Nucleic Acids Research, 2005, Vol. 33, No. 13 ***4357–4367***
doi:10.1093/nar/gki746

An Oct-1 binding site mediates activation of the *gata2* promoter by BMP signaling

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Received May 5, 2005; Revised July 5, 2005; Accepted July 13, 2005

ABSTRACT

The *gata2* gene encodes a transcription factor implicated in regulating early patterning of ectoderm and mesoderm, and later in numerous cell-specific gene expression programs. Activation of the *gata2* gene during embryogenesis is dependent on the bone morphogenetic protein (BMP) signaling pathway, but the mechanism for how signaling controls gene activity has not been defined. We developed an assay in *Xenopus* embryos to analyze regulatory sequences of the zebrafish *gata2* promoter that are necessary to mediate the response to BMP signaling during embryogenesis. We show that activation is Smad dependent, since it is blocked by expression of the inhibitory Smad6. Deletion analysis identified an octamer binding site that is necessary for BMP-mediated induction, and that interacts with the POU homeodomain protein Oct-1. However, this element is not sufficient to transfer a BMP response to a heterologous promoter, requiring an additional more proximal cooperating element. Based on recent studies with other BMP-dependent promoters (*Drosophila* vestigial and *Xenopus* Xvent-2), our studies of the *gata2* gene suggest that POU-domain proteins comprise a common component of the BMP signaling pathway, cooperating with Smad proteins and other transcriptional activators.

INTRODUCTION

Bone morphogenetic proteins (BMPs) are members of the TGF-β superfamily of extracellular ligands, which signal by binding to heterodimeric serine/threonine kinase receptor complexes. Much progress has been made identifying the molecular components that regulate transduction of a BMP signal from the plasma membrane to the nucleus (1,2). Ligand binding activates the receptor complex, which relays the signal by recruiting downstream mediators. A commonly used subset of mediators are known collectively as Smads, which are categorized into three functional groups: (i) receptor regulated Smads (R-Smads) that are directly phosphorylated by the activated receptor; (ii) Smad4, which cooperates with R-Smads to form an active signaling complex; and (iii) inhibitory Smads (I-Smads), which serve as negative regulators of the pathway (3). Although there may be exceptions (4,5), generally R-Smads 1, 5 and 8 are phosphorylated by the BMP pathway, whereas R-Smads 2 and 3 function instead to transduce a TGF-β, nodal or activin signal. The R-Smad/Smad4 complex interacts with specific nuclear transcription factors to activate gene transcription. There are also Smad-independent mechanisms to mediate BMP signaling, e.g. by p38 MAPK (6).

Defining the mechanisms by which BMP-induced Smads activate specific target genes is an important goal (7) given the wide range of developmental and physiological responses that are under the control of the pathway. BMP signaling patterns early germ layers to establish a dorsal/ventral mesoderm axis, the anterior/posterior endoderm character and the distinction of neural/epidermal ectoderm (8–12). BMP signaling also regulates lineage and morphogenetic programs relevant to bone, cartilage, kidney, heart and reproductive organ development. Thus, a wide range of highly specific gene expression programs is coordinated by the action of this common signaling pathway, presumably by the presence or absence of intersecting signaling pathways and specific nuclear co-factors.

An R-Smad/Smad4 complex binds DNA weakly on its own, relying on interaction with other nuclear partners to achieve stable and functional binding (13). A paradigm of Smad–cofactor interaction was established for TGF-β/activin signaling by the identification of winged helix proteins, such as FAST-1 as DNA-binding partners that interact with Smad2/3 at target promoters (14,15). With respect to the BMP pathway, an analogous example is the 30-zinc finger protein OAZ, shown to interact with BMP-induced Smads to activate the promoter for the homeobox gene Xvent2 (16). Most, but not

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all, known BMP response elements (BREs) are also Smad binding elements (SBEs), including those in the promoters for the genes encoding Vestigial (17), xVent2B (16,18), Smad6 (19), Id1 (20,21), Dlx3 (22) and Hex (23). A BMP-4 syn-expression motif was identified (TGGCGCC) as a conserved BRE (7), and SBEs have in some cases (17,24) been associated with GC-rich elements (GCCGnCGC) or as other defined short (GTCT or GCAT) motifs (18,25). However, there is no single consensus sequence that can readily predict a functional Smad1 binding site. Given that Smads have weak affinity for DNA, it is necessary to define the DNA-binding cofactor(s) to understand how Smads are targeted to any particular BRE.

One of the best characterized BMP-regulated pathways relates to the induction of ventral/posterior cell fate during *Xenopus* development. The homeobox transcription factor Xvent2 was the first target gene of BMP signaling to be investigated mechanistically, and several elements that contribute to BMP responsiveness have been defined, including an SBE/OAZ BRE, GCAT motifs, Vent2 auto-regulatory binding sites, and most recently, binding sites for the POU-domain protein Oct-25 (26). Another gene important to the ventral pathway is *gata2*, encoding a zinc finger transcription factor that is expressed throughout ventral ectoderm and ventral and lateral mesoderm (27–29). The *gata2* gene is induced by BMP-4 (30,31) and forced expression of an engrailed-Gata2 fusion protein is sufficient to dorsalize *Xenopus* embryos (32), providing strong evidence that Gata factor activity is essential for ventral cell fate. Gata2 and xVent2 cooperate to activate the xVent1 gene, placing these two transcription factors within a common ventral network (32,33). Here, we investigate the mechanism by which the *gata2* gene is activated by BMP signaling. We establish a reporter assay to define sequences of the *gata2* promoter that mediate induction by BMP-4 during embryonic development. We find that an octamer binding site, interacting with Oct-1, is necessary for induction, but only functions in the context of a separate more proximal DNA sequence. The results provide an independent example of a POU-homeodomain protein mediating induction by BMP signaling, and implicate a common network controlling regulatory genes of the ventral pathway.

MATERIALS AND METHODS

Identification of the zebrafish *gata2* transcriptional start site

RNA was isolated from zebrafish embryos (75% epiboly) with Tri-reagent (MRC, Inc.). Superscript Reverse Transcriptase (BRL) was used to generate cDNA, followed by treatment with RNAse. Adapters were ligated to the cDNA ends using the Invitrogen 5′ RACE kit as per the manufacturer's instructions. A 3′ gene-specific reverse primer, corresponding to position +92 (relative to the translation start site) of the *gata2* transcript, was used to amplify cDNA by PCR. The primer sequence was 5′-GTAAGCCCGTGATGATGTGACTCT (TE872). A single PCR product was identified in this analysis and subcloned in pCR-2.1 TOPO (Invitrogen). Sequence analysis confirmed that the clone included the previously characterized *gata2* cDNA, with additional 5′-untranslated region. The start site and exon/intron boundaries were determined by comparing this new cDNA sequence with the genomic sequence.

Reporter plasmids and deletion constructs

A genomic region including ~7.1 kb of sequence upstream of the initiation methionine (6.5 kb upstream of transcriptional start site) was provided by Shuo Lin (UCLA), and transferred to the Kpn1 site of the pGL3basic luciferase reporter plasmid (Promega). The deletion to −1070 was facilitated by a unique MluI restriction site at that position. The other 5′ deletion constructs were generated by PCR, incorporating in the upstream primer a KpnI site and using a reverse primer corresponding to position +58 of the *gata2* promoter. PCR products were cloned into the pCR-2.1 TOPO vector (Invitrogen). Subsequent cloning steps took advantage of an internal BssH2 site located at position −97. Therefore, each PCR-derived clone was digested with KpnI/BssH2 and the fragment used to substitute the full-length *gata2* promoter sequence in the context of the pGL3basic luciferase reporter. PCR primers used to generate the progressive deletions were (KpnI sites underlined): −890 Kpn I forward, 5′-AAAGGTACCGCCTGCTGCTTGTTTTGCC (TE851); −819 KpnI forward, 5′-AAAGGTACCGGCTTTATTTGGACCTGC (TE876); −751 KpnI forward, 5′-AAA GGTACCAAGTGACGTTTGATCGCT (TE878); −728 KpnI forward, 5′-AAAGGTACCCGGCATACAAAGACAGTG (TE852); +58 reverse, 5′-CCCGATTGTTAAAGTCTCCC (TE837).

To transfer a 'minimal' BRE region upstream of the SV40 promoter, a ClaI site at position −443 was used with promoter constructs truncated at −819 or −751. Plasmids were digested with KpnI/ClaI, transferred first into pBluescript KS digested with KpnI/ClaI, re-excised with KpnI and SmaI, and finally transferred into similarly digested SV40 pGL3promoter luciferase plasmid (Promega). This resulted in reporter plasmids that transfer from −819 to −443 (377 bp) or −751 to −443 (308 bp) of the *gata2* promoter, respectively. Additional minimal promoter constructs (which transfer 233, 184 or 134 bp) were generated by PCR, first cloning products into pCR-2.1 TOPO, followed by re-excision with KpnI and SacI, and transfer into the KpnI/SacI sites of the SV40 pGL3promoter luciferase plasmid (Promega). This resulted in the transfer of regions −819 to −586 (233 bp), −819 to −635 (184 bp), or −819 to −685 (134 bp), respectively. The PCR primers used for this purpose were (restriction sites underlined): −819 KpnI forward, 5′-AAAGGTACCGGCTTTATTTGGACCTGC (TE 876); −685 SacI reverse, 5′-AAAGAGCTCGTCACCACATTCTTCTTG (TE 1127); −635 SacI reverse 5′-AAAGAGCTC CTCTAAATTGGGGGCTATTG (TE1161); −586 SacI reverse, 5′-AAAGAGCTCCTCAA CTCCTAGCACCTC (TE1128).

For site-directed mutagenesis, the GeneEditor *in vitro* mutagenesis system (Invitrogen) was used with the following oligonucleotides (mutations underlined): mutation #1, 5′-TCGATAGGTACCACTTTTATTTGGACC (TE1009); mutation #2, 5′-ACCGGCTTTATTGCTACCTGCCCATGC (TE1012); mutation #3, 5′-GGCTTTATTTGGGAGTGCCCATGCGAC (TE1013); mutation #4, 5′-TGGACCTGCCCAAAGGACCTGTCGGCAC (TE1016); mutation #5, 5′-CACCTCCAAGAGACTAGTTCGCTATTAATATGTAAAGTG (TE1134); mutation #6, 5′-AGAGACGGGCTCGC-

TCTAGATATGTAAAGTGACG (TE1135); mutation #7, 5′-GGGCTCGCTATTAAGCTTTAAAGTGACGTTTGATCG (TE1147).

In vitro transcription

RNA used for micro-injection was obtained by *in vitro* transcription using linearized pCS2 vectors. The vectors were pCS2-lacZ, pCS2-xSmad1 (from J. Thomsen), pCS2-zSmad5 (from M. Mullins), pCS2-xSmad6 (from J. Christian), pCS2-xSmad8 (34) and pCS2-BMP4 (from C. Wright) were used as templates for *in vitro* transcription. One microgram of linearized template was used to generate capped mRNAs with the mMessage mMachine kit (Ambion), followed by precipitation with LiCl. RNA was quantified by optical density, and integrity was confirmed by gel electrophoresis.

Microinjection and luciferase assays

Eggs were obtained from female *Xenopus* by standard gonadotropin induction protocols. Eggs were fertilized *in vitro* and dejellied in a solution of 2% cysteine (pH 8.0). Microinjection was performed with embryos in 0.1× MBS and 5% Ficoll. Each injection mix contained 62.5 pg of RNA encoding either BMP-4 or an irrelevant control RNA (such as lacZ), 25 pg of luciferase reporter DNA and 0.10 pg of RNA encoding *Renilla* luciferase (to normalize reporter activity). In some experiments, 250 pg of RNA encoding either xSmad1, zSmad5 or xSmad8 was included in place of BMP-4 RNA. In a standard assay, mixes were injected into the two dorsal blastomeres of a four-cell stage embryo. To generate lysates for gel mobility shift experiments, RNA was injected at the two-cell stage.

Embryos were staged according to Nieuwkoop and Faber (35). Following microinjection, embryos were cultured in 0.1× MBS until stage 13, at which point embryos were collected and lysed in 1× Passive Lysis Buffer (Promega). Luciferase assays were performed according to the manufacturer's instructions. For each assay, four embryos were lysed in 100 µl of buffer, and typically 3–5 sets were used to generate independent data points for each independent experiment, using 5 µl of lysate per 50 µl of luciferase substrate, measured in a Turner TD-20e luminometer. The firefly luciferase values were divided by corresponding values for the control *Renilla* luciferase. Normalized data from embryos injected with BMP-4 was divided by normalized numbers obtained from embryos injected with control RNA to determine 'BMP-4 fold induction'. All luciferase data represent averages of at least three independent microinjection experiments. Error bars in the figures indicate standard error of the mean.

Preparation of *Xenopus* nuclear and total cell extracts

Total cell extracts were prepared by freezing embryos in liquid N_2, followed by homogenization in total cell extract buffer (50 mM Tris–HCl, pH 8.0, 400 mM KCl, 1 mM DTT, 25% glycerol, 50 mM NaF and protease inhibitors). Extracts were cleared by centrifugation three times for 10 min at 8160 *g* 10–20 µg per reaction was used in gel mobility shift reactions. To generate nuclear extracts, a nuclear pellet was obtained from stage 13 embryos as described (36). The pellet was resuspended in Buffer C (20 mM HEPES–KOH, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT and protease inhibitors) on ice and centrifuged at 10 000 *g* in a microfuge for 1 min at 4°C to clear contaminating pigment granules: 1–5 µg was used for each gel mobility shift assay. To obtain lysates from embryos expressing BMP-4, embryos were injected at the two-cell stage with 500 pg of RNA and harvested at stage 13. Total protein concentration for all experiments was obtained using a modified Bradford assay (BioRad).

Gel mobility shift assays

Oligomers were gel-purified prior to use as probes or cold competitors. For probes, the top strand was end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase, followed by purification thorough a G-25 Sepharose column. The radiolabeled top-strand was annealed with the complementary bottom strand by heating to 95°C for 5 min in 0.1 M NaCl, and then gradually cooling the reaction to room temperature. Cold competitors were prepared identically, without labeling. For super-shift experiments, reactions included 0.5 µg of anti-Oct-1 antibody (sc–8024, Santa Cruz) or 0.5 µg of control isotype-matched antibody (various, but the one shown is anti-Chicken Gata2, sc-267). Gels were run and analyzed as described (37).

The sequences of the top strand of the oligomers are as follows: −763 to −737, 5′-AGCTTATTAATATGTAAAGTGACGTTTGATA (TE1175); −763 to −737 (mutated), 5′-AGCTTATTAAGCATTAAAGTGACGTTTGATA (TE1191); Octamer consensus, 5′-CGAATATGCAAATAAGGC (TE1189); CCAAT, 5′-ACTGGCTGGCGGAGGCTTGTGATTGGCTGGCCCGG (TE1207).

RESULTS

The *gata2* promoter is induced by BMP signaling

The *gata2* gene is activated at the transcriptional level by BMP signaling, but the mechanism for induction is not known. Previous transgenic reporter experiments demonstrated that 7.3 kb of sequences upstream of the zebrafish *gata2* initiation ATG are sufficient to direct appropriate expression of a reporter to the early ventral domain (38). We sought to define the specific DNA regulatory elements that mediate transduction of the BMP signal to the *gata2* gene. The *gata2* gene structure has been studied in a variety of organisms, including human (39,40), mouse (41), chicken (42) and *Xenopus* (39,43). Both the mouse and the chicken genes contain two alternative first exons (proximal and distal), and the different transcriptional start sites are used depending on the cell type (41,42). Alternative first exons are not described for either the *Xenopus* or human genes, but it remains possible that multiple promoters are a common feature of the *gata2* locus, and this could complicate any mapping experiments. Therefore, before attempting to map BMP responsive sequences, we first identified the transcriptional start site for the z*gata2* gene in order to define the proximal promoter.

Sequence comparison of the zebrafish *gata2* putative promoter region with DNA sequences from exon 1 of the *gata2* gene from a variety of species failed to identify regions of homology (data not shown). Therefore, to identify the

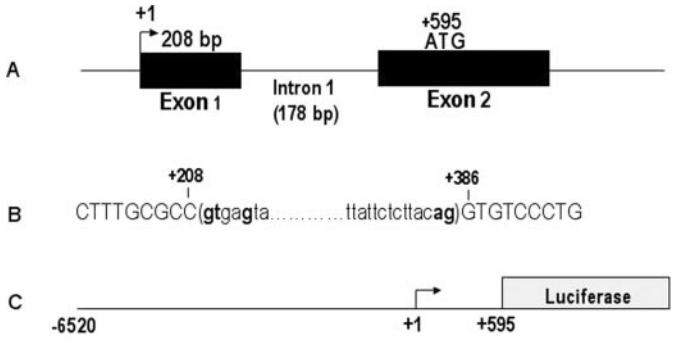

Figure 1. Structure of the zebrafish *gata2* gene. (**A**) The diagram illustrates the exon/intron structure at the 5′ end of the zebrafish *gata2* gene, determined by comparing the 5′-RACE product with the genomic sequence. Exons are shown as boxes and +1 indicates the transcription start site. (**B**) The position of the first intron relative to the genomic sequence is shown, with intronic sequences in lower case, and conserved intronic splice junction sequences in bold. (**C**) To generate a reporter gene, the sequences from the initiation ATG at +595 (relative to the transcription start site) to −6520 were placed upstream of the coding sequences for the firefly luciferase gene. This construct is analogous to a transgenic reporter shown to recapitulate the early *gata2* expression pattern in zebrafish embryos (38).

zebrafish *gata2* transcriptional start site, 5′ rapid amplification of cDNA ends (RACE) was performed on RNA isolated from embryos at 75% epiboly, the first stage at which *gata2* transcripts are readily detected (44). A single PCR product was obtained from this analysis, and subsequent sequencing and comparison with the genomic sequence determined that the transcription start site is located 595 bp upstream of the initiation methionine (Figure 1A). The analysis identifies a short non-coding first exon and places the ATG initiation codon within the second exon (Figure 1A and B). The cDNA isolated by RACE includes sequences just upstream of an independently isolated *gata2* cDNA clone (45). No alternative 5′ sequences that would suggest an alternative exon were identified in the expressed sequence tag database. The exon 1 sequence does not show homology with *gata2* genomic sequences from other species. The result does not rule out that additional promoters might be used in specific cell types or later stages of development, but indicates that a proximal promoter functions during early embryogenesis, initiating transcription at ∼595 bp upstream of the initiation ATG.

Therefore, we considered the ∼6.5 kb of sequences upstream of this region as putative regulatory sequences and developed an assay to determine if they are able to mediate a BMP response. Although it should be possible to do this experiment in the homologous zebrafish system, *Xenopus* embryos provide a unique advantage in that the dorsal/ventral axis is discernable as early as the four-cell stage. Furthermore, the primary components of the early embryonic BMP signaling pathway are thought to be highly conserved. Therefore, the genomic sequence including the ATG was cloned upstream of a firefly luciferase reporter gene (Figure 1C) and this plasmid was injected into developing *Xenopus* embryos. This approach allows activity of the reporter to be analyzed *in vivo* during the early stages of embryogenesis when *gata2* expression is known to be dependent on BMP signaling. In the *Xenopus* system, blastomeres committed to dorsal-anterior or ventral-posterior fates can be distinguished already at the four-cell stage, by virtue of pigment differences that arise following cortical rotation. A reporter sensitive to the endogenous BMP program is expected to be relatively more active when injected at the four-cell stage into presumptive ventral-posterior blastomeres compared to dorsal-anterior blastomeres. The reporter was co-injected with control RNA expressing *Renilla* luciferase. Preliminary experiments demonstrated that the control luciferase is equally active regardless of the injected blastomere (data not shown). The approach of using *Renilla* luciferase RNA (rather than a *Renilla* luciferase expression plasmid) avoids any potential concern that BMP-4 might also influence a control promoter. Embryos were harvested at stage 12–13 (during early neurulation) and protein lysates were tested for the relative amount of firefly luciferase, normalized to *Renilla* luciferase.

We found that the *gata2*:luciferase reporter is more active when injected into the two ventral-posterior blastomeres of a four-cell embryo compared to activity when injected into the two dorsal-anterior blastomeres (Figure 2A). The difference is ∼2-fold, consistent with the gradient of BMP signaling across the dorsal/ventral axis. Importantly, co-injection of RNA encoding *Xenopus* Smad6 (xSmad6), which block BMP signaling (46–48), reduces the activity of the reporter when injected into ventral blastomeres to a level below what is found when the reporter is injected into dorsal-anterior blastomeres (Figure 2A). Thus, the reporter responds to the embryonic BMP program in a manner consistent with the endogenous pathway. To demonstrate directly that the reporter is activated by the BMP pathway, the *gata2* and control reporter constructs were co-injected with RNA encoding BMP-4 into the two dorsal blastomeres. In this assay, the activity of the injected BMP-4 is confirmed by the subsequent ventralized phenotype of injected embryos (data not shown). As shown in Figure 2B, BMP-4 expression results in an average 5-fold induction of reporter activity compared to activity of the reporter when coinjected with control RNA. Co-injection of Smad6 is sufficient to block this activation to the basal level activity of the promoter. We conclude that 6.5 kb of upstream *gata2* genomic sequence is sufficient to respond to BMP signaling, and that the developing *Xenopus* embryo can be used as a model system to define the relevant *cis* elements.

Deletion analysis defines an upstream region required for activation by BMP4

To define the BREs, a series of progressive 5′ deletion constructs were each injected into developing *Xenopus* embryos with either control RNA or RNA encoding BMP-4. In each

case, the activity of the reporter was measured in luciferase assays following injection into the two presumptive dorsal blastomeres of a four-cell embryo, and normalized to *Renilla* luciferase activity from co-injected *Renilla* luciferase RNA.

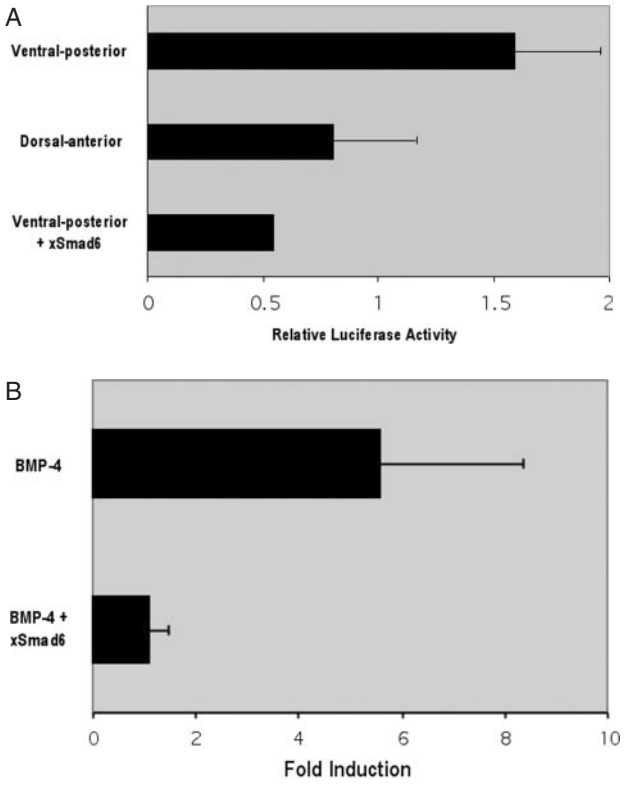

Figure 2. The *gata2* promoter directs reporter gene activity that is induced by BMP4 and dependent on Smad signaling. (**A**) The luciferase reporter was injected at the four-cell stage into blastomeres that contribute preferentially to ventral or dorsal regions of the embryo, as indicated. The reporter is more active in the ventral-posterior derivatives, but this is abrogated by co-injection of RNA encoding the inhibitory Smad6 (the error bar for this sample is present, but it is so small that it is not evident). The results indicate that the reporter responds as predicted to the endogenous Smad-dependent BMP signaling pathway. (**B**) The *gata2* promoter is induced ~5-fold by co-injection of RNA encoding BMP4. This activation is also blocked by co-expression of Smad6.

As shown in Figure 3, deletion of sequences from −6520 to −819 has no effect on the levels of induction caused by BMP-4. However, removing a 68 bp region located between −819 and −751 (relative to the start of transcription) results in a significant loss of induction, indicating that this region mediates a response to BMP-4 and might contain one or more BREs.

BMP-dependent Smads can activate the *zgata2* promoter via the 68 bp BRE

We showed that the BMP response on the *gata2* promoter is sensitive to Smad6 (the inhibitory Smad), so it is expected that BMP-dependent R-Smads should be at least part of the mechanism that mediates the induction between −819 and −751, although this could be direct or indirect. We therefore tested whether injection of RNA encoding BMP regulated Smads 1, 5 or 8 could function through the *gata2* BRE to induce reporter activity. The reporter either contained the BRE (−819) or lacked the BRE (−751), as illustrated in the diagram of Figure 3. As shown in Figure 4, injection of mRNA encoding *Xenopus* Smad1 (xSmad1) or zebrafish Smad5 (zSmad5) was sufficient to activate the promoter containing the 68 bp BRE (−819). Injection of *Xenopus* Smad8 (xSmad8) failed to activate the same reporter, indicating that not all BMP regulated Smads can mediate this effect and that there are functional distinctions between Smads 1, 5 and 8 [shown also in other studies (34,49)]. Importantly, Smad1 failed to activate a reporter construct deleted of the 68 bp BRE (−751), indicating that this region is required for both BMP-4 signaling and Smad1 activity to induce the *gata2* promoter. The experiment does not distinguish whether Smads function directly by binding this promoter, but only that their activity is mediated by the 68 bp sequence.

An Oct-1 binding site is a necessary element within the 68 bp zGATA2 BRE

We first considered whether sequences similar to previously defined SBEs exist within the 68 bp *gata2* BRE identified by the deletion analysis. Alignment of the region to the syntenic region of the *Fugu rubripes gata2* gene showed that the

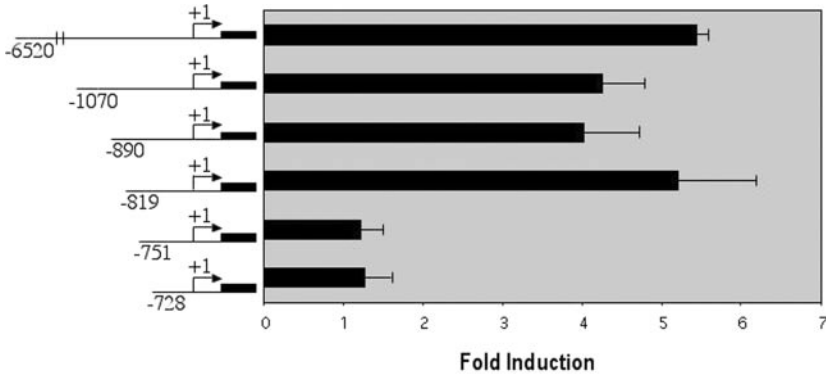

Figure 3. Deletion analysis maps a BRE to a 68 bp region upstream of the *gata2* promoter. A progressive series of 5′ deletions was used to measure the relative activity of the promoter when injected with RNA encoding BMP4, compared to the same reporter when injected with non-coding control RNA, as described in Materials and Methods. For each construct, the site of truncation is indicated, the +1 indicates the transcriptional start site, and the box represents the firefly luciferase reporter. The fold difference (induced/uninduced) is plotted and shows a significant drop when sequences are deleted between −819 and −751. This 68 bp sequence is designated BRE1.

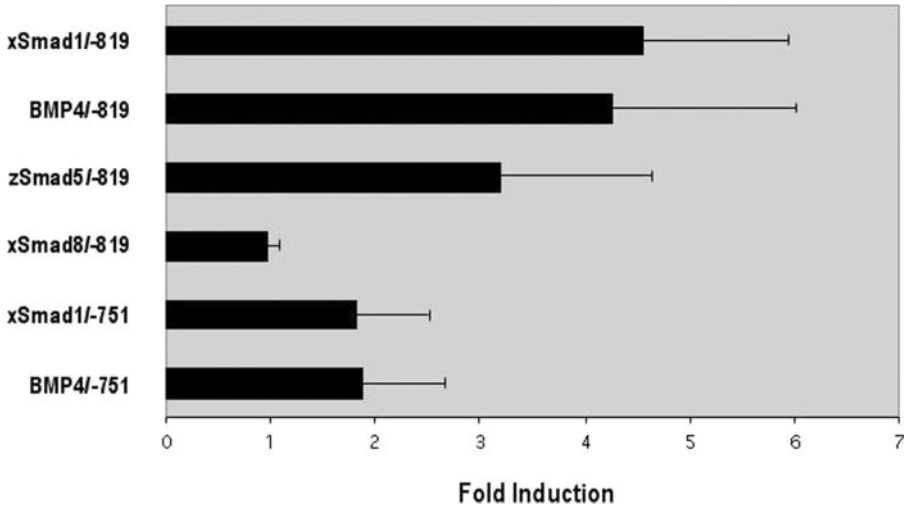

Figure 4. BRE1 mediates activation by BMP4 or Smads. The inducible reporter (−819) or the reporter lacking BRE1 (−751), as diagrammed in Figure 3, was co-injected into *Xenopus* embryos with RNA encoding Smad1, BMP4, Smad5 or Smad8, as indicated. Smad1 activates the reporter equivalent to BMP4, and in both cases this is dependent on the presence of the BRE1 sequences. Smad5 is only slightly less active, while Smad8 fails to induce reporter activity.

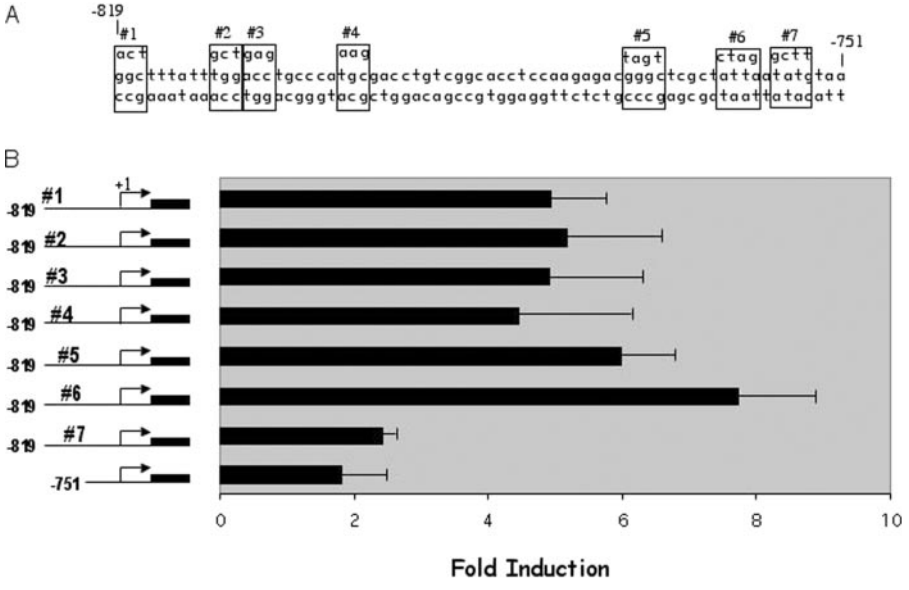

Figure 5. Mutation of an octamer element located at the 3′ end of the BRE1 is sufficient to block BMP-induced activation of the promoter. (**A**) A series of specific mutations were introduced into the BRE1 by site-directed mutagenesis for potential Smad-binding sites (mutations #1–5) or for an A/T-rich sequence (mutation #6), or a putative octamer binding site (mutation #7). (**B**) When compared to the BMP-inducible reporter containing the intact BRE1 (−819), none of the first six mutations had a significant effect on induction. In contrast, mutation of the putative octamer binding site (mutation #7) blocked BMP-mediated induction, similar to deletion of the BRE1 (−751).

sequence is 69% conserved (data not shown), but with no obvious clustering, and with no sequences that match perfectly a known SBE. However, we tested several potential candidate sequences by site-specific mutagenesis (Figure 5A). The sequence 5′-TGGAGC is important for mediating BMP-4 activation of the xVent2 promoter by directing the assembly of a Smad1/Smad4/OAZ transcriptional complex (16). The zebrafish *gata2* BRE contains a related sequence: 5′-TGGACC (Figure 5A, #2 and 3). Also, *Drosophila* Mad binds to the GC-rich sequence 5′-GCCGnCGC (17), and a related sequence 5′-GGGCTCGC is present in the zebrafish *gata2* promoter (Figure 5A, #5). Both these elements and several other sequences with some similarity to previously identified SBEs (e.g. GGCT, Figure 5A, #1), or A/T-rich sequences (Figure 5A, #6) were altered using site-directed mutagenesis. However, none of these mutations (#1–6) had any effect on the ability of BMP-4 to induce the promoter (Figure 5B).

Gel mobility shift assays using the 68 bp BRE as a probe failed to reveal any specific protein complexes using whole cell or nuclear extracts from *Xenopus* embryos injected with xSmad1 or mSmad4 (data not shown). Neither GST–Smad1 nor GST–Smad4 fusion proteins were able to bind the 68 bp

sequence (data not shown). Although these are negative results, they indicate that Smad proteins might not function directly by binding sequences in this region. Therefore, we considered other potential binding sites, and identified a strong match with the consensus binding site (5′-ATGCAAAT) for the POU homeodomain transcription factor Oct-1 (50–52). The related sequence 5′-ATGTAAAG is truncated precisely at the junction of the 68 bp deletion. Interestingly, Oct-1 binding is maximal if the flanking sequences are A/T rich (51), which is the case in the *gata2* sequence. Mutation of this putative Oct-1 binding site in the *gata2* promoter nearly eliminated induction of the promoter by BMP-4, equivalent to truncation of the promoter at −751 (Figure 5B, mutation #7). It is important to point out that the mutation does not alter the basal activity of the promoter, but only the activation by BMP. Thus, the Oct-1 site mediates BMP responsiveness in this system.

In gel mobility-shift assays, nuclear extracts derived from stage 13 *Xenopus* embryos generate a single predominant complex with a probe that is centered on the putative Oct-1 binding site (arrow in Figure 6A). This complex was not detected using probes from the original 68 bp BRE, since the octamer site is truncated on those sequences. This binding activity is competed in the presence of excess unlabeled probe corresponding to the wild-type sequence, but not with an unlabeled oligomer probe corresponding to the mutated sequence that fails to support a BMP response. Furthermore, this complex is fully competed by an oligomer probe containing an Oct-1 consensus binding site (52). Finally, the complex that forms with the wild-type probe derived from the *gata2* BRE is super-shifted specifically by addition of a monoclonal antibody raised against the C-terminus of the *Xenopus* Oct-1 protein (Figure 6B). Incubation with control isotype-matched antibodies does not alter migration of the complex. Thus, Oct-1 binds to the *gata2* BRE (designated BRE1), and a mutation that abolishes Oct-1 binding abrogates the ability of BMP-4 to induce the *gata2* promoter.

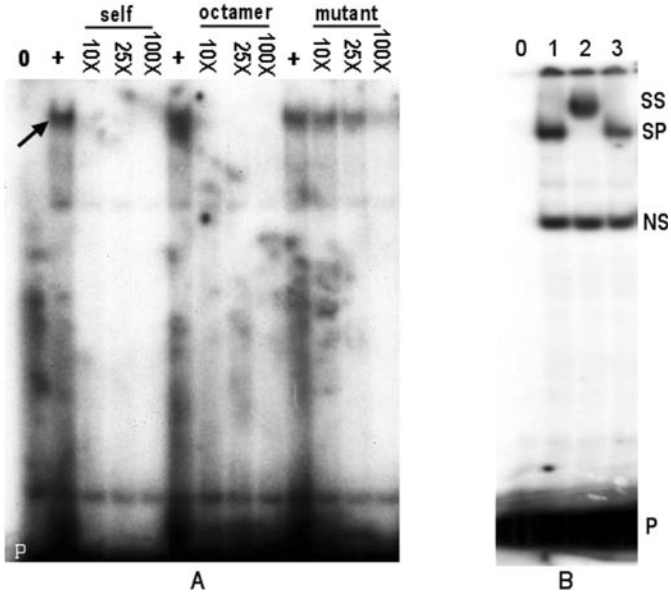

Figure 6. The octamer site of BRE1 binds Oct-1 in *Xenopus* nuclear lysates. (**A**) Gel mobility shift assays were performed using a labeled probe containing the BRE1 sequences including the putative octamer binding site. Lane 0 represents probe alone and the position of the free probe is indicated (P). In lanes marked with a '+', a specific complex forms using nuclear extracts derived from stage 13 embryos, as indicated by the arrow in the second lane. The complex is competed specifically by the addition in the reaction of excess (10×, 25× or 100×) unlabeled probe DNA (self) or DNA containing the octamer consensus (octamer), but not by DNA containing the same mutation of the octamer that blocks the induction by BMP4 (mutant). (**B**) Similar gel mobility-shift assays were performed. In this case, the lanes include probe alone (0), nuclear extract (1), nuclear extract and antibody to *Xenopus* Oct-1 (2) or a control isotype-matched antibody (3). The free probe is indicated (P) as are the positions of a non-specific complex (NS), the specific complex (SP) and the complex that is super-shifted by addition of the Oct-1 antibody (SS).

We tested if BMP signaling modifies the interaction of Oct-1 with the BRE1 site. For this purpose, fertilized eggs were injected with BMP-4 RNA or control RNA. Nuclear extracts were subsequently prepared from the embryos at stage 13 and compared for binding activities. Although extracts from BMP-4 injected embryos appeared to have a modest relative decrease in Oct-1 binding activity, this difference is essentially the same difference found for a distinct complex formed with a control oligomer containing a CCAAT binding site (data not shown). Therefore, the amount and activity of normalized Oct-1 complex formation is not obviously altered by BMP-4 signaling.

The Oct-1 site is not sufficient for mediating the BMP response, but cooperates with a downstream element

Since BMP signaling does not appear to directly alter the Oct-1 interaction with the BRE1, we next considered whether Oct-1 cooperates with one or more additional complexes, which themselves might be direct targets of BMP signaling. Note also that the deletion of the BRE1 or mutation of the Oct-1 site does not eliminate entirely the BMP response, typically reducing the effect from 5- to 2-fold, indicating that additional proximal sequences might contribute to activity. We first tested whether the BRE1 was sufficient to function autonomously upstream of a heterologous promoter. Because the Oct-1 binding site spanned the 3′ junction of the 68 bp region, a larger region of the *gata2* promoter including the Oct-1 site and flanking sequences (134 bp, from −819 to −685) was tested for the ability to mediate BMP responsiveness when placed as a single copy or in triplicate upstream of the SV40 promoter. These sequences were unable to mediate a BMP response with the SV40 promoter, indicating that the Oct-1 dependent BRE1 is necessary but not sufficient for mediating induction (Figure 7A).

When a larger region including sequences downstream of the BRE was tested (377 bp, from −819 to −443), BMP-4 induction was conferred to the SV40 promoter. This result is consistent with the presence of one or more cooperating *cis*-elements located within the more proximal sequences. Therefore, progressive 3′ deletions were tested to define the 3′ boundary of this activity. As shown in Figure 7A, sequences between positions −666 and −646 (BRE2) are required for conferring a BMP-4 response onto the SV40 promoter. To confirm that this sequence cooperates with the original BRE1 defined by the 5′ deletion series (the Oct-1 site), the upstream sequences were removed from the context of the BRE2. Deletion of 68 bp from the larger 377 bp

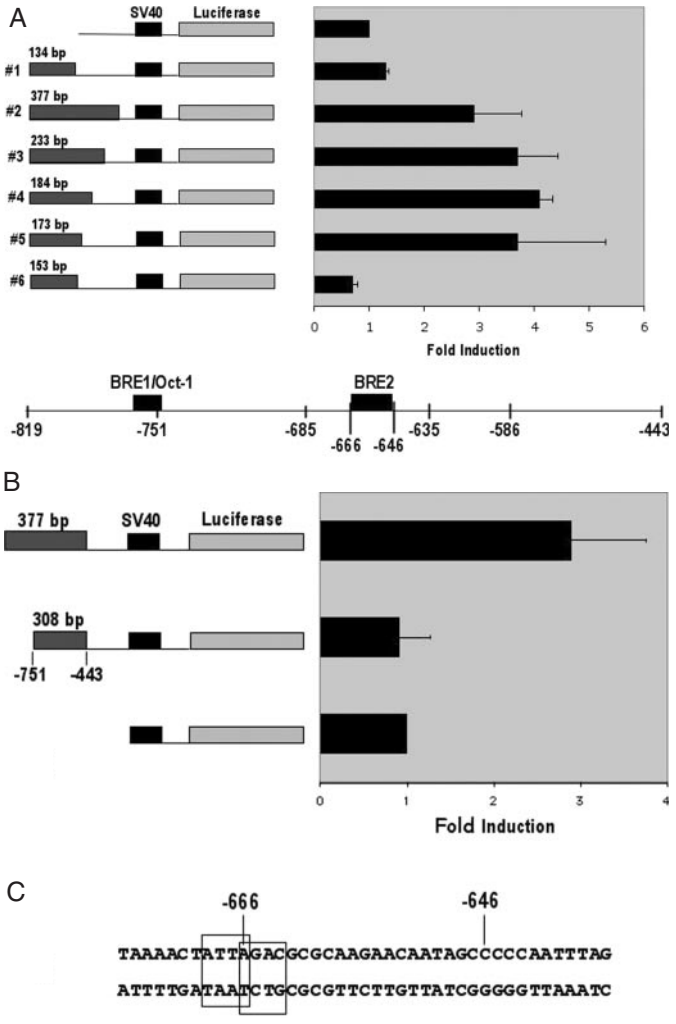

Figure 7. The BRE1 cooperates with a distinct BRE2 element located at ~100 bp proximal to the Oct-1 binding site. (**A**) The SV40 minimal promoter was used to test if the BRE activity could be transferred to a heterologous promoter. The SV40 promoter itself is not induced by BMP4. Shown below this construct are six additional reporters containing various regions of the *gata2* upstream region as indicated on the map below, placed upstream of the SV40 promoter. Sequences from −819 to −685 containing just the BRE1 (construct #1, 134 bp) does not function as a BRE, whereas the entire region from −819 to −443 (construct #2, 377 bp) is sufficient to mediate ~3-fold activation of the SV40 promoter by BMP-4. Subsequent constructs delineated a 20 bp sequence (between −666 and −646) that is necessary for BRE activity (designated BRE2). Dark grey boxes indicate *gata2* genomic sequences, black boxes indicate the SV40 promoter, and the light grey box represents the luciferase reporter gene. Black boxes on the map below represent the BRE sequences. (**B**) BRE2 is not active in the absence of BRE1. This is shown by transferring sequences from −751 to −443 upstream of the SV40 promoter, which fails to support BMP-mediated induction. Therefore, neither BRE1 nor BRE2 has activity on its own, but the two regions cooperate to mediate the response. Boxes represent sequences as in (A). (**C**) Sequences around the BRE2, defined so far by the 20 bp region between −646 and −666. Potential Smad (AGAC) or Vent-2 (ATTA) binding sites are indicated, although we have so far been unable to confirm that they are functional, either for binding or activity (data not shown).

region (308 bp, from −751 to −443) removes the Oct-1 site and eliminates induction of the SV40 promoter by BMP-4 (Figure 7B). We conclude that BMP induction requires both the distal Oct-1 site (BRE1) and sequences within or flanking a 20 bp region of a proximal element (BRE2).

DISCUSSION

Gata2 is a transcription factor expressed in multiple tissues including those of the hematopoietic (31,53–55) and nervous systems (56–58). The gene is likely to be regulated by distinct pathways in diverse tissues, but the early activation in ventral and lateral embryonic mesoderm and ectoderm is fully dependent on the BMP signaling pathway. The *gata2* gene is therefore a component of the BMP syn-expression network. Our results show that BMP signals activate the *gata2* promoter, dependent on the participation of R-Smad proteins, and requiring a binding site for the POU homeodomain transcription factor Oct-1, which functions in conjunction with a more proximal regulatory region. It is important to point out that the *gata2* gene is expressed broadly throughout the BMP syn-expression domain, at the stages of development we are investigating. The Oct-1 binding site is therefore presumably involved in mediating this broad activation of the *gata2* promoter, and our experiments do not address whether it continues to be important for the regulatory mechanisms that later restrict expression of the *gata2* gene to defined cell lineages, e.g. in the hematopoietic system.

Although originally considered a ubiquitous transcription factor (50), *Xenopus* Oct-1 is expressed during embryogenesis in a restricted pattern (59) in regions of both ectoderm and mesoderm coincident with *gata2* expression. Furthermore, mis-expression of Oct-1 in developing *Xenopus* embryos causes axis defects (59), suggesting that Oct-1 might be a component of early dorsal–ventral patterning. Axis defects are similarly caused by expression of a dominant-negative Gata2 isoform (32). There is precedence for mediation of the BMP pathway by POU domain proteins, provided first by studies of the Dpp-regulated *vestigial* gene in *Drosophila* (60). A sequence from the *vestigial* locus, known as the vgQ enhancer, is directly regulated by Dpp signaling through binding of Mad to this element (17). However, over-expression of the POU domain protein Drifter (DFR) expands the domain of LacZ expression in transgenic flies that carry a vgQ-LacZ reporter (60). Subsequent promoter deletion analysis identified a DFR binding element in close proximity to the already characterized Mad binding element, consistent with the idea that full Dpp-mediated transcriptional activity requires the cooperation of both DFR and Mad proteins at the *vestigial* promoter (60). Ectopic expression of Dpp or a constitutively activated form of the Dpp receptor Thick Veins fails to activate DFR expression, implying that DFR itself is not a direct target of the Dpp signaling pathway (60).

Recently, Cao *et al*. (26) found using a one-hybrid assay that Oct-25 binds to and stimulates the activity of the Xvent-2B promoter, and that Oct-25 can also bind to components of the BMP signaling pathway, including Smad1, Smad4 and Xvent-2. Interestingly, Oct-25 expression is not sufficient for activation of the Xvent-2B promoter, but requires the *cis*-elements that interact with Smad1/4 and OAZ. This is analogous to the situation we describe with the *gata2* promoter, in that Oct-1 binding is not sufficient but requires in addition the more proximal BRE2 sequences, which we propose are direct or indirect targets for Smads and/or other activators. Our preliminary attempts at further defining the

key sequences of BRE2 by site-directed mutagenesis have not yielded consistent results, perhaps indicating the presence of overlapping or redundant elements that will require alternative approaches to unravel.

There are two adjacent octamer sequences present in the Xvent-2 promoter, neither of which matches exactly the single site of the *gata2* promoter. POU transcription factors bind DNA via their unique POU domain, consisting of two sub-domains: a POU-type homeodomain (POU_H) and a POU-specific domain (POU_S), tethered to each other by a linker of variable length and sequence (61). In the case of Oct-1, both these domains are indispensable for DNA binding to the octamer consensus sequence 5′-ATGCAAAT-3′ (62). The Oct-1 binding site in the *gata2* BRE1, 5′-ATGTAAAG, retains the four (underlined) essential bases, and overall is identical at six of the eight nucleotides. Furthermore, supershift experiments using an antibody specific to Oct-1 suggest that Oct-1 is the protein in *Xenopus* nuclear extracts that binds to the *gata2* BRE1. Mutations that abolish this binding also block BMP-4 mediated induction of the *gata2* promoter. However, at this point it seems feasible that Oct-25 could also bind to the *gata2* BRE1 (and also that Oct-1 might be able to interact at the Xvent-2 promoter). Until specificity is further documented, it seems reasonable to consider that POU-domain octamer binding proteins represent functional components that mediate BMP signaling.

Based on the studies of the *vestigial* and Xvent-2 promoters, and our data showing that Smad1 can substitute for BMP4, while Smad6 blocks the BRE response, it seems likely that Oct-1 functions with Smad1/4 and perhaps Vent homeodomain proteins for activation of the *gata2* promoter. Our attempts to demonstrate that Smad1/4 or Vent2 bind directly to sequences in the proximal BRE2 element have so far yielded negative results, but this may be due to relatively low binding affinities or the requirement of additional co-factors. Although the cooperating elements in the *vestigial* locus were located within 25 bp of each other (60), it is conceivable that proteins bound to the *gata2* elements could interact over larger distances, presumably facilitated by DNA bending, since the Oct-1 POU domain can bend DNA *in vitro* (63).

The 20 bp region defined as the BRE2 has sequences that might be capable of binding Smad (5′-AGAC) and/or Vent (5′-TAAT) proteins (Figure 7C). The core sequence 5′-TAAT-3′ has been previously shown to be absolutely required for binding and activity of Xvent-2 (64,65). Xvent-2 is an excellent candidate as a co-modulator of the *gata2* response to BMP signaling. First, it is a direct target of BMP-4 (66). Second, zebrafish embryos in which the Vent-related homeobox genes *vox* and *vent* are inactivated by mutation have reduced expression of *gata2* at mid-gastrula stages (67). Third, Xvent-2 can associate with Smad1 to regulate the transcription of downstream target genes (68). Fourth, Xvent-2 and *gata2* co-regulate the expression of Xvent-1, placing both these genes in the same ventralizing pathway (33). Collectively, these data are consistent with the hypothesis that *gata2* expression is induced by a combination of Smad1/4, Oct-1 and Xvent-2 (and perhaps other co-factors), followed by cooperation of these transcription factors to activate other proteins important for ventral fate and the specification of ventrally derived tissues.

ACKNOWLEDGEMENTS

The full-length zebrafish *gata2* promoter was provided by Shuo Lin (UCLA) and the initial set of deletions was generated based on the sequences analyzed previously in his laboratory in transgenic animals. We are also grateful to J. Thomsen (SUNY, Stony Brook), J. Christian (Oregon Health Sciences University), M. Mullins (University of Pennsylvania) and C. Wright (Vanderbilt University) for providing expression constructs. These studies were supported by grants to T.E. from the National Institutes of Health (HL056182) and the Irma T. Hirschl Trust. Funding to pay the Open Access publication charges for this article was provided by the National Institutes of Health.

Conflict of interest statement. None declared.

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