratinocytes; BMP-2 is expressed in proliferative basal and differentiated suprabasal keratinocytes. BMP-2 induced transcription of Dlx3 within 12 h of treatment of keratinocytes cultured *in vitro*. We proceeded to delineate the BMP-2-responsive region to an area between –1917 and –1747 in the Dlx3 promoter. Gel shift assays with recombinant Smad1 and Smad4 demonstrated that this DNA fragment (–1917 to –1747) was competent in the formation of protein–DNA complexes. By deletion and mutational analyses we localized a Smad1/Smad4-binding site containing a GCAT motif, which showed similarity to other TGF-β family responsive elements. Supershift assays with keratinocyte nuclear extracts and antibodies against members of the Smad family showed that this motif was able to form a complex with Smad1. Mutation of the Smad1/Smad4-binding site inhibited transcriptional activation of the Dlx3 gene by BMP-2. In the hair follicle, where Dlx3 is expressed in the hair matrix cells, BMP-2 also activates Dlx3 transcription. These results provide a possible mechanism of action for the BMP signaling pathway on the regulation of Dlx3.

INTRODUCTION

Dlx3 is a homeodomain transcription factor and member of the Dlx vertebrate family that is expressed in differentiating epidermal cells. The stratified epidermis is characterized by the outward migration of keratinocytes from the proliferative basal compartment to the upper, terminally differentiated cornified layer of the skin. The differentiation process is associated with sequential induction of stratification-specific markers and enzymes (1).

Dlx3 is a transcriptional activator that is primarily expressed in the differentiated granular layer of the epidermis and in the hair matrix cells of the hair follicle and has an AT-rich consensus DNA-binding site (2). Transgenic temporal and spatial misexpression of Dlx3 in the basal layer causes an abnormal skin phenotype, characterized by cessation of proliferation and premature differentiation of the basal cells (3). Disruption of the DLX3 coding sequence has been associated with the autosomal dominant human tricho-dento-osseous syndrome, characterized by defects in ectodermal derivatives such as hair and teeth, and craniofacial bone abnormalities (4). The expression of Dlx3 is controlled by transcription factors NF-Y and Sp-1 (5) and is up-regulated by Ca^{2+} through the protein kinase C (PKC) signaling pathway. Dlx3 function is potentially regulated through post-translational phosphorylation by PKC (6).

The bone morphogenetic proteins (BMPs) were originally identified as inducers of bone and cartilage formation in ectopic tissues (7). BMPs constitute the largest subfamily in the transforming growth factor-β (TGF-β) superfamily of growth and differentiation factors, and exert pleiotropic biological effects ranging from regulation of early developmental processes to organogenesis (7–8). BMPs play important roles in many aspects of vertebrate embryogenesis. Transgenic mice misexpressing a constitutively active BMP receptor-1B caused bifurcation, duplication and posterior transformation of digits in the mouse limb (9). Patterning of embryonic ectoderm is dependent on transcription factors such as Dlx3 and Msx1, which have differential responses to BMP signaling in the ectoderm (10). In *Xenopus*, ventral ectoderm transcription of Dlx3 is regulated by BMP signaling. This is demonstrated by the negative response of Dlx3 to BMP antagonists, such as the neural inducer chordin, or a truncated BMP receptor (11). Several BMPs are expressed in hair follicle cells as well as the interfollicular epidermis. BMP-6 is expressed in the developing intermediate layers at 15.5 d.p.c. (12) and located in the suprabasal layer (13). In the mature hair follicle, BMP-2 transcripts are normally seen only in precortex cells at the base of the hair shaft (14).

BMPs bind to two types of transmembrane receptors, type I and type II, with serine/threonine kinase activity (15). Upon ligand binding, type II receptors phosphorylate the GS domain of type I receptors. The activated type I receptors then phosphorylate the Smads downstream substrates. Smads are grouped into three classes based on structure and function.

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Smad1, Smad5 and Smad8 propagate BMP signals and Smad2 and Smad3 are activated by TGF-β or activin. Smad4 is the only common Smad and can associate with receptor-regulated Smads (1–3 and 5), while Smad6 and Smad7 belong to the inhibitory class Smads. Receptor-regulated Smads are directly phosphorylated by type I receptors and then associate with Smad4. The heteromeric complexes translocate to the nucleus, where they regulate transcription of target genes in association with several nuclear partners (FAST-1, FAST-2, STAT3, Hoxc-8 and AP-1; 16–21; reviewed in 22,23). Smads mediate their effects through protein–protein and protein–DNA binding interactions and several DNA-binding motifs for Smads have been identified. PCR-based screening of random sequences identified palindromic GTCTAGAC as a Smad-binding motif (24) and a Smad3-binding motif (CAGACA) has been identified (25,26). The first demonstration that Smads can directly bind to DNA was reported in *Drosophila* (27). The Smad binding GCCG$_n$CGC (GCCG motif) sequence was also found in mammalian BMP-regulated genes (28). Recently it was reported that a GCAT motif was responsible for Smad1 binding in the Xvent-2B promoter (29).

In this report we show that BMP-2 is expressed in mouse keratinocytes and transactivates Dlx3 gene expression. We have identified a BMP-2-responsive region containing Smad1- and Smad4-binding sites (GCAT motif) in the Dlx3 promoter. We also show that this transactivation occurs in hair follicle cells through the same DNA sequence. We propose that the BMP signaling pathway is one possible mechanism of regulation of Dlx3 during epidermal differentiation.

MATERIALS AND METHODS

Plasmid construction

The –2317 to +160 upstream sequence of the Dlx3 gene was inserted into a pCAT-basic (Promega) and designated p2317CAT. All 5′-deleted clones were constructed by PCR using the oligonucleotides 5′-cgcgtcgacAGCTGGGGGCTCCAAAACAGGCCCTCC-3′ for p1917CAT, 5′-cgcgtcgacGAAGAACAGAGGGCATCCAGAGCGGG-3′ for p1797CAT, 5′-cgcgtcgacACGTTTTTCCAGGTTCAGCATCCTGTG-3′ for p1565CAT and 5′-cgctctagaAACGGGCGGAGGAGCCCAGGT-3′ (160R) with the p2317CAT vector as template. The lower case letters correspond to extensions to the Dlx3-specific sequence with underlined letters corresponding to restriction enzyme sites for *Sal*I and *Xba*I. Each PCR product was digested with *Sal*I and *Xba*I, and inserted into the pCAT-basic vector. The p1747CAT clone was made by using the *Sac*I enzyme site located in the Dlx3 promoter. To make the p1917mCAT construct containing a mutant GCAT sequence, site-directed mutagenesis was performed using the QuickChange kit following the conditions suggested by the manufacturer (Stratagene).

For expression of the Smad proteins in bacteria, the cDNA of each Smad gene was amplified by PCR using mouse keratinocyte RNA and gene-specific oligonucleotides: Smad1, 5′-cgcgtcgacATGAATGTGACAAGTTTATTTTCCTTTACAAGTCC-3′ and 5′-cgcgcggccgcTTAAGATACAGATGAAATAGGATTATGAGG-3′; Smad2, 5′-cgcgtcgacATGTCGTCCATCTTGCCATTCACGCCGCCAG-3′ and 5′-cgcgcggccgcTTATGACATGCTTGAGCAACGCACTGAAGGGG-3′; Smad3, 5′-cgcgtcgacATGTCGTCCATCCTGCCCTTCACCCCCCCGATCG-3′ and 5′-cgcgcggccgccTAAGACACACGGAACAGCGGTAGCTCGGGG-3′; Smad4, 5′-cgcgtcgacATGGACAATATGTCTATTACGAATACACCAAC-3′ and 5′-cgcgcggccgcTCAGTCTAAAGGTTGTGGGTCTGCAATCGG-3′. The PCR reaction was 36 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 68°C for 90 s. Each amplified DNA was digested with *Not*I and *Sal*I and directionally cloned into the pGEX-5X-3 GST fusion vector (Amersham).

Cell culture and transfection

Primary mouse keratinocytes were isolated from BALB/c newborn mouse skin and grown in Eagle's minimal essential medium lacking Ca^{2+}, with 8% chelex-treated fetal bovine serum (30,31). Ca^{2+} concentrations were determined by analysis in an atomic absorption spectrophotometer. Unless otherwise indicated, the Ca^{2+} concentration of the medium was adjusted to 0.05 mM to maintain a basal cell-like population of undifferentiated cells. Hair follicle cells were isolated from the dermis of newborn mouse skin using a Ficoll gradient. Briefly, neonatal skin was treated with 0.25% trypsin overnight at 4°C, after which the dermis and epidermis were separated. The dermis was treated with collagenase (0.35%) and DNase for 30 min at 37°C. The suspension was passed through Nytex gauze and centrifuged at 800 r.p.m. for 5 min. The pellet was resuspended in 8 ml of 4.5% Ficoll in 1.2 mM Ca^{2+} EMEM, laid over 5 ml of 9% Ficoll and centrifuged for 5 min at 400 r.p.m. The hair follicle buds were washed and centrifuged three times in 1.2 mM Ca^{2+} EMEM and plated on fibronectin and collagen (1%)-coated plates. Cells were grown in 1.2 mM Ca^{2+} EMEM until time of transfection.

Primary mouse keratinocytes and hair follicle cells were transfected using the FuGene6 reagent (Roche). Typically, 1 μg each CAT construct and 3 μl of FuGene6 reagent were used to transfect cells plated and cultured in 6-well plates coated with rat tail collagen (0.1 mg/ml). For hair follicle cells, 6 μl of FuGene6 reagent was used for 1 μg DNA transfection. After 4 h incubation, cells were treated with 15% glycerol in KSF medium (Gibco BRL) for 3.5 min and then maintained in growth medium with 0.05 mM Ca^{2+}. CAT activities were determined 48 h after transfection and normalized to the protein value at OD_{595}. For treatment with BMP-2, different concentrations of BMP-2 (Genetics Institute) were added to the growth medium for 24 h before the CAT assay. CAT activities were measured by the fluor diffusion CAT assay using Econofluor premixed scintillation fluid and [^{3}H]acetyl-CoA (Dupont NEN) in the linear range of the assay. Results are expressed as c.p.m. transferred to the organic solvent phase. Each experiment was done in duplicate and repeated at least three times.

RT–PCR analysis

The total RNA was prepared from cells using Trizol reagent (Gibco BRL) and the first cDNA strand was synthesized. To perform RT–PCR of Dlx3, gene-specific primers were used: 5′-TCTGGTTCCAGAACCGCCGCT-3′ and 5′-TCAGTACACAGCCCCAGGGTTA-3′. These primers result in a 340 bp PCR product. For detection of the BMP-2 transcript, the specific primers 5′-GCTAGATCTGTACCGCAGGCACTCA-3′ and 5′-GGAAATTTCAAGTTGGCTGCTGCAGGC-3′ were used to generate a 311 bp PCR product. PCR reactions were performed

for 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 68°C for 90 s. All PCR reactions were in 20 µl of 1.8 mM $MgCl_2$, 200 µM each dATP, dGTP and dTTP, 50 µM dCTP, 10 µCi [^{32}P]dCTP (3000 Ci/mmol), 20 pmol each primer and elongase enzyme mix from Gibco BRL. The RT–PCR products were separated on 6% TBE gels and exposed to X-ray film.

Preparation of nuclear extracts

Nuclear extracts were prepared from undifferentiated and differentiated primary mouse keratinocytes as described by Andreas and Faller (32). All steps were carried out at 4°C. The cells (1×10^7) were harvested, washed three times with ice-cold PBS and pelleted. The keratinocytes were resuspended in 0.4 ml of buffer A [10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM dithiothreitol (DTT) and 1 mM phenylmethylsulfonyl fluoride (PMSF)], incubated on ice for 15 min and then homogenized with a Dounce homogenizer B pestle. The homogenate was centrifuged at 14 000 r.p.m. and the nuclear pellet was resuspended in 0.2 ml of buffer B (20 mM HEPES, pH 7.9, 25% v/v glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT and 1 mM PMSF) and incubated on ice for 30 min. After centrifugation at 14 000 r.p.m. for 20 min at 4°C, the supernatant fraction was aliquoted and stored at –70°C. The protein concentration was determined using the Bradford assay following the methods described by the manufacturer (Bio-Rad).

Preparation of GST fusion proteins

GST-tagged fusion proteins were prepared according to the manufacturer's guide using glutathione–Sepharose 4B (Pharmacia). Briefly, 100 ml of cultured *Escherichia coli* BL21 (DE3) cells (Novagen) were induced with 1 mM isopropyl-β-D-thiogalactopyranoside for 3 h at 37°C, collected and lysed by sonication in 5 ml of binding buffer (20 mM Tris–HCl, pH 7.9, 500 mM NaCl and 5 mM imidazole). After centrifugation, the lysate was incubated with glutathione–Sepharose 4B and washed three times with PBS. GST fusion proteins were eluted by adding elution buffer (50 mM Tris–HCl and 10 mM reduced glutathione).

Gel shift analysis

Gel shift analysis was carried out according to Ausubel *et al.* (33). ^{32}P-end-labeled DNA fragments (~1 ng DNA) were incubated for 15 min at 4°C with nuclear extracts or recombinant proteins in a total volume of 15 µl of binding buffer (10 mM HEPES, pH 7.9, 60 mM KCl, 1 mM EDTA, 100 µg BSA, 10% glycerol and 10 mM $MgCl_2$) containing 2 µg poly(dI·dC). The products of the DNA–protein binding reaction were separated by electrophoresis on a non-denaturing 6% polyacrylamide gel in low salt TBE buffer containing 44 mM Tris, 44 mM boric acid and 1 mM EDTA. DNA–protein complexes and unbound DNA probe were visualized in the gel by autoradiography on X-ray film. For gel mobility supershift analysis, the binding reactions were performed as described above, except that the nuclear extracts were incubated for 30 min at 4°C with 2 µg each specific anti-Smad antibody (Santa Cruz) prior to addition of the radiolabeled probe.

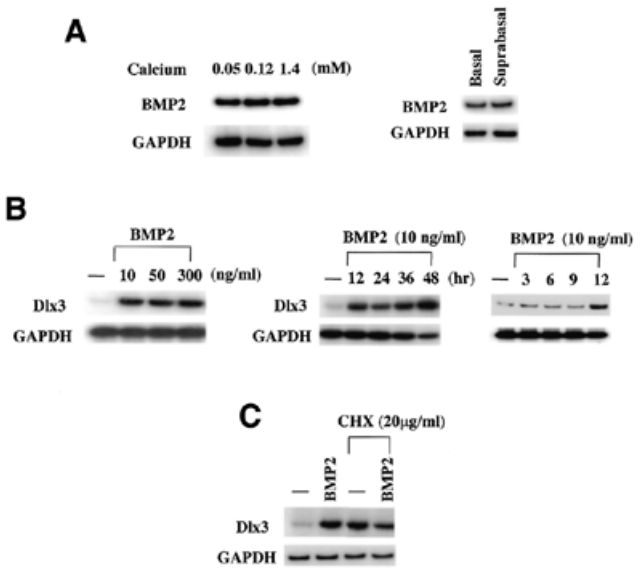

Figure 1. BMP-2 is expressed in mouse keratinocytes and activates the transcription of Dlx3. (**A**) RT–PCR analysis was performed with cDNA made from total RNA prepared from mouse keratinocytes cultured in 0.05, 0.12 or 1.4 mM Ca^{2+} using specific primers for BMP-2. (Right) RT–PCR analysis of cDNA from basal and suprabasal cells of mouse neonatal skin separated on a Percoll gradient. For normalization of the amount of RNA used for RT–PCR, GAPDH RT–PCR products are shown on the bottom line. (**B**) Total RNA was prepared from mouse keratinocytes grown in 0.05 mM Ca^{2+}, which were treated with 10, 50 or 300 ng/ml BMP-2 for 24 h and then RT–PCR analysis was performed with specific primers for Dlx3 (left). RNA from keratinocytes was treated with 10 ng/ml BMP-2 for 12, 24, 36 and 48 h (center) and 3, 6, 9 and 12 h (right). (**C**) Mouse keratinocytes were incubated with 20 µg/ml cycloheximide (CHX) for 30 min and cultured in the absence or presence of 10 ng/ml BMP-2 for 24 h and then RT–PCR analysis was performed with Dlx3-specific primers.

RESULTS

RT–PCR analysis of BMP-2 in murine keratinocytes *in vitro*

To determine the expression pattern of BMP-2 in keratinocytes, we performed RT–PCR using specific primers for murine BMP-2 from mouse keratinocytes cultured in 0.05, 0.12 and 1.4 mM Ca^{2+}. At these concentrations of calcium the keratinocytes present a basal (0.05 mM Ca^{2+}) or a differentiated phenotype (0.12 or 1.4 mM Ca^{2+}) (30). We found that BMP-2 was expressed in mouse keratinocytes independent of Ca^{2+} concentration (Fig. 1A). We also tested whether BMP-2 is expressed in basal and suprabasal keratinocytes separated on a Percoll gradient. Expression of BMP-2 is also detected in both cell types (Fig. 1A, right). To examine whether Dlx3 is a target for BMP-2 signaling in keratinocytes, we treated mouse primary keratinocytes grown in 0.05 mM Ca^{2+} (which maintains the basal condition) with increasing concentrations of BMP-2 and performed RT–PCR analysis with specific primers for Dlx3. At this Ca^{2+} concentration Dlx3 is not expressed or is present at very low levels in normal mouse keratinocytes cultured *in vitro* (5). As shown in Figure 1B, Dlx3 transcription increased upon treatment with 10 ng/ml BMP-2, with no further increments at higher BMP-2 concentrations. BMP-2 at 10 ng/ml was the concentration used for future experiments. The levels of Dlx3 mRNA were strongly induced 12 h after

treatment with BMP-2 and remained elevated throughout the duration of experiment (Fig. 1B, center). To further delineate the earliest time point of Dlx3 induction by BMP-2, we carried out assays 3, 6, 9 and 12 h after treatment. As shown in Figure 1B (right), the induction level of Dlx3 mRNA increased dramatically after 12 h treatment.

To examine whether treatment with BMP-2 affected the transcription of structural terminal differentiation markers in keratinocytes, RT–PCR analyses were performed with specific primers for keratin1, filaggrin and transglutaminase K. There was no change in the transcription of terminal differentiation markers (data not shown). Cycloheximide treatment of keratinocytes grown in 0.05 mM Ca^{2+} (basal conditions) resulted in activation of Dlx3 expression, as shown in Figure 1C, which suggests that there is an inhibitory factor regulating Dlx3 expression under basal culture conditions. Pretreatment of the keratinocytes with cycloheximide and consequent BMP-2 treatment did not show additional activation of Dlx3 transcription (Fig. 1C). This result suggests that BMP-2-mediated activation of Dlx3 might also require new protein synthesis and/or that BMP-2 treatment results in the removal of an inhibitor of Dlx3 expression.

Identification of the BMP-2-responsive region in the Dlx3 promoter

Several deletion mutants were constructed and tested for BMP-2 responsiveness (Fig. 2A). Each deletion construct was transfected into primary mouse keratinocytes and the cells were treated with 10 ng/ml BMP-2 for 24 h. The results of the CAT assays are shown in Figure 2B. The Dlx3 promoter construct containing nucleotides –1213 to +160 (5) did not respond to BMP-2 treatment. Therefore, we extended the sequence of the Dlx3 promoter region analyzed to nucleotides –2317 to +160. p2317CAT showed a 2-fold induction of transcriptional activity after BMP-2 treatment. The deletion construct p1917CAT exhibited induced CAT activity, while p1747CAT, p1565CAT and p1213CAT were unaffected. These results indicate that the BMP-2-responsive element resided in the region between –1917 and –1747.

Smad1 and Smad4 bind to the BMP-2-responsive region in the Dlx3 promoter

Because Smads are the intracellular transducers of BMP signaling, we analyzed whether the BMP-2 response region had consensus Smad-binding sequences. Analysis of the –1917 to –1747 region of the Dlx3 promoter showed four minimal Smad recognition sequences (GNCT) and two GCAT motifs that are known to be binding motifs for Smad1 (25,28). The elements were clustered in the region between –1917 and –1747 (Fig. 3A, upper). To determine whether Smad1 and Smad4 were capable of directly binding to this region, bacterially expressed full-length Smad1, Smad4 and Smad3 were incubated with the ^{32}P-end-labeled –1917 to –1747 fragment. As shown in Figure 3A, both Smad1 and Smad4 were capable of binding this fragment in a concentration-dependent manner, but Smad3 was unable to bind under the same experimental conditions. To test the binding activity of Smad3 protein, we performed gel shift assays using a Smad3/4 consensus oligonucleotide (Santa Cruz). The Smad3 protein was capable of binding to the probe and the shift was competed with unlabeled oligonucleotide competitor (Fig. 3A, right) The lack of a DNA

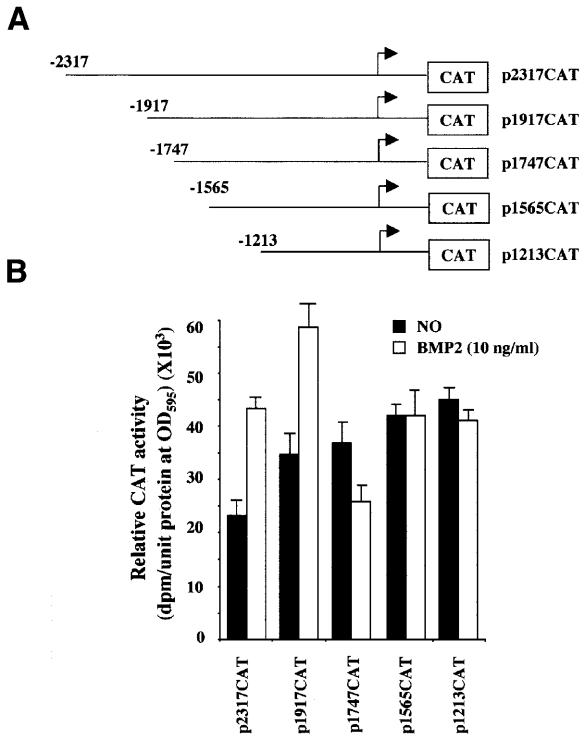

Figure 2. Delineation of the BMP-2-responsive sequence in the Dlx3 promoter. (**A**) Schematic diagram of each 5′-end deletion construct of the Dlx3 promoter. (**B**) Primary mouse keratinocytes were transfected with 1 µg each deletion construct and treated with 10 ng/ml BMP-2 for 24 h. Forty-eight hours after transfection, cells were harvested and CAT activity was measured. CAT activity was normalized to the protein concentration measured by the Bradford assay. The bars represent the average normalized CAT activity of duplicate plates from three experiments for each construct.

shift by Smad3 and binding by Smad1 suggested a specific role for BMP signaling.

To further delineate the Smad-binding sites, four overlapping 50mer oligonucleotides covering the –1917 to –1747 fragment were prepared and used as probes in gel shift assays. As shown in Figure 3B, Smad1and Smad4 bound to SBE2 and SBE4 specifically. SBE2 and SBE4 contain the GCAT motif. Although SBE1 also contained a GCAT motif, no specific shift was observed with either Smad1 or Smad4. To test the specificity of the Smad-dependent complex, we performed competition assays with the SBE4 oligonucleotide probe. As shown in Figure 3B (right), although the bottom shifted complex was competed by the unlabeled SBE4 competitor, the upper nonspecific complex was unaffected. To delineate which parts of SBE2 and SBE4 were responsible for binding to Smad1 and Smad4, each element was divided into two parts and used as a competitor in gel shift assays. The 5′- and 3′-parts of SBE2 partially competed the complexes formed between SBE2 and Smad1 or Smad4 (Fig. 4A). In contrast, the 5′-part of SBE4 completely competed the complexes, while the 3′-part of SBE4 did not (Fig. 4B). To confirm specific binding of Smad proteins to SBE4-1, supershift assays were performed with anti-Smads antibodies and nuclear extract of mouse keratinocytes pretreated with BMP-2. Several specific complexes, which were competed with cold SBE4-1, were formed and a supershifted complex was shown in the presence of

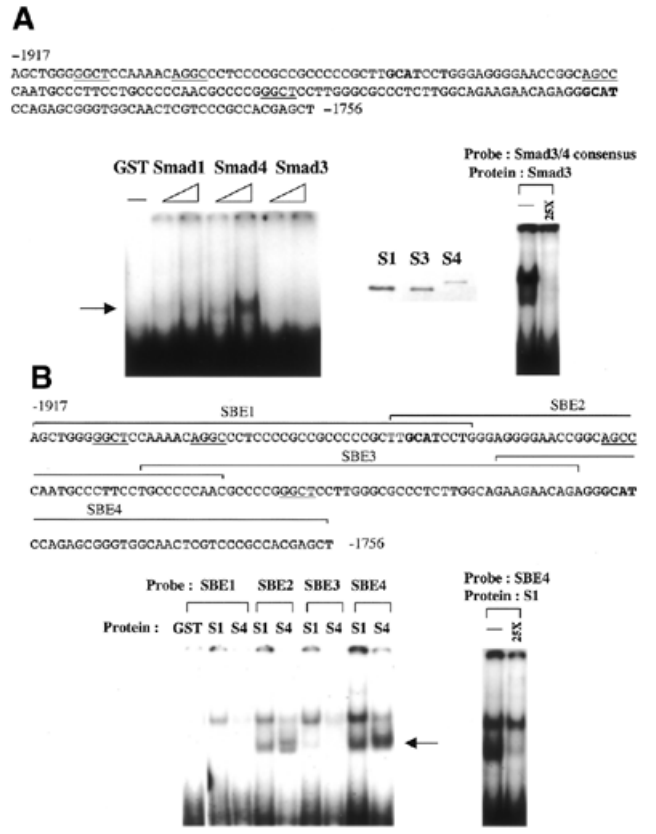

Figure 3. Smad1 and Smad4 bind to the BMP-2-responsive region in the Dlx3 promoter. (**A**) Gel shift assays were performed using increasing amounts of GST–Smad1, GST–Smad4 and GST–Smad3 fusion proteins and radiolabeled DNA fragment (–1917 to –1747). The GST fusion proteins used in gel shift assays are shown in the center panel (S1, Smad1; S3, Smad3; S4, Smad4). The right panel shows a gel shift assay to demonstrate the binding capability of Smad3. The arrow indicates the complex formed by GST–Smad1 or GST–Smad4 and the DNA fragment. The putative Smad-binding motifs are indicated by underlined and bold letters. (**B**) Gel shift assays were performed with four overlapping oligonucleotides covering the Smad-binding region (–1917 to –1756) and GST–Smad1 or GST–Smad4. The right panel shows a gel shift assay using SBE4 and Smad1 to determine the specificity of each complex. Each oligonucleotide sequence is shown with the putative Smad-binding motifs indicated by bold and underlined letters.

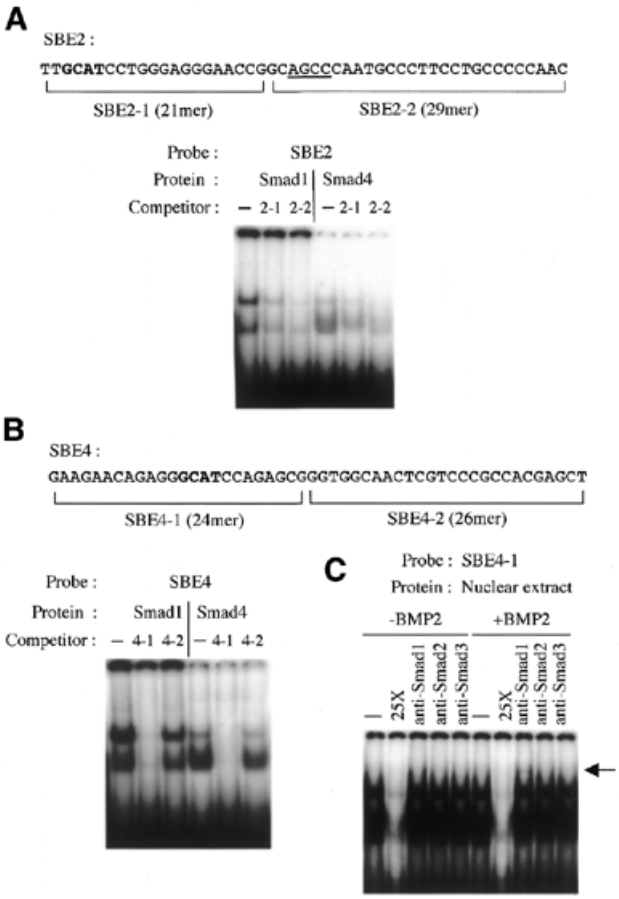

Figure 4. Smad1 and Smad4 bind to the SBE4-1 element. (**A**) Gel shift assays were performed with radiolabeled SBE2 and GST–Smad1 or GST–Smad4. As cold competitors, SBE2-1 and SBE2-2 were used at a 25-fold molar excess. (**B**) Gel shift assays were performed with radiolabeled SBE4 and recombinant GST–Smad1 or GST–Smad4. SBE4-1 and SBE4-2 were used as cold competitors at a 25-fold molar excess. (**C**) The SBF4-1 oligonucleotide was used as probe for gel shift assays with nuclear extract from mouse keratinocytes untreated or treated with BMP-2. In the supershift assay, each anti-Smads antibody was added to the reaction before addition of probe. The supershifted complex is indicated with an arrow.

anti-Smad1 antibody (Fig. 4C). This supershifted complex was not readily detectable in assays performed with nuclear extracts from untreated (–BMP-2) cultured cells (Fig. 4C). We were unable to detect a supershift when using a Smad4-specific antibody (Santa Cruz). Together, these results suggest that Smad1 and Smad4 bound specifically to the 5′-part of SBE4. A possible explanation for the results with SBE2 is that binding of Smad1 and Smad4 requires the whole region of SBE2 or that the specificity for binding is not as strong as for SBE4. We focused on SBE4-1 for further examination.

Smad1 and Sma4 bind to the GCAT sequence in SBE4

To determine the specific sequence responsible for Smad1 and Smad4 binding, serial 4 bp mutations were made in SBE4-1 (Fig. 5A, left). Each mutant oligonucleotide was used as a probe in gel shift assays with Smad1 and Smad4 recombinant proteins. As shown in Figure 5A, Smad1 and Smad4 binding to SBE4-1 was unaffected by the mutations in the M1, M2 and M4 probes. However, the M3 mutant probe, mutated in the GCAT sequence, showed decreased binding activity for both Smad1 and Smad4. To investigate whether the GCAT sequence contributes to BMP-2-induced transcriptional activation of the Dlx3 promoter, the site was mutated in p1917CAT. The mutated p1917mCAT was transfected into mouse keratinocytes, after which the cells were treated with BMP-2 (Fig. 5B). Mutation of the GCAT sequence consistently reduced transcriptional activation by BMP-2 treatment when compared to the wild-type p1917CAT (Fig. 5C). To confirm the role of the GCAT sequence, we investigated the response of p1917mCAT and p1917CAT to co-transfection with Smad1 and Smad4 expression vectors. The transcriptional activity of p1917CAT was induced with Smad1 and Smad4. However, p1917mCAT showed a reduction in transcriptional activation by Smad1/Smad4 (Fig. 5D). These results strongly suggest that the GCAT sequence located in the –1797 to –1747 region of the Dlx3

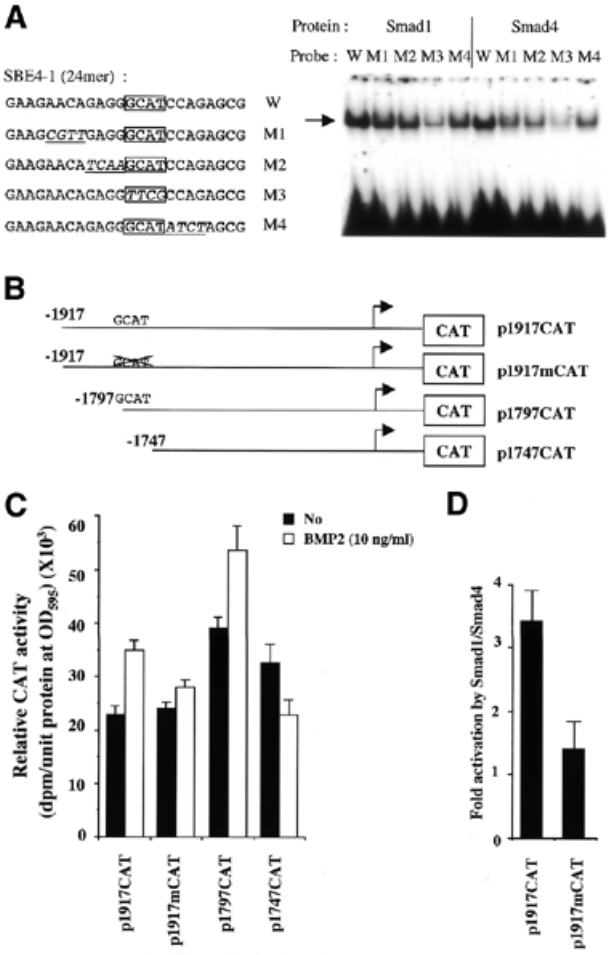

Figure 5. A GCAT sequence is responsible for Smad1 and Smad4 binding and activation of Dlx3 expression by BMP-2. (**A**) Gel shift analysis was performed with radiolabeled mutated SBE4-1 oligonucleotides and GST–Smad1 or GST–Smad4. Each mutated oligonucleotide sequence is shown on the left. The GCAT sequence is boxed. (**B**) Schematic diagram of constructs used in the CAT assays. (**C**) Primary mouse keratinocytes were transfected independently with 1 µg p1917CAT, p1917mCAT, p1797CAT or p1747CAT and treated with 10 ng/ml BMP-2 for 24 h. Forty-eight hours after transfection, cells were harvested and CAT activity was measured. (**D**) The GCAT mutant construct p1917mCAT and the wild-type, p1917CAT, were co-transfected into primary mouse keratinocytes with Smad1/Smad4 expression vectors. Fold activation was calculated based on the CAT activity of each construct co-transfected with the parental vector.

promoter is responsible for binding of Smad1 and Smad4 and for transcriptional activation of Dlx3 by BMP-2.

Transcriptional activation of Dlx3 by BMP-2 in hair follicle cells

By the end of embryonic development, Dlx3 is expressed in the stratified epidermis and in the matrix cells of hair follicles. In addition to keratinocytes, we tested the transcriptional activation of Dlx3 by BMP-2 in hair follicle cells. We prepared RNA from hair follicle cells cultured *in vitro* after treatment with BMP-2 at increasing concentrations. Using total RNA, RT–PCR was performed with Dlx3-specific primers. As shown in Figure 6A, transcription of Dlx3 was induced by 10 ng/ml BMP-2 and reached a maximum at 100 ng/ml BMP-2. To determine whether the identified GCAT sequence, which is

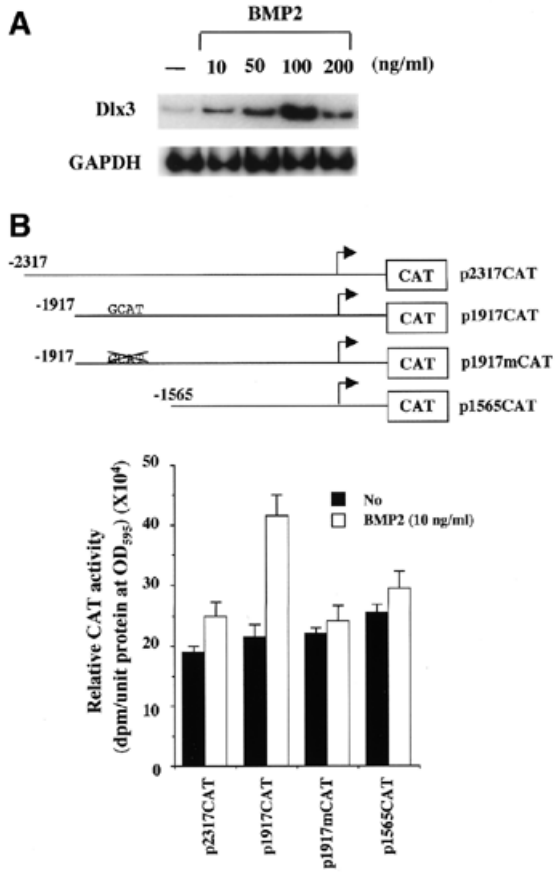

Figure 6. BMP-2 induced the transcription of Dlx3 in hair follicle cells. (**A**) RT–PCR analysis was performed with specific primers for Dlx3 using total RNA from mouse hair follicle cells treated with 10, 50, 100 or 200 ng/ml BMP-2 for 24 h. (**B**) Primary mouse hair follicle cells were transfected independently with 1 µg p2317CAT, p1917CAT, p1917mCAT or p1565CAT and treated with 10 ng/ml BMP-2 for 24 h. Forty-eight hours after transfection, cells were harvested and CAT activity was measured.

responsible for transcriptional activation of Dlx3 by BMP-2 in keratinocytes, also has an important role in activation in hair follicle cells, several constructs, including the GCAT mutation construct p1917mCAT, were transfected into hair follicle cells. After treatment with BMP-2, extracts from transfected cells were used in CAT assays. The wild-type p1917CAT construct showed induction of transcriptional activity by BMP-2, while p1917mCAT did not (Fig. 6B). Even though the longest promoter construct (p2317CAT) showed weak activation by BMP-2, the GCAT sequence in the Dlx3 promoter has an important role in the transcriptional activation of Dlx3 by BMP-2 in hair follicle cells.

Taken together, the *in vitro* binding experiments demonstrate that the GCAT motif located between –1797 and –1747 of the Dlx3 promoter is required for BMP-2-induced transcriptional activation mediated by direct binding of Smad1 and Smad4 in keratinocytes and hair follicle cells.

DISCUSSION

BMP signaling plays an essential role in vertebrate embryogenesis (8). Although it has been established that activation of

Dlx3 and two other family members (Dlx5 and Dlx6) requires BMP signaling (34), the responsive sequences in the promoter region have not been delineated. We examined expression of BMP-2 in mouse keratinocytes isolated from neonatal epidermis and cultured *in vitro*, and show that BMP-2 is expressed independent of the differentiation state of the keratinocyte. We also show up-regulation of Dlx3 expression after treatment with BMP-2, and specific binding and transactivation activities of Smad regulators on the Dlx3 gene, indicating an alternative mechanism for induction of Dlx3 than the PKC-dependent Ca^{2+} activation of epidermal differentiation factors (5,35).

BMP signal transduction is mediated by direct phosphorylation of Smad1 protein. Upon phosphorylation, Smad1 interacts with a common partner, Smad4, which then translocates to the nucleus where the complex recruits a transcription factor(s) to activate specific gene transcription (15). Evidence indicates that homeobox genes (Dlx5, Xvent2 and Tlx2; 29,36,37) mediate events downstream of the BMP signal. In other cases, Smads directly interact with homeobox proteins and indirectly regulate their function. Hoxc-8, which functions as a transcriptional repressor, interacts with Smad1 and is dislodged from the Hoxc-8-binding site in the osteopontin gene, in this way activating gene transcription in response to BMP stimulation (38).

Recent work has delineated the BMP-responsive region of the Xvent-2B proximal promoter (–275 to –152) (29), which contains a Smad1-binding region with two GCAT motifs and AGNC binding sites for Smad4. We have delineated the BMP-2-responsive sequences in the Dlx3 promoter to the region between –1917 and –1747 and found an element that contains a GCAT sequence, SBE4, able to bind Smad1 and Smad4. Four other GNCT motifs were also found in the –1917 to –1747 region but these do not contribute to the responsiveness to BMP-2. The BMP-2 response of the –1917 to –1747 promoter region was weak when compared to the dramatic response shown for the endogenous Dlx3 gene. One possible explanation is that treatment with BMP-2 not only induces Dlx3 expression but also affects the stability and/or half-life of the mRNA. A second possibility is that besides the BMP-2-responsive elements that have been delineated in the –1917 to –1747 region, another element(s) located further upstream is required for the full response to BMP-2 shown by endogenous Dlx3.

Crystal structure studies have shown that sequence-specific DNA recognition is facilitated by the interaction of amino acids with only three bases of a Smad-binding element (39). As these residues are invariant among the Smads, the GNCT sequence is expected to be recognized by several members of the Smad family. Furthermore, biochemical and biological studies have established at least two distinct TGF-β family pathways: one shared by TGF-β and activin and the other by the BMP-2 and BMP-4 subfamily. The promoters of TGF-β- and activin-responsive genes have been shown to contain GNCT Smad motifs. We tested the transcriptional activation of Dlx3 in mouse keratinocytes by TGF-β and found no effect (G.-T.Park, unpublished results). Therefore, transcription of Dlx3 responds to BMP through direct binding of Smad1 and Smad4 to a GCAT sequence in the Dlx3 promoter.

BMPs also regulate expression of another member of the Dlx family, Dlx5, in chick embryos (36). In *Xenopus*, during early development expression of Dlx3 is localized to the ventral ectoderm and interference in BMP signaling inhibits expression of Dlx3 (11,40). We have previously shown that in differentiating mouse keratinocytes Dlx3 expression is dependent on the activity of Sp1 family members and NF-Y (5) and that the inductive process requires Ca^{2+} signaling through a PKC-dependent pathway (5,35). We present evidence to support the hypothesis that BMP signaling could potentially be one of the regulatory pathways that control Dlx3 transcription in mouse keratinocytes.

Dlx3 is also expressed during embryonic development in the hair follicle and dental and mammary gland primordia, sites derived from epithelium–mesenchyme interactions (41,42). Within the hair follicles, Dlx3 is expressed in the matrix cells at the base of the follicle. It is possible that the BMP pathway may exert an important function in this ectodermal derivative when compared to the stratified epidermis, where the Ca^{2+} induction-dependent pathway plays a crucial role.

Since BMP-2 is expressed in basal and differentiated keratinocytes, there must be a regulatory mechanism that prevents activation of Dlx3 expression by BMP signaling in basal cells of the epidermis, where it is not normally found *in vivo*. It remains to be determined how this activation pathway is regulated and how it interacts/overlaps with other inductive signaling pathways, such as the PKC/Ca^{2+}-dependent pathway.

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