Like other genes of the transforming growth factor-\(\beta\) family, the BMP-4 gene is regulated by an autocatalytic loop. In Xenopus embryos this loop can be ectopically induced by injection of BMP-2 RNA. However, cycloheximide treatment subsequent to BMP-2 overexpression revealed that BMP signaling is not direct but requires additional factor(s). As putative mediator we have identified Xvent-2 which is activated by BMP-2/4 signaling and, in turn, activates BMP-4 transcription. Using promoter/reporter assays we have delineated Xvent-2 responsive elements within the BMP-4 gene. We further demonstrate that Xvent-2 which has recently been characterized as a transcriptional repressor can also act, context dependent, as an activator binding two copies of a 5′-CTAAAT-3′ motif in the second intron of the BMP-4 gene. Replacement of Xvent-2 target sites within the goosecoid (gsc) promoter by the BMP-4 enhancer converts Xvent-2 caused repression of gsc to strong activation. This switch is obviously due to adjacent nucleotides probably binding a transcriptional co-activator interacting with Xvent-2. A model is presented describing the mechanism of BMP-4 gene activation in Xenopus embryos at the early gastrula stage.

Induction and patterning of germ layers in vertebrate embryogenesis depends on intercellular signaling and intracellular signal transduction pathways triggered by various growth factors or growth factor-like molecules. Investigations performed with Xenopus laevis embryos have shown that bone morphogenetic protein 4 (BMP-4), a member of the transforming growth factor-\(\beta\) superfamily, is a key signal for ventralizing the mesoderm, for the inhibition of dorsalizing and neuralizing factors and for converting ectodermal to epidermal cell fate (1, 2). Thus it is evident that the activation of the BMP-4 gene at the ventral side of late blastula/early gastrula stage in Xenopus embryos is of general importance for dorso/ventral pattern formation. However, the molecular nature of factors being responsible for the zygotic activation of this gene in vivo is still not clear. Even in the case of invertebrate homologues, like the Drosophila gene decapentaplegic (dpp), the mechanism governing the initial activation at blastoderm stage is not completely understood. It could be shown that the second intron contains elements which contribute to the correct spatial blastoderm pattern (3–5). A gradient of the protein dorsal, the homologue of c-Rel in vertebrates, suppresses dpp in the ventral half, but the factors involved in transcriptional activation of dpp in the dorsal half remain to be elucidated. Subsequent expression of dpp in visceral mesoderm is regulated by ultrabithorax (ubx) which itself is up-regulated by dpp (6, 7) and, at dorsal closure, the dpp target Fos (FosD) cooperates with Jun (JunD) by regulating the expression of dpp (8).

Autoregulatory loops have also been reported for transforming growth factor-\(\beta\) (9) and Xenopus BMP-4 (10). While auto-induction of transforming growth factor-\(\beta\) involves activatory protein 1, it is unknown, whether the autoregulatory loop of BMP-4 is direct or requires additional factors. We here show that the activation of the Xenopus BMP-4 gene depends upon BMP signaling, but this activation is not observed in the presence of cycloheximide, i.e. in the absence of protein synthesis. In search of putative mediators we have analyzed the role of transcription factors activated in ventral mesoderm, like the homeodomain proteins Xvent-1 (closely related to Xvent-1B and PV.1) (11–13), Xvent-2 (identical or closely related to Vox, Xom, Xbr, and Xvent-2B) (12, 14–17), and the zinc finger factor GATA-2 (18, 19). While all these genes are known to be activated by ectopic expression of BMP-4, only Xvent-2 up-regulates BMP-4 transcription in vivo, and hence is a candidate to function within the autocatalytic loop.

Deletion mutant/reporter gene assays of the Xenopus BMP-4 gene (20, 21) have shown that enhancer elements located within the second intron and the 5′-flanking region contribute to transcriptional activation by BMP signaling. We now have further delineated these regions and demonstrate by co-injection experiments that the same regions which respond to BMP signaling are activated by Xvent-2. The direct interaction of Xvent-2 with a corresponding target site within the second intron was demonstrated by mobility shift and DNase I footprint experiments. In contrast to the previous characterization of Xvent-2 as a transcriptional repressor (22–24) we here document that this factor can additionally work as a transcriptional activator. This dual activity is context dependent and obviously requires a co-activator interacting with Xvent-2 and binding to an adjacent target site. The results suggest a model, in which the autoregulatory loop of BMP-4 is triggered by maternal BMP-2 activating Xvent-2 and is subsequently maintained by BMP-4 via Xvent-2 as a mediator. It is consistent with the observation that Xvent-2 and BMP-4 show identical spatial expression patterns throughout embryogenesis (14, 25).

**ExPERIMENTAL PROCEDURES**

Preparation of Deletion Mutants—All deletion mutants were synthesized as described previously (20) by PCR using DNA of phage \(\times B\) as template. To facilitate the directed cloning into the luciferase pGL3 basic vector (Promega), the forward primers were \(5′\) elongated with a restriction site for BanHI (upstream) or KpnI (intron) and all reverse primers with one for HindIII (upstream) or SmaI (intron). Indicated

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The Homeodomain Transcription Factor Xvent-2 Mediates Autocatalytic Regulation of BMP-4 Expression in Xenopus Embryos*

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The abbreviations used are: BMP, bone morphogenetic protein; RT-PCR, reverse transcriptase-polymerase chain reaction; CHX, cycloheximide; bp, base pair(s).
numbers for all mutants refer to the BMP-4 gene sequence (EMBL AC: AJ005076). Goosecoid promoter DNA fragments were amplified from genomic DNA by using primers which were either KpnI or XhoI elongated (forward) and either Xhol or HindIII elongated (reverse). Forward −226: 5′-GGGCTGAGCCATTAATCGATTAAAGGTGAGC-3′; forward −103: 5′-CCGCTCGAGGAGATCTTTCCTCTCCTCACCACC-3′; reverse +3: 5′-GGGCCATCGTGCCCTCTGGGTTTGG-3′; reverse −128: 5′-CCGCTCGAGCCTGCTCTCCATCCATGTTGCTC-3′. Fragments were subcloned both directly or as cassettes in combination with corresponding fragments of the BMP-4 gene in pGL3.

Preparation of RNAs—RNA used for microinjection experiments was transcribed in vitro with SP6 or T3 polymerase in the presence of GpppG (Cap Scribe Kit, Roche Molecular Biochemicals) using the following templates: BMP-2 in pSP64T, EcoRI linearized, SP6 for sense RNA; BMP-4 in pSP64T, BamHI linearized, SP6; Xvent-2 in pSP64T, EcoRI linearized, SP6; Xvent-1 in pSP64T, BamHI linearized, SP6; Chordin in pRN, NotI linearized, T3; Xvent-2 (P40) in pRN, PstI linearized, T3. Capped RNAs were purified using RNeasy columns (Qiagen). The GAL4-AD/Xvent-2 construct was generated by PCR amplification of the GAL4 activation domain of pGAD424 (CLONTECH) (forward primer: 5′-GGCGTGGAGGTGATAAGGGGAGGATATCCC-3′; reverse primer: 5′-CGGCTAGATATCCCTCCCTGCTTGGTGTTGGTGAGG-3′; synthetic stop codon underlined) which was inserted into Xhol and XhoI sites in-frame to Xvent-2 coding sequence in pCS2. The natural stop codon had been replaced by a Xhol restriction site. RNA was synthesized from NotI-linearized template by SP6 polymerase.

Whole Mount in Situ Hybridization—RNA probes were synthesized from cDNA templates using the DIG labeling kit (Roche Molecular Biochemicals). Whole mount in situ hybridizations were performed with staged embryos as described (26) with slight modifications. Embryos where fixed in MEMFA after staining, bleached in H2O2/methanol, and documented with a DP10 digital camera (Olympus).

Embryo Injections and Luciferase Assay—5′ promoter deletion and intron-2 fusion constructs were injected at 20 pg/blastoctome into two-cell stage embryos in both blastomeres or into four-cell stage embryos in the dorsal or in the ventral blastomeres. When indicated, RNA was co-injected into individual blastomeres. Embryos were collected at stage 12.5 (staging according to Nieuwkoop and Faber (27)) and frozen in liquid nitrogen. Embryos injected with luciferase reporter constructs were processed as described previously (20).

Cycloheximide Treatment—Embryos were injected at the four-cell stage with indicated RNAs and grown until stage 7. Cycloheximide concentration for whole embryos was determined to be sufficient at 30 μg/ml. Embryos were kept in cycloheximide until control embryos had reached stage 10.5 and then fixed for in situ whole mount hybridization. Yeast Transcriptional Assay (28)—Xvent-1 and Xvent-2 were integrated in-frame into the BamHI recognition site of the bacteria/yeast shuttle vector pAS2 (CLONTECH). Subsequently, the constructs have been transformed according to established procedures (yeast protocols handbook, CLONTECH) into yeast strain Y187 employing selection markers trp1 and leu2 (29) and plated on drop-out plates missing tryptophan. Colonies were grown overnight in minimal medium in the absence of tryptophan and the next day inoculated in YEPD medium. Cells were harvested at 0.6 OD (600 nm), disintegrated, and assayed for reporter gene activity by means of a colorimetric assay employing ONPG (o-nitrophenyl-β-D-galactopyranoside) as a substrate at 420 nm (30). The amount of expression was verified by Western blot analysis due to a hemagglutinin A epitope tag provided by pAS2 and by using mouse anti-HA monoclonal antibodies (Roche Molecular Biochemicals). Visualization was performed with goat anti-mouse antibodies using the ECL detection kit (Amersham Pharmacia Biotech).

Mobility Shift Assays and DNase I Footprinting—The isolation procedure of bacterially expressed Xvent-2 homeodomain protein and the
conditions for mobility shift and DNase I footprint experiments are as described previously (31).

RESULTS

Activation of BMP-4 Expression in the Gastrula Stage Embryo—Since the related proteins BMP-2 and BMP-4 are known to bind and activate the same receptors (32, 33), we first investigated whether the BMP-4 autoregulation in *Xenopus* might be initiated by maternal BMP-2. Injection of BMP-2 RNA into the dorsal blastomeres of four-cell stage embryos with subsequent in situ whole mount hybridization for BMP-4 transcripts reveals ectopic activation of BMP-4 in the most dorsal mesoderm, the dorsal lip or Spemann organizer (Figs. 1, A and B). In contrast, radial injection of the truncated BMP type I receptor RNA leads, in a dose dependent response, to an inhibition of BMP-4 transcription (Fig. 1C), thereby demonstrating that BMP signaling is required for BMP-4 gene activation. Thus, the initial activation of the BMP-4 autoregulatory loop could be triggered by endogenous BMP-2. The BMP-2 gene is maternally transcribed and high levels of transcripts are detected until the early gastrula stage (34, 35); BMP-2 protein is present at the late blastula/early gastrula stage (36, 37), when BMP-4 transcription is initiated.

However, treatment of BMP-2 injected embryos with cycloheximide (CHX) prior to midblastula transition prevents transcription of the *BMP-4* gene (Fig. 1D). As controls we used the *Xvent-2* gene (Fig. 1E) which is a direct target for BMP signaling and the *Xvent-1* gene (Fig. 1F) which does not directly respond to BMP-2/4 (12). Thus it seems that the autoregulatory loop is not direct but requires additional newly synthesized proteins. Such factors should be activated by BMP-2/4 signaling and, in turn, up-regulate the *BMP-4* gene. *Xvent-1* was excluded, since it is neither directly activated by BMP-4 (Fig. 1F) nor is it able to activate BMP-4 (data not shown). To investigate the role of *Xvent-2* and GATA-2 on BMP-4 transcription, the corresponding RNAs were injected into dorsal

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Fig. 2. The 5'-flanking region and the second intron of the *Xenopus BMP-4* gene respond to BMP-2/4 signaling. A, genomic structure of the *Xenopus BMP-4* gene and schematic representation of mutants used for luciferase reporter gene assays. All mutants are numbered according to nucleotide position +1 of the transcription start site (indicated at the first exon) and all contain a +116/-54 minimal promoter fragment (shadowed) (additional mutants are described in Fig. 5). Arrows indicate sites responding to Xvent-2. B, DNA (20 pg) of upstream (B) or intron/reporter fusion constructs (C) were co-injected with 200 pg of BMP-2 or BMP-4 RNA, respectively, into both blastomeres of two-cell stage embryos. Luciferase activity was measured when un.injected control siblings had reached stage 12.5.

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2 A. Schuler-Metz, S. Knöchel, E. Kaufmann, and W. Knöchel, unpublished results.
blastomeres of four-cell stage embryos which were subsequently analyzed for the presence of BMP-4 transcripts. Fig. 1, G and J, show that in case of Xvent-2 ventral and lateral expression of BMP-4 is expanded and leads to circumferential expression around the blastoporus, but not in the case of GATA-2. Vice versa, dorsal injections of BMP-4 RNA or BMP-2 RNA lead to a strong increase of Xvent-2 or GATA-2, respectively (Fig. 1, I and L). In conclusion, that the activation obtained with 300 pg of BMP-2 RNA is strongly inhibited by co-injection with 1.3 ng of Xvent-2 (P40) RNA.

**Upstream and Intron Enhancers Regulate the BMP-4 Gene**—We have previously shown by injection of promoter/reporter DNA constructs that the Xenopus BMP-4 gene is activated by several enhancer elements within the 5′-flanking region and within the second intron (20). The upstream region between −206 and −156 responded to BMP signaling, in that a −206/+54 mutant but not a −156/+54 mutant showed a significant increase in reporter gene activity after co-injection with BMP-4 RNA and a down-regulation following co-injection with truncated BMP receptor RNA. Noteworthy, a mutant containing 116 nucleotides of the upstream region fused to complete intron-2 exhibited higher reporter gene activity than approximately 5 kilobases of the 5′-flanking region. Thus, it is evident that intron-2 contains an enhancer which significantly contributes to the activation of the BMP-4 gene. Moreover, this mutant was stimulated by co-injection with either BMP-4 or BMP-2 RNA, whereas co-injection with chordin or truncated BMP receptor RNA led to a drastic decrease of reporter gene activity.

By using serial deletion mutants we have now further delineated the nucleotide element which contributes to the observed activity. Fig. 2A shows the deletion mutants which have been used and a compilation of reporter activities generated by various deletions of the upstream or intron regions after injection into both blastomeres of two-cell stage embryos in the absence or presence of co-injected BMP-2/4 RNA. While the 5′ border of the BMP responsive element within the upstream region was
reduced to 20 bp between positions −176 and −156 (Fig. 2B), major activity of intron sequences was localized between positions +1815 and +1918 (Fig. 2C).

**Xvent-2 as Mediator of BMP Signaling**—We next investigated whether the observed stimulations by BMP-2/4 can also be obtained by Xvent-2. Fig. 3A shows reporter gene activities determined after co-injection of 5′ flanking and intron mutants with Xvent-2 RNA. Both the upstream and the intron mutants behaved similarly or even identical when co-injected with BMP-4 or Xvent-2 RNA, respectively (compare with Fig. 2, B and C). By using a 200-bp intron deletion mutant (I-2Δ+1750/+1951) we observe a reduced basal activity and a loss of stimulation both for BMP-2/4 (not shown) as well for Xvent-2 (Fig. 3A). These findings suggest that Xvent-2 serves as the required component in mediating the autoregulatory regulation of the BMP-4 gene. Additional support for this notion was rendered by the finding that the +1815 mutant was strongly inhibited both by chordin or a dominant negative Xvent-2 mutant (P40) (22), respectively, but could be rescued in both cases by co-injection with wild type Xvent-2 (Fig. 3B). Moreover, the activation obtained with BMP-2 was drastically reduced by co-injection with the P40 mutant. This negative interference supports the notion that Xvent-2 is required for the activation of BMP-4, and it is consistent with the observation that Xvent-2 (P40) inhibits BMP-4 transcription in vivo (data not shown).

The activation observed with Xvent-2 is specific as it was never observed with Xvent-1. This result does not agree with previous studies using the Xvent-1 related PV.1 (21) but is consistent with the finding that only Xvent-2, but not Xvent-1, is able to activate BMP-4 transcription (38). An intron mutant comprising only 232 nucleotides (+1738 to +1969), but including the BMP response element, behaved similarly, in that it was activated by Xvent-2 and inhibited by chordin or Xvent-2 (P40), respectively (Fig. 3C). In conclusion, these results are consistent with Xvent-2 as a mediator of BMP signaling on the −176/−156 upstream and +1815/+1951 intron regions.

**Xvent-2 Interacts with a BMP-4 Gene Enhancer**—To test whether the biological effects observed upon co-injection of deletion mutants with Xvent-2 RNA reflect direct binding of this homeobox protein to DNA we have performed gel retardation assays and DNase I footprint analyses with the Xvent-2 homeodomain. While bacterially expressed Xvent-2 homeodomain readily shifts the intron 2 region which had been shown to elicit a biological response (Fig. 4A), we repeatedly failed to demonstrate such an behavior with the corresponding upstream region (data not shown). This result can be explained by the assumption that an additional factor which might be synthesized under control of BMP-signalizing via Xvent-2 is binding to this region.

Fig. 4B shows a DNase I footprint of the Xvent-2 homeodomain protein on both strands of the intron 2 region, for which we could demonstrate a stimulation upon Xvent-2 co-injection and band retardation. The protected region corresponds for both strands and contain two copies of a motif 5′-CTAATT-3′. This binding site defines an AT-rich homeobox target sequence and it is rather similar to the 5′-CTAATT-3′ motif we have previously shown to bind to Xvent-1 (31). Even more important, this motif is fully compatible to the recently published Xom (Xvent-2) target sequence derived from random oligonucleotides by PCR selection (24) revealing two copies of a TATA/ATTAT motif separated by six or seven nucleotides and the first copy most frequently being found as 5′-CTAATT-3′. In the case of the BMP-4 enhancer, the distance was found to be six nucleotides, but the core motif 5′-CTAATT-3′ is two times directly repeated, whereas the PCR approach is reported to yield in 75% of investigated sequences in antiparallel orientation. Search for this element in the human and mouse BMP-4 genes (39–42), which contain 5 instead of 3 exons, revealed the existence of a conserved 13-bp element within the proximal promoter region (Fig. 4C). This region has also been implicated in transcriptional activation (40, 42). However, mammalian orthologues to Xvent-2 have so far not been isolated; thus, it remains an open question whether these sites are necessary in mammals and whether they bind to similar or other homeodomain proteins.

The Core Enhancer is Required for Binding but Not Suffi-
cient for Activation—We have further delineated the enhancer by additional 5′ and 3′ deletions to a 62-bp fragment extending from nucleotide positions +1823 to +1884. This fragment is shifted by the Xvent-2 homeodomain and, after fusion to the −116/+54 basal promoter, results in a distinct increase of reporter gene activity upon co-injection of Xvent-2 (Fig. 5). Thus, all molecular and biological data suggest that this region contributes to the activation and autocatalytic regulation of the BMP-4 gene. Accordingly, deletion of the two core motifs (+1842 to +1857) led to a loss of binding, to reduced basal activity, and loss of stimulation by Xvent-2. Interestingly, dissection of the 62-bp fragment into a 5′ fragment (28 bp: +1823/+1850) and a 3′ fragment (33 bp: +1852/+1884) revealed that, while both fragments contain a core motif and still bind to the Xvent-2 homeodomain, only the 5′ fragment responds to co-injection of Xvent-2. These findings demonstrate a necessity of 5′-flanking nucleotides for transcriptional activation and that the core motif is required for Xvent-2 binding but not sufficient to up-regulate the BMP-4 gene.

Xvent-2 Serves as Transcriptional Activator—Recent reports demonstrated that constructs containing VP16 or GAL4 activation domains (GAL4-AD) fused to Xvent-2 behave as anti-morphs in acting as repressors for ventral and as activators for dorsal genes (22–24). Accordingly, Xvent-2 was postulated to serve as a repressor. To explain the activation of ventral genes by Xvent-2, a mechanism was suggested according to which Xvent-2 acting as a repressor inhibits transcription of a yet unknown ventral suppressor. Since these conclusions apparently contradict our present model for Xvent-2 as an activator, we have prepared a GAL4-AD/Xvent-2 fusion construct in pCS2 (23) to analyze the effect of microinjected RNA on the BMP-4 gene promoter. Our results confirm the previous findings, but the following experiments demonstrate that Xvent-2 has a dual role in that it can also serve as transcriptional activator. First, all intron deletion mutants lacking the Xvent-2 responding region show a reduced activity as compared with the wild type sequence which is not compatible with a suppressor model. Second, while ventral injection of GAL4-AD/Xvent-2 fusion construct leads to an already described duplication of posterior axis (23), dorsal injection causes a loss of anterior structures suggesting ventralizing activity (Fig. 6A). Third, co-injection of GAL4-AD/Xvent-2 at low concentration with the intron-2/reporter mutant leads to a stimulation of reporter activity, whereas, at higher concentration, an inhibition is observed (Fig. 6B). Fourth, this concentration dependent dual effect can also be observed for the wild type BMP-4 gene. In situ hybridizations show that low concentrations lead to a weak but distinct activation at the dorsal side, while high concentrations applied to the ventral side even lead to clearance of BMP-4 transcripts (data not shown). Thus, at least at low concentrations, this construct behaves as an activator of BMP-4 gene transcription. Fifth, the difference between Xvent-2 and Xvent-1 regarding their activatory potential was finally also demonstrated in the yeast GAL4 assay (28). Fusions of the two proteins to the GAL4 DNA-binding domain revealed that Xvent-2, but not Xvent-1, like the GAL4
The experimental results suggest that the activatory function of Xvent-2 is context-dependent and requires additional sequence motifs which probably bind to a co-activator. If this hypothesis holds true, we would expect that the Xvent-2 function by itself is not sufficient to up-regulate BMP-4 expression. We therefore have analyzed the ability of Xvent-2 to activate BMP-4 gene transcription in the presence of CHX. RT-PCR of RNA from Xvent-2-injected embryos and from uninjected control embryos grown in the absence or presence of CHX (Fig. 8) as well as an in situ whole mount hybridization of corresponding embryos (data not shown) clearly demonstrate that CHX, in contrast to untreated embryos, significantly diminishes or even prevents BMP-4 transcription. This result allows the conclusion that activation of BMP-4 by Xvent-2 requires a co-activator which is either de novo synthesized or whose recruitment is blocked by treatment with CHX. An alternative could be that Xvent-2 directs synthesis of another protein whose recruitment is blocked by treatment with CHX.

**DISCUSSION**

We here show that zygotic activation of the BMP-4 gene at late blastula/early gastrula stage can be triggered by BMP-2 which, in vivo, is translated from maternal transcripts being present until the gastrula stage within the embryo (35).
In contrast, the intron 2 enhancer (1) percentage. dorsal blastomeres at the four-cell stage are set as 100%, the values obtained upon co-injection of 500 pg of Xvent-2 RNA are given as relative activity, depending on the context. Reporter activities determined after injection of 20 pg of DNA constructs into BMP-4 expression prevents maintenance but also in the activation of the gene. Similar conclusions can be drawn from zebrafish mutants. At least the maintenance of zBMP-2 and zBMP-4 is affected in swirl (zBMP-2) mutant embryos; zBMP-4 expression in the ventral marginal region depends on zBMP-2 as indicated by the reduced initial expression and subsequent loss of the marginal zone BMP-4 expression in swirl mutant embryos (44). Also, the requirement of BMP signal transducers has been documented. BMP2b and Smad5 (somitabun: sbn) double mutant analysis and RNA injection experiments have shown that sbn acts downstream of BMP2b signaling to mediate BMP2b autoregulation during early dorsoventral pattern formation (45).

addition, maternal BMP-4 transcripts at the gastrula stage. Thus it is likely that BMP signaling is a major component not only in the maintenance but also in the activation of the Xenopus BMP-4 gene. Similar conclusions can be drawn from zebrafish mutants. At least the maintenance of zBMP-2 and zBMP-4 is affected in swirl (zBMP-2) mutant embryos; zBMP-4 expression in the ventral marginal region depends on zBMP-2 as indicated by the reduced initial expression and subsequent loss of the marginal zone BMP-4 expression in swirl mutant embryos (44). Also, the requirement of BMP signal transducers has been documented. BMP2b and Smad5 (somitabun: sbn) double mutant analysis and RNA injection experiments have shown that sbn acts downstream of BMP2b signaling to mediate BMP2b autoregulation during early dorsoventral pattern formation (45).

However, cycloheximide treatment of BMP-2-injected Xenopus embryos prior to midblastula transition prevents BMP-4 transcription; thus it seems clear that the activation and/or the autoregulatory loop are not direct but indirect. As putative mediators we have investigated several genes which are activated by BMP-2/4. In turn, corresponding proteins should also be able to activate the BMP-4 gene when overexpressed within the embryo. We found that Xvent-2, but not Xvent-1, GATA-2, or Xwnt-8 (46) fulfill both of these requirements. Therefore, the results suggest that Xvent-2 might be directly involved in the transcriptional regulation of the BMP-4 gene. Also, the activatory potential of Xvent-2 for BMP-4 as well as for Xvent-1 transcription (12) strongly suggests that Xvent-2 does not only work as a repressor as recently suggested (22–24), but additionally serves as a transcriptional activator. We have demonstrated that the GAL4 activator domain/Xvent-2 fusion protein behaves for the BMP-4 promoter in a dose-dependent manner as a transcriptional activator which then is converted to a repressor at higher concentrations. The inhibition of ventral genes observed after injection at high concentrations might be explained by an artificial activation of dorsal genes, like goosecid and chordin, which are known to suppress ventral genes. Also, a comparison of the activatory potential of Xvent-2 and Xvent-1 in the yeast system clearly indicates that Xvent-2, in contrast to Xvent-1, behaves as an activator. Finally, swapping of the BMP-4 intron enhancer to the gsc promoter converts Xvent-2 from acting as a repressor to a transcriptional activator. However, the activatory potential of Xvent-2 on BMP-4 gene transcription requires an additional co-activator, because
Moreover, it displays a high degree of conservation to the notion that this motif serves as a natural Xvent-2-binding site. This strongly supports the biological effects observed in reporter gene activation assays needing the action of another, further downstream factor. The expression of BMP-2/4 reactive mutants and can be rescued by Xvent-2. This co-injection with BMP-2/4 to those obtained with Xvent-2 we quantitatively level, Xvent-2 mimics the action of BMP-2/4 and their extent of stimulation. Thus, on a qualitative and autoregulatory loop (20).

Finally, we have investigated the ability of Xvent-2 to interact with DNA motifs found to be essential in reporter gene activation. We demonstrate that the Xvent-2 responsive element in intron 2 is retarded by the Xvent-2 homeodomain, but we failed in mobility shifts using the upstream Xvent-2 responsive region. Thus, we have to conclude that the action of Xvent-2 on the upstream region is not direct but indirect and requires the action of another, further downstream factor. The intron target was subjected to DNase I footprinting. The result corresponds for both strands and reveals a duplicated 4′-CTA-ATT-3′ motif as a target motif for Xvent-2. This strongly supports previous findings of a Xom (Xvent-2) target consensus sequence derived by a PCR-based oligonucleotide selection procedure containing exactly this motif (24). The fact that the biological effects observed in reporter gene activation assays coincide with the presence of this element strongly supports the notion that this motif serves as a natural Xvent-2-binding site. Moreover, it displays a high degree of conservation to the 5′-CTATT-3′ motif, which we have recently described as a Xvent-1 target site within the XFD-1′ promoter (31).

In summary, we show both for the wild type gene and for promoter/reporter constructs that the autoregulatory regulation of the BMP-4 gene is mediated by Xvent-2. Results obtained from biological and molecular investigations are compatible with the notion that the autoregulatory loop of BMP-4 is initially triggered by maternal BMP-2 signals activating Xvent-2 and maintained during early development by Xvent-2. The major contribution of intron 2 to transcriptional activation of BMP-4 coincides with two copies of a 5′-CTAATT-3′ target motif which have the potential to bind to Xvent-2. Although our results do definitely not rule out the possibility that other factors being activated by BMP-4, e.g. msx1 or Xvex-1 (49, 50), might participate in the autostabilization loop, we provide the first insight into the regulatory mechanisms governing the transcription of the BMP-4 gene in Xenopus embryos.

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