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Transcriptional regulation of BMP4 synexpression in transgenic *Xenopus*

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Synexpression groups are genetic modules composed of genes that share both a complex expression pattern and the biological process in which they function. Here we investigate the regulation of BMP4 synexpression by studying the enhancers of *bambi*, *smad7* and *vent2* in *Xenopus*. We find that a BMP4 synexpression promoter module is compact and (i) requires direct BMP responsiveness through Smad and Smad-cofactor binding motifs, (ii) may contain an evolutionary conserved BMP-responsive element, bre7 (TGGCGCC), that is crucial for expression of *bambi* and *smad7* and is highly prognostic for novel BMP-responsive enhancers (BREs); and (iii) requires a narrow window of BMP inducibility, because minor enhancement or reduction of BMP responsiveness abolishes synexpression. Furthermore, we used a bioinformatic model to predict *in silico* 13 novel BREs, and tested five of them that were found in the *id1-4* genes. The results highlight that *in vivo* analysis is required to reveal the physiological, spatio-temporal regulation of BMP-responsive genes.

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Introduction

Recent large-scale analyses have revealed coordinate expression of genes functioning in the same biological process. Such clusters of coexpressed genes were found by microarray experiments in yeast and in mammalian cultured cells, and in a systematic gene expression screen by RNA *in situ* hybridization, we have previously discovered four clusters of about 50 coexpressed genes during early *Xenopus* development (Gawantka *et al*, 1998). Such clusters, or synexpression groups (Niehrs and Pollet, 1999), were linked to distinct molecular processes, such as protein secretion, chromatin assembly and growth factor signaling. More recently, synexpression groups were also identified in mouse and zebrafish embryos, indicating that they are a widespread phenomenon (Tsang *et al*, 2000; Grotewold *et al*, 2001; Niehrs and Meinhardt, 2002). Member genes of synexpression groups show tight spatio-temporal RNA coexpression over various stages of embryogenesis and, wherever investigated, these genes cofunction in the respective molecular pathway (Onichtchouk *et al*, 1996, 1999). The tight coexpression of member genes of a synexpression group raises the question of how coregulation is achieved. A likely possibility is that genes of a synexpression group share similar enhancers, which in its simplest case may be regulated by one master control gene. A well-characterized example is the BMP4 synexpression group (Figure 1A), members of which are expressed like this growth factor—dorsally in the eye, heart and proctodeum of tailbud stage *Xenopus* embryos (Figure 1B). This group consists of eight members, which all encode components of the BMP signaling cascade as studied in early development, including ligands, receptors and downstream components of the pathway.

BMPs belong to the large family of TGF-β signaling molecules and are important regulators of early embryonic patterning, organogenesis and tissue homeostasis. BMP ligands bind and induce heterodimerization of two classes of receptors with Ser/Thr kinase activity that transmit the signal mainly via the Smad pathway (Heldin *et al*, 1997; Miyazawa *et al*, 2002; Shi and Massagué, 2003). Smad proteins belong to three distinct functional classes. The receptor activated Smads (R-Smads) are phosphorylated by the activated receptors and transduce the BMP (Smad1, 5 and 8) or the TGF-β/activin type of signals (Smad2 and 3). In the cytoplasm, R-Smads form heteromeric complexes with the Co-Smad (Smad4) and translocate to the nucleus, where they regulate target genes' expression in complexes with other transcription factors. Inhibitory Smads (Smad6 and 7) attenuate the signaling cascade by inhibition of R-Smad phosphorylation and oligomerization or by targeting the receptors to degradation (Shi and Massagué, 2003).

Smads can bind directly to certain DNA motifs, but with relatively low affinity and specificity (ten Dijke *et al*, 2000; Shi and Massagué, 2003; Zwijsen *et al*, 2003). R-Smads and Smad4 recognize the so-called Smad binding element (SBE) motif GTCT (Shi *et al*, 1998) optimally: GTCTG, which is found in the promoters of most TGF-β and BMP target genes. Another motif, GCAT, was described as a Smad1 binding element in the promoter of *Xenopus vent2* (Henningfeld *et al*, 2000). Furthermore, *Drosophila* R-Smad (Mad) was shown to bind GC-rich sequences with a GCCG core consensus (Kim *et al*, 1997). Recently, GC-rich elements were described as Smad binding motifs in several vertebrate BMP-responsive promoters, for example, *smad6* (Ishida *et al*, 2000) and *id1* (Korchynskyi and ten Dijke, 2002; Lopez-Rovira *et al*, 2002). Multimerized GCCG elements were used as BMP-specific reporters in cell culture assays (Kusanagi *et al*, 2000).

Smads alone bind to DNA with low affinity and selectivity and therefore need to associate with various transcription factors (TF) and it is these R-Smad/Co-Smad/Co-TF

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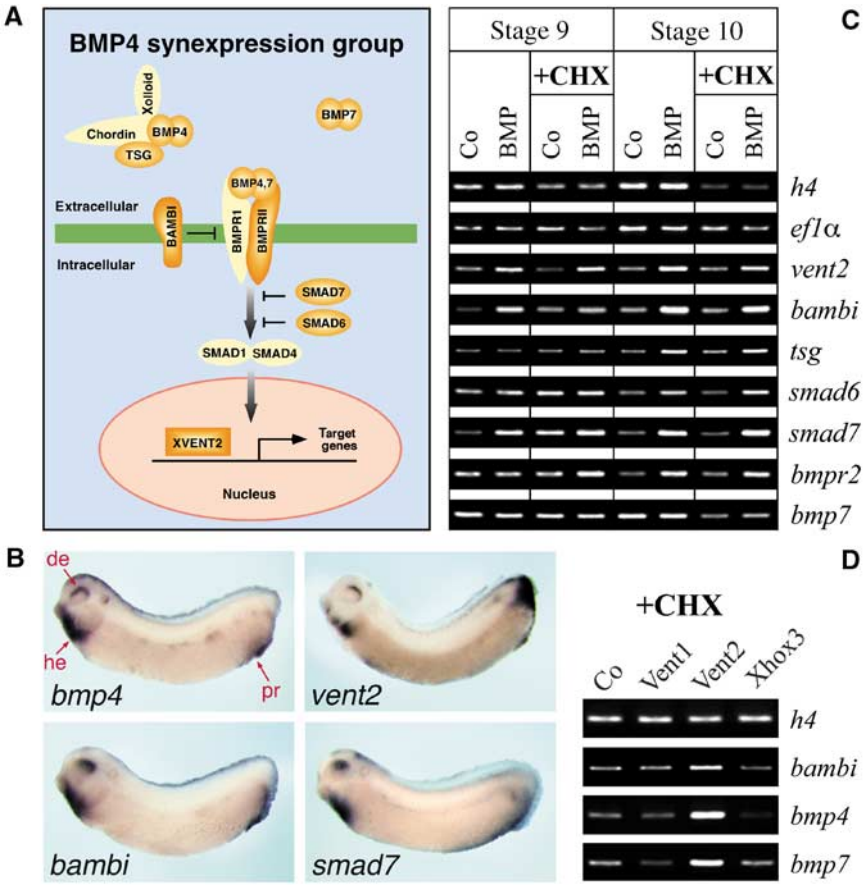


Figure 1 BMP4 synexpression group. (**A**) Simplified scheme of the BMP signaling pathway with coexpressed components (synexpression group member genes) indicated in orange. (**B**) Whole-mount *in situ* hybridization for *bmp4, vent2, bambi* and *smad7* in *Xenopus* tailbud embryos reveals their highly similar expression pattern in the dorsal eye (de), heart region (he) and proctodeum (pr). (**C**) RT–PCR analysis of ectodermal explants (animal caps) from embryos injected animally with 2 ng *bmp4* RNA and either treated or untreated with cycloheximide (CHX) from stage 8 onwards. Animal caps were collected at stage 9 (late blastula) or 10 (early gastrula) and analyzed for expression of the indicated RNAs. The samples were normalized by *ef1*α and histone *h4* expression. (**D**) RT–PCR analysis of animal caps from embryos injected animally with 2 ng *vent1, vent2* or *xhox3* RNA and treated with CHX from stage 8 for 3 h.

complexes that ensure promoter and cell-type specific activation of TGF-β target genes (Miyazawa *et al*, 2002; Zwijsen *et al*, 2003). Many transcription factors (Fast, AP1, ATF2, TFE3, Mixer, Lef/TCF and others) were found as Smad partners in the activin/TGF-β pathway (Shi and Massagué, 2003) and a common Smad interacting motif (SIM), which could be utilized for bioinformatic prediction of other Smad cofactors, was identified in some of them (Germain *et al*, 2000). Unlike TGF-β-responsive enhancers, little is known at present about how Smad/Co-TF complexes form and regulate BMP-responsive enhancers (BREs). The best-studied case is the *Xenopus vent2* gene. OAZ (Hata *et al*, 2000) and Vent2 itself (Henningfeld *et al*, 2002) were shown to interact physically with Smad complexes and DNA elements on the *vent2* promoter.

The coordinate transcriptional regulation of members of the BMP4 synexpression group raises the question of its molecular basis. Some of the genes, such as *vent2* and *smad6*, are direct targets of the BMP/Smad signaling pathway. It is unknown whether other BMP synexpression group genes are also directly regulated by Smads, thus making BMP4 the master regulator of the entire synexpression group. Are there common elements in the enhancers of member genes and if so which? If the enhancers are composed of different modules, what is their individual contribution to the domains of expression? Is it possible to derive a consensus structure for the regulatory elements of this synexpression group? Importantly, it is unclear whether BMP responsiveness alone is sufficient to confer synexpression: despite the many studies on BREs there is little information regarding their physiological relevance for *in vivo* expression.

To address these questions, we have cloned and characterized three BREs from *Xenopus bambi* and *smad7* and performed comparative analyses of these BREs with that of *vent2* in transgenic frogs. Combining experimental and bioinformatic methods, we identified a GC-rich phylogenetically conserved motif as a critical BMP-responsive element that can be used for *in silico* identification of novel BREs.

Results

Synexpression group members are immediate early BMP4 target genes

The BMP4 synexpression group consists of eight genes that encode ligands (BMP4 and 7), receptors (BMPR2, BAMBI), extra- and intracellular modulators (Tsg, Smad6, Smad7), and transcription factors (Vent2) involved in BMP signaling

(Figure 1A). The similar expression pattern of these genes (Figure 1B) suggests that they may be under common transcriptional regulation. Indeed, *Xenopus vent2* and mouse *smad6* genes are direct targets of BMP signaling (Hata *et al*, 2000; Ishida *et al*, 2000). To address whether other members of the synexpression group are also immediate BMP targets, *Xenopus* embryos were injected with *bmp4* RNA and analyzed by RT–PCR for target gene expression at the late blastula (st. 9) and early gastrula stage (st. 10). In order to distinguish between direct and indirect induction, we treated embryos with or without the protein synthesis inhibitor cycloheximide (CHX). In *Xenopus* embryos, RNA starts being translated immediately after injection, whereas zygotic transcription begins at the mid-blastula stage when embryos were transferred to CHX. RT–PCR analysis (Figure 1C) revealed that *vent2, bambi, tsg, bmpr2, smad6* and *smad7* are induced by *bmp4* in the presence of CHX, albeit with subtle kinetic differences, and hence are direct targets of BMP signaling. In contrast, *bmp7* is not induced by *bmp4*. Similar results were obtained when embryos were injected with RNA for downstream components of the pathway, namely constitutively active BMP receptor—CABR (Candia *et al*, 1997)—or Smad1 plus Smad4, and CHX treated (data not shown). To test whether downstream effectors of the BMP signaling cascade can also induce BMP4 synexpression group genes, *vent1, vent2* and *xhox3* RNAs were injected and the embryos were treated with CHX (Figure 1D). Only Vent2 was capable of directly inducing the tested genes *bambi*, *bmp4* and *bmp7*. *bmp4* contains Vent2 binding sites that are required for its autoregulation (Schuler-Metz *et al*, 2000). As *bambi* is induced both by BMP4 as well as its transcriptional target Vent2 in the presence of CHX, it is both a direct as well as an indirect (via Vent2) BMP target gene. These results suggest that developmental synexpression results from transcriptional coregulation by the BMP signaling cascade.

Identification of the BRE of bambi

Xenopus bambi encodes a pseudoreceptor that negatively regulates TGF-β signaling (Onichtchouk *et al*, 1999) and is tightly coexpressed with BMP4 during development in *Xenopus* (Figure 1B), mouse (Grotewold *et al*, 2001) and zebrafish (Tsang *et al*, 2000). To analyze its enhancer and to compare it with already known BREs, a 13 kb clone, containing around 6 kb from the 5′ flanking region of *bambi*, was isolated from a *Xenopus* genomic library (Figure 2A). When the upstream region of *bambi* was sequenced and compared with the corresponding genomic segment of the human ortholog *nma*, a single highly conserved fragment of around 200 bp was found 2 kb upstream of *bambi* and 0.5 kb upstream of *nma* (Figure 2A). Conserved noncoding sequences (CNCS) are reliable indicators of regulatory elements, especially when found in distant species, due to the selective pressure exerted on functionally important DNA regions (exons, promoters, enhancers, etc.).

We further analyzed this putative enhancer for potential TF binding sites that are linked to BMP signaling. Interestingly, eight Smad binding sites were detected, five of them fully conserved between the frog and the human DNA sequence. Most represent the common SBE motif GTCT (or AGAC in reverse orientation). In addition to SBE, two Smad1 binding motifs (GCAT) and one fully conserved GC-rich element (potential Smad1/5 motif) were found in the putative *bambi* enhancer (Figure 2A). Consensus binding sites were also found for GATA, Lef/TCF and Vent2 transcription factors. Of special interest is the presence of four putative Vent2 binding motifs (C)TAATT, because we observed direct induction of *bambi* by Vent2 in RT–PCR experiments and since Vent2 interacts with Smad1 to activate its own promoter (Henningfeld *et al*, 2002).

To confirm that this 200 bp fragment represents a functional enhancer, we carried out a systematic deletion analysis of the *bambi* upstream genomic region via luciferase reporter assays (Figure 2B). Deletion constructs were coinjected in *Xenopus* embryos either with constitutively active (CABR) or truncated (dominant-negative) BMP receptor (tBR), to enhance or suppress embryonic BMP signaling. This analysis showed that the evolutionary conserved 200 bp segment was indeed the BMP-responsive enhancer of *bambi* (compare constructs −2197 and −2007). The −154 construct harbors BMP-independent, basal promoter activity driven by two evolutionary conserved, overlapping Sp1 sites, deletion of which eliminates this activity (data not shown). In addition, a silencer element is located around −1 kb (compare −1323 to −767 constructs) that reduces the basal activity of the proximal promoter only. The *bambi* enhancer is active not only on its own proximal promoter but also on a heterologous minimal promoter from the collagen X gene (Harada *et al*, 1997). Strong stimulation of pE + Xcol was observed by CABR mRNA coinjection, which was significantly reduced when 5′ and, especially, 3′ deletions in the enhancer were introduced (Figure 2C).

Characterization of the BRE of bambi

We next dissected the *bambi* enhancer through deletions (Figure 3A) and point mutations (Figure 3B) of the predicted TF binding sites. Both 5′ and 3′ deletions of the enhancer confirmed the importance of most Smad and Vent2 consensus motifs. Multimerization of the 5′ 30 bp segment containing such motifs led to strong BMP responsiveness (p11). However, BMP inducibility is partially retained when deleting the 5′ Smad/Vent2 motifs (e.g. p3), but almost abolished by deleting the 3′ motifs (e.g. p9, p15 and Figure 2C). The greater importance of the 3′ enhancer part is supported by multi-species sequence alignment (see below).

While deletions of the putative GATA and the 5′ SBE motifs did not affect reporter activity, elimination of the consensus Lef/TCF binding site strongly reduced reporter levels (compare p3 and p4 constructs). Lef/TCF are HMG box transcription factors that mediate Wnt/β-catenin signaling, but may also serve an architectural function in the assembly of TF complexes by DNA bending. In the case of the *bambi* enhancer regulation, Wnt signaling is not involved, since neither overexpression of XWnt8 (by plasmid injection) nor the Wnt inhibitor Dkk1 affects reporter activity (data not shown). This suggests that Lef/TCF or related proteins may have architectural rather than signaling function in this case. This is further supported by substituting a 60 bp fragment containing this element with irrelevant DNA, which strongly reduced expression (p13). In contrast, its deletion did not affect significantly reporter activity (p12), possibly because proximity of the 5′ and 3′ enhancer parts can compensate for lack of DNA bending.

While point mutations in the 5′ sites only reduce enhancer activity, mutating the GC-rich box (potential Smad1/5

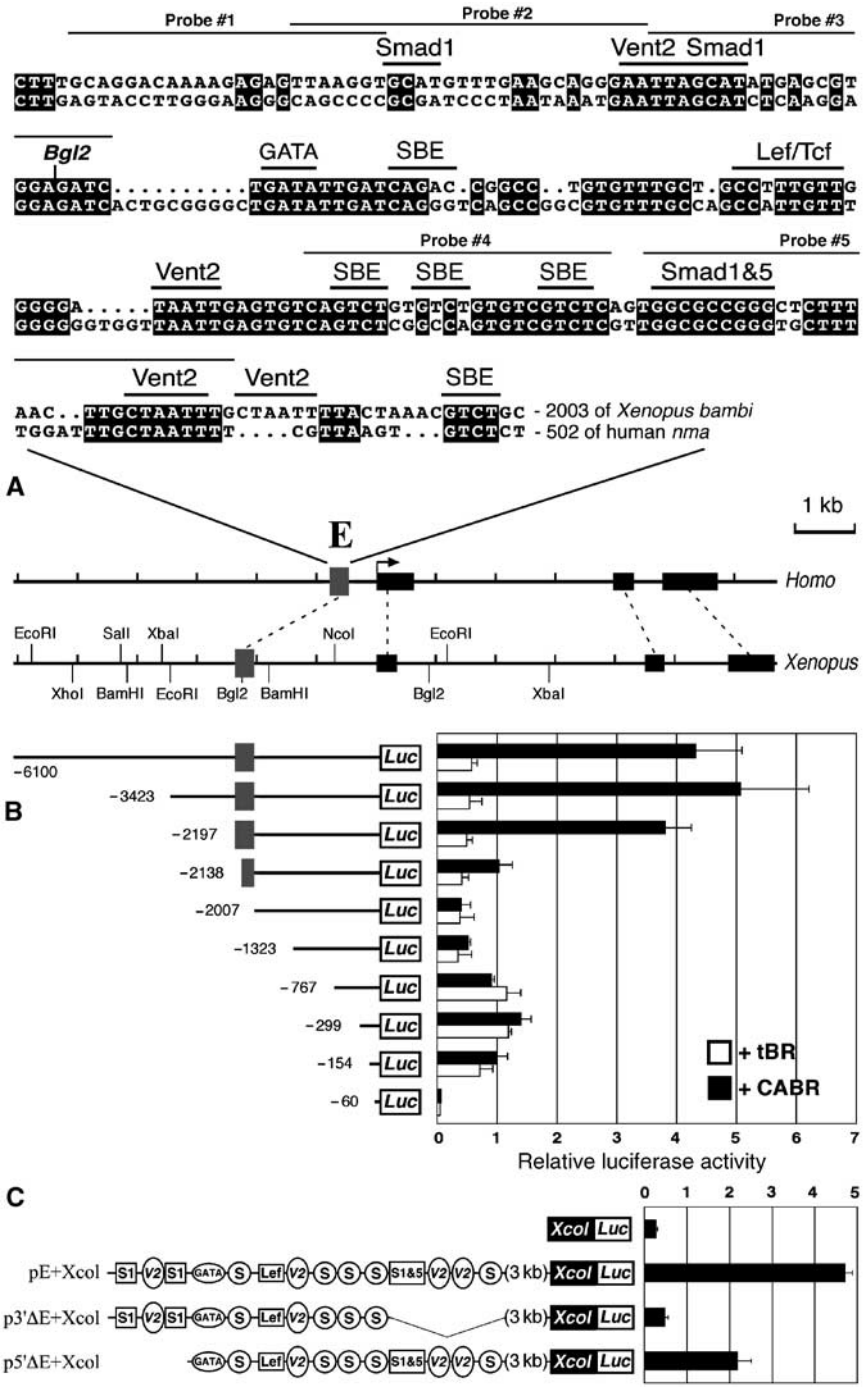


Figure 2 Isolation of the BRE of *bambi*. (**A**) Comparative map of *Xenopus bambi* and human *nma* genomic regions. The exons are shown as black bars, a conserved 200 bp fragment (putative enhancer) is labeled 'E' (gray box) and its *Xenopus/Homo* sequence alignment is shown. The putative transcription factor binding sites are shown above the alignment. Also highlighted are sequences used to derive probes for EMSA assays. (**B**) Deletion analysis of the 5′ flanking region of *bambi* confirms that E is a BRE. *Xenopus* embryos were coinjected with the indicated promoter constructs and either tBR or CABR mRNA and harvested at the gastrula stage for luciferase reporter assay. (**C**) The *bambi* enhancer confers BMP inducibility to a heterologous (collagen X) minimal promoter. *Xenopus* embryos were coinjected with the indicated promoter constructs and CABR. Putative TF binding sites from panel A are represented schematically: S, SBE; S1, Smad1; V2, Vent2; S1&5, Smad1 and 5 motifs.

binding sequence, pM11) completely abolishes BMP inducibility (Figure 3B). This is in agreement with the deletion experiments (p15, Figure 3A) and points to the critical importance of this putative Smad1/5 binding motif. Mutating the three adjacent SBEs (pM8/9/10) also significantly reduces reporter activity. One of the two consensus Vent2 elements adjacent to the Smad1/5 motif is also essential (pM12 and p7, Figure 3A).

Consistent with the greater importance of the 55 bp 3′ BMP-responsive element (3′ BRE), it is phylogenetically highly conserved (Figure 3C). A heptanucleotide TGGCGCC in the critical Smad 1/5 motif is fully conserved, but the

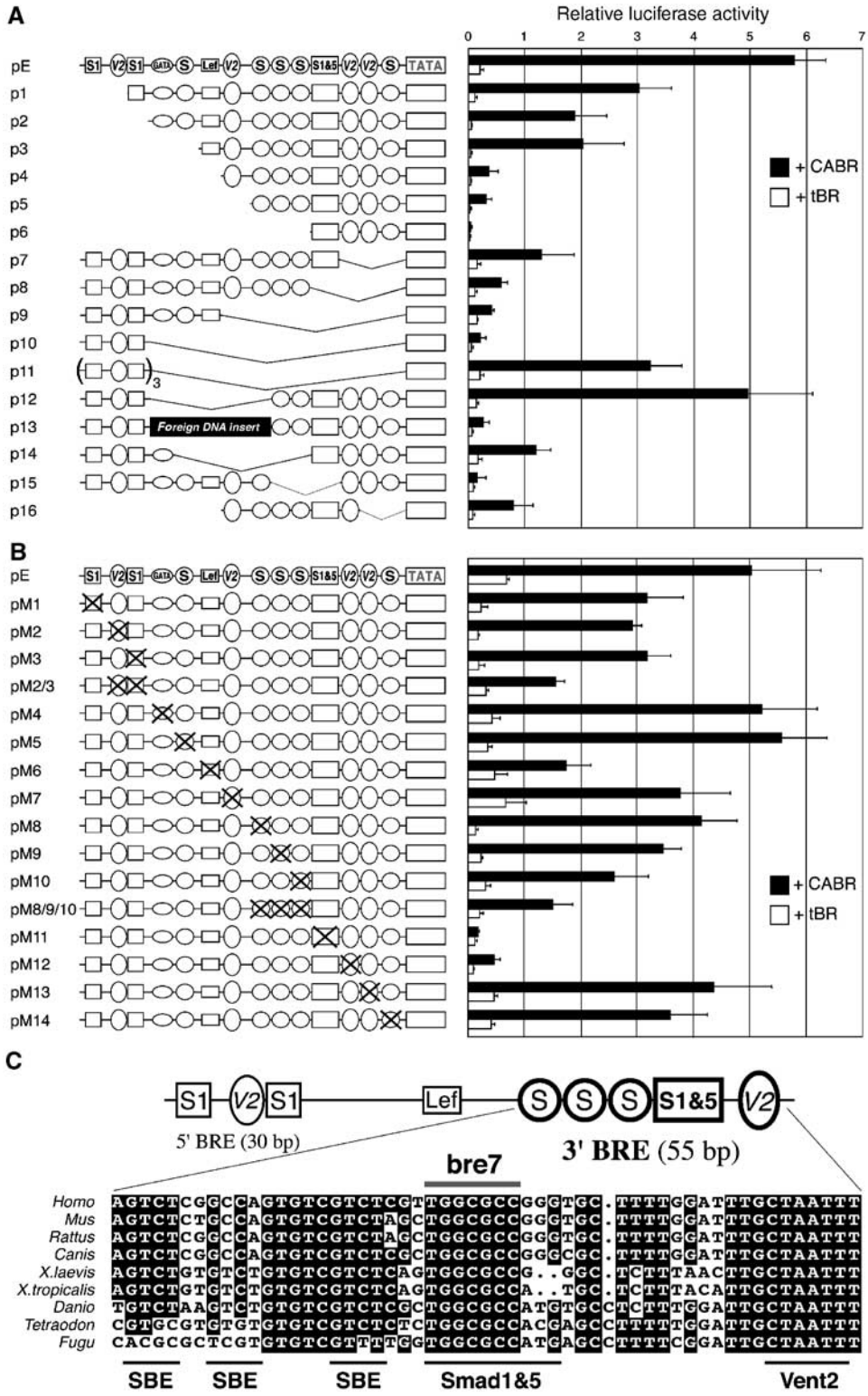
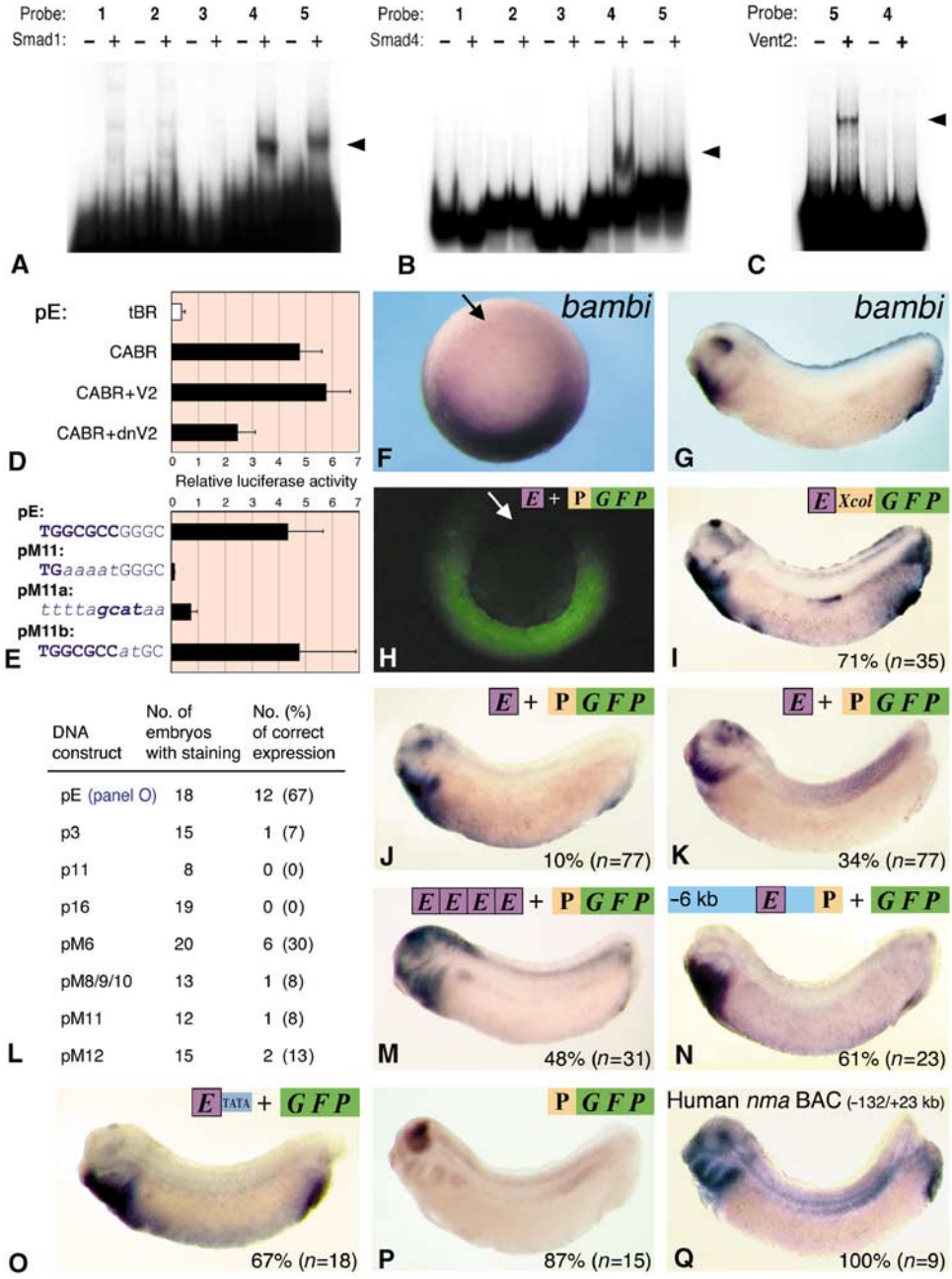


Figure 3 Functional dissection of the *bambi* enhancer. (**A**) Deletion and (**B**) point-mutation analyses of the 200 bp enhancer. Frog embryos were injected with the indicated constructs together with tBR or CABR mRNA and collected at gastrula stage for luciferase reporter assays. Putative TF binding sites are represented schematically: S, SBE; S1, Smad1; V2, Vent2; S1&5, Smad1 and 5 motifs. 'TATA' represents the heterologous 13 bp core adenovirus E1b promoter. (**C**) Structure of the *bambi* enhancer with functionally relevant TF binding sites. The major 3′ BMP-responsive element (3′ BRE) is highlighted and its phylogenetic conservation in nine vertebrate species is shown. Putative TF binding sites are underlined. Sequences were extracted from the assembled genomic data for *Homo* (human), *Mus* (mouse), *Rattus* (rat), *Danio* (zebrafish) and *Fugu* (pufferfish), or from the whole-genome shotgun trace archives for *Canis* (dog), *X. tropicalis* (frog) and *Tetraodon* (pufferfish).

overlapping GCCG core, believed to be essential for Smad1/5 binding, is not. There is no consensus SBE in *Fugu*, one in *Tetraodon*, and two or three copies are found in the higher vertebrates. The region upstream of the 3′ BRE is partially conserved in *Xenopus tropicalis*, zebrafish and mammals, but not in the pufferfish species. Thus, there is a trend toward more Smad motifs and presumably greater BMP responsiveness in higher vertebrates.

We next tested the ability of recombinant Smad and Vent2 proteins to bind to the predicted motifs in EMSA assays. Smad1 interacts with the probe containing the putative Smad1/5 binding site (#5), as well as with the 3× SBE probe (#4, Figure 4A). However, no Smad1 binding to the GCAT-containing probes was observed (#2 and #3), which may reflect the reported importance of flanking sequences within the *vent2* promoter (Henningfeld *et al*, 2000).



DNA construct	No. of embryos with staining	No. (%) of correct expression
pE (panel O)	18	12 (67)
p3	15	1 (7)
p11	8	0 (0)
p16	19	0 (0)
pM6	20	6 (30)
pM8/9/10	13	1 (8)
pM11	12	1 (8)
pM12	15	2 (13)

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Figure 4 *In vitro* and transgenic characterization of the *bambi* enhancer. EMSA assays with recombinant Smad1 (**A**), Smad4 (**B**) and Vent2 proteins (**C**) of probes 1–5 (see Figure 2A). Specific bandshifts are indicated with arrowheads. (**D**) *Xenopus* embryos were injected with the 200 bp enhancer construct pE together with the indicated RNAs. V2, Vent2; dnV2, dominant-negative Vent2. (**E**) Luciferase reporter assay in *Xenopus* embryos of enhancer constructs with mutations in the crucial GC-rich element. All constructs were coinjected in embryos with CABR mRNA. Constructs pE (wild-type) and pM11 are shown in Figure 3B. The replaced nucleotides and the alternative Smad1 motif GCAT are highlighted. (**F, G**) *In situ* hybridization of *bambi* at gastrula (vegetal view) and tailbud stage embryos. The arrows in panels F and H point to the blastopore. (**H–Q**) Transgenic *Xenopus* assays with the indicated DNA constructs. Transgene expression was visualized by fluorescence (H) or *in situ* hybridization for *gfp* (I–P) and *nma* mRNA (Q). The percentage of embryos with the depicted pattern is indicated. E, 200 bp *bambi* enhancer; Xcol, collagen X minimal promoter; P, *bambi* minimal promoter (−154/+60). (L) Results from transgenic experiments with constructs from Figure 3. The correct expression pattern referred to (heart + proctodeum) is the one shown in panel O.

Expectedly, recombinant Smad4 interacted only with the 3 × SBE probe (#4) and Vent2 with the probe containing the CTAATT motif (Figure 4B and C). The *in vivo* role of Vent2 in the regulation of *bambi* was confirmed in a reporter assay where dominant-negative Vent2 mRNA was co-injected with CABR, which significantly reduced reporter activity (Figure 4D).

Next we asked whether the crucial Smad1/5 GC-rich element is functionally interchangeable with the other known Smad1 motif GCAT, found in the 5′ BRE and in the *vent2* promoter. When the GC element was replaced by a GCAT motif, enhancer activity was only partially rescued (compare pM11 and pM11a; Figure 4E), consistent with the EMSA data showing that GCAT has a lower Smad binding affinity than the GC-rich element (Figure 4A), at least *in vitro*. In line with the poor evolutionary conservation of the GCCG core within the GC-rich element (Figure 3C), we found that its integrity was not important (pM11b) but that the conserved TGGCGCC motif, referred to as bre7 is required for enhancer activity (pM11, Figure 4E). As it turns out this motif is found in many other BMP-responsive genes (see below).

Transgenic analysis of the bambi enhancer

An important question is whether the 200 bp enhancer is capable of reproducing the characteristic expression pattern of the endogenous *bambi* gene. We therefore performed transgenic experiments in *Xenopus* (Kroll and Amaya, 1996) with GFP-based reporter constructs and monitored the expression by GFP fluorescence or *in situ* hybridization. In most experiments, we mixed enhancer DNA fragments and promoter/GFP constructs ('cotransgenesis'; Hartley *et al*, 2001).

In gastrula stage embryos, the 200 bp enhancer is sufficient to drive reproducibly GFP transgene expression similar to *bambi* in the ventral and lateral marginal zone (Figure 4F and H). For later embryonic stages, GFP expression was monitored not by fluorescence but by *in situ* hybridization, because the stability of the GFP protein caused an overlap of the early and late expression. At the tailbud stage, the characteristic eye–heart–proctodeum synexpression pattern (Figure 4G) was seen fully reproduced in around 10% ($n = 77$) of the transgenic embryos (Figure 4J), suggesting that the 200 bp enhancer is in principle capable of successfully driving synexpression. However, likely due to position effect variegation (PEV), the majority of embryos showed a combination of correct and ectopic expression (example in Figure 4K), also when using a heterologous minimal promoter from the collagen X gene (Figure 4I). Multimerization of the enhancer to reduce PEV indeed leads to a 5-fold more reproducible expression pattern, but adds ectopic expression in the brain (Figure 4M) where low-level BMP signaling is present. This indicates that synexpression requires a certain threshold of BMP inducibility.

Expression in the heart and proctodeum was present in the majority of transgenic embryos, whereas expression in the dorsal eye was rarely seen and only when the *bambi* enhancer was combined with its own proximal promoter (Figure 4J). Using the entire 6 kb *bambi* promoter improved the reproducibility of pattern except for dorsal eye expression, as was the case for a 155 kb bacterial artificial chromosome containing the human *bambi* ortholog *nma* (Figure 4Q). We therefore conclude that the *bambi* enhancer is sensitive to PEV, especially concerning expression in the dorsal eye.

We also tested some of the deletion and point-mutation constructs from Figure 3 in transgenic frogs. The intact enhancer in front of a TATA box drives reproducible, albeit weak expression in the heart and proctodeum (Figure 4O). Mutation in the consensus Lef/TCF site (pM6) reduced the percentage of correct expression twofold, while mutations in the 3′ BRE elements SBE (pM8/9/10), bre7 (pM11) and Vent2 (pM12) strongly affected it (Figure 4L), in agreement with the reporter assay data (Figure 3B). Interestingly, deletion of the 5′ BRE (p3), which only moderately reduced overall luciferase reporter activity and retained BMP inducibility (Figure 3A), had a dramatic effect on transgene expression (Figure 4L). Likewise, neither 3′ BRE alone (p16) nor the multimerized 5′ BRE (p11) was able to drive correct expression, although they display BMP inducibility (Figure 3A). We conclude that (i) the full-length *bambi* enhancer is required for proper developmental expression and (ii) a minor change in BMP responsiveness has a profound effect on synexpression.

Transgenic analysis of the vent2 promoter

The transcriptional regulation of the *Xenopus vent2* gene was extensively studied by several groups and is a paradigm for a BMP-responsive promoter. It contains a core 50 bp BMP-responsive element (BRE), composed of one SBE and an adjacent motif for the cofactor OAZ, which are critically important for BMP stimulation in cell culture assays (Hata *et al*, 2000). Furthermore, two GCAT Smad1 binding motifs upstream of the BRE are necessary for full promoter activity in *Xenopus* embryos (Henningfeld *et al*, 2000). In addition, Vent2 autoregulates its own expression in a complex with Smad1 (Henningfeld *et al*, 2002). However, the role and importance of these elements for the synexpression pattern of *vent2* (Figure 5B) have not been previously analyzed. We tested the 300 bp *vent2* promoter (Figure 5A) in transgenic frogs and found it sufficient to reproduce robustly the BMP4-like pattern of the endogenous *vent2* gene except for a relatively weaker expression in the tailbud (Figure 5C). Point mutations in the Smad1 motifs (Figure 5D,E) and even deletion of the entire region upstream from the BRE (Figure 5I), while weakening the expression, did not significantly affect the pattern. However, mutating the SBE or OAZ motifs resulted in a complete loss of correct expression and in a weak ectopic expression in the somites (Figure 5F and G). Likewise, a 3′ deletion including the proximal Vent2 binding site also failed to produce the wild-type pattern (data not shown). Sequence comparison of the promoters of *vent2* and *vent1* (a closely related homeobox gene) revealed a conserved consensus Lef/TCF binding sequence and its mutation in the *vent2* promoter decreased the strength and reproducibility of transgene expression (Figure 5H). We conclude that, similar to *bambi*, binding sites for Smads and transcription cofactors are required in the *vent2* promoter for synexpression.

We also tested three artificial, multimerized and *in vitro* strongly BMP-inducible BREs for their ability to confer BMP4 synexpression. BRE × 4 (Hata *et al*, 2000) transgenic embryos displayed variable expression and even the few embryos with correct pattern showed additional ectopic expression in the somites (Figure 5J). Likewise, 3GC2 (Ishida *et al*, 2000) and

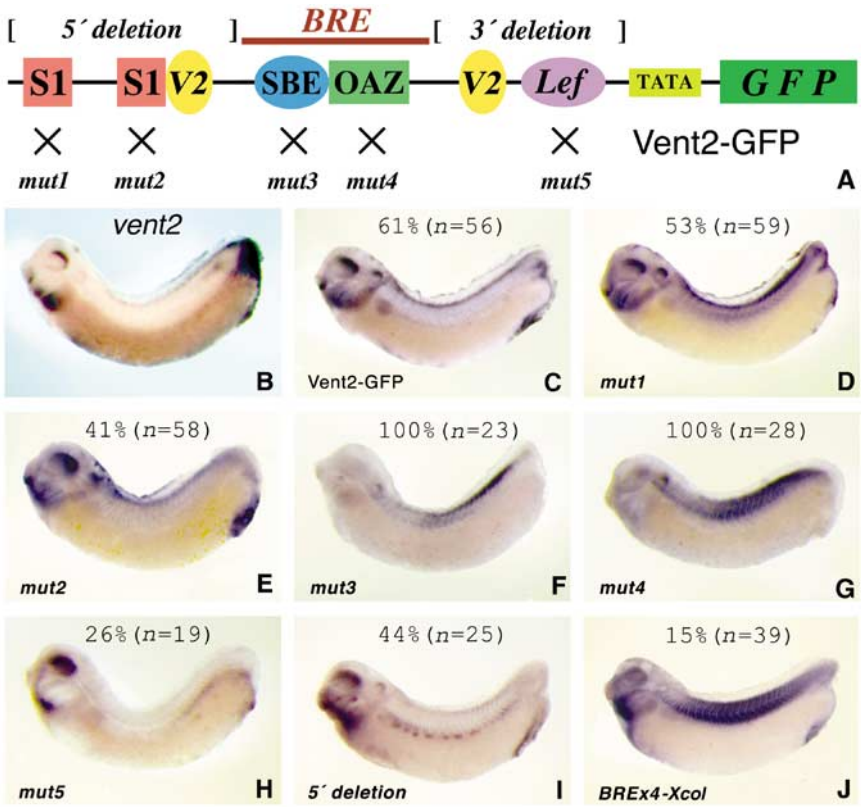

Figure 5 Transgenic analysis of the *vent2* promoter. (**A**) Scheme of the *vent2* promoter (Hata *et al*, 2000; Henningfeld *et al*, 2000, 2002). S1, Smad1 motif (GCAT); OAZ, binding site (TGGAGC) for the Smad cofactor OAZ; Lef, putative Lef/TCF motif (CCTTTGAT); V2, Vent2 motifs. (**B**) *In situ* hybridization of *vent2* at the tailbud stage. (**C**–**J**) *gfp in situ* hybridization of tailbud stage embryos transgenic for the indicated constructs (see panel A). *BREx4-Xcol*, four copies of the *vent2* BRE in front of the collagen X minimal promoter. The percentage of embryos with the depicted expression pattern is shown.

12 × GCCG (Kusanagi *et al*, 2000) transgenic frogs failed to reproduce the BMP4 synexpression pattern (data not shown). These results further highlight that different BREs drive very different expression patterns.

Characterization of BMP-responsive enhancers in the Xenopus smad7 gene

To study a third member of the synexpression group, we chose *smad7*, a negative feedback regulator of BMP and qTGF-β signaling (Nakao *et al*, 1997), which is coexpressed with BMP4 during *Xenopus* development (Figure 1B; Casellas and Hemmati-Brivanlou, 1998). *smad7* expression is induced by both TGF-β and BMP signaling in various cell types and organisms (Zwijsen *et al*, 2000; Locklin *et al*, 2001; Ota *et al*, 2002) and our RT–PCR experiments showed that it is a direct transcriptional target of BMP signaling in *Xenopus* (Figure 1C). We isolated, sequenced and compared a *Xenopus smad7* genomic clone with the corresponding human and *Fugu* DNA regions. Two highly conserved non-coding segments were detected, one in the 5′ flanking region (E1) and one near the 3′ end of intron1 (E2) (Figure 6A). The mouse *smad7* promoter region containing E1 was previously described as only activin/TGF-β-responsive (Nagarajan *et al*, 1999). Phylogenetic footprinting of E1 shows perfect conservation of the octanucletide palindrome composed of two SBEs and adjacent putative binding sites for the transcription factors EKLF, USF and AP1. A GC-rich sequence at the 3′ end of E1 is another potential Smad1/5 binding site. The predicted intronic enhancer E2 contains conserved binding motifs for Smads (SBE and GC-rich element), GATA and NFY transcription factors. Remarkably, the GC-rich element contains the same bre7 motif that we found to be critically important in the *bambi* enhancer. In E2, bre7 is present on both strands, forming a perfect palindrome (Figure 6A, red arrows). Similar to *bambi*, there is a GC-rich stretch 3′ of the bre7 motif.

Transgenic *Xenopus* assays demonstrated that either E1 (Figure 6C) or intron1 (Figure 6D) could drive an expression pattern reminiscent to that of *smad7*, but with ectopic expression in somites (E1) or the lateral plate (intron1). Only when both enhancers were combined, ectopic expression was suppressed and the majority of transgenic embryos showed a pattern very similar to that of the endogenous gene (Figure 6E). We conclude that *smad7* synexpression requires both the upstream and intronic enhancers, even though both are individually BMP-responsive in luciferase assay (Figure 6F,G).

To further analyze the two enhancers, reporter and transgenic assays were performed with intact and mutated constructs (Figure 6F–H). Mutations of all predicted TF binding sites in E1 and E2 affected luciferase reporter and GFP transgene expressions. For E1, complete loss of correct transgene expression was observed when the 2 × SBE or the putative USF/AP1 motifs were disrupted (mut.2 and 3, Figure 6H), although BMP responsiveness in luciferase assay was only partially reduced (Figure 6F). Mutating the

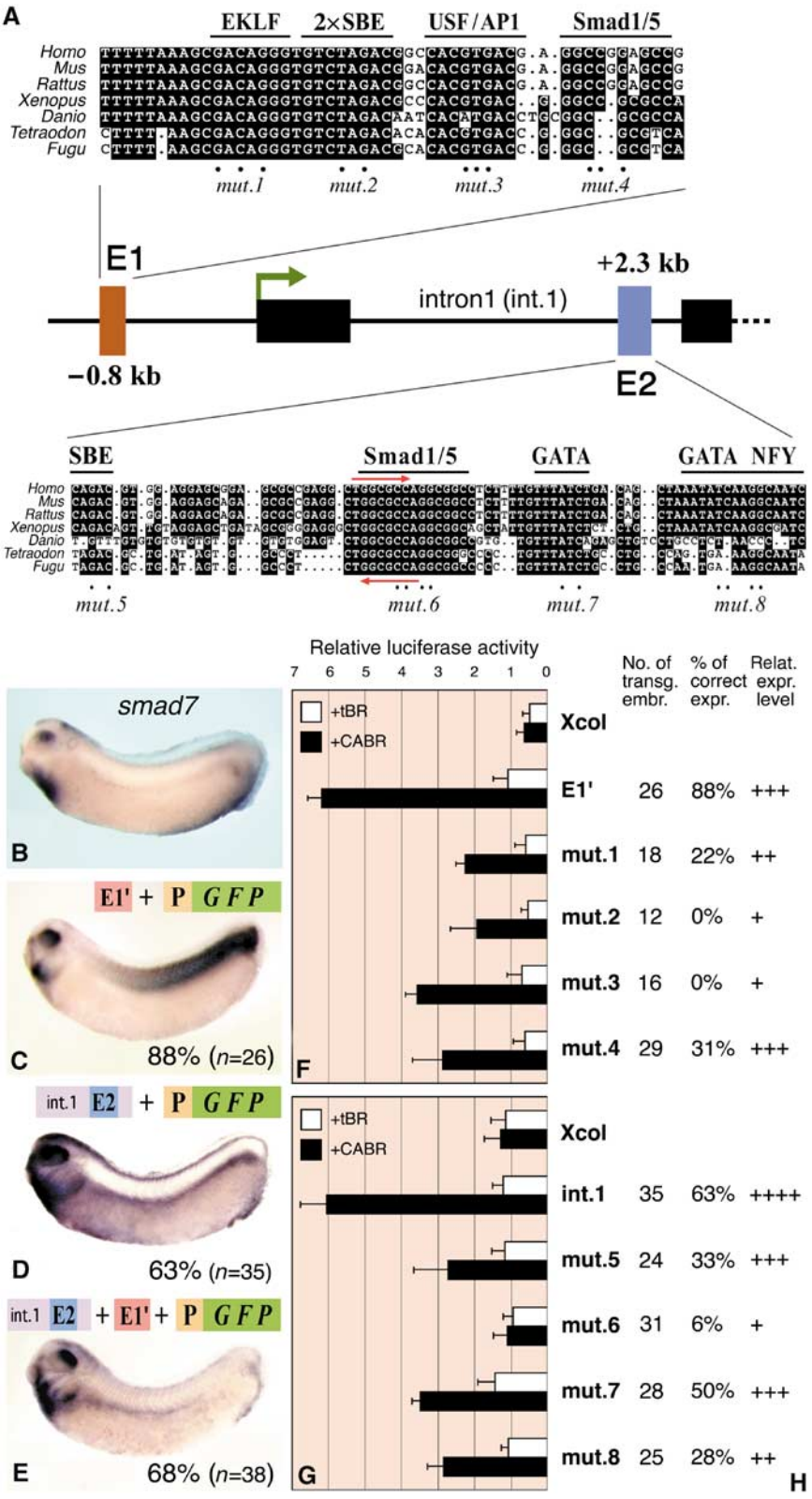

Figure 6 Characterization of the BREs of *smad7*. (**A**) Map of the *Xenopus smad7* gene with the phylogenetically conserved putative enhancers E1 and E2. Multi-species sequence alignment with putative TF binding sites is shown for each enhancer. Red arrows in E2 indicate the bre7 motif also found in the *bambi* enhancer. Mutated nucleotides are indicated with dots. For transgenic and luciferase reporter assays, E1 was cloned with 24 bp upstream and 58 bp downstream nonconserved flanking sequences (E1′). (**B**) *In situ* hybridization of *smad7* mRNA at the tailbud stage. (**C–E**) *gfp in situ* hybridization of transgenic embryos with the depicted constructs. P is the minimal promoter of *bambi* (−154/+60). The percentage of embryos with the depicted expression pattern is shown. (**F, G**) Data from luciferase reporter assays of the *smad7* enhancers. E1′, intron1 or their mutated versions (see panel A) were cloned upstream of the Xcol minimal promoter and injected in embryos together with tBR or CABR mRNA as indicated. (**H**) Tabular results of embryos transgenic for the intact or mutated constructs (see panel A). The pattern referred to as correct is the one depicted in panel C (for E1′) and D (for intron1). The number of transgenic embryos analyzed, the percentage of embryos with correct pattern and the relative strength of transgene expression for each construct are shown.

GC-rich (bre7) motif in E2 resulted in a complete loss of enhancer activity in the luciferase assay and in only very few transgenic embryos with correct, but much weaker expression (mut.6, Figure 6G and H). These data again demonstrate (i) the critical importance of the bre7 motif for BMP responsiveness and (ii) the nonequivalence of BMP inducibility and synexpression.

In silico prediction of novel BREs

The conservation of the bre7 motif in the BREs of *bambi* and *smad7* encouraged us to search *in silico* for other putative BREs in known BMP target genes. We specifically looked for adjacent SBE and bre7 motifs in phylogenetically conserved regions that are close to the transcription start sites of the genes of interest. This resulted in 15 hits in 13 BMP target genes (Table I and Supplementary Figure 2).

One class of genes identified are *id* (inhibitors of differentiation) genes (Figure 7), which are immediate targets of BMP signaling (Hollnagel *et al*, 1999). A bre7 motif was recognized previously as a 6 bp GGCGCC palindrome in the *id1* promoter (Korchynskyi and ten Dijke, 2002; Supplementary Figure 1A). In addition to this known BRE, we identified a second putative BRE downstream of the *id1* gene (Figure 7B), containing a GGCGCC palindrome and an SBE motif 5 bp apart, which are fully conserved from *Fugu* to *Homo*. Analysis of *id2*, *3* and *4* genomic regions also revealed a single highly conserved noncoding segment in each gene (Figure 7A). These regions are located upstream of the genes (*id2, id3*) or inside an intron (*id4*). Remarkably, all of them contain conserved SBE and bre7 motifs in the same orientation and with the same spacing, despite a lack of overall homology. A second putative BRE for *id3* is located inside an intron (Figure 7B). When cloned and tested, all five *in silico* predicted enhancers proved to be BMP-responsive in frog embryos, with inductions between 3- and 6-fold (Figure 7C). We conclude that clusters of evolutionary conserved bre7 and SBE motifs are highly prognostic for BREs.

Discussion

One shortcoming of the many studies dealing with BREs is the lack of *in vivo* analysis to characterize promoters in whole animals. Thus, prior to this study it was unknown whether the identified BREs are sufficient to drive faithful expression of the gene in question in transgenic animals. Here we have addressed this issue.

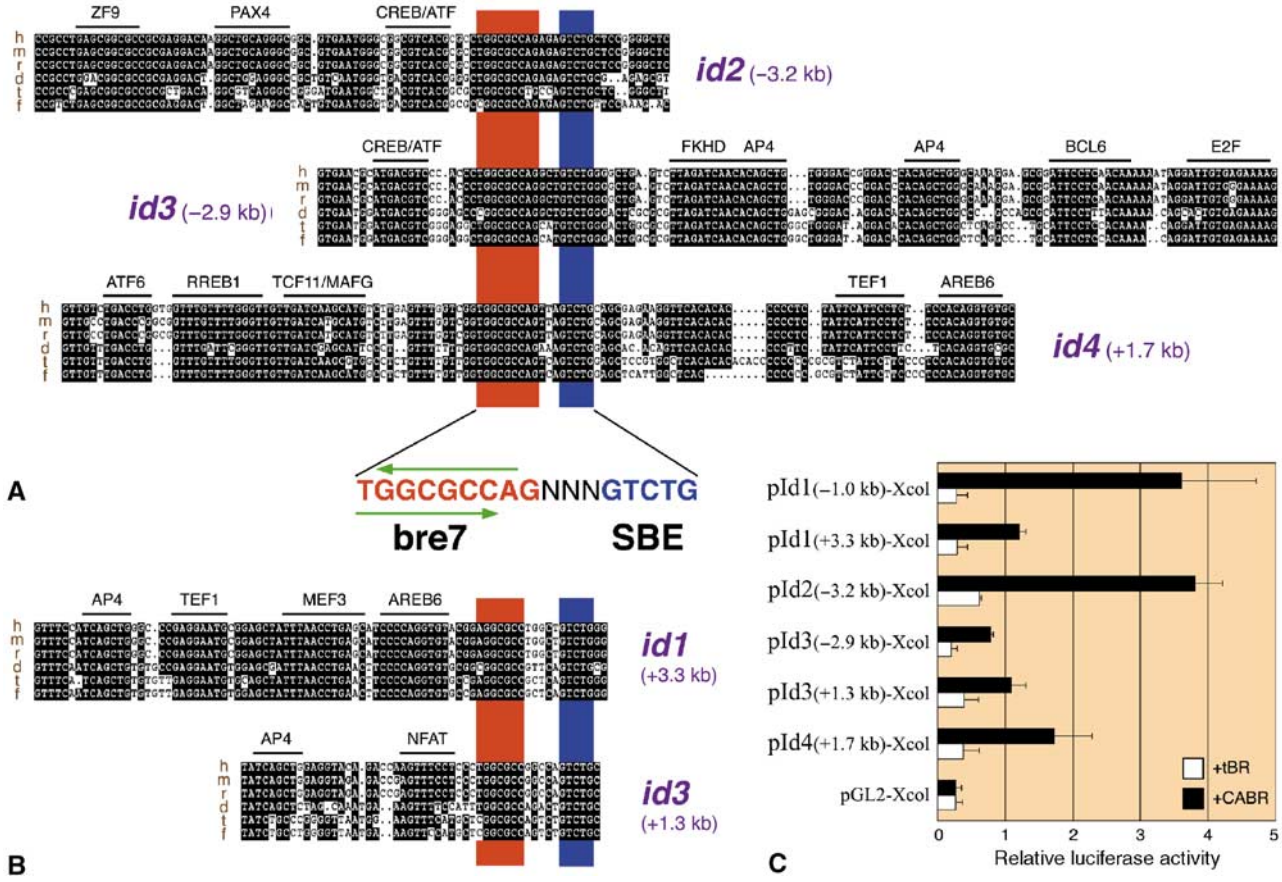

Figure 7 Bioinformatic prediction and experimental verification of five novel BREs for *id1-4* genes. (**A, B**) Multi-species sequence alignment of the *in silico* identified novel *id* enhancers. The SBE and bre7 motifs are highlighted in blue and red, respectively. Putative TF binding sites are indicated. The currently available genomic sequences of *Homo* (h), *Mus* (m), *Rattus* (r), *Danio* (d), *Tetraodon* (t) and *Fugu* (f) are aligned. (**C**) Luciferase reporter assay of the five novel and the known (*id1* –1.0 kb) BREs, cloned upstream of the XcoI minimal promoter (see Supplementary Figure 1C for fragments cloned). Frog embryos were injected with the indicated constructs together with tBR or CABR mRNA and harvested at the gastrula stage for luciferase assay.

Common architecture of the BREs of bambi, smad7 and vent2

bmp4 and *-7* are subject to complex regulation and integrate a variety of inputs, which control their developmental expression pattern (Schuler-Metz *et al*, 2000). In contrast, all other member genes of the BMP4 synexpression group proved to be direct transcriptional targets of the BMP/Smad signaling pathway in *Xenopus* (Figure 1). Therefore, developmental synexpression in the BMP4 group seems to result from the common, BMP/Smad-dependent transcriptional regulation of the member genes where the BMP ligands are the master regulators.

The 200 bp enhancer of *bambi* has a complex structure, consisting of 5′ BRE and 3′ BRE that contain multiple Smad and Vent2 binding sites. Both BREs and the linker region between them synergize in eliciting BMP-induced activation. Consistent with the leading importance of the 3′ BRE, it is highly conserved in nine vertebrate species from fish to mammals and is the only conserved region in a *Fugu/Homo* sequence comparison. However, neither of the two BREs alone is capable of driving strong reporter activity or conferring the correct pattern of expression; only the intact enhancer can mimic the expression pattern of *bambi* in transgenic assays.

The *smad7* expression pattern is driven by two BREs (upstream and intronic). Crucial for expression are the $2 \times$ SBE and putative USF/AP1 motifs in the upstream enhancer, and the bre7 element in the intronic enhancer. While this manuscript was in preparation, an independent study described the mouse *smad7* intronic enhancer as GATA and Smad dependent, but the role of bre7 was not examined there (Benchabane and Wrana, 2003).

For the *vent2* promoter, both the SBE and OAZ motifs are strictly required for correct developmental expression, but sequences outside the SBE and OAZ sites also affect expression, since the related gene *vent1* has the same motifs (Hata *et al*, 2000) and is not coexpressed with *vent2* at the tailbud stage (Gawantka *et al*, 1998).

Thus, all enhancers studied are relatively compact and contain at least one functional SBE motif. In addition, the evolutionary conserved GC-rich element bre7 was found to be critically important in the *bambi* and the *smad7* intronic enhancers. This element is located close to conserved SBE and cofactor binding motifs. Smads can bind to certain GC-rich sequences (Shi and Massagué, 2003; Zwijsen *et al*, 2003) and bre7-containing probes specifically bind recombinant Smad5 (Korchynskyi and ten Dijke, 2002). Substitution of the bre7 element with a GCAT Smad1 motif partially rescues the *bambi* enhancer activity. Collectively, these data suggest that bre7 may function *in vivo* as a binding site for the BMP-specific R-Smads and, in combination with SBE motifs, can be utilized to predict novel BREs. Interestingly, bre7 (TGGCGCC) has similarity to the proposed OAZ motif TGGAGC (Hata *et al*, 2000), raising the possibility that Smad complexes may bind to bre7 through or synergistically with OAZ. On the other hand, *id* genes, which contain bre7 in their enhancers, are rapidly induced by BMP stimulation in C2C12 myoblast cells, which lack OAZ (Korchynskyi and ten Dijke, 2002), and the *vent2* reporter is unresponsive to BMPs in these cells, unless OAZ is exogenously supplied (Hata *et al*, 2000). Furthermore, we find a bre7 motif in the *tlx2* BMP-responsive promoter, reported to be OAZ independent (Hata *et al*, 2000). The possible interaction of OAZ with bre7 therefore needs further clarification.

Table I BMP target genes with conserved clusters of SBE and bre7 motifs

Gene (BRE)	CNCS (kb)	SBE	Spacing (bp)	bre7
bambi (nma)	−0.5	GTCT	3	TGGCGCC
smad7	+2.1	GTCTG	28	TGGCGCCA
id1	−1.0	GTCTG	58	TGGCGCC
id1	+3.3	GTCTG	5	AGGCGCC
id2	−3.2	GTCTG	4	TGGCGCCA
id3	−2.9	GTCTG	4	TGGCGCCA
id3	+1.3	GTCTG	5	TGGCGCC
id4	+1.7	GTCTG	4	TGGCGCCA
msx1	−2.8	GTCTG	16	TGGCGCC
msx2	−3.3	GTCT	18	TGGCGCC
gata2	+5.5	GTCTG	16	TGGCGCCA
gata3	−0.1	GTCTG	16	TGGCGCC
bmpr2	−1.8	GTCTG	9	TGGCGCCA
tlx2 (hox11L1)	−1.2	GTCT	12	TGGCGCC
vox (vega1)	−0.2	GTCTG	5	TGGCGCC
vent (vega2)	−0.3	GTCTG	4	TGGCGCC
tbx2	−2.9	GTCTG	60	TGGCGCCA

The position of the conserved noncoding segments (CNCS) is shown according to the annotation of the NCBI human genomic browser, except for *vox* and *vent* (zebrafish). BREs experimentally analyzed in this study are in bold. Previously known BREs are underlined.

Enhancer model for in silico prediction of novel BREs

Based on the phylogenetic conservation of bre7 and SBE motifs in the *bambi* and *smad7* enhancers, we built an enhancer model and predicted a dozen novel BREs in other BMP target genes (Table I). A prominent case are *msx* genes, which are co-expressed with BMPs in many tissues and are immediate targets of BMP signaling (Hollnagel *et al*, 1999). *msx1* also participates in a positive feedback loop, regulating *bmp4* expression during mammalian palatogenesis and tooth development (Zhang *et al*, 2002). Another important hit were *id* family genes, which are BMP-responsive (Hollnagel *et al*, 1999; Locklin *et al*, 2001; Ota *et al*, 2002) and crucial for many developmental processes and for the maintenance of ES cell pluripotency. One of the six BREs predicted in *id1-4*, that upstream of *id1*, was previously characterized (Korchynskyi and ten Dijke, 2002; Lopez-Rovira *et al*, 2002). It harbors functional bre7 and SBE motifs, which are essential for BMP inducibility and Smad binding. For the five newly predicted *id1-4* enhancers, we experimentally confirmed that they are also functional BREs, proving the usefulness of our bioinformatic search. The results highlight the utility of combining experimentally based enhancer modeling with phylogenetic footprinting to predict *in silico* novel pathway-specific regulatory elements.

BMP inducibility and synexpression

While there is no simple BMP4 synexpression promoter module shared by all genes, they have common features which are crucial for BMP inducibility and synexpression: (i) one or more functional SBE and/or alternative Smad motifs (bre7), (ii) adjacent functional binding sites for transcription factors that can physically interact with Smad proteins (Vent2, OAZ, AP1), and (iii) a restricted window of BMP inducibility. The member genes of the BMP4 synexpression group are a subset of all BMP target genes. In order to be

coexpressed with BMP4, they should have similar thresholds of BMP inducibility. This is important because BMP4 in *Xenopus* like Dpp in *Drosophila* acts as a morphogen in a graded fashion, inducing target genes at different signaling thresholds. For example, *vent1* is a BMP-responsive gene whose expression requires higher levels of BMP signaling than the expression of *vent2* (Dosch *et al*, 1997). While both share a similar BRE, *vent1* is not synexpressed but has a more restricted pattern in the marginal zone of the blastula embryo, as well as in later stages. Conversely, artificial, multimerized BREs, which are highly sensitive to BMP signaling, typically show ectopic sites of transgene expression. Collectively, the results indicate that synexpression promoter modules respond to similar levels of BMP signaling.

In general, we conclude that different BREs that show BMP inducibility *in vitro* confer different spatiotemporal expression *in vivo*, highlighting the importance of transgenic analysis for understanding developmental regulation in general and synexpression in particular.

Materials and methods

Embryo manipulation and RT–PCR

In vitro fertilization, embryo culture, staging, microinjection and explantation were carried out as described (Gawantka *et al*, 1995). Cycloheximide (CHX) treatment was performed from stage 8 onwards with 30 μg/ml until control embryos reached stage 9 or 10. CHX treatment was effective since cell division was arrested and H4 mRNA levels were reduced. RT–PCR assays on animal cap explants were carried out as described previously (Gawantka *et al*, 1995). Primers for *h4*, *vent1*, *vent2* and *bmp4* were as described (Gawantka *et al*, 1995; Onichtchouk *et al*, 1996). Other primers are listed in Supplement 3.

Enhancer cloning

A total of 2×10^6 clones from the Lambda FIX II *Xenopus laevis* genomic library (Stratagene) were screened with 5′ cDNA probes for *bambi* and *smad7*. Several genomic clones were isolated, partially sequenced (GenBank accession numbers: AY499615—*bambi*, AY499616—*smad7*) and subcloned in the promoter or enhancer cloning sites of the luciferase reporter vectors pGL2-Basic (Promega), pGL2-Xcol (Harada *et al*, 1997) and pE1b-luc (Song *et al*, 1998). Deletion and point mutation constructs were generated by PCR and verified by sequencing. The point-mutations in the *bambi* and *vent2* enhancers are listed in Supplement 3. The 51 bp insert in construct p13 (Figure 3A) was cut from the MCS of the pTimer vector (Clontech) with BglII and BamHI. The six *id* BREs were cloned from human gDNA by PCR (see Supplementary Figure 1C), sequence verified and subcloned in the enhancer cloning site of pGL2-Xcol.

Xenopus transgenesis and in situ hybridization

Transgenesis was performed using the REMI nuclear transplantation method as described (Kroll and Amaya, 1996; Sive *et al*, 2000). When cointegration of two or three DNA pieces was performed (Hartley *et al*, 2001), they were produced with the same NotI overhangs and added in equimolar concentrations to sperm nuclei. A total of 200 ng of the BAC RP11-48B24 (RZPD), containing the human *nma* gene and linearized with NotI, was used as a transgene in Figure 4Q. The frequency of BAC transgenics was around 6% (9/140 embryos). *Xenopus* whole-mount *in situ* hybridization and bleaching of pigment was performed as described (Sive *et al*, 2000).

Luciferase reporter and EMSA assays

Embryos were injected at the four-cell stage in each blastomere with luciferase reporter constructs (50 pg/blastomere) plus synthetic mRNAs for CABR (300 pg/blastomere), tBR (600 pg/blastomere), Vent2 (1 ng/blastomere) or dominant-negative Vent2 (1 ng/blastomere). CABR is an ALK3 (Q228D) constitutively active variant (Candia *et al*, 1997); tBR is a kinase domain-deficient dominant-negative BMP receptor (Graff *et al*, 1994); dnVent2 harbors a point mutation in the homeodomain and may sequester wild-type Vent2 protein (Onichtchouk *et al*, 1998). For each sample, three pools of three embryos were collected at gastrula stage 11, homogenized in passive lysis buffer (Promega) and assayed for luciferase activity. Data were normalized per embryo. Electrophoretic mobility shift assay (EMSA) and the production of recombinant proteins were carried out as described previously (Henningfeld *et al*, 2000). All DNA oligonucleotides used as probes for EMSA (shown in Figure 2A) had additional eight-nucleotide PCR primer sequences at the ends: 5′-CCGAGCTC and GTCGACGC-3′.

Bioinformatic tools

Described in Supplementary Figure 1A legend.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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