A phylogenetically conserved cis-regulatory module in the *Msx2* promoter is sufficient for BMP-dependent transcription in murine and *Drosophila* embryos

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Summary

To understand the actions of morphogens, it is crucial to determine how they elicit different transcriptional responses in different cell types. Here, we identify a BMP-responsive enhancer of *Msx2*, an immediate early target of bone morphogenetic protein (BMP) signaling. We show that the BMP-responsive region of *Msx2* consists of a core element, required generally for BMP-dependent expression, and ancillary elements that mediate signaling in diverse developmental settings. Analysis of the core element identified two classes of functional sites: GCCG sequences related to the consensus binding site of Mad/Smad-related BMP signal transducers; and a single TTAATT sequence, matching the consensus site for Antennapedia superclass homeodomain proteins. Chromatin immunoprecipitation and mutagenesis experiments indicate that the GCCG sites are direct targets of BMP restricted Smads. Intriguingly, however, these sites are not sufficient for BMP responsiveness in mouse embryos; the TTAATT sequence is also required. DNA sequence comparisons reveal this element is highly conserved in *Msx2* promoters from mammalian orders but is not detectable in other vertebrates or non-vertebrates. Despite this lack of conservation outside mammals, the *Msx2* BMP-responsive element serves as an accurate readout of Dpp signaling in a distantly related bilaterian – *Drosophila*. Strikingly, in *Drosophila* embryos, as in mice, both TTAATT and GCCG sequences are required for Dpp responsiveness, showing that a common cis-regulatory apparatus can mediate the transcriptional activation of BMP-regulated genes in widely divergent bilaterians.

Key words: BMP, *Msx2*, Homeodomain, Smad, Transcription, Transgenic mouse, Evolution

Introduction

A striking property of morphogens is that they act through a small number of signaling pathways, yet elicit a wide variety of cellular responses in different developmental settings. How this diversity is achieved remains an unanswered question. It is clear that part of the solution is in the complement of transcription factors present in a responding cell. Such factors, through interactions with cis-regulatory elements, transform a morphogen signal into a pattern of gene expression. To understand morphogen action, it is therefore crucial to identify these transcription factors and to understand how their interactions on target promoters control gene expression. Here, we address the problem of morphogen specificity by investigating a BMP-responsive enhancer of the murine *Msx2* gene.

BMPs are a large class of TGF-β related ligands with diverse activities in embryonic development. They can function as classical morphogens, eliciting changes in cell fate in a concentration-dependent manner (Gurdon and Bourillot, 2001). Interaction of BMPs with their cognate receptors leads to the phosphorylation of members of the receptor-regulated Smad family (R-Smads) (Heldin et al., 1997; Massague, 1998). Upon phosphorylation, R-Smads associate with Smad4 and translocate to the nucleus, where they serve as effectors of transcription (Kretzschmar et al., 1997; Lagna et al., 1996; Liu et al., 1997). Smads 1, 5 and 8 function in the BMP pathway, Smads 2 and 3 in the activin and TGF-β pathways, and Smad4 in all three branches of the TGF-β superfamily (Hoodless et al., 1996; Liu et al., 1996; Massague and Chen, 2000; Shi and Massague, 2003; Suzuki et al., 1997; Wiersdorff et al., 1996).

Although Smads are crucial for the transcriptional activation of BMP target genes, they bind DNA weakly and are expressed broadly in embryonic tissues (Shi et al., 1998; Wrana, 2000). Therefore, the tissue-specific activation of BMP target genes

probably involves sequence-specific transcription factors that cooperate with Smads. Despite the importance of such factors, few have been identified and little is known about how they interact with Smads to regulate transcription (Hata et al., 2000; Henningfeld et al., 2002). The *Msx2* promoter provides an ideal starting point in the search for such factors.

Msx2 is one of three closely related genes in mammals (Bell et al., 1993; Davidson, 1995; Maxson et al., 2003; Pollard and Holland, 2000). A well-documented BMP target, *Msx2* is expressed in a subset of known domains of BMP function (Furuta and Hogan, 1998; Graham et al., 1994; Hollnagel et al., 1999; Vainio et al., 1993). Targeted mutations in *Msx2* have verified that it acts downstream of BMPs and is required for a subset of BMP-mediated cellular responses (Satokata and Maas, 1994; Satokata et al., 2000). The regulatory elements controlling *Msx2* expression thus provide a useful model for investigating how BMP signals are processed at the level of the promoter to produce different BMP responses in specific developmental settings.

Here, we report the identification and fine-structure analysis of a BMP-responsive enhancer (BMPRE) located upstream of the murine *Msx2* gene. We demonstrate that consensus Smad elements and a consensus homeodomain element control the temporal and spatial pattern of BMP-dependent transcription directed by this enhancer. We show that the nucleotide sequence of this enhancer is highly conserved within mammals, but not in other vertebrate classes or nonvertebrates. Despite this lack of conservation outside mammals, this *Msx2* BMPRE accurately interprets Dpp signals in *Drosophila* embryos and imaginal discs. Furthermore, as in mouse embryos, it exhibits a dependence on the consensus homeodomain as well as consensus Smad sites. These results demonstrate that the *Msx2* BMPRE can respond to BMP/Dpp signals in two widely divergent animal groups, and suggest the functional design represented by this enhancer originated early in the evolution of the bilateria.

Materials and methods

DNA constructs

For transient transfections, segments of the murine *Msx2* 5′ flanking sequence were fused with a *TK* minimal promoter and subcloned into PGL2 (Promega). The *Δ3Msx2luciferase* construct contained a 1.8 kb *Bam*HI fragment located between –5082 and –3298 bp upstream of the translation start site. The 560 bp *Δ4Msx2luciferase* construct contained a fragment (–3862 to –3303) generated by PCR. The insert in the *560Δ52bpMsx2luciferase* construct was created by ligating two PCR products together, with the 52 bp BMP-responsive region being omitted. The 560 bp homeodomain mutant (hdm) insert was generated using the QuickChange Site-Directed Mutagenesis Kit (Stratagene) and the primers 5′-GCCGGGCCGAGAGCTCGTACCGCGGCTC-CGGCGCG-3′ (forward) and 5′-CGCGCCGGAGCCGCGGTACG-AGCTCTCGGCCCGGC-3′ (reverse). This insert was subcloned into TK-PGL2 to generate *560bpMsx2-hdm-luciferase*.

For the production of transgenic mice, *Msx2* promoter fragments were fused with the *hsp68* minimal promoter and a *lacZ* reporter (Kothary et al., 1989). The 1.8 kb *Δ3Msx2-hsplacZ* and 560 bp *Δ4Msx2-hsplacZ* transgenic mice have been described (Kwang et al., 2002). The *Δ5Msx2-hsplacZ* construct contained a 220 bp PCR fragment located between –3521 and –3310 bp upstream of the *Msx2* translation start site. The 52 bp *Δ6Msx2-hsplacZ* construct contained a tetramer repeat of the sequence between –3523 and –3471. This construct, and those bearing mutations in the Smad and homeodomain sites, were generated using dimer repeat oligos that were ligated together immediately upstream of the *hsp68-lacZ*-SV40 cassette. The *560Δ52bpMsx2-hsplacZ* and *560bpMsx2-hdm-hsplacZ* transgenes were generated as described previously.

Msx2 promoter fragments used for producing transgenic flies were cloned into the CPLZN P-element vector. For the 560, 480 and 220 bp promoter fragments, inserts were generated by PCR. The 52 bp transgene consisted of a tetramer repeat.

Cell culture and transfection

10T1/2 cells were propagated in DMEM with 10% FBS. The cells were transfected with the reporter constructs using Superfect (Qiagen) and 24 hours later induced with BMP4 (60ng/ml final) (R&D). After another 24 hours in culture, the cells were harvested and luciferase assays performed using the dual luciferase system (Promega).

Bead implantations

Affigel agarose beads (BioRad) were washed in PBS prior to incubating in 0.1% BSA with 100 ng/μl BMP4 (R&D), 5 ng/μl TGFβ1 (R&D) or BSA (0.1%) for 30 minutes at 37°C. Beads were placed on tissues in transwell membranes, immersed in DMEM. For limb-bud implantations, BMP4 beads were placed on the dorsal side of left forelimbs prior to overnight incubation in 6% CO_2. Tissues were then fixed for 10 minutes in 4% paraformaldehyde and stained for *lacZ* activity.

Chromatin immunoprecipitation (ChIP) assays

MLB13MYC-clone-14 (C-14) cells (Rosen et al., 1994) were cultured in DMEM (10% FBS) and induced with 60 ng/ml BMP4 (R&D) for 30 minutes prior to fixation with formaldehyde (1.0% final concentration) at room temperature for 10 minutes. ChIP assays were performed as described (Ma et al., 2003) using an anti Phospho-Smad1 antibody (Upstate). PCR amplification was performed using primers that span the 52 bp BMP-responsive region of the *Msx2* promoter (32 cycles). Primer sequences were: BMPRE (forward) 5′-TCT GCC CAG TTG GAG GTT TGA-3′ and (reverse) 5′-GCC GCG TTA ATT GCT CTC G-3′; upstream control (forward) 5′-GCA ACA AAC ATC CCT GAG A-3′ and (reverse) 5′-CTG CCT CCT AAC CTT CAT AG-3′.

Electophoretic mobility shift assays

GST-Smad4 (MH-1 domain) fusion proteins were expressed in BL21 bacteria and purified by chromatography on glutathione beads (Pharmacia). Prx1b protein was expressed from a mouse cDNA (Norris and Kern, 2001) by coupled in vitro transcription-translation (TNT kit, Invitrogen). All probes used were labeled with α-^{32}P-dCTP. EMSA was performed as previously described (Kwang et al., 2002).

β-galactosidase staining, in situ hybridization and immunohistochemistry

Mouse embryos were fixed in 4% paraformaldehyde at 4°C prior to staining for *lacZ* (Liu et al., 1994). Whole-mount in situ hybridization on mouse embryos was performed as described previously (Hogan et al., 1994; Kwang et al., 2002). In situ hybridization and detection of β-galactosidase expression in *Drosophila* embryos was carried out as described in Arora et al. (Arora et al., 1994). To detect phosphorylated R-Smads, tissue was fixed in 4% paraformaldehyde and equilibrated in 30% sucrose prior to freezing in Histo Prep (Fisher). Frozen sections were cut (8 μm) and fixed in cold acetone for 10 minutes. Primary anti phospho-Smad1, 5, 8 antibody (Cell Signaling Technology) was diluted 1/50 in PBST/1.0% BSA and incubated at 4°C overnight. The secondary antibody, anti-Rabbit Rhodamine (Calbiochem), diluted 1/200 in PBST/1.0% BSA, was incubated for 1 hour at room temperature. Sections were counterstained with DAPI and cover slipped using Vectashield (Vector Labs). *lacZ* staining of frozen sections was performed as described in Ishii et al. (Ishii et al., 2003).

Production of transgenic mice and flies

Transgenic mouse embryos and lines were generated by pronuclear injections as described by Liu et al. (Liu et al., 1994). Transgenic flies were produced by germline transformation as described by Arora et al. (Arora et al., 1994).

Genotyping

DNA was prepared from mouse tails or embryo yolk sacs as described by Hogan (Hogan, 1994). *Msx2-lacZ* transgene genotypes were determined by PCR using primers to *lacZ* as described by Kwang et al. (Kwang et al., 2002).

Results

A 52 bp sequence is necessary for BMP responsiveness of *Msx2* transgenes in murine embryos and 10T1/2 cells

Previously we identified a 1.8 kb fragment of the 5′ flanking sequence of *Msx2* (Fig. 1A, *Δ3Msx2*) that was sufficient to confer BMP responsiveness on a reporter gene in cultured cells (Daluiski et al., 2001). A closely related fragment directed reporter gene expression to most sites in the developing embryo where endogenous *Msx2* is expressed (Kwang et al., 2002). A 560 bp subfragment of the 1.8 kb fragment (Fig. 1A, *Δ4Msx2)* responded to BMP4 stimulation in 10T1/2 cells to approximately the same extent as the parental 1.8 kb fragment (Fig. 1B). A transgene comprising the 560 bp fragment fused with *hsp68-lacZ* also was sufficient for correct spatiotemporal expression in embryos (Fig. 1C-F).

To determine whether the 560 bp fragment was able to respond to BMP signals in murine embryos, we carried out BMP bead implantations in mice bearing the *560bpMsx2-hsplacZ* transgene (Fig. 1E,G). Beads soaked in BMP4 were implanted on E11.5 limb buds, and after 16 hours in culture, *lacZ* activity was assessed. A halo of *lacZ* activity appeared around implanted beads within 15 minutes of staining and grew significantly darker by 1 hour (Fig. 1G). Control beads soaked

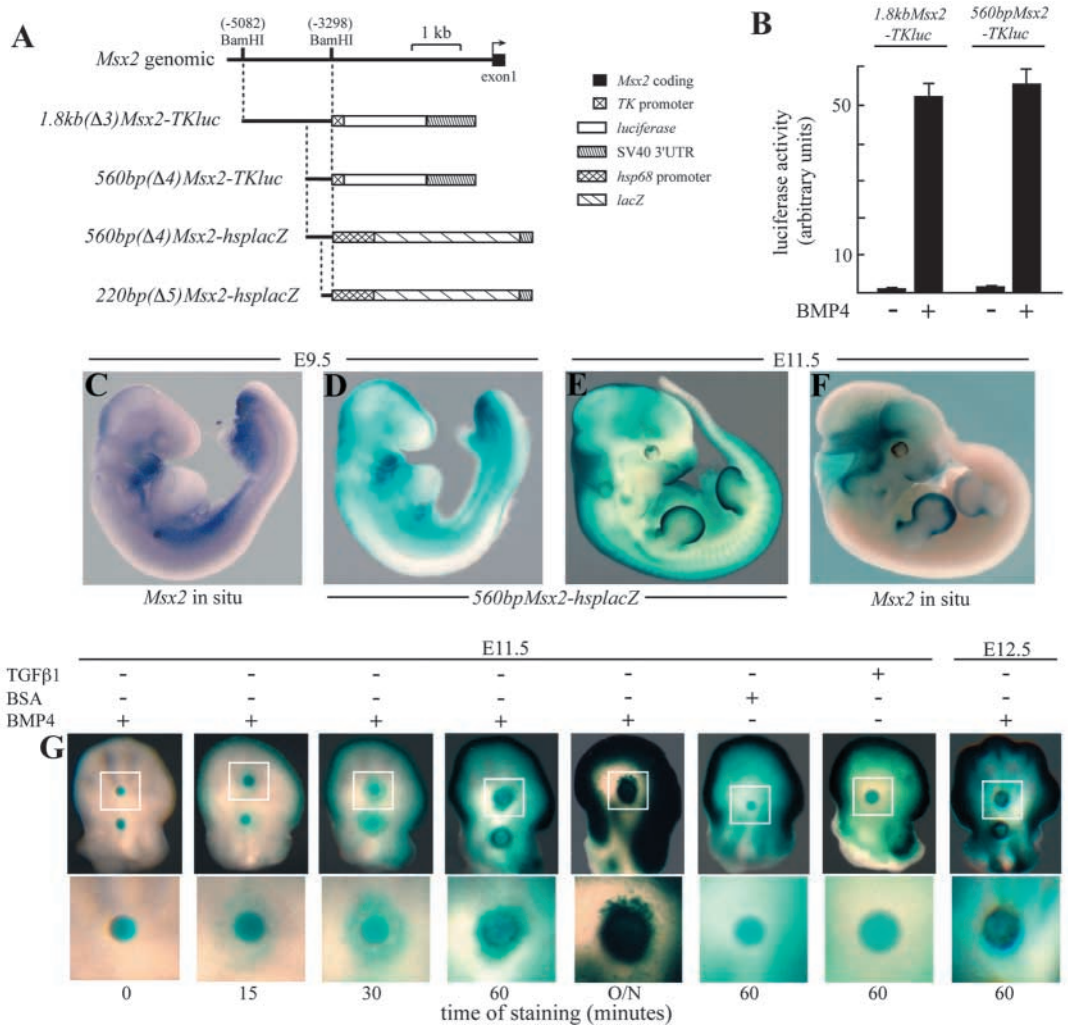

Fig. 1. A 560 bp fragment of the *Msx2* promoter is sufficient for BMP responsiveness in 10T1/2 cells and murine embryonic limbs. (A) Genomic map showing fragments of murine *Msx2* 5′ flanking sequence used in transgenic and transfection experiments. (B) Results of transient transfections of 10T1/2 cells with *Msx2* promoter-*TK-luciferase* vectors bearing either the 1.8 kb or 560 bp fragment. (C-F) Comparisons of endogenous *Msx2* expression by whole-mount in situ hybridization (C,F) and *Msx2* promoter-*hsplacZ* transgene expression (D,E). (G) Limbs derived from E11.5 *560bpMsx2-hsplacZ* transgenic embryos implanted with Affigel agarose beads soaked in BMP4, TGFβ1 or BSA. Limbs were stained for β-galactosidase activity for the indicated times. Anterior towards the right. The beads are light blue; a positive response to ligand produces dark-blue staining around the bead.

either in BSA or TGFβ1 did not elicit a *lacZ* signal (Fig. 1G), demonstrating the response was specific for BMP. When bead implantations were performed at E12.5, a weaker response was evident (Fig. 1G), indicating the response was stage-specific. BMP4-soaked beads also stimulated β-galactosidase activity in the head, mandible and tail (data not shown).

Alignments of *Msx2* loci from mouse, rat, rabbit, human and chimpanzee revealed a 480 bp block of homology positioned between 3 and 4 kb upstream of the translation start sites (Fig. 2). Located within the 560 bp BMP-responsive region, this block exhibited an average identity between different orders of mammals of ~90%. By contrast, the 3.3 kb promoter region located between the conserved block and the translation start site had an average identity of 56%, suggesting that selection has maintained the sequence of the 480 bp block. No significant matches with this 480 bp region were evident in non-mammals. Nor did mammalian sequences outside the *Msx2* locus match the 480 bp sequence. A survey (MatInspector 7.0, 2003) of consensus cis-regulatory elements within the conserved region revealed several consensus binding sites with potential roles in BMP signaling. These included Smad1 and Smad4 elements, as well as sites for OAZ, Creb, Tcf/Lef1 and Hnf/Fox (Fig. 2) (Ionescu et al., 2004; Ishida et al., 2000; Johnson et al., 1999; Rice et al., 2003; Shim et al., 2002; Zawel et al., 1998).

We tested a series of *Msx2* promoter fragments for their ability to mediate BMP responsiveness in transgenic embryos (Fig. 3A). A 220 bp subfragment of the 560 bp fragment drove reporter expression in a near-normal spatial pattern in embryos, with the only significant difference being in the posterior limb bud (Fig. 3C; Table 1). Like the 560 bp fragment, this 220 bp fragment conferred BMP responsiveness on a *lacZ* reporter in limb buds (Fig. 3F), as well as in tails and mandibles (data not shown). Limb buds bearing BSA and TGFβ1 control beads were negative (data not shown).

Fragments responding to BMP treatment had in common a 52 bp region (Fig. 3A, asterisk). This sequence included Smad and homeodomain consensus sites (Fig. 2). To determine whether this sequence was important for BMP responsiveness, we deleted it in the context of the 560 bp fragment, and tested the activity of the mutant transgene (*560Δ52bpMsx2*) in transgenic mice and 10T1/2 cells. In transgenic embryos at E11.5 (eight independent lines), the mutated 560 bp fragment drove *lacZ* expression at much lower levels (Fig. 3D) compared with the parental fragment (Fig. 3H), as evidenced by the greatly extended staining time required for the *560Δ52bpMsx2* transgene to achieve a similar relative intensity as the transgenes containing the 560 or 220 bp fragments (Fig. 3B,C). Expression in both the limb bud mesenchyme and AER was reduced substantially (compare Fig. 3G with Fig. 1G). Bead implantations on limbs carrying the mutant transgene elicited no detectable *lacZ* activity (Fig. 3G), whereas implantations on limbs carrying the wild-type *560bpMsx2* transgene resulted in robust *lacZ* signals (Fig. 3E). In 10T1/2 cells, the 52 bp deletion caused a near-complete loss of BMP responsiveness without affecting the basal level of promoter activity (Fig. 3I). Together, data from transgenic embryos and cultured cells suggested that the

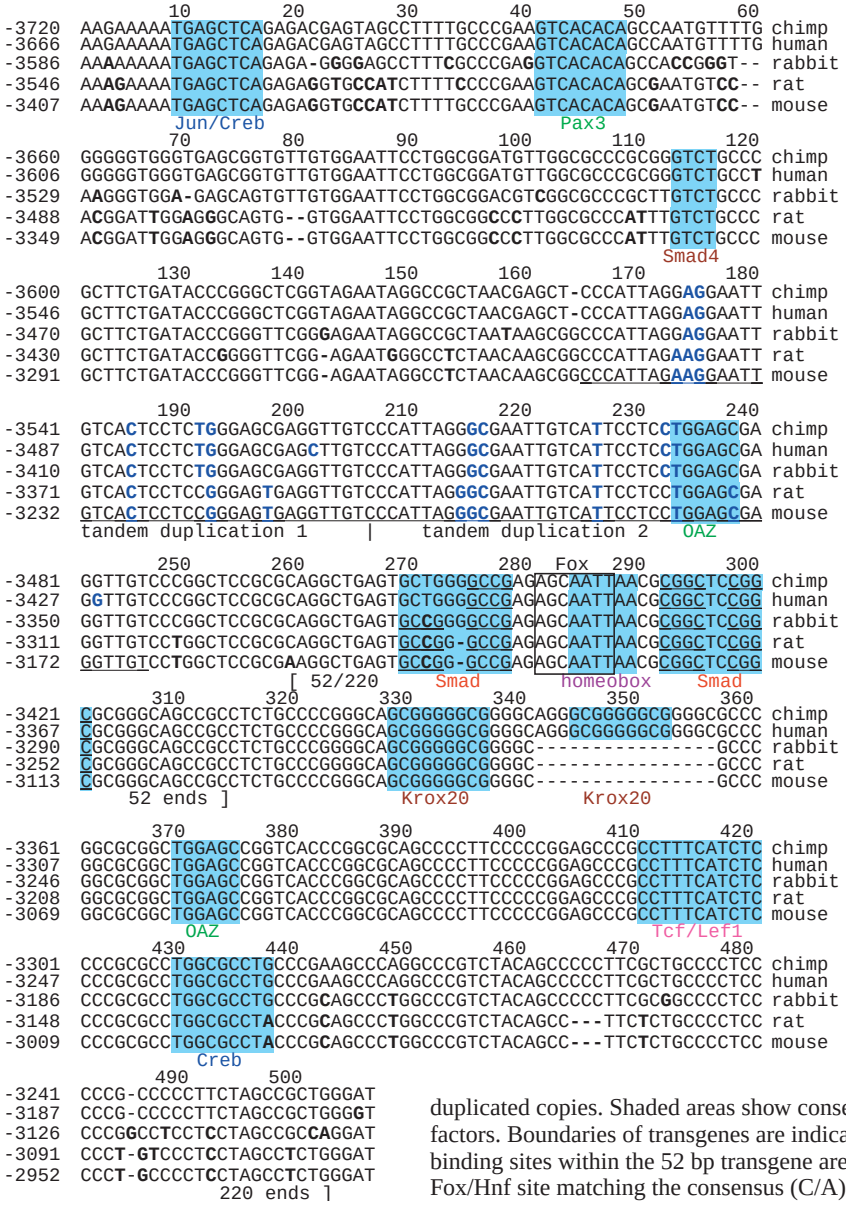

Fig. 2. The *Msx2* BMP-responsive region is highly conserved among mammals. BLAST was used to search for sequence identity between the murine *Msx2* locus and *Msx2* loci of other vertebrates. Alignments of a 480 bp homology block are shown in different mammalian species, with coordinates of transcription start sites at left. Sequence differences relative to chimpanzee are in bold. The underlined sequence (164-246) is a tandem duplication; bases in blue are those that differ between the two duplicated copies. Shaded areas show consensus binding sites for the indicated transcription factors. Boundaries of transgenes are indicated by brackets below the sequence. Putative Smad-binding sites within the 52 bp transgene are underlined. The boxed area indicates a putative Fox/Hnf site matching the consensus (C/A)(T/C)(C/A)AA(T/C)A in 5/7 positions.

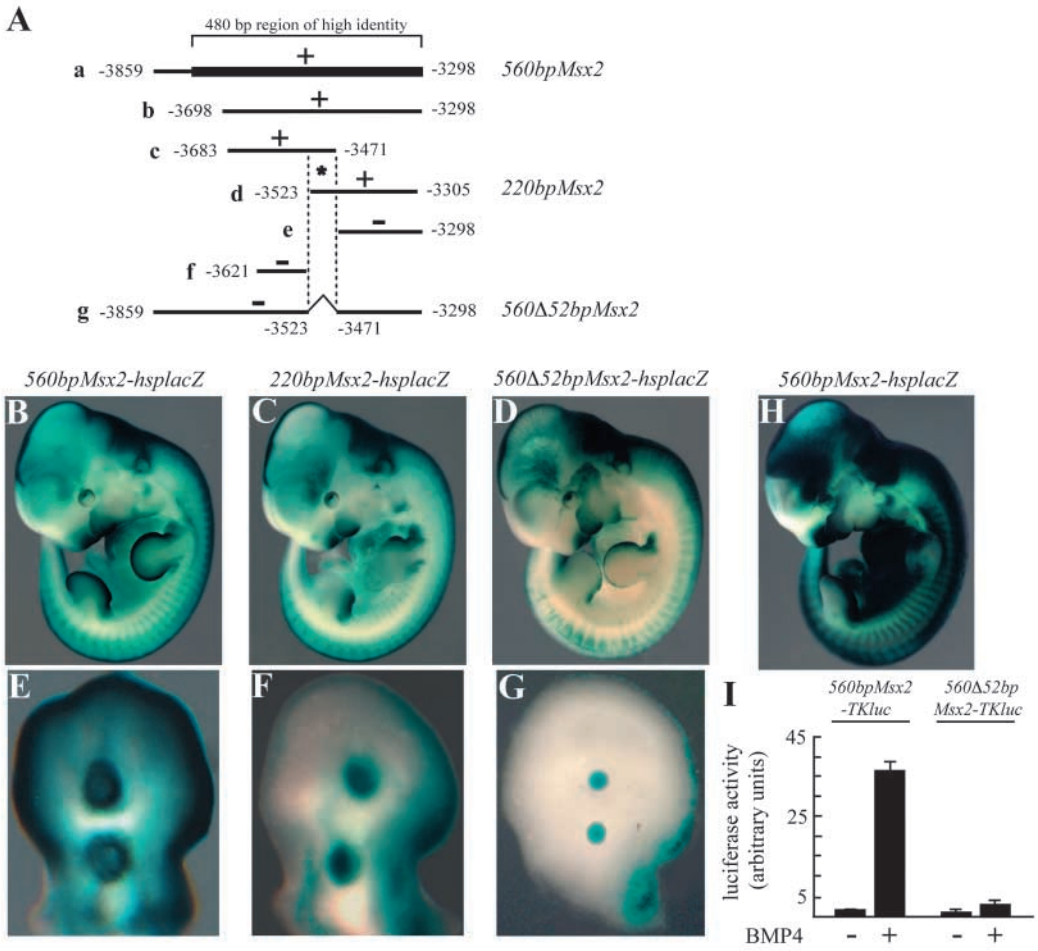

Fig. 3. Deletion analysis defines a 220 bp minimal *Msx2* element and a 52 bp element required for BMP signaling. (A) Deletion series of *lacZ* constructs bearing fragments of the 560 bp promoter sequence. The BMP responsiveness of each transgene was assessed by bead implantation; positive response (+), negative response (–). (B,C,E,F,H) *lacZ* stains of 560 and 220 bp transgenic embryos at E11.5. Expression patterns (B,C) and BMP responsiveness (E,F) are similar. BMP-responsive constructs shared a 52 bp region (A, *), deletion of which in the context of the 560 bp transgene resulted in reduced expression (D), and loss of BMP responsiveness in embryos (G) and in 10T1/2 cells (I). Because of diminished transgene activity, the embryo in D was stained overnight (compare with H), while embryos in B and C were stained for 1 (B) and 2 (C) hours.

52 bp sequence is required for BMP responsiveness in limb bud mesenchymal cells and in 10T1/2 cells.

We next asked whether the 52 bp sequence was sufficient to respond to a BMP signal in vivo. To minimize position effect, we produced a tetramer of this sequence, which we inserted upstream of the *hsp68-lacZ* reporter. In transgenic embryos, this construct was expressed in a subset of the sites where the parental *560bpMsx2-hsplacZ* transgene and the endogenous *Msx2* gene are expressed (Fig. 4A-I; Table 1) (10 independent lines). These included the cardiac outflow tract, pharyngeal arches, genital region, allantois, limb buds, mandible, nasal process, midbrain, eye, otic vesicle and hair follicles. An extended staining time was also required to achieve signal intensities comparable with those of larger *Msx2* constructs, indicating reduced transgene activity.

That the 52 bp fragment responded to BMP4 in a tissue-restricted manner compared with the parental 560 bp fragment was evident from experiments in which beads were placed in various tissues and at different locations in limb buds of E11.5 transgenic embryos. A BMP4-soaked bead placed in the center

Table 1. Summary of expression of *Msx2* promoter *hsplacZ* constructs

	Construct/line						
Region	560 bp	220 bp	560Δ 52 bp	560 bp hdm	52 bp	52 bp sm	52 bp hdm
Hindbrain	4/4*	4/4	4/4	4/4	4/5	2/3	0/4
Midbrain	4/4	4/4	4/4	3/4	5/5	2/3	2/4
Eye	4/4	4/4	4/4	4/4	5/5	2/3	3/4
Otic vesicle	4/4	4/4	4/4	3/4	5/5	1/3	1/4
Nasal region	4/4	4/4	4/4	4/4	5/5	2/3	0/4
Pharyngeal arch	4/4	4/4	3/4	2/4	5/5	0/3	0/4
Outflow tract	4/4	4/4	3/4	2/4	5/5	0/3	0/4
Anterior limb bud	4/4	4/4	3/4	1/4	5/5	0/3	0/4
AER	4/4	4/4	1/4	3/4	0/5	0/3	0/4
Genital region	4/4	4/4	4/4	4/4	3/5	1/3	0/4

*Number of transgenic lines expressing in a given tissue at E11.5, out of the total number of lines that expressed the transgene in a consistent pattern (sm, Smad mutant; hdm, homeodomain mutant).

or anterior of the limb elicited a *lacZ* signal comparable to that of the *560bpMsx2* transgene (Fig. 4J,K; 60 minutes). When a BMP4-soaked bead was placed in either the proximal or posterior regions of the limb, it did not stimulate *lacZ* activity (Fig. 4K; data not shown). By contrast, the *560bpMsx2* transgene responded to BMP4 beads placed in each of these locations (Fig. 4J, Fig. 6N). Furthermore, while the 560 bp fragment responded to beads placed on the head or tail, the 52 bp fragment did not. The BMP responsiveness of the *52bpMsx2* transgene was also restricted temporally. Whereas the 560 bp fragment responded to beads implanted in E12.5 limbs (Fig. 1G), the 52 bp fragment did not (Fig. 4K). TGFβ1 beads elicited no *lacZ* response (Fig. 4K), demonstrating the *52bpMsx2* transgene remained specific for the BMP pathway.

When transfected into 10T1/2 cells, a multimerized 52 bp fragment did not respond to BMP stimulation (data not shown), suggesting that 10T1/2 cells, like much of the limb mesenchyme, lacked the transcriptional machinery necessary to activate expression of the BMP-responsive element. Transgenic and transfection approaches thus showed that the 52 bp fragment directed expression and mediated BMP responsiveness in a subset of tissues and developmental stages compared with the parental 560 bp fragment.

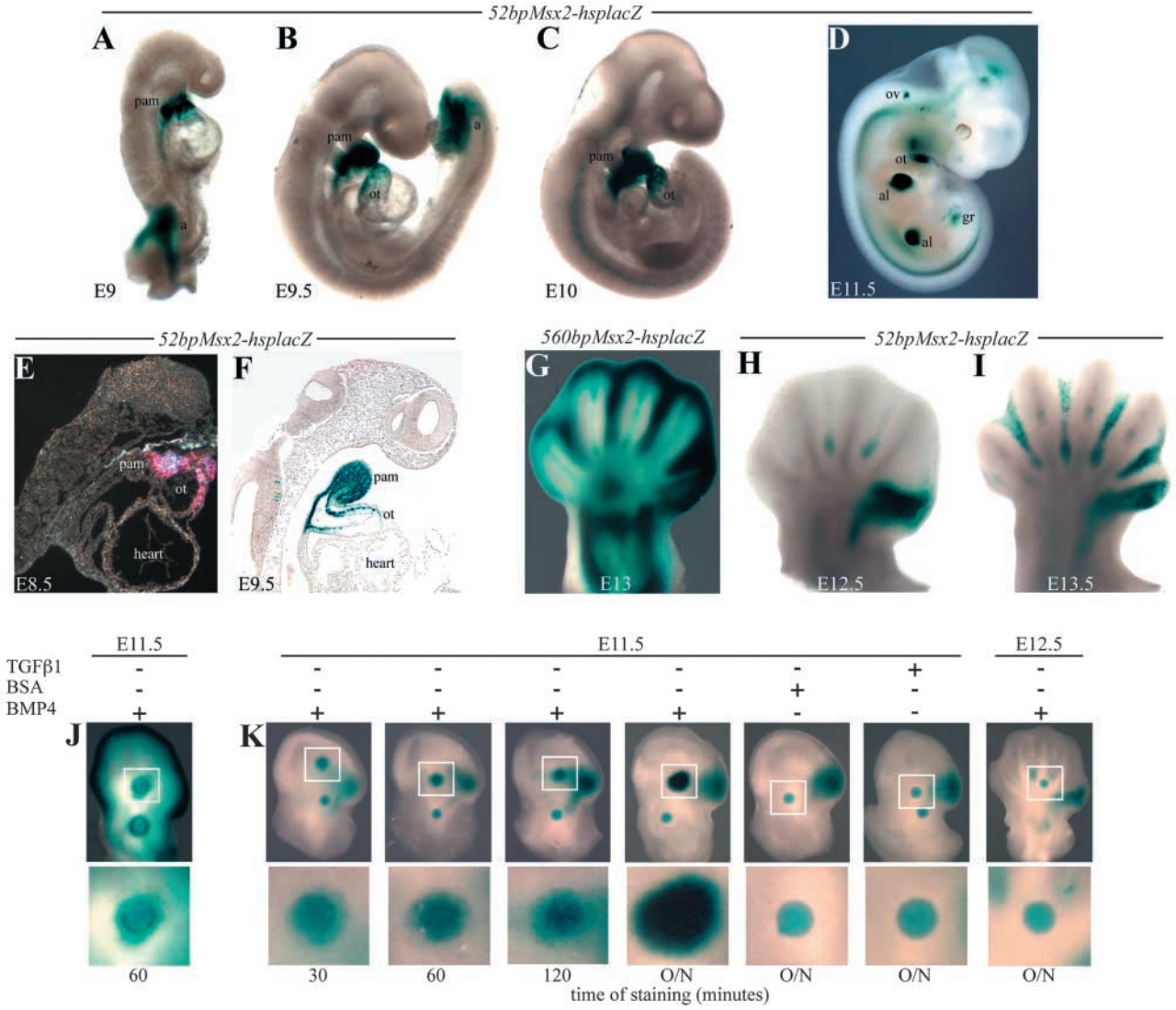

Fig. 4. The 52 bp element responds to BMP signaling in a subset of sites relative to the parental 560 bp fragment. Expression and BMP-responsiveness of the *52bpMsx2-hsplacZ* transgene were assessed in a developmental series of embryos and tissues from E8.5 to E13.5. A total of five transgenic lines were compared, with closely similar results (Table 1). (A-D) Overnight *lacZ* stains of embryo whole mounts. (E,F) Midsagittal sections of whole mounts stained for *lacZ*. The embryo in E was embedded in plastic and photographed in dark field, showing *lacZ* activity in pink. Note expression in the allantois (A,B), pharyngeal arches (A-C,E,F) and cardiac outflow tract (A-F). Expression in the pharyngeal region was restricted to tissues adjacent to the pharyngeal endoderm and cardiac outflow tract (E,F). At E11.5, expression persisted in the outflow tract, and was evident in the genital region, the anterior limb bud mesenchyme, the otic vesicle and the eye (D). (G-I) High-magnification views of forelimbs of E12.5-E13.5 embryos stained for *lacZ* (anterior to right). (G) Expression of the parental 560 bp transgene. (H,I) Expression of the 52 bp transgene in the anterior and interdigital limb mesenchyme. (J,K) *lacZ* expression in bead-implanted limbs. a, allantois; pam, pharyngeal arch mesenchyme; ot, outflow tract; al, anterior limb; gr, genital region; ov, otic vesicle; e, eye.

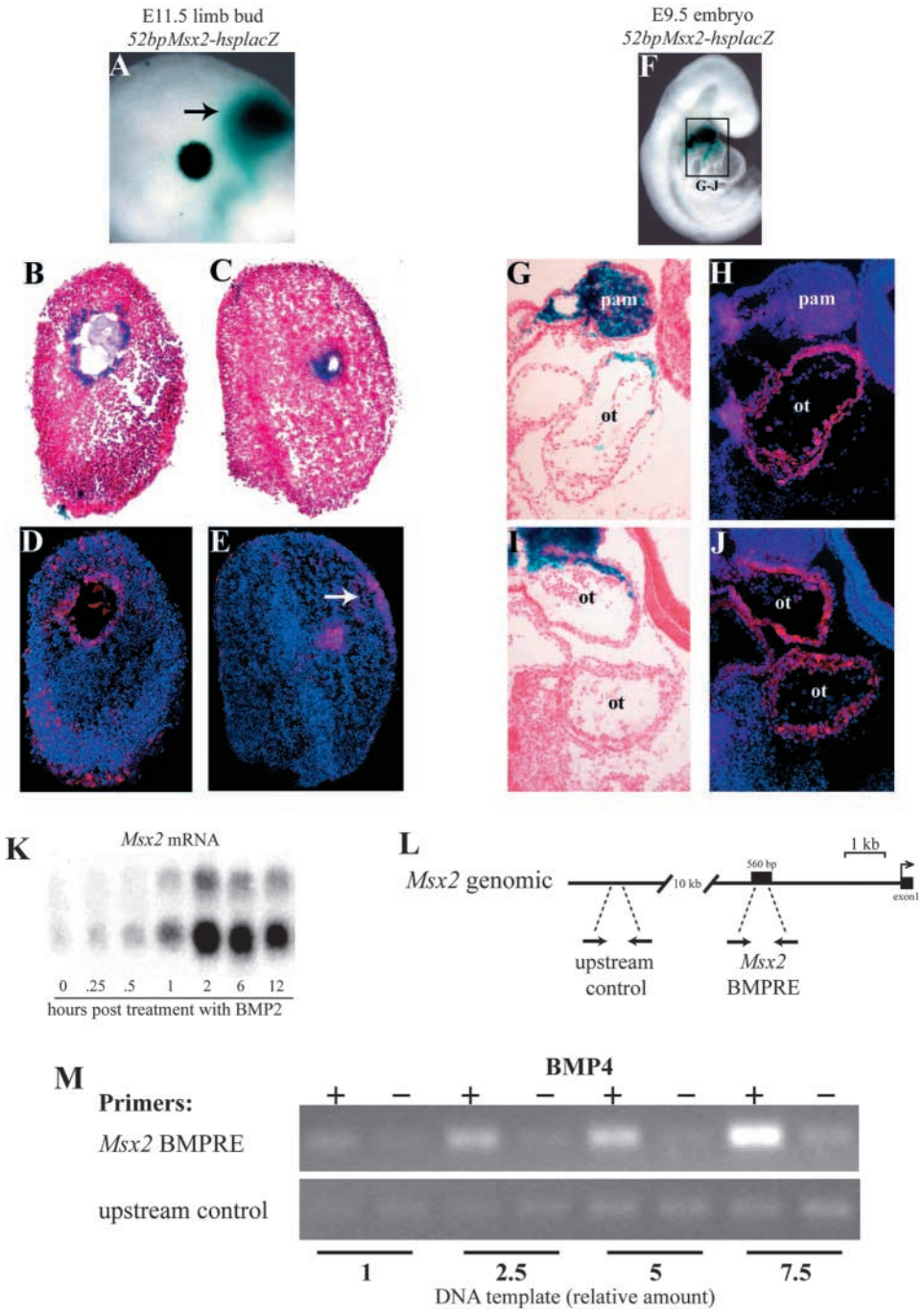

Fig. 5. Phosphorylated forms of BMP-dependent R-Smads colocalize with 52 bp *lacZ* transgene expression in the limb and cardiac outflow tract, and are recruited to the *Msx2* BMP-responsive region in native chromatin. Beads soaked in BMP4 were implanted on limbs of E11.5, *52bpMsx2-hsplacZ* transgenic embryos. (A) Whole-mount *lacZ* stain showing expression and BMP response in the anterior limb (arrow). (B-E) Adjacent frozen sections along the dorsoventral axis of the limbs were either stained for *lacZ* activity (B,C; blue against Nuclear Fast Red counterstain), or immunostained with an antibody against the phosphorylated forms of the BMP R-Smads 1, 5 and 8 (D,E; pink against DAPI counterstain). (F-J) *lacZ* (G,I) and phospho-Smad (H,J) staining of adjacent midsagittal sections through cardiac region of a *52bpMsx2-hsplacZ* embryo at E9.5 (F). A subset of phospho-Smad stained cells are positive for *lacZ* activity. (K-M) ChIP assay showing association of phosphorylated R-Smads with the *Msx2* BMPRE in C14 limb mesenchymal cells. (K) Autoradiogram of RNA derived from BMP2-treated C14 cells, probed for *Msx2*. Upstream control and BMPRE primers (L) were used to interrogate chromatin immunoprecitated with anti phospo-Smad1 antibody (M).

Phosphorylated BMP R-Smads colocalize with 52 bp transgene expression in embryos and are recruited to the *Msx2* BMP-responsive region in native chromatin

That the 52 bp transgene is expressed at sites of active BMP signaling was confirmed by staining with an antibody against the phosphorylated (active) form of the BMP restricted Smads 1, 5, 8 (R-Smads). Limbs exhibited nuclear staining in a halo surrounding implanted BMP4-soaked beads (Fig. 5D,E), coincident with *lacZ* expression (Fig. 5B,C). Nuclear staining was also evident in the anterior limb, overlapping with *52bpMsx2* transgene expression (Fig. 5A,E, arrows). Similarly, in the cardiac region, nuclear staining was apparent in pharyngeal arch mesenchyme and the outflow tract (Fig. 5H,J). Thus, in the pharyngeal region the 52 bp fragment was expressed in a subset of R-Smad positive cells (Fig. 5G,I).

The correlation between R-Smad nuclear localization and *Msx2* transgene expression suggested that *Msx2* is an immediate early target of R-Smads. If so, R-Smads should associate with the *Msx2* BMPRE in a BMP-dependent manner. To test this hypothesis, we carried out a chromatin immunoprecipitation (ChIP) analysis on C14 limb bud mesenchymal cells (Rosen et al., 1994). We chose these cells because of their tissue of origin and because their endogenous *Msx2* mRNA is upregulated robustly by BMPs (Fig. 5K). An anti Phospho-Smad1 antibody was used to immunoprecipitate chromatin from BMP-treated and control cells. Semi-quantitative PCR showed that a 350 bp fragment encompassing the 52 bp BMPRE (Fig. 5L) was enriched several fold in chromatin derived from cells treated with BMP4 compared with untreated cells (Fig. 5M), consistent with the view that BMP treatment causes association of BMP R-Smads with the *Msx2* BMPRE.

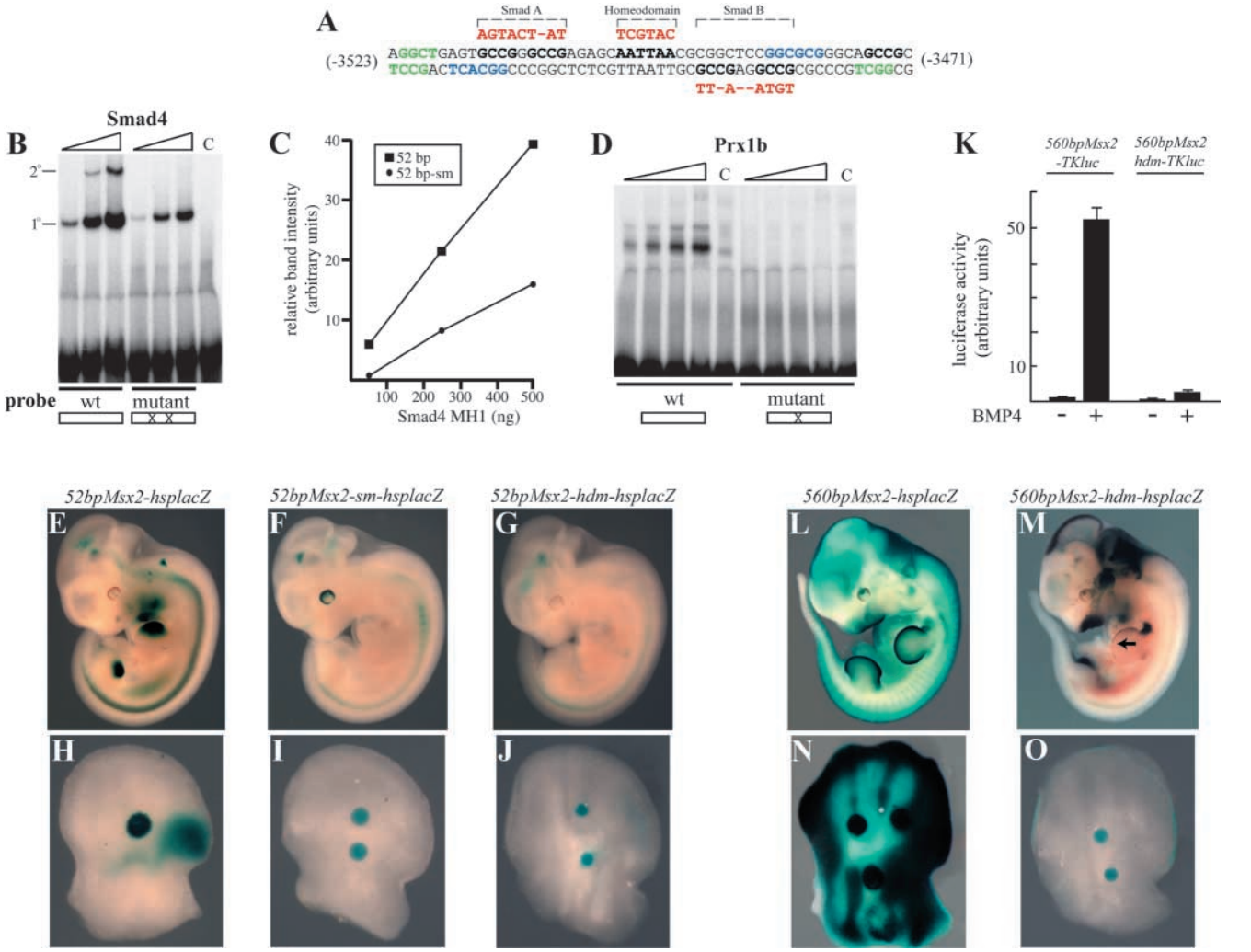

Fig. 6. Smad and homeodomain consensus sites are necessary for BMP responsiveness of *Msx2* transgenes. (A) Smad or homeodomain consensus sites (bold) were mutated in the 52 bp element as shown above and below the sequence (red). Potential Brinker (blue) and Smad4 (green) sites are identified. (B-D) EMSA was used to assess the effects of the mutations on binding of bacterially expressed Smad4 MH1 domain (B), and in vitro transcribed/translated Prx1b, a paired-class homeoprotein (D). Amounts of GST-Smad4 protein used were 50, 250 and 500ng (wild-type control; 500 ng GST). The relative amounts of Prx1b lysate used were 1, 2, 4 and 10 μl (controls; 20 μl). Band intensities of the faster-migrating, primary Smad4-DNA complexes are plotted in C. (E-J) Effect of Smad site and homeodomain site mutations on *52bpMsx2-hsplacZ* transgene expression in transgenic embryos at E11.5 (E-G), and BMP responsiveness in limbs (H-J). (K-O) We introduced the homeodomain site mutation into the 560 bp fragment (Fig. 1A, Fig. 3A) and examined the expression and BMP responsiveness of this construct in 10T1/2 cells (K) and in transgenic embryos (L-O) at E11.5. Embryos in E-G,M were stained for *lacZ* activity overnight, while that in L was stained for 1 hour.

GCCG and TAAT sequences are necessary for BMP responsiveness of *Msx2* transgenes

The 52 bp fragment contained a GC-rich partial inverted repeat flanking an AT-rich region (Fig. 2, Fig. 6A). Each repeat contained two GCCG sequences, which resembled the GCCGNCGC consensus sequence to which the *Drosophila* Mad protein binds (Kim et al., 1997; Raftery and Sutherland, 1999; Szuts et al., 1998; Xu et al., 1998). The GCCG sites flanked the sequence AGAGCAATTAACG, which matched closely the consensus site for several Antennapedia superclass homeodomain proteins, as well as paired class homeodomain proteins (de Jong et al., 1993; Florence et al., 1991; Gehring et al., 1994; Laughon, 1991) (MatInspector 7.0, 2003). As homeodomain proteins are candidates for tissue- and stage-specific modulators of the BMP response (Marty et al., 2001), the AATTAA site was of particular interest.

To test the function of the GCCG and AATTAA sequences in vivo, we produced two mutant *52bpMsx2-hsplacZ* transgenes. One contained mutations in the four GCCG sequences, the other a mutation in the AATTAA site (Fig. 6A). Gel shift experiments verified that mutations in the GC-rich inverted repeats significantly reduced binding by the Smad4 MH1 domain, a DNA-binding motif conserved among several Smad proteins (Massague, 1998). Low protein inputs produced a single complex, higher inputs an additional, more slowly migrating complex (Fig. 6B). The latter complex probably resulted from the binding of additional Smad4 molecules. Mutation of the four GCCG sequences abolished the slower

migrating (2°) complex and reduced substantially (60-90%) the faster migrating (1°) complex (Fig. 6B,C). That the primary complex was not abolished may have been due to an additional GCCG sequence located 3′ of the inverted repeat, or to several GNCT motifs, both of which might bind Smad4 (Fig. 6A) (Ishida et al., 2000; Johnson et al., 1999; Kusanagi et al., 2000; Zawel et al., 1998). Despite this residual binding of Smad4 in vitro, the transgene bearing mutations in the four GCCG sites (7 independent lines) was expressed at much lower levels and in fewer regions than its non-mutated counterpart (Fig. 6E,F; Table 1). In addition, the mutant transgene did not respond to BMP4-soaked beads implanted on the limb bud (Fig. 6I) or mandible (data not shown).

Protein titrations showed that oligonucleotides containing the TTAATT site and flanking sequence were capable of binding the homeodomain proteins Prx1b (Fig. 6D) and Msx2 (data not shown). A mutation in the TTAATT site (Fig. 6A) reduced the binding of Prx1b to an undetectable level (Fig. 6D), prompting us to test this same mutation in vivo. Analysis of 14 independent lines revealed a dramatic reduction in transgene expression in the limb bud and in most other sites (Fig. 6E,G; Table 1). Bead implantations in the limb bud showed that the mutant transgene was not BMP responsive (Fig. 6J). This profound change in the expression of the *52bpMsx2* transgene led us to test the role of the TTAATT element in a larger genomic context. We used the 560 bp fragment because it contained all or most of the cis-regulatory information needed for expression and BMP responsiveness of *Msx2* (Kwang et al., 2002).

In 10T1/2 cells, the homeodomain site mutation resulted in near-complete loss of BMP-inducibility of a transfected 560 bp construct (Fig. 6K), similar to the effect of deleting the entire 52 bp sequence in the 560 bp fragment (Fig. 3I). Analysis of six transgenic lines bearing *560bpMsx2-hdm-hsplacZ* mutant transgenes showed that the mutation resulted in a general reduction of transgene expression (compare Fig. 6M with Fig. 3H) as well as a site-selective loss of expression (Table 1). For example, expression was reduced substantially in limb bud mesenchyme but retained in the AER (Fig. 6M, arrow). Bead implantations showed that the mutation abolished BMP-responsiveness in limb bud mesenchyme of E11.5 embryos (Fig. 6O). We conclude that the TTAATT element is crucially important for BMP responsiveness of *Msx2* both in cultured cells and in a subset of sites in murine embryos.

The *Msx2* BMP-responsive element can interpret Dpp signals accurately in *Drosophila*

That the homeodomain and Smad consensus sites are required coordinately for the function of the *Msx2* BMPRE in murine embryos, and that the sequence of the BMPRE is highly conserved among mammalian groups, raised the question of whether this combination of homeodomain and Smad consensus sites reflects an ancient mechanism for BMP-dependent transcriptional activation. Cis-regulatory elements can undergo mutational turnover yet maintain their function (Ludwig et al., 2000). Thus, despite the apparent lack of sequence conservation of the BMPRE outside mammals, the possibility remained that it might function in a more diverse group of organisms.

To test this idea, we asked whether the *Msx2* BMPRE was capable of responding to Dpp signals in *Drosophila*. We chose *Drosophila* because its distant relation to mouse would provide a stringent test of the hypothesis that the BMPRE can function over a large phylogenetic distance. In addition, a large number of mutants in components of the Dpp pathway are available, enabling us to rigorously test whether the murine element was responding appropriately to Dpp signals.

We produced transgenic flies bearing the 560, 480, 220 and 52 bp sequences driving nuclear-localized *lacZ*. We examined expression in imaginal discs and embryos, both in wild-type flies and in mutants in which Dpp signaling was perturbed. Examples of transgene expression are shown in Fig. 7. The *480bpMsx2-lacZ* transgene was expressed in wing imaginal discs in a pattern that closely resembled that of *vestigial*, a known Dpp target (Fig. 7B,C). Ectopic activation of Dpp signaling with *A9Gal4>TkvA* resulted in a corresponding expansion of *Msx2* transgene expression (Fig. 7D). In stage 13 embryos, the *220bpMsx2-lacZ* transgene was expressed in parasegments (ps) 3 and 7 of embryo visceral mesoderm in a pattern closely matching that of *dpp* (Fig. 7E,G). This pattern was expanded throughout the gut in embryos in which *dpp* expression was driven ectopically by a heat shock promoter (Fig. 7I). In *dpp (S11/S22)* regulatory mutants, in which *dpp* signaling is lost specifically in ps3 (Fig. 7F, arrow), the *220bpMsx2-lacZ* transgene was also downregulated in ps3 (Fig. 7H, arrow).

The 52 bp sequence drove expression on the dorsal side of early blastoderm embryos in a pattern that matched Dpp signaling (Fig. 7J,K). Expanded transgene expression was evident in *dorsal* mutant embryos (Fig. 7L), consistent with the finding that dorsal represses *dpp* transcription in ventral cells, thus restricting Dpp signaling to the dorsal region of the embryo (Huang et al., 1993). Expression was also lost in *screw* mutant embryos (Fig. 7M), in which levels of Dpp signaling are reduced (Arora et al., 1994). Conversely, ectopic activation of Dpp signaling using *Tub Gal4>UAS Dpp* resulted in expansion of transgene expression in the dorsal half of the embryo (Fig. 7N). Similarly, embryos mutant for *brinker*, a repressor of Dpp signaling (Jazwinska et al., 1999), exhibited expanded *lacZ* activity (Fig. 7O).

When we tested the expression of *52bpMsx2-lacZ* transgenes bearing the same GCCG or TTAATT site mutations used for transgenic mice, we found that each mutation resulted in a profound loss of transgene expression (Fig. 7P,Q) (five independent lines each). Similar results were apparent in stage 13 embryos bearing these mutant transgenes (data not shown). Together, these data show: (1) that sequences within the *Msx2* promoter can respond to Dpp signaling in *Drosophila* embryos and wing imaginal discs; and (2) that both TTAATT and GCCG sites are crucial for expression of the *Msx2* BMPRE in *Drosophila* embryos as they are in mouse embryos.

Discussion

Although several BMP responsive elements have been characterized in cultured cells (Benchabane and Wrana, 2003; Henningfeld et al., 2000; Hullinger et al., 2001; Jonk et al., 1998), few have been studied in a developing mammalian embryo (Liberatore et al., 2002; Lien et al., 2002; Theil et al., 2002). The *Msx2* BMP-dependent enhancer is of particular interest because of the importance of *Msx2* in the BMP pathway and because *Msx2* is frequently employed as a readout

of BMP action, despite the fact that the mechanisms controlling its BMP responsiveness have not been elucidated.

Distinct cis-regulatory elements are required for BMP-dependent activation of *Msx2* transgenes in different developmental settings

A long-term goal in the analysis of the *Msx2* BMPRE has been to identify trans-regulators that cooperate with Smads to modulate BMP responsiveness in different developmental settings. We provide evidence here that a homeodomain consensus site is required, together with Smad consensus sites, for BMP responsiveness of *Msx2* transgenes, and that these sites function together with other cis-regulatory elements to control differential BMP responsiveness and expression in subregions of limb mesenchyme and in other structures in the developing embryo. That the consensus homeodomain site plays such a major role in the BMP responsiveness of the *Msx2* promoter was unexpected. Although Smad sites are typically found to be functionally crucial in vertebrate BMP responsive elements (Lopez-Rovira et al., 2002; Park and Morasso, 2002), similar crucial roles for homeodomain sites have not been documented. We note, however, that Smad1 interacts with Hoxc8 during activation of the osteopontin promoter (Shi et al., 1999) and that in *Xenopus* embryos, the Xvent-2 homeodomain protein can act as a Smad1-specific co-activator during maintenance of its own transcriptional regulation (Henningfeld et al., 2002).

It is clearly of interest to know the identities of proteins that interact with the TTAATT site in the *Msx2* BMPRE. Homeodomain-containing proteins of the Nk (including Msx), paired and Hox classes are capable of binding this element in vitro (Fig. 6; S.M.B. and R.M., unpublished). Representatives of each class are expressed in patterns consistent with a role in the regulation of *Msx2*, though genetic proof that any such proteins participate in the BMP-dependent activation of *Msx2*

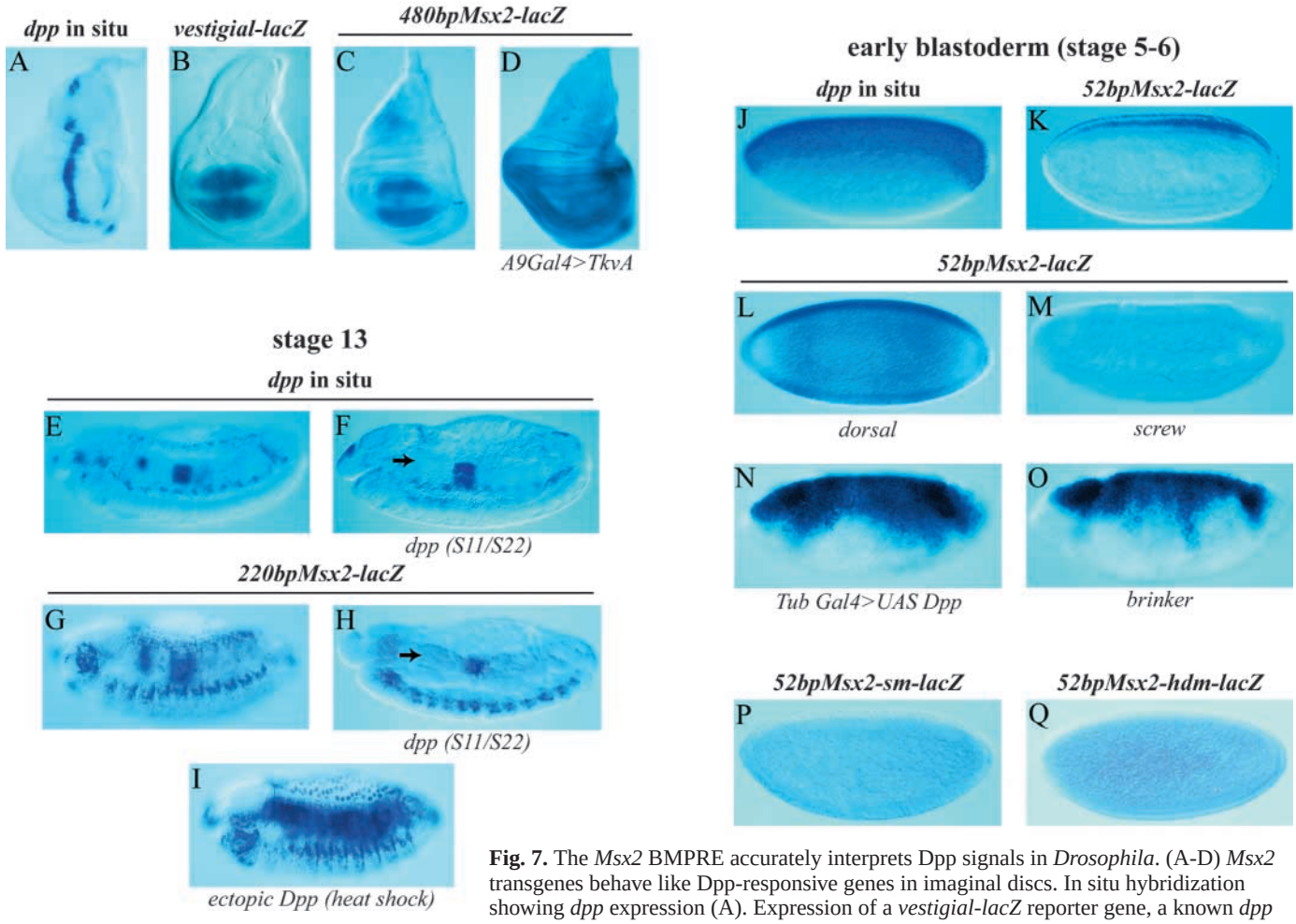

Fig. 7. The *Msx2* BMPRE accurately interprets Dpp signals in *Drosophila*. (A-D) *Msx2* transgenes behave like Dpp-responsive genes in imaginal discs. In situ hybridization showing *dpp* expression (A). Expression of a *vestigial-lacZ* reporter gene, a known *dpp* target (B). Expression of the *480bpMsx2-lacZ* transgene (C). The 480 bp fragment is the conserved region identified in Fig. 2, Fig. 3A. Expression of the *480bpMsx2-lacZ* transgene in the wing pouch with ectopic activation of the Dpp pathway by *A9Gal4>TkvA* (D). (E-I) *dpp* and *220bpMsx2-lacZ* transgene expression in stage 13 embryos (lateral view). *dpp* in situ hybridization showing wild-type expression pattern in parasegments (ps) 3 and 7 of the embryonic mid-gut (E) and in *dpp (S11/S22)* mutant (F), in which *dpp* expression is lost in ps3 (arrow), but not in ps7. *220bpMsx2-lacZ* transgene in wild type (G) and *dpp (S11/S22)* mutant (H). Expression of *220bpMsx2-lacZ* transgene throughout the gut in response to ectopic expression of Dpp driven by a heat-shock promoter (I). (J-Q) *dpp* and *52bpMsx2-lacZ* transgene expression in early blastoderm embryos (lateral view). *dpp* in situ of a stage 5 embryo showing restriction of *dpp* expression to the dorsal part of the embryo (J). Expression of the *52bpMsx2-lacZ* transgene in wild-type (K) and *dorsal* mutant (L) embryos. Expression of *52bpMsx2-lacZ* transgene in *screw* (M) and *brinker* (O) mutant embryos, and in an embryo with ectopic activation of Dpp using *Tub Gal4>UAS Dpp* (N). *52bpMsx2-lacZ* transgene expression is lost when the Smad sites (P) or homeodomain site (Q) are mutated.

is lacking. Interestingly, *Xenopus* Xmsx-1 has been shown to physically associate with pathway-restricted Smads and Smad4 (Yamamoto et al., 2001), suggesting that like Xvent2, Msx proteins may participate in an autoregulatory loop.

In addition to homeodomain-containing proteins, members of the Fox family of forkhead/winged helix transcription factors have emerged as candidate regulators of the BMP-responsiveness of *Msx2*. Foxc1 is required for the BMP-dependent induction of *Msx2* in calvarial tissues of murine embryos (Rice et al., 2003). Inspection of the sequence around the TTAATT site revealed a close match (5/7) to a consensus derived for Hnf3/Fox class proteins (Fig. 2) (Gao et al., 2003). Whether Fox proteins can bind this sequence, and, if so, whether such binding is disrupted by mutations in the TTAATT site are unanswered questions. It is intriguing that Fox proteins can form heterodimers with homeodomain-containing proteins (Foucher et al., 2003), and that the Fox protein Fast1 can interact with Smads and modulate the activity of TGFβ-responsive promoters (Chen et al., 1996; Chen et al., 1997; Weisberg et al., 1998).

Deletion analysis showed sequences flanking the 52 bp element control the location, sensitivity and timing of BMP responsiveness in limbs and other sites in mouse embryos. Within these sequences are consensus binding sites for Creb, OAZ and Tcf/Lef1 (Fig. 2), each implicated in BMP signaling in widely divergent organisms (Hata et al., 2000; Ionescu et al., 2004; Theil et al., 2002). In the *Drosophila Ubx* promoter, for example, Dpp responsiveness in parasegments 3 and 7 of the embryonic midgut depends on a CRE site acting together with Mad sites (Waltzer and Bienz, 1999). In addition, Tcf/Lef1 sites located 270 and 420 bp upstream (at positions –4130 and –4280) of the *Δ4Msx2* fragment, participate in BMP responsiveness in ES cells (Hussein et al., 2003). Mutation of these sites in mouse embryos will provide insight into their roles in modulating the BMP responsiveness of *Msx2* in different tissues and cell types. We showed previously that Pax3 acts through a site in the 560 bp fragment to repress *Msx2* expression in the dorsal neural tube (Kwang et al., 2002). We have not yet identified sequences required for upregulated neural tube expression of *Msx2* in the absence of *Pax3* function, but it is possible that these sequences include the *Msx2* BMPRE. Finally, we note that YYI has been implicated in the activation of *Msx2* expression in craniofacial structures, but in a manner independent of the BMP pathway (Tan et al., 2002).

In *Drosophila*, *brinker* and *schnurri* are crucial for the Dpp-dependent expression of a number of genes. Brinker (Brk) acts through GC-rich sites to repress target gene expression (Rushlow et al., 2001; Sivasankaran et al., 2000). Schnurri represses *brinker* expression in a Dpp-dependent manner (Marty et al., 2000; Torres-Vazquez et al., 2000). That *Msx2* transgene expression in *Drosophila* embryos expands in a *brinker* mutant is consistent with results showing that Brk acts through Mad-like binding sites for its repressive function (Kirkpatrick et al., 2001; Saller and Bienz, 2001), and with our findings that the *Msx2* BMPRE is probably a direct target of Smad1 (Fig. 5). Our demonstration that mutations in Mad/Smad1-binding sites cause loss of transgene expression in *Drosophila*, together with the expansion of transgene expression in the *brinker* mutant, suggest that the *Msx2* BMPRE is subject to positive and negative regulation in *Drosophila*, as expected for direct targets of Mad.

Whether *Msx2* transgenes are similarly regulated by positive and negative inputs in mouse embryos is not clear. Orthologs of *brinker* have not been found in vertebrates. However, Zeb-2, a zinc-finger transcription factor, has been identified as a negative regulator of BMP signaling (Postigo, 2003). This protein binds to a GC-rich sequence and can interact with Smads (Verschueren et al., 1999), as well as inhibit expression of the BMP target, *Xmsx1*, in *Xenopus* (Postigo et al., 2003).

Evolution of the *Msx2* BMP-responsive element

Despite the lack of demonstrable sequence homology between the *Msx2* BMPRE and Dpp-responsive enhancers in flies, our transgenic experiments show that the *Msx2* element provides an accurate readout of Dpp activity in *Drosophila*. This is not merely a result of the activity of Mad, as a mutant transgene bearing a disabled TTAATT sequence but intact GCCG consensus sites was not expressed in *Drosophila* embryos. We note that the requirement of the TTAATT site for BMP responsiveness of *Msx2* transgenes in mice and flies is reminiscent of the dependence of the *labial*, *tinman* and *even-skipped* Dpp-activated enhancers of *Drosophila* on the homeodomain proteins Labial and Tinman (Knirr and Frasch, 2001; Marty et al., 2001; Xu et al., 1998).

That several BMP-responsive enhancers spanning a wide phylogenetic distance exhibit a dual requirement for homeodomain and Smad sites suggests synergistic interactions between homeodomain and Smad proteins is an ancient feature of the BMP/Dpp pathway. Such interactions may represent a general mechanism for integrating BMP/Dpp signaling inputs with tissue-specific transcription factors.

Few cis-regulatory elements are known to function over an evolutionary distance comparable with that separating mammals and insects. One such element is the eye enhancer of the *Drosophila eyeless* gene, a *Pax6* ortholog. In transgenic mice, this enhancer directs expression that partially recapitulates that of endogenous *Pax6* (Xu et al., 1999). Another is the murine *Hoxa2* rhombomere 2-specific enhancer, which is expressed in head segments of transgenic flies, paralleling the expression of the *Hhoxa2* ortholog, *proboscipedia* (Frasch et al., 1995). Similarly, a *Hox4b* cis-regulatory element mediates correct spatial expression in *Drosophila* (Malicki et al., 1992), and the orthologous *Drosophila* autoregulatory element from the homeotic gene, *Deformed*, functions appropriately in mice (Awgulewitsch and Jacobs, 1992).

These examples notwithstanding, careful comparative analyses within nematode and echinoderm lineages suggest that cis-regulatory elements usually evolve rapidly, becoming non-alignable within 35-50 million years (Ruvinsky and Ruvkun, 2003; Wray et al., 2003). Intriguingly, such divergent promoter elements frequently maintain their function, suggesting they are subject both to drift and stabilizing selection, which promote rapid sequence divergence while maintaining regulatory function (Ludwig et al., 2000; Ruvunski and Ruvkun, 2003; Wray et al., 2003). It seems probable that the ability of the *Msx2* BMPRE to function in flies, despite its lack of significant sequence identity to known Dpp-responsive enhancers, can be explained by a similar process of drift and stabilizing selection. We suggest this process differs from that operating on a typical promoter element only in its slow pace, reflecting the fundamental and

conservative role of the machinery that controls the transcription of crucial effectors of the BMP pathway.

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