Cooperative Interaction of Xvent-2 and GATA-2 in the Activation of the Ventral Homeobox Gene Xvent-1B*

Received for publication, February 23, 2002, and in revised form, April 16, 2002
Published, JBC Papers in Press, April 18, 2002, DOI 10.1074/jbc.M201831200

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The Xvent family of homeobox transcription factors is essential for the establishment of the dorsal-ventral body axis during Xenopus embryogenesis. In contrast to Xvent-2B and other members of the Xvent-2 subfamily, Xvent-1B is not a direct response gene of bone morphogenetic protein-4 signaling. Xvent-1B is activated by Xvent-2, but CHX experiments revealed the requirement of additional factors. In this study, we report on the cooperative effect of Xvent-2 and the zinc finger transcription factor GATA-2 on the promoter of the Xvent-1B gene. We show that GATA-2 is a direct target gene of bone morphogenetic protein-4 and that GATA-2 interacts with Xvent-2 to activate transcription of Xvent-1B. Both transcription factors bind to distinct elements within the Xvent-1B promoter, and GATA-2 physically interacts with the C-terminal domain of Xvent-2. Promoter reporter studies in Xenopus embryos revealed that full activation of Xvent-1B requires both Xvent-2 and GATA-2. Moreover, the two factors are sufficient to direct transcription of Xvent-1B in the presence of CHX at the ventral side of the embryo. The failure of both factors to activate Xvent-1B on the dorsal side suggests the existence of a dorsal inhibitor. This inhibitor is likely a component of the dorsal Wnt signaling pathway because nuclear translocation of β-catenin before midblastula transition results in a suppression of Xvent-1B transcription.

The establishment of the dorsal-ventral body axis in vertebrate embryogenesis is the result of antagonisms among different growth factors and/or their mediators. One of the first steps in the early development in Xenopus laevis is the accumulation of β-catenin in the future dorsal signaling center, the Nieuwkoop center (1). The interaction of β-catenin with high mobility group box transcription factors of the LEF/TCF family leads to the activation of other dorsal factors such as the homeobox transcription factor siamois (2). All of these molecules are components of the dorsal Wnt signaling pathway and result, when ectopically expressed on the ventral side, in a dorsalization of the embryo and formation of a second axis (3).

Another important signaling pathway in early embryogene-

sis is the BMP pathway which, in contrast to the dorsal Wnt signal, is responsible for the activation of ventral molecules (4, 5). In Xenopus, it has been shown that the autoregulatory loop of BMP-4 expression is mediated by the ventral homeobox protein Xvent-2 (6). Because Xvents can mimic all early BMP-4 effects, the Xvent transcription factors are regarded as downstream effectors of BMP-4 signaling in early amphibian development (7). Although most of our knowledge about Vents is derived from experiments with Xenopus and zebrafish (8–15), molecular cloning of a human Vent-like gene has recently been reported (16).

Based upon amino acid sequence comparisons, members of the Xvent family are divided into two subfamilies, the Xvent-1 (Xvent-1 (8), PV.1 (9), and Xvent-1B (10)) and Xvent-2 (Xvent-2 (11), Xbr-1B (12), Xom (13), Vox 15 (14) and Xvent-2B (10)) subfamilies. Besides their sequence divergence, the two groups differ clearly in their expression patterns, which already suggests different regulatory mechanisms. It has been shown that Xvent-2B is a direct target gene of BMP signaling (10). The BMP mediator Smad1, which is phosphorylated by the BMP type 1 receptor, interacts with Smad4 to build a transcriptionally active complex on the Xvent-2B promoter (17, 18). This is part of a subsequent, indirect autoregulatory loop in which BMP-4 induces its own expression by using Xvent-2 as a mediator (6) and a direct autoregulatory loop in which Xvent-2 activates its own expression (19). In contrast, Xvent-1B is not up-regulated by BMP signaling in the absence of de novo protein synthesis and, therefore, is not regarded as a direct BMP-4 target (10). Instead, Xvent-1B can be activated by Xvent-2, and because it can rescue the phenotype caused by the dominant-negative Xvent-2 P(40) mutant, it has been suggested that Xvent-1 acts downstream of Xvent-2 (10). However, Xvent-2, like BMP-4, is not capable of activating members of the Xvent-1 family in the presence of cycloheximide (CHX). This suggests that either another or an additional factor is necessary for the activation of Xvent-1B. This factor could either be produced by a target gene of Xvent-2, or it might synergize with Xvent-2 in a cooperative manner. One possible candidate factor seemed to be the zinc finger protein GATA-2 (20), which activates Xvent-1, and when overexpressed as a dominant-interfering construct, had been shown to suppress specifically expression of the Xvent-1 but not of the Xvent-2 gene (21). The GATA-2 gene is induced by BMP-4 (22), and GATA-2 expression in ventral mesoderm starts at the early gastrula stage (23), i.e. at the right time and at the right place to suggest this factor as an additional player in the in vivo regulation of Xvent-1 transcription.

In the present report we have investigated the transcriptional regulation of the Xvent-1B gene by Xvent-2 and GATA-2. We have found that GATA-2 is able to activate the Xvent-1B promoter and that it rescues the effects induced by a dominant-negative Xvent-2 mutant. In contrast, blocking BMP signaling

*This work was supported by Deutsche Forschungsgemeinschaft Grant SFB497/A1 and by Fonds der Chemischen Industrie. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.
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‡ The abbreviations used are: LEF/TCF, lymphoid enhancer factor/TCF cell factor; BMP, bone morphogenetic protein; CHX, cycloheximide; CMV, cytomegalovirus; GST, glutathione S-transferase; MBT, midblastula transition; RT, reverse transcription; tBR, truncated BMP type I receptor; Tm, promoter mutated at a TCP target site.
at the level of the BMP receptor, which leads to an inhibition of endogenous Xvent-2 gene activity, cannot be compensated for by GATA-2. Therefore, it seems unlikely that GATA-2 operates downstream of Xvent-2. Instead, this observation favors a model of a cooperative action between Xvent-2 and GATA-2 in the activation of Xvent-1B. To test this model we have performed protein-DNA binding assays. We could define target elements for both Xvent-2 and GATA-2 factors on the Xvent-1 promoter. It could also be shown that GATA-2 interacts directly with the C-terminal domain of the Xvent-2 protein.

Analysis of the Xvent-1B promoter by microinjection of different deletion mutants further supports the cooperative function of these two transcription factors. Whereas activation by GATA-2 is strictly dependent on Xvent-2 binding elements, Xvent-2 also seems to activate Xvent-1B in a GATA-2 independent manner, albeit at a much lower extent. Furthermore, although Xvent-2 is unable to activate Xvent-1B in the presence of CHX, a combination of Xvent-2 and GATA-2 is sufficient to up-regulate Xvent-1B expression if injected on the ventral side. However, overexpression of GATA-2 and Xvent-2 on the dorsal side does not lead to an activation of Xvent-1B transcription in CHX-treated embryos, even if coinjected with BMP-4. This suggests that activation of Xvent-1B on the ventral side of embryos in vivo is the result not only of the presence of Xvent-2 and GATA-2 proteins, but also the absence of dorsal inhibitors.

**EXPERIMENTAL PROCEDURES**

**Constructs and Plasmids—**Xvent-1B promoter fragments were created by PCR using the following primers: upstream, [−249 (5′-CGGGATCCATGGGATGGAGGGAGGTAAGCTG-3′), −164 (5′-CGGGATCCATGGGATGGAGGGAGGTAAGCTG-3′), downstream, +52 (5′-CCCAAGCTTGGGTAGGGAAGG-AGGCTG-3′), −164 (5′-CGGGATCCATGGGATGGAGGGAGGTAAGCTG-3′)]. Nucleotide positions refer to the published sequence (10). The resulting PCR products were digested with BamHI/HindIII and cloned into pGemHI/HindIII of the pG5-basic vector (Promega). The Xvent-1B/pG3 construct −55/+52 was obtained by digesting the −164/+52 PCR fragment with Sau3A and the −249/−164 and −55/+52 fragments. The GATA-mutated (Gm) Xvent-1B promoter fragment (−249/Gm/+52) was constructed by fusion of two PCR-generated fragments via an artificial EcoRI site using the following primers: upstream primer, 5′-GGATCCGGATCCGATCGAGG-3′, downstream primer, 5′-GGATCCCGGATCCGATCGAGG-3′; downstream fragment, −197 (5′-GGATCCGGATCCGATCGAGG-3′) and +52 (as described). The PCR products were digested with BamHI/EcoRI (−249/−192) or with EcoRI/HindIII (−197/−52) and ligated at their EcoRI restriction sites. A mutation of the putative LEF/TCF binding site was generated by PCR amplification using the −249 upstream primer (see above) and a mutated reverse primer starting at −52 (5′-CGGGATCCATGATAGGTAGGGAAGCTG-3′; mutated positions are underlined).

**Microinjections and Luciferase Assays—**Microinjections were performed with *in vitro* fertilized Xenopus embryos, dejellied in 2% cysteine hydrochloride in 0.1 × MBSS (10 mM HEPES pH 7.4, 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO3, 0.82 mM MgSO4, 0.11 mM CaCl2, 0.66 mM KNO3) and staged according to Nieuwkoop and Faber (24). Before injection, embryos were placed into 1 × MBSS containing 4% Ficoll. Deletion mutants were injected dorsally or ventrally at the four-cell stage (20 pg/blastomere). In *in vitro* transcribed capped mRNAs (Messager-mMachine™ SP6 Kit, Ambion) were purified over RNAeasy columns (Qiagen) for microinjection. Linearization and transcription of DNA were performed as indicated: pSp64T3-BMP-4 (Xenopus BMP-4, BamHI, SP6); pSp64T3Xvent-2 (Xvent-2, EcoRI, SP6); pSp64-GATA-2 (NofI, SP6). RNA was injected at the following concentrations: 500 pg/blastomere BMP-4, 200–400 pg/blastomere Xvent-2, 50 pg/blastomere GATA-2. As an internal control, the pRL-CMV renilla reporter plasmid (Promega) was co-injected to allow for normalization of firefly luciferase values.

Injected embryos were cultured until stage 11 and snap-frozen in liquid nitrogen. Luciferase assays were performed according to the manufacturer’s protocol, except that 10 μl of passive lysis buffer was used per embryo (Dual Luciferase Assay System, Promega). Luciferase activities of firefly and renilla were determined separately using 20 μl of supernatant (centrifuged for 10 min at 4 °C).

**Protein Preparation—**Fusion proteins were expressed in E. coli BL21 (DE3) Plus (Stratagene) and purified as described recently by Henningfeld et al. (19). 35S-Labeled proteins were prepared using the TNT-coupled transcription-translation system (Promega).

**Preparation of Embryonic Extracts—**Protein extracts containing Myc-tagged Xvent-2 (MT-Xvent-2) were essentially prepared as reported previously (19).

**GST Pull-down Assays—**To 20 μl of glutathione-Sepharose (Amerham Biosciences) in 500 μl of binding buffer (50 mM Tris-HCl, pH 8.0, 50 mM KCl, 5 mM MgCl2, 1 mM dithiothreitol, 0.2% Nonidet P-40, and 10% glycerol) an equal amount (5 μl) of purified GST or GST fusion proteins and 5 μl of 35S-labeled proteins were added. After incubation at 4 °C (rotation slowly), the reactions were washed four times with wash buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, and 0.2% Nonidet P-40), and 30 μl of 2 × SDS loading buffer was added. The samples were boiled for 5 min and analyzed on a 10% SDS-polyacrylamide gel. The gel was Coomassie stained to visualize GST proteins, dried, and subjected to PhosphorImager analysis. For pull-down assays from cell extracts, experiments were performed with 25 μg of total protein from Xenopus. Western analysis was performed with the anti-Myc antibody 9E10.

**RT-PCR—**Xenopus embryos were injected into both dorsal and ventral blastomeres at the four-cell stage with 500 pg of BMP-4, 400 pg of Xvent-2, or 50 pg of GATA-2 RNA. Lithium treatment was started at stage 5 for 15 min with 300 mM lithium chloride. At stage 7.0 embryos were treated with 25 μg/ml CHX (Sigma). CHX-treated embryos reached stage 10.5. Total RNA was isolated using RNAeasy minicolumns (Qiagen; RNAeasy protocol for isolation of total RNA from animal tissues). DNase digestion was performed by adding 1.5 μl of RNase-free DNase I (Roche Molecular Biochemicals) and 5 μl of 25 mM MgCl2 to 50 μl of total RNA. The reaction was incubated for 20 min at 37 °C followed by inactivation of DNase by heating for 10 min at 75 °C. cDNA synthesis was performed under the following conditions: 1 × RT reaction buffer (Amerham Biosciences), 10 ng of (dT)12–18, 10 ng of random primer, 0.2 μM dNTPs, 26.8 units of RNAguard™ RNA inhibitor (Amerham Biosciences), 10 units of Moloney murine leukemia virus reverse transcriptase (Amerham Biosciences), and 600 ng of total RNA.

For PCRs the following primers and annealing conditions were used. For histone H4, the upstream primer was 5′-CGGGATCCATGCTCGTAGACTACT-3′, and the downstream primer was 5′-ATCATGCTCGTAGACTACT-3′ with 56 °C annealing temperature. For Xvent-1B, the upstream primer was 5′-TCCCTCTAGAGTCTTTCTCT-3′, and the downstream primer was 5′-GATCTCTAGAGTCTTTCTCT-3′ with 56 °C annealing temperature. For GATA-2, the upstream primer was 5′-CGGGATCCATGCTCGTAGACTACT-3′, and the downstream primer was 5′-CGGGATCCATGCTCGTAGACTACT-3′ with 56 °C annealing temperature.

**LightCycler (Roche Molecular Biochemicals) reactions** were set up according to the manufacturer (SYBR-green fast start protocol) and using the following primers: ODC (upstream primer, 5′-CAAAGCTTGTTCTACAGGATAAGC-3′; downstream primer, 5′-CCGTTTGATCTCGACTGACGAGAGG-3′; and the downstream primer was 5′-CGGGATCCATGCTCGTAGACTACT-3′ with 56 °C annealing temperature.

**Electrophoretic Mobility Shift Assay—**The desired promoter fragments were excised from the pBS KS II (+) (−249/−13), XbaI/DdeI; −249/−164, XbaI/EcoRI) or pG3 vector (−164/−13, NheI/DdeI) and 3′-labeled on one strand by a fill-in reaction with [α-32P]dCTP and Klenow DNA polymerase. Binding reactions were carried out on ice for 30 min in 30 μl of binding buffer (30 mM Tris-HCl, pH 7.5, 30 mM KCl, 1 mM MgCl2, 1 mM dithiothreitol, 12.3% glycerol) containing 1 μg poly(dI-dC) and 1 ng of the gel-purified probe. After 5 min of preincubation, the protein was added and incubated for 30 min. The probes were separated on 7% native acrylamide gels in 0.5 × Tris-borate.

**DNase I Footprinting—**Binding reactions for DNase I footprinting were prepared and incubated as described under “Electrophoretic Mobility Shift Assay.” The concentration of MgCl2 was subsequently raised to 5 mM for DNase I footprinting, and 0.065 unit (free DNA) or 0.195 unit (DNA + protein) of DNase I was added at room temperature for 45 s. The DNase I digestion was stopped by the addition of an equal volume of sample buffer (60% deionized formamide, 20 mM EDTA, 660 mM sucrose). Sequencing reactions were performed according to the method of Maxam and Gilbert (25). After preelectrophoresis for 2 h at 70 W, samples were analyzed by 7% denaturing PAGE at 60 W in 1 × Tris-borate/EDTA.

**Whole Mount in Situ Hybridization—**Localization of Xvent-1B transcripts in gastrula stage embryos was demonstrated by whole mount in situ hybridization using a digoxigenin-labeled antisense RNA (10).
GATA-2 Is a Direct Target of BMP-4 and an Activator for Xvent-1B—Because Xvent-2 has been shown not to be sufficient to activate Xvent-1B in the presence of CHX (10), we have been searching for additional factors. One candidate was the zinc finger transcription factor GATA-2, which is induced by BMP signaling (22). It has been shown that GATA-2 activates Xvent-1 and that a dominant-interfering GATA-2 (G2en) specifically suppresses Xvent-1 but has no effect upon Xvent-2 expression (21). This prompted us to analyze whether GATA-2 is a direct BMP target gene and to investigate the effects of GATA-2 on the Xvent-1 promoter.

First, RT-PCR analysis was performed to study the BMP-4 induced activation of GATA-2. BMP-4 RNA was injected into embryos at the four-cell stage, CHX was added (10) at stage 7.0 prior to MBT, and the embryos were cultured until control embryos had reached midgastrula (stage 10.5). Fig. 1A shows that BMP-4 leads to an increase of GATA-2 transcripts in both the absence and presence of CHX. This indicates that BMP-4 being translated at early cleavage stages is sufficient to activate zygotic transcription of GATA-2 after MBT even in the absence of protein biosynthesis. We next have analyzed whether GATA-2 is a regulator of Xvent-1B expression. In agreement with previous reports (21) we find that GATA-2 leads to a distinct up-regulation of Xvent-1B; however, no transcripts are detected when injected embryos were treated with CHX (Fig. 1B). Dorsal activation of Xvent-1B in GATA-2 or Xvent-2 RNA-injected embryos was also demonstrated by whole mount in situ hybridization (Fig. 1, C–E). Although both factors led to an ectopic expression of Xvent-1B RNA within the dorsal marginal zone, only Xvent-2 renders expression within the most dorsal located region, the Spemann organizer. Taken together, our results suggest that GATA-2 is a direct target of BMP-4 signaling and that GATA-2 activates Xvent-1B, but that additional factors are required because activation occurs only in the presence of protein biosynthesis.

GATA-2 Rescues the Effect of Xvent-2 P(40)—To investigate the involvement of GATA-2 in the activation of the Xvent-1B promoter and to find out whether it acts downstream of Xvent-2 or in a parallel, cooperative mode, we have used the previously described −249/+52 Xvent-1B promoter fragment fused to a luciferase reporter (10) and performed coinjections of GATA-2 with the dominant-negative Xvent-2 P(40) RNA (26) or with truncated BMP type I receptor (tBR) RNA (27), respectively. As shown in Fig. 2, GATA-2 can rescue the suppression caused by Xvent-2 P(40). This would still be in line with the downstream as well as with the parallel mechanism for the activation of Xvent-1B. However, coinjections of GATA-2 RNA with tBR RNA do not lead to an increase of luciferase activity compared with the lowered activity caused by injections of tBR. Because tBR injection suppresses Xvent-2 transcription, these results suggest that GATA-2 action needs endogenous Xvent-2 protein. They further argue for the cooperative model because blocking the BMP signaling cascade at the level of the BMP receptor leads to a loss of Xvent-2 protein in the affected cells and therefore prevents GATA-2 from up-regulating the Xvent-1B promoter.

GATA-2 Interacts Directly with Xvent-2—To find out whether Xvent-2 physically interacts with GATA-2, we prepared a GST-Xvent-2 fusion construct and performed pull-down assays with radiolabeled GATA-2 protein. Fig. 3A shows that GATA-2 can interact with the GST-Xvent-2 fusion protein, whereas GST alone is not able to bind to GATA-2 under these conditions. To confirm this result and to investigate whether in vivo translated Xvent-2 binds to GATA-2, a GST-GATA-2 fusion protein was constructed and incubated with Xenopus extracts containing Myc-tagged Xvent-2 protein. As shown by immunoblotting in Fig. 3B, binding of Xvent-2 and GATA-2 was also detected under these circumstances. Additional experiments revealed that GST-GATA-2 was also able to bind to radiolabeled Xvent-2 P(40) (see Fig. 3C). This is an important finding because GATA-2 RNA injection resulted in a rescue of
leads to a shift. Using the Xvent-2 P(40) protein, no binding from positions –Xvent-1B promoter fragments, respectively. When the 3′/H11002 Xvent-2 proteins. As shown schematically in Fig. 4, shifts with different Xvent-1B promoter fragments to- length protein, binds to GATA-2, Xvent-2 distinct elements in the Xvent-2 suggests that both transcription factors should bind to this interaction is not disrupted by the P(40) mutation within the homeodomain.

GATA-2 and Xvent-2 Bind to Distinct Elements on the Xvent-1B Promoter—The cooperative action of GATA-2 and Xvent-2 suggests that both transcription factors should bind to distinct elements in the Xvent-1B promoter. We performed gel shift assays with different Xvent-1B promoter fragments together with bacterially expressed full-length GATA-2 and Xvent-2 proteins. As shown schematically in Fig. 4A, Xvent-2 and GATA-2 proteins bind to –249/–13 and –249/–164 Xvent-1B promoter fragments, respectively. When the 3′-part from positions –164 to –13 was used, only Xvent-2 protein leads to a shift. Using the Xvent-2 P(40) protein, no binding could be detected to any of these promoter fragments. These results suggest that the dominant-negative effect of this mutant is caused by a loss of DNA binding activity.

To gain more detailed information about the binding sequences of GATA-2 and Xvent-2, we performed DNase I protection assays using the Xvent-1B promoter fragment from position –249 to –13 (Fig. 4, B and C). Fig. 4B shows that GATA-2 protected only one region between positions –292 and –175 containing a canonical GATA-2 binding site 5′-TGATA-3′ (28). In contrast, Xvent-2 protects two regions (Fig. 4C), a proximal region within positions –111 and –74 and a more distal region between –229 and –175. These findings confirm the results obtained from the gel shift experiments. The distal region, which overlaps with the GATA-2-protected region, was resolved further into three distinct elements by using a truncated –249/–164 promoter fragment (Fig. 4D). Inspection of all the Xvent-2 binding sites revealed the accumulation of nine motifs (see boxes in Fig. 4) that can be aligned to the consensus sequence 5′-CC/TAAT-3′. This sequence is in good agreement with results obtained from random oligonucleotide selection (29) as well as the recently reported 5′-CTAAT-3′ motif as an Xvent-2 target site on the BMP-4 and Xvent-2 genes (6, 19).

Activities of Xvent-2 and GATA-2 Elements on the Xvent-1B Promoter—DNA-protein binding assays have revealed that the Xvent-1B promoter contains binding elements for Xvent-2 and GATA-2. To analyze the biological relevance of these elements, we have coinjected different promoter deletions with RNAs for these factors (Fig. 5). The longest mutant used for these experiments (–249/+52 Xvent-1B) contains the GATA-2 as well as the proximal and the distal Xvent-2-binding elements. Coinjection of this promoter fragment with GATA-2 or Xvent-2 RNA result in an increase of luciferase activity. A 5′-deletion starting at position –164 and missing the GATA-2 and distal Xvent-2 binding sites cannot be activated by Xvent-2 but is still activated by Xvent-2 RNA injection. This activation is most likely the result of the proximal binding site because a –55/+52 promoter fragment was not activated. Vice versa, a promoter fragment with an internal deletion (–249/Δ164 –52 Xvent-1B) containing the GATA-2 as well as the proximal Xvent-2-binding site is also activated by Xvent-2 RNA. Surprisingly, GATA-2 fails to activate this mutant, even if the GATA-2-binding site is present. To ensure that the canonical target site in the distally located GATA-2-binding element is required for the activity of this factor, we have mutated this site and coinjected this mutant with GATA-2 or Xvent-2 RNA, respectively. As shown in Fig. 5A, Xvent-2 activates this promoter fragment, whereas GATA-2 is completely ineffective.

The results obtained from microinjection experiments and from DNA-protein binding assays support the idea that GATA-2 requires its own binding site as well as elements responsible for Xvent-2 binding. In contrast, Xvent-2 seems not only to act in a GATA-2-dependent manner but can also activate Xvent-1B in the absence of the GATA-2-binding site. Therefore, we analyzed whether GATA-2 has any effect on Xvent-2-caused activation of the Xvent-1B promoter when the GATA-2 binding site is mutated. Coinjections of Xvent-2 and GATA-2 RNA were performed with the wild type as well as with the mutated promoter fragments (Fig. 5B). As could already be expected from results presented in Fig. 5A, the wild type promoter was activated strongly by injection of both RNAs. However, the mutated promoter was repressed slightly by coinjection with GATA-2 RNA, and even more importantly, GATA-2 also leads to a distinct suppression of activation caused by Xvent-2.

GATA-2 and Xvent-2 Are Sufficient to Activate Xvent-1B on the Ventral Side—The results presented so far imply that Xvent-2 and GATA-2 cooperate in the activation of the
Xvent-1B promoter. To analyze whether they are also sufficient to activate transcription of the Xvent-1B gene if protein synthesis is inhibited, we have co-injected GATA-2 and Xvent-2 RNA into Xenopus embryos at the four-cell stage and treated the embryos with CHX before MBT. Total RNA was isolated for RT-PCR analysis when control embryos had reached stage 10.5. It has been shown previously that dorsal injection of Xvent-2

FIG. 4. GATA-2 and Xvent-2 bind to the Xvent-1B promoter. A, schematic summary of results from electrophoretic mobility shift assay with 32P-labeled fragments of the Xvent-1B promoter and Xvent-2, Xvent-2 P(40), or GATA-2 proteins (as indicated). + indicates retardation of the DNA fragment; − indicates no retardation. B–D, DNase I footprinting analysis of GATA-2 (B) or Xvent-2 protein (C) to the 32P-labeled −249/−13 or of Xvent-2 to the 32P-labeled −249/−164 Xvent-1B promoter fragment (D). G/A indicates guanine and adenine residues from chemical sequencing reactions; triangles show increasing amounts of protein (from 40 to 160 ng). Protected regions are indicated by vertical lines, and GATA-2 and Xvent-2 target motifs are shown in boxes.
RNA does not result in Xvent-1B transcription when the embryos are treated just before MBT with CHX (10). Fig. 6A shows that this failure also holds true for ventral injection of Xvent-2 and for dorsal coinjection of Xvent-2 and GATA-2. However, Xvent-1B transcripts are clearly detected in CHX-treated embryos when Xvent-2 and GATA-2 RNAs are coinjected into ventral blastomeres. This means that GATA-2 cooperates with Xvent-2 and that, although neither of these factors by itself is sufficient, the two factors together are able to trigger transcriptional activation of Xvent-1B. There are two possible explanations for the failure of these factors to evoke the activation after injection at the dorsal side. First, an additional factor, which is necessary to activate Xvent-1B, might be missing on the dorsal side, or second, dorsal inhibitors do not allow transcription of this gene. To address this question, we have analyzed whether additional ventral signals, i.e., BMP signaling, could result in an activation of Xvent-1B. We have coinjected Xvent-2, GATA-2, and BMP-4 RNAs into both dorsal or ventral blastomeres, respectively. Although ventral injection leads to an expression of the Xvent-1B gene, no transcripts were detected after dorsal injection of these RNAs (Fig. 6B). Therefore, even if the existence of additional factors cannot be excluded, it is more likely that dorsal inhibitors prevent Xvent-1B transcription at the dorsal side.

Dorsal Wnt Signaling Inhibits Xvent-1B Transcription—Dorsalization during early development is achieved mainly by the canonical Wnt signaling pathway, resulting in the nuclear translocation of β-catenin because of an inactivation of glycogen synthase kinase-3β (30). It has also been shown that treatment of embryos with lithium leads to an inhibition of glycogen synthase kinase-3β and that this procedure, therefore, phenocopies Wnt signaling (31). To analyze the effect of lithium on the regulation of Xvent-1B, Xvent-2 and GATA-2 RNA were injected into both ventral blastomeres of four-cell stage embryos. At stage 5, some of the embryos were treated with lithium chloride. At stage 7, they were transferred to CHX-containing medium and cultured until stage 10.5. Because the activation of Xvent-1B by Xvent-2 and GATA-2 in the presence of CHX is difficult to quantify by conventional methods, we studied the effects of lithium by using real time PCR on the Roche LightCycler system. Fig. 7 shows that treatment with lithium chloride in the absence of CHX suppresses transcription of Xvent-1B in Xvent-2- and GATA-2-injected as well as in uninjected embryos. Noteworthy, the activation of Xvent-1B in the presence of CHX by coinjection of Xvent-2 and GATA-2 (see also Fig. 6) was abolished completely when embryos were treated with lithium chloride before CHX treatment. This find-
ing suggests that the ubiquitous activation of Wnt signaling throughout the embryo before MBT results in a suppression of the Xvent-1B gene.

Different Mechanisms of Dorsal and Ventral Wnt Pathways Regulate Xvent-1B—The canonical Wnt pathway results in an interaction of \(\beta\)-catenin with a high mobility group box transcription factor of the LEF/TCF family (32). To analyze a possible role of Wnt signaling on the regulation of the Xvent-1B gene, we first searched for LEF/TCF binding motifs within the Xvent promoters and found a putative LEF/TCF element (\(5'\)CTTTGAT\(3'\)) at similar positions in both the Xvent-1B \((65/59)\) and Xvent-2B \((76/70)\) promoters (10). The sequence of this element is identical to those found in the S1 and S3 sites of the siamois promoter, which have been shown to interact with XTCF-3 (33). We mutated this putative LEF/TCF element in the Xvent-1B promoter and performed coinjections with Xvent-2/GATA-2 and/or \(\beta\)-catenin RNA (Fig. 8A). Interestingly, not only the wild type \((-249/+52)\), but also the mutated \((-249/Tm/+52)\) promoter is suppressed significantly by injection of \(\beta\)-catenin RNA, suggesting an inhibitory mechanism that is independent of this putative LEF/TCF element.

In contrast to dorsal Wnts, Xwnt-8 is expressed zygotically in the ventro/lateral part of the embryo and has been described as a ventral activator of the Xvent genes (34). Therefore, we have also investigated the effect of Xwnt-8 DNA injection on the Xvent-1B promoter. DNA must be injected because RNA present before MBT evokes a dorsalizing response similar to overexpression of \(\beta\)-catenin. The cytomegalovirus promoter renders ubiquitous activation of the Xvent-8 gene within the embryo after the onset of zygotic transcription. In contrast to injection of \(\beta\)-catenin RNA, Xwnt-8 protein synthesized after MBT activates the wild type Xvent-1B promoter and depends upon the LEF/TCF binding site because there was no effect on the mutated \(-249/Tm/+52\) promoter (Fig. 8B). In addition, the activa-

![Fig. 6. Direct activation of Xvent-1B by Xvent-2 and GATA-2.](image)

![Fig. 7. Inhibition of Xvent-1B by dorsal Wnt signaling.](image)

| mRNA injected | Xvent-2 ventral | Xvent-2/GATA-2 dorsal | Xvent-2/GATA-2 ventral | PCR contr. |
|----------------|----------------|-----------------------|------------------------|-----------|
| cycloheximide  | -              | +                     | -                      | +         |
| Xvent-1B       |                |                       |                        |           |
| Histone H4     |                |                       |                        |           |

| BMP-4/Xvent-2/GATA-2 | - | + dorsal | + ventral | PCR contr. |
|-----------------------|---|----------|----------|-----------|
| cycloheximide         | - | +        | -        | +         |
| Xvent-1B              |   |          |          |           |
| Histone H4            |   |          |          |           |

**FIG. 6.** Direct activation of Xvent-1B by Xvent-2 and GATA-2. Xenopus embryos were injected with (A) 400 pg of Xvent-2 RNA or Xvent-2 and 50 pg of GATA-2 RNA or (B) Xvent-2, GATA-2, and 500 pg of BMP-4 RNA at the four-cell stage as indicated. At stage 7, half of the embryos were treated with CHX. Total RNA was extracted when control embryos had reached stage 10.5 and subjected to RT-PCR to evaluate Xvent-1B transcripts. Histone H4 transcripts were determined as an internal control.

**FIG. 7.** Inhibition of Xvent-1B by dorsal Wnt signaling. Microinjection of 200 pg of Xvent-2 and 50 pg of GATA-2 RNA into both dorsal blastomeres of Xenopus embryos at the four-cell stage is shown. Embryos were treated with 300 mM lithium chloride at stage 5 (LiCl St. 5) and/or 25 \(\mu\)g/ml CHX at stage 7 and cultured until control embryos reached stage 10.5. cDNA synthesis was performed by using total RNA. Xvent-1B transcripts were detected using the Roche LightCycler system and quantified in relation to ornithine decarboxylase.

**TABLE 1.** Effect of BMP-4/Xvent-2/GATA-2 and cycloheximide on Xvent-1B expression.

| BMP-4/Xvent-2/GATA-2 | - | + dorsal | + ventral | PCR contr. |
|-----------------------|---|----------|----------|-----------|
| cycloheximide         | - | +        | -        | +         |
| Xvent-1B              |   |          |          |           |
| Histone H4            |   |          |          |           |
**Regulation of Xvent-1B by Xvent-2 and GATA-2**

To investigate the epistatic relationship between *Xvent* genes and to learn about their regulation, we have analyzed here the transcriptional control mechanism of the *Xvent-1B* gene in Xenopus embryos during gastrulation. Unlike the members of Xvent-2 subfamily, *Xvent-1B* is not a direct target of BMP signaling. Although it can be activated by Xvent-2 and is regarded as a downstream target of Xvent-2, Xvent-2 is not sufficient for this activation process because it does not occur in the presence of CHX (10). In a search for additional factors, we show here that the zinc finger transcription factor GATA-2, which previously had been reported to be expressed upon BMP-4 signaling and to affect *Xvent-1* but not *Xvent-2* expression (21, 22), functions as another regulator of the *Xvent-1* promoter. We demonstrate that GATA-2 is a direct target gene of BMP signaling and that, together with Xvent-2, it is also essential for the activation of *Xvent-1B*. This finding contrasts to a previous report showing that PV.1, a closely related member of Xvent-1, and GATA-2 repress each other during blood formation (35). Although this discrepancy may be the result of the different developmental context, it is evident from our work and that of others (21) that GATA-2 at the early gastrula stage is able to activate the *Xvent-1B* promoter, if endogenous Xvent-2 protein is present.

To investigate a direct physical interaction between Xvent-2 and GATA-2, pull-down experiments using GST fusion proteins were performed. GATA-2 interacted with the full-length Xvent-2 protein as well as with truncated versions containing the C-terminal domain. Obviously, this type of interaction is different from the previously described binding between the homeoprotein Nkx2.5 and the zinc finger transcription factor GATA-4, where the interaction depended primarily upon amino acids within the homeodomain (36).

The dominant-negative *Xvent-2* P(40), which is characterized by a L/P exchange preceding the third helix of the homeodomain (26), also exhibited a strong binding behavior for GATA-2. This result is interesting because GATA-2 rescued *Xvent-2* P(40)-induced suppression of *Xvent-1B* promoter activity. Consistent with the fact that both factors, GATA-2 and Xvent-2, activate the *Xvent-1B* promoter, gel shift experiments identified binding sites for Xvent-2 and GATA-2. Although Xvent-2 P(40) cannot bind to the Xvent-2 elements on the *Xvent-1B* promoter, it has, nevertheless, an inhibitory effect on the transcription of the *Xvent-1B* gene. These results support the idea that GATA-2 and Xvent-2 cooperatively activate *Xvent-1B* and that the dominant-negative action of Xvent-2 P(40) results from a loss of endogenous GATA-2 available to bind to endogenous Xvent-2 because it is sorted out by an excess of Xvent-2 P(40). This effect can be compensated for by an overexpression of GATA-2. The protein binds to the dominant-negative as well as to the remaining endogenous wild type Xvent-2 and therefore rescues expression of *Xvent-1B*.

The binding sites of GATA-2 and Xvent-2 were determined by gel shift and subsequent DNase I footprinting experiments. The *Xvent-1* promoter region contains a canonical GATA core motif including the flanking nucleotides preferred by GATA-2 (28). This motif was clearly shown to bind to GATA-2. In the case of Xvent-2, we have found two protected regions containing various elements with the consensus sequence, 5′-CC/TAAAT-3′, which is identical to those identified within the BMP-4 and Xvent-2B promoters (6, 19). Furthermore, this sequence is very similar to the target site of the closely related Xvent-1 protein (5′-CTATT/TCC-3′), which was identified within the XFD-1′ promoter (37). The results suggest that these two transcription factors have similar binding properties.

The requirement of Xvent-2 and GATA-2 binding sites...
within the Xvent-1B promoter was studied by microinjection experiments. Mutations of the promoter affecting the GATA-2 and the proximal Xvent-2 binding sites clearly affect the ability of GATA-2 to activate Xvent-1B. Obviously, GATA-2 requires its own as well as the proximal Xvent-2-binding element. However, Xvent-2 injections led in all cases to an increase of luciferase activity except for a minimal promoter fragment lacking the distal as well as the proximal binding elements. The presence of the GATA-2-binding site appears to be important for full activation of the Xvent-1B promoter because coinjection of GATA-2 suppresses Xvent-2 induced activation if the GATA-2 site is mutated. This means that GATA-2-dependent activation of the Xvent-1B promoter requires a proximal Xvent-2 binding site and that full activation by Xvent-2 requires an intact GATA-2 binding site. These results clearly demonstrate that both factors cooperate and support the hypothesis that the effect of overexpressing the Xvent-2 P(40) mutant is the result of a loss of endogenous GATA-2.

The question of whether Xvent-2 and GATA-2 are sufficient to activate the Xvent-1B promoter could not be answered by performing promoter/luciferase assays because these experiments require translation of the reporter enzyme. However, RT-PCR analysis of Xvent-1B transcripts in embryos treated with CHX clearly demonstrated that Xvent-2 and GATA-2 are sufficient for Xvent-1B activation in the gastrulating embryo, albeit only at the ventral and not at the dorsal side.

To investigate the suppression of Xvent-1B in the dorsal hemisphere of the embryo even in the presence of exogenous Xvent-2 and GATA-2, we focused on inhibitory mechanisms provided by the Wnt pathway. A pre-MBT Wnt signal results in the nuclear translocation of β-catenin at the future dorsal region. RT-PCR analysis using lithium-treated embryos displayed and mimicked all of the effects of early pre-MBT Wnt signaling on Xvent-1B repression. Although Xvent-2 and GATA-2 induce transcription of this gene on the ventral side, treatment with lithium chloride completely represses this activation. Identical results are obtained by ventral injection of Xwnt-8 and β-catenin RNA into four-cell stage embryos. In summary, an artificial pre-MBT Wnt signaling on the ventral side leads to an inhibition of Xvent-1B. Therefore, it is reasonable to assume that the dorsal pre-MBT Wnt signal is responsible not only for the suppression of BMP-signaling and for neural development (38), but also for the dorsal suppression of the Xvent-1B gene. It should be mentioned, however, that the molecular mechanism and the components involved in this suppression are not yet identified. It is also not clear whether the inhibition is a direct or an indirect response to Wnt signaling. In any case, this mechanism is independent of BMP-4 gene suppression because coinjection with BMP-4 does not overcome this failure (Fig. 6).

The effects of Wnt signaling are changed dramatically after MBT. Xwnt-8, which is expressed in the ventral/lateral mesoderm, has already been shown to be involved in the activation of the Xvent genes (34). We show here that the identified LEF/TCF site responds specifically to this activating signal because the Xvent-1B promoter is up-regulated by coinjection of ubiquitously transcribed Xwnt-8 DNA, and mutational disruption of this element abolishes this response. In contrast, the inhibitory potential of the pre-MBT signal also being observed for the Xvent-1B promoter does not require this site because mutation of the LEF/TCF-binding element did not result in the loss of Xvent-1B repression caused by β-catenin.

Unfortunately, there is not much known about the differences between early (pre-MBT) and late (post-MBT) Wnt signaling mechanisms. Although it has been suggested recently that a difference in XTcf-3 dependence accounts for this change (39), experiments using a hormone-inducible XTcf-3 led to the conclusion that the same components of the canonical Wnt signaling pathway including XTcf-3 are required before and after MBT and that the competence of Wnts to induce a dorsal axis is lost in the nucleus as a result of changes in the responsiveness of target promoters (40). Another explanation for the repressing function of this signaling pathway before the beginning of zygotic transcription might be an interaction of Xvent-2 and/or GATA-2 with molecules such as CtBP or groucho (41, 42), which are released from TCF-3 after interaction with β-catenin. On the other hand, the inducing capacity of Xvent-8 might be regarded as a classical Wnt signaling process because the LEF/TCF element in the Xvent-1B promoter is only responsible for activation but not for suppression. The characterization of Xvent-1B as a gene being suppressed by pre-MBT and activated by post-MBT signaling and the identification of the activating element will now facilitate a more detailed analysis of factors and cofactors that are required for the change in response to Wnt signaling in the Xenopus blastula.

Acknowledgments—We are grateful to R. Patient (London), C. Niehrs (Heidelberg), and S. Rastegar (Strasbourg) for generously providing the DNA constructs used in this study. We also thank A. Schuler-Metz (Ulm) for the Xvent-2 deletion mutants and K. Dillinger and D. Weber for excellent technical assistance.

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