The POU Factor Oct-25 Regulates the *Xvent-2B* Gene and Counteracts Terminal Differentiation in *Xenopus* Embryos*

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Running title: *Xvent-2B* gene is regulated by Oct-25

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The Xvent-2B promoter is regulated by a BMP-2/4 induced transcription complex comprising Smad signal transducers and specific transcription factors. Using a yeast one-hybrid screen we have found that Oct-25, a Xenopus POU domain protein related to mammalian Oct-3/4, binds as an additional factor to the Xvent-2B promoter. This interaction was further confirmed by both in vitro and in vivo analyses. The Oct-25 gene is mainly transcribed during blastula and gastrula stages in the newly forming ectodermal and mesodermal germ layers. Luciferase reporter gene assay demonstrated that Oct-25 stimulates transcription of the Xvent-2B gene. This stimulation depends on the Oct-25 binding site and the BMP responsive element. Furthermore, Oct-25 interacts in vitro with components of the Xvent-2B transcription complex, like Smad1/4 and Xvent-2. Overexpression of Oct-25 results in anterior/posterior truncations and lack of differentiation for neuroectoderm- and mesoderm-derived tissues including blood cells. This effect is consistent with an evolutionarily conserved role of class V POU factors in the maintenance of an undifferentiated cell state. In Xenopus, the molecular mechanism underlying this process might be coupled to the expression of Xvent proteins.
Xvent genes are homeobox genes of the Nkx/Bar subfamily which are mainly expressed at the ventral side of Xenopus embryos starting at the blastula stage of development (1-5). The corresponding proteins serve as transcription factors and mediate some major effects of bone morphogenetic proteins (BMPs) in germ layer formation and dorsoventral patterning within the early embryo (6, 7). Ectopic overexpression of Xenopus BMPs (8, 9) or of Xvents at the dorsal side of embryos leads to complete ventralization and both, BMPs and Xvents, have been shown to antagonize dorsalizing genes or signals expressed within the most dorsal mesoderm, the organizer. The Xvent-2B gene or its pseudoallelic variant, the Xvent-2 gene, have been characterized as direct target genes of BMP-2/4 signaling (10, 11). This signaling cascade involves receptor specific Smads, like Smad1, 5 or 8, which after phosphorylation by a type I BMP receptor associate with receptor unspecific Smads, like Smad4, and are translocated to the nucleus, where they recruit specific transcription factors to activate their target genes (12). The closely related promoters of the Xvent-2 and Xvent-2B genes contain a BMP response element (BRE) which is composed of Smad binding sites (SBE) for Smad4 and Smad1 as well as binding sites for specific transcription factors, like OAZ or Xvent-2 itself (10, 11, 13). These factors have been shown to interact with the Smads and they are required for the transcription complex to be targeted to the BRE, since binding affinity of Smads alone to DNA is rather weak. Xvent-2 has also been shown to mediate the autocatalytic loop of BMP-4 expression, because it strongly activates BMP-4 transcription by binding to an intron-located enhancer (14, 15). Therefore, Xvent-2 has a dual autoregulatory effect on its own expression. First, it stimulates BMP-4 transcription, thereby enhancing BMP-4 expression and signaling, and second, it is part of the transcription complex regulating its own promoter (13).

To gain further insights into the molecular architecture of the Xvent-2B transcription complex and to characterize additional compounds we have screened a gastrula stage cDNA expression
library for proteins, which bind to a selected region of the Xvent-2B promoter (one-hybrid screening). This procedure led to the identification of Oct-25, a previously described class V member of POU domain proteins in Xenopus (16). The gene had been reported to be mainly expressed during gastrulation and the protein shares a high degree of sequence conservation with the mammalian Oct-3/4 factor (17-19). We show here that Oct-25 transcripts are highly abundant in the forming ectoderm and mesoderm, persist during early neurulation in the posterior neuroectoderm and are still present within the tip of the tail at tadpole stage. While neither the temporal nor the spatial expression patterns exactly correlate to those reported for mammalian Oct-3/4, we show by RNA microinjection experiments that Oct-25 inhibits terminal differentiation of mesodermal and neuroectodermal derivatives. Such an inhibition of differentiation is reminiscent to the role of Oct-3/4 in pluripotent embryonic stem cells (20). Overexpression of Oct-25 leads to an upregulation of BMP-4 and Xvent genes. We further show that Oct-25 binds to the Xvent-2B promoter not only in vitro but also in vivo and that it interacts with Xvent-2, another component of the transcription complex. Reporter gene expression directed by the Xvent-2B promoter is upregulated by Oct-25. This activation requires the Oct-25 binding site but is also dependent upon the BMP-signaling pathway. These studies indicate for the first time a functional role of this POU domain protein in BMP signaling and they suggest evolutionary conserved mechanisms in the primary inhibition of terminal differentiation.
EXPERIMENTAL PROCEDURES

Construction of cDNA expression library and one-hybrid screen—Total RNA was isolated from gastrula stage *Xenopus* embryos by Trizol (GIBCO) extraction and subsequently enriched for poly-A RNA by affinity chromatography on poly-dT cellulose. RNA was transcribed into cDNA and integrated into the multicloning site of plasmid pGADT (clontech) generating GAL-4 activation domain fusion constructs under the direction of the strong ADH1 promoter. The primary library yielded $1 \times 10^6$ cfu, the insert size varying between 0.8 and 5 kb. The library was amplified in semi-solid SeqPlaque agarose generating about $10^9$ total transformants (Stratagene plasmid library construction kit).

The reporter for the yeast one-hybrid screening was constructed by unidirectional multimerization of Ava I-site flanked recognition site oligomers (from position -267 to -214 of the Xvent-2B gene (5)) followed by insertion into pHISi (clontech) and pLacZi (clontech) (21). Library and reporter constructs were co-transformed into yeast strain YM4271 (MATa, ura3-52, his3-200, ade2-101, lys2-801, leu2-3,112, trp1-901, tyr1-50, gal4-Δ512, gal80-Δ538, ade5::hisG). Clones indicating protein-DNA interaction are isolated on minimal yeast medium lacking histidine (pHISi) and uracil (pLacZi), respectively. The application of two reporter constructs reduces the number of false positive results (22). Low-level expression of the HIS3 in a leaky manner is prevented by titration with the competitive inhibitor 3-aminotriazole between 0 and 40 mM. For further experimental details see clontech yeast protocols handbook (www.clontech.de).

Constructs, RNA synthesis and microinjection—*Xenopus* Oct-25 cDNA including the 5’ and 3’ UTRs was subcloned to the Eco RI site of pCS2+ (23) to generate the expression construct pCS2+ Oct-25. To fuse the myc tag in frame to the N-terminus of Oct-25, coding sequence of Oct-25 was PCR amplified and subcloned to the Eco RI/Xho I sites of pCS2+ MT to generate pCS2+ MT-Oct-25.
pCS2+ Oct-25 or pCS2+ MT-Oct-25 was cut with Not I. Capped mRNA was synthesized with mMessage mMachine™ Kit (Ambion). For overexpression of Oct-25, the RNA was injected in a total dose of 150 pg, 300 pg or 1 ng, respectively, into all four blastomeres at 4-cell stage. For ChIP analysis, MT-Oct-25 RNA was injected in a total dose of 600 pg into all four blastomeres at 4-cell stage. For functional knock-down of Oct-25, an antisense morpholino oligonucleotide against Oct-25 (Oct-25 MO) with the sequence 5’-GTACATGGTGTCCAAGAGCTTGCAG-3’ was injected in a dose of 10, 30 or 50 ng.

RT-PCR—Synthetic Oct-25 mRNA was injected in a total dose of 600 pg into all four blastomeres at the 4-cell stage. Injected and uninjected control embryos were collected at stage 19 or 30, respectively, and frozen in liquid nitrogen. Total RNA was extracted with RNeasy kit (Qiagen), followed by DNase I treatment and purification again with RNeasy kit. First strand cDNA was synthesized with RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas). Primers were as follows: ODC forw.: 5’-GGGCAAAGAGGCTTAATGTG-3’, rev.: 5’-CATTGGGCAGCATCTTCTTCA-3’; BMP-4 forw.: 5’-GTCAGTCTAATCCCGCGAGA-3’, rev.: 5’-ATCGTGAGCTCATCTTCTTCT-3’; Xvent-2 forw.: 5’-AATCCAAGATGCGAGACCGCAG-3’, rev.: 5’-CGGGAGGAGCGAGAATGACAT-3’; N-CAM forw.: 5’-GCCCTCTTTGTGGATCTTGA-3’, rev.: 5’-ACAGCGCGAGGTAGCAGTT-3’; epidermal keratin forw.: 5’-ACTATCGCGCAGCCTAGAGGA-3’, rev.: 5’-ACACTTCAGGATCTCAGGGAAG-3’; α-globin forw.: 5’-AAGCCCAACTAGCTCCCTTG-3’, rev.: 5’-GAATTGTCCAAGACGACAT-3’; cardiac α-actin forw.: 5’-GGAAATGAACGTTTCCGTG-3’, rev.: 5’-TTGCTTGGAGAGTGTGTGTGTG-3’; XAG forw.: 5’-GCTTTTGTCGCTGATCAAT-3’, rev.: 5’-TTTGGGAGTGCTCTCTCTGG-3’
Preparation of fusion proteins—Fusion proteins were expressed in E. coli BL21 (DE3) (Stratagene) and purified in batch under native conditions. His-tagged Oct-25 or the His-tagged DNA binding domain of Oct-25, a POU/homeodomain construct, were purified using Ni-NTA-agarose (Qiagen) and GST (glutathione S-transferase)-tagged proteins were isolated using glutathione-Sepharose (Amersham Bioscience) according to the manufacturer's protocols. The purified His-tagged proteins were dialyzed overnight at 4°C (50 mM Tris-HCl, pH 7.0, 10 mM KCl, 10 mM MgCl₂, 1 mM DTT, 20% glycerol) and the GST-tagged proteins were dialyzed overnight at 4°C (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT, and 20% glycerol). ³⁵S-labeled proteins were prepared using the TNT-coupled transcription-translation system (Promega).

DNase I footprinting—Xvent-2B promoter fragment (-305/-123) was generated by PCR and cloned by use of the Qiagen PCR cloning kit into the pDrive vector in both orientations. After cleavage with Bam HI/Hind III the dephosphorylated fragments were 5’-labeled and cleaved with Sal I. Binding reactions were carried out on ice for 30 min in 30 µl binding buffer (30 mM Tris-HCl, pH 7.5, 30 mM KCl, 1 mM MgCl₂, 1 mM DTT, 13.2% glycerol) containing 1 µg poly(dI-dC) and the Oct-25 protein. After 5 min of pre-incubation, 1 ng of the gel-purified probe was added and incubated for 30 min. The concentration of MgCl₂ was subsequently raised to 5 mM for DNase I footprinting, and 0.065 U (free DNA) or 0.195 U (DNA + protein) of DNase I were added at room temperature for 45 s. The DNase I digestion was stopped by addition of an equal volume of sample buffer (66% de-ionized formamide, 20 mM EDTA, 660 mM sucrose). Chemical sequencing reactions were performed for sequence alignment. After pre-electrophoresis for 2 h at 70 W, samples were analyzed by 8% and 6% denaturing PAGE at 60 W in 1X Tris-Borate-EDTA.
Electrophoretic mobility shift assay (EMSA) — Electrophoretic mobility shift assays (EMSA) were performed with wild type or mutated sense and antisense oligonucleotides ranging from position -234 to -204. The oligonucleotides were 5’-end labeled with γ-ATP using T4 polynucleotide kinase and subsequently annealed. The gel-purified probes were incubated with the POU/homeodomain of Oct-25 protein on ice for 25 min in 30 µl binding buffer (25 mM Tris (pH 8.0), 50 mM KCl, 6.25 mM MgCl₂, 0.5 mM EDTA, 10% glycerol, 0.5 mM DTT, 0.25 µg poly(dI-dC)).

Luciferase assay — For promoter analysis, the -275/+44 Xvent-2B sequence or 5’ as well as internal deletions were cloned in pGL3 vector (Promega) and injected into both blastomeres at 2-cell stage. In the same way the promoter/reporter constructs were co-injected with 250 pg Oct-25 RNA or 300 pg BMP-4 RNA. Embryos injected with luciferase reporter constructs were collected when control embryos had reached stage 12 (24) and homogenized in 1 x lysis buffer (Promega) using 10 µl buffer/embryo. Samples were left on ice for 10 min followed by a 5 min centrifugation (10 000 rpm) at 4 °C to pellet debris. Luciferase assays were performed with 20 µl lysate (two embryo equivalents) and with 20 µl luciferase substrate (Promega) using a Lumat LB 9507 luminometer (Berthold). Internal controls for transcriptional activities were performed by using the CMV-pRL vector (cytomegalovirus promoter in front of the Renilla luciferase) and the instructions given by the supplier (Promega).

Chromatin immunoprecipitation (ChIP) — ChIP was performed according to a recently published procedure (25) and modified for Xenopus (K. Mansperger and R. Rupp, personal communication). About 200 of MT(myc-tagged)-Oct-25 injected embryos, of NLS-MT(nuclear localization signal) injected embryos or uninjected embryos were fixed in 0.5 x MBSH (1 x MBSH: 88 mM NaCl, 2.4 mM NaHCO₃, 1 mM KCl, 0.82 mM MgSO₄, 0.41 mM CaCl₂, 0.33 mM Ca(NO₃)₂, 10 mM HEPES, pH 7.4) containing 1% formaldehyde at room temperature for
30 min to crosslink proteins to DNA. Crosslinking was then quenched by adding 2.5M glycine to a final concentration of 0.125 M. Embryos were washed with PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4) and homogenized in low salt buffer (150 mM NaCl, 50 mM HEPES, 10% glycerol, 1 mM EDTA, 0.1% Triton, 1 mM DTT, Roche complete protease inhibitor cocktail) with French Press three times at 850 psi. Homogenates were precipitated and supernatants were precleared with pre-blocked protein G-agarose (Roche). 15 µl of rabbit anti-c-myc antibody (Santa Cruz Biotechnology) was added to chromatin supernatants, while leaving an aliquot without antibody for negative control, and incubated overnight. To precipitate chromatin, blocked protein G-agarose was added and incubated for 2-3 hours. Agarose beads were spin down and washed with once of low salt buffer, twice of high salt buffer (500 mM NaCl, 50 mM HEPES, 10% glycerol, 1 mM EDTA, 0.1% Triton, 1 mM DTT, Roche complete protease inhibitor cocktail), twice of LiCl-solution (10 mM Tris, pH 8.0, 250 mM LiCl, 1 mM EDTA, 1% NP40, 1% Na Desoxycholate), and again twice of low salt buffer. Protein/DNA complex was then eluted twice with low salt buffer containing 1% SDS at room temperature, and crosslinking was reversed at 65°C overnight. After digestion with proteinase K, DNA was phenol extracted, ethanol precipitated, and dissolved in 50 µl of TE (10 mM Tris, 0.1 mM EDTA). Xvent-2B promoter was assayed with the immunoprecipitated DNA and the supernatant DNA, using the Xvent-2B promoter primers forw.: 5’-TGAGCATCACAGATGGCATT-3’ (position -364) and rev.: 5’-AGGGAGGAAGAGCCCATCTA-3’ (position -4).

**GST pull-down assay**—GST (glutathione S-transferase) pull down assays were performed as recently described (26). The ΔN and ΔC mutants of Xvent-2 have been reported elsewhere (13), Oct-25 mutants were generated by PCR using the following primers: Oct-25ΔC forw.: 5’-GGAATTCATGTACAGCCAACAGCCCTT-3’, rev.: 5’-CCGCTCGAGTTAGCGTCGTTCTCCTCAACGCT-3’ including a termination signal; Oct-
25ΔN forw.: 5’-GGAATTCAATGAGCGAATCGAATGGAGCA-3’ (this primer encodes an additional methionine), rev.: 5’-CCGCTCGAGTCAGCCAATGTGGCCCCC-3’. The mutant Oct-25ΔPH that excludes both the POU and the homeodomain was synthesized in two parts. The N-terminal part was created using the Oct-25ΔC forward primer and a reverse primer 5’-GGGTACCAGGGAACCTCCTCATT-3’. The C-terminal part was generated using a forward primer 5’-GGGTACCAGGGAACCTCCTCATTGTTGCACA-3’ and the Oct-25ΔN reverse primer. Both fragments were fused via the included Kpn I site (underlined).
RESULTS

One-hybrid screen—The present investigation was initiated by the observation, that the activity of the Xvent-2B promoter as judged by reporter gene expression after injection into Xenopus embryos was markedly reduced when nucleotides from -275 to -220 were deleted (5, 27). This region does neither contain the identified Smad1/4 binding sites nor the OAZ binding site required for activation (Fig. 1A) (10, 11). Instead, it was shown to contain an enhancer element (27) and to bind to Smad1 as well as to Xvent-2 (11, 13). In an attempt to identify further factors that influence Xvent-2/2B gene activity, we have performed a one-hybrid screen (21) by using the -267 to -214 region (Fig. 1B) as bait to be targeted by proteins expressed from gastrula stage RNA. Since these proteins had been fused to the GAL-4 transcriptional activation domain, binding of fusion proteins to the Xvent-2B promoter element led to the expression of His or LacZ genes in corresponding yeast deficiency strains. Subsequent usage of two different reporter genes has proven as a valuable tool to eliminate false positives which had been isolated after the first round in using only one reporter (22). The procedure led to the isolation of seven clones, one of which was identified to code for the POU domain protein Oct-91/XLPOU91 (16, 28) and the remaining six to code for the POU domain protein Oct-25 (16). Noteworthy, the Oct-25 sequences had obviously been derived from different cDNAs. They all encoded the complete POU specific domain (POU₅) and the POU homeodomain (POUᵢ₅) but some of them varied and were not complete with respect to their length at the 3’-end. To obtain a full size Oct-25 transcript we next have screened a gastrula stage cDNA library by using the incomplete sequence as labeled probe. This led to the isolation of the complete sequence (sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession No. AJ699165) which differed at the nucleotide level by 0.6% and at the amino acid level by five out of 449 amino acids from the previously published sequence (16) and led to an extension or completion of the 5’- and
3′-UTRs. Based upon its homology to POU factors from other species, Oct-25 had been classified as a member of the Class V POU domain proteins (29).

Spatial expression of Oct-25 in the Xenopus embryo—A previous report on the temporal expression had shown that very low levels of Oct-25 transcripts are present in oocytes and early cleavage embryos (16). The amount of transcripts is dramatically increased during gastrulation. Transcripts persist during early neurulation but were no longer detectable by the end of neurulation. We now have analysed the spatial distribution of Oct-25 transcripts by using the whole mount in situ hybridization technique (30). While we could not detect any transcripts in pre-blastula stage embryos (data not shown), zygotic transcription is strongly activated after midblastula transition within the animal half both in the forming mesoderm and ectoderm (Fig. 2A-C). In support of the reported temporal pattern, most intense staining is observed at stage 11 (Fig. 2B). Subsequent expression is gradually restricted to the dorsal neuroectoderm with a sharp boundary at the anterior front (Fig. 2D). A transverse section through an early neurula shows no expression in the notochord and the presomitic mesoderm but strong expression in the overlaying neuroectoderm (Fig. 2M). The anterior boundary was examined by double whole mount hybridizations using Krox 20 or en-2 as additional probes (Fig. 2E,F). Expression of Oct-25 is observed to be more anterior than that of Krox 20, which is transcribed in rhombomeres 3 and 5, but coincides with that of en-2, which demarcates the hindbrain/midbrain boundary. Therefore, expression of Oct-25 in neuroectoderm at the early neurula stage extends to the future hindbrain/midbrain boundary. However, as neurulation proceeds, Oct-25 transcripts become localized to the posterior part of the CNS (Fig. 2G-K). We here demonstrate that they persist during development and are clearly present within the tip of the tail even at stage 35 (Fig. 2L).

In summary, the Oct-25 gene is zygotically activated at the blastula stage within future mesoderm and ectoderm; transcription reaches a maximum at the midgastrula stage and thereafter
becomes restricted to the future neuroectoderm. Initial expression within the neuroectoderm extends until the future hindbrain/midbrain boundary but, later on, it is progressively shifted towards the posterior pole where it persists within the tip of the tail in hatching embryos.

**Functional analysis of Oct-25 by gain of function and loss of function**—To gain insights into the biological function of Oct-25, ectopic overexpression and antisense morpholino oligonucleotide based knock-down strategies were applied. Injection of Oct-25 RNA into all four blastomeres of 4-cell stage embryos led in a dose dependent manner to an increased rate of mortality during gastrulation. While more than 90% (n = 72) of embryos survived at an injected amount of 150 pg, only 80% (n = 45) or 50% (n = 82) continued development at 300 pg or 1 ng, respectively. The surviving embryos suffered from severe differentiation defects along the body axis (Fig. 3A-C). They show a loss of head structures including brain and eyes, no spinal cord which is obviously due to loss of closure of the neural tube, no somite structures and no extension of the tail. Moreover, nearly all injected embryos exhibit an accumulation of darkly pigmented cells either at their lateral or ventral sides. Except for the formation of the cement gland which is present in all embryos injected with 150 or 300 pg, respectively, it seems that ectopic overexpression of Oct-25 results in a failure of differentiation for most tissues.

Injection of different doses of the morpholino into embryos did not induce any phenotypic effect (data not shown). At the present moment we cannot decide whether this is due to functional redundancy by closely related genes or to the possibility that the morpholino does not react with the pseudo-allelic version of Oct-25.

**Overexpression of Oct-25 prevents the differentiation of tissues**—To analyse the influence of Oct-25 overexpression on the regulation of distinct genes we have investigated the amount of transcripts for some mesoderm or ectoderm specific marker genes. RNA from Oct-25 injected embryos and from uninjected control embryos was submitted to RT-PCR, when controls had
reached developmental stage 19 and 30, respectively. As shown in Figure 4, the neural marker N-CAM is down regulated upon injection of Oct-25 RNA which is consistent with the lack of neural structures in injected embryos (Fig. 3A-C). The epidermal keratin gene is not affected. The cement gland marker XAG is converted from an initial decrease at stage 19 to a significant increase at stage 30. The lack of differentiation of somitic mesoderm in injected embryos is clearly reflected by a down-regulation of \( \alpha \)-actin. The same holds true for the \( \alpha \)-globin gene that is expressed in ventral mesoderm derived blood islands. In contrast, the early ventral and ventrolateral genes BMP-4 and Xvent-2 are up-regulated.

In summary, overexpression of Oct-25 in the early Xenopus embryo leads to an extensive alteration of the genetic program. Whereas some of the early ventral genes are activated, it is evident, that all markers for terminally differentiating tissues, like N-CAM for brain or CNS, \( \alpha \)-actin for somites and \( \alpha \)-globin for blood islands, are downregulated. Thus, overexpression of Oct-25 interferes with normal development in that it prevents the activation of genes required for terminal differentiation of tissues.

Oct-25 binds to the Xvent-2B promoter—To ascertain the result of the one-hybrid screening and to determine the binding site, we have performed a DNase I footprint analysis with the bacterially expressed Oct-25 protein on both the sense and antisense strands of a -305/-123 Xvent-2B promoter fragment. Results from both experiments demonstrate an Oct-25 binding site located between -231 and -207 (Fig. 5). This region contains two times an octamer motif containing the 5’-located ATGC/T motif, which had been shown to bind the POU specific domain (POUS) followed by 3’-located A-T rich sequence motifs bound by the POU homeodomain (POUH). Also, we find in both cases an A in the fifth position which had been suggested to be important for cooperative binding of the POUS and POUH domains (31). Interestingly, the protected area on the Xvent-2B promoter extends for seven nucleotides at its
3′-end, which were not present within the target used for the one-hybrid screening. Obviously, these seven nucleotides were not required for binding the Oct-25/GAL-4 AD to the triplicated target site (-267 to -214) and for activation of the reporter genes in yeast. It is possible that these nucleotides are protected from degradation by the protein although not being directly engaged in protein/DNA contacts. To evaluate the contribution of the two putative binding sites we have performed electrophoretic mobility shift assays (EMSA) of the Oct-25 POU/homeodomain on targets containing various point mutations (Fig. 6). It is evident that the binding reaction requires the second motif, which is located within the center of the protected area (Fig. 5), whereas the first motif seems to be of minor importance. While the alteration of the second motif completely inhibits an interaction with the Oct-25 POU/homeodomain, the binding reaction is only slightly reduced by mutation of the first motif. Binding of the wild type sequence or of a mutant affecting only the first motif is effectively competed for by corresponding unlabeled targets but not by a mutant containing alterations of the second motif.

Oct-25 stimulates the Xvent-2B gene promoter—The binding studies can not answer the question, if and how Oct-25 might influence transcriptional activity of the Xvent-2B gene. Therefore, promoter/luciferase reporter fusion constructs were coinjected with Oct-25 RNA and/or with BMP-4 RNA (Fig. 7). It is evident that Oct-25 is able to stimulate the luciferase activity of the -275 construct, but not that of the -174 deletion that is missing the Oct-25 binding site. The stimulation observed for Oct-25 does not seem to potenti ate but to add to that observed for BMP-4. However, activation by Oct-25 seems to be dependent upon the target sites of the BMP-4 signaling pathway, because internal deletion of the Smad1/4 binding elements and the OAZ binding site (see Fig. 1A) does not only result in a lack of BMP-4 inducibility but also in a lack of stimulation caused by Oct-25. Thus, Oct-25 behaves as a transcriptional
activator that requires interaction with other factors being probably involved in the BMP pathway.

*Oct-25 is part of the Xvent-2B transcription complex in vivo and interacts with Xvent-2*—Binding in yeast cells (one-hybrid screening) and binding *in vitro* is indicative but is no proof for binding *in vivo*. Therefore, we have analysed by chromatin immunoprecipitation (ChIP) whether the Oct-25 protein is part of the transcription complex assembled on the *Xvent-2B* promoter *in vivo*. In lack of Oct-25 specific antibodies, we have injected myc-tagged Oct-25 (MT-Oct-25) RNA or, for control, RNA encoding the myc-tag coupled to a nuclear localization signal (NLS-MT). After fixation of chromatin by formaldehyde, anti-myc antibodies can readily precipitate the fusion proteins. The interaction with a specific DNA target sequence is demonstrated by its presence after amplification by PCR. Provided the target site to be analysed neither contains a direct or indirect myc binding site, this is an efficient method to demonstrate *in vivo* the presence of a protein as a component of a transcription complex within chromatin. Figure 8 shows that the -364/-4 *Xvent-2B* promoter fragment can be amplified from antibody precipitated chromatin of MT-Oct-25 injected embryos in contrast to that of NLS-MT injected or of uninjected embryos and that no amplification occurs, if the antibody precipitation was omitted. Thus it is evident, that Oct-25 is bound to the upstream region of the *Xvent-2B* gene also *in vivo*.

We next have investigated the ability of Oct-25 to physically interact with other components of the transcription complex that had already been reported to regulate *Xvent-2B* expression (10, 11, 13). GST pull-down assays with [*35S*] labeled Oct-25 and, vice versa, with [*35S*] labeled Xvent-2 demonstrate that Oct-25 can interact with Xvent-2 (Fig. 9). It is also shown, that the interaction between Xvent-2 and Oct-25 requires the C-terminus of Xvent-2, because a truncation at the C-terminus in contrast to that of the N-terminus prevents binding to Oct-25.
Vice versa, the binding site on the Oct-25 protein seems to require the POU and the
homeodomain. A mutant lacking these domains (Oct-25ΔPH) does not react with Xvent-2,
whereas a complete deletion of the N- or C-terminal part of the Oct-25 protein does not abolish
the interaction with Xvent-2. Moreover, it is demonstrated that [35S] labeled Oct-25 interacts
with Smad1 as well as with Smad4 and vice versa. In summary, these results suggest an
interaction between Oct-25 and other components of the transcription complex, like Xvent-2 and
Smad signal transducers, in the regulation of Xvent-2B promoter activity.
The promoter of the Xvent-2B gene is subject to direct regulation by the BMP-2/4 signaling cascade. By serial promoter deletion and point mutation experiments, various cis-regulatory elements have been identified which turned out to represent binding sites for Smads or for Smad interacting transcription factors, like the zinc finger protein OAZ or the homeodomain protein Xvent-2 (10, 11, 13). While the Smad binding element (SBE) IV characterized by a 5′-CAGAC-3′ motif is indispensable for the BMP response, full activation of the Xvent-2B gene is augmented by putative Smad1 binding sites (5′-GCAT-3′) being also present in other, more recently characterized BMP target genes (32, 33). The transcription factors Xvent-2 and OAZ have been demonstrated to bind to the Xvent-2/2B promoter. As they can simultaneously bind to Smads, they may recruit the Smads and stabilize their interaction with DNA within the transcription complex. The OAZ binding site has been assigned to a 5′-TGGAGC-3′ motif located a few nucleotides downstream of the decisive SBE. Interestingly, a comparative study on conserved sequence elements within promoters of genes belonging to the BMP-4 synexpression group and being regulated by BMP-4 led to the identification of a 5′-TGGCGCC-3′ motif, termed bre 7 (33), which is very similar to the OAZ binding site. Therefore, it will be interesting to analyse whether OAZ or related factors will bind to this site. OAZ has recently been found to interact with poly(ADP-ribose) polymerase 1 (Parp1) which serves as a transcriptional coactivator of OAZ in BMP-dependent gene regulation (34).

The one-hybrid screening of a 54 bp region (-267/-214) of the Xvent-2B promoter with proteins expressed from gastrula stage RNA has now revealed an additional component of the Xvent-2B transcription complex. The POU domain factor Oct-25 (16) has been identified as a potent binding partner not only in the yeast system but also in Xenopus embryos.
binding was analysed by EMSA and DNase I footprinting, \textit{in vivo} binding in \textit{Xenopus} was demonstrated by ChIP. The footprint shows a DNase I protected region that corresponds for both the sense and antisense strands. It comprises a tandem arrangement of two Oct binding sites, one of which turned out to be absolutely essential in EMSA studies by using point mutated target sites.

Injection of Oct-25 RNA into \textit{Xenopus} embryos does not only stimulate endogenous \textit{Xvent-2} gene transcription but also luciferase reporter gene expression directed by the \textit{Xvent-2B} promoter. The stimulation of the reporter depends upon the presence of the identified Oct-25 target site, because a promoter deletion mutant lacking this site does not respond to co-injection with Oct-25 RNA. On the other hand, deletion of the BRE including the decisive Smad and the OAZ binding sites does also not respond to Oct-25, although the Oct-25 binding site is present. This argues for an interaction between Oct-25 and other components of the BMP signaling pathway. Indeed, GST pull-down assays show that Oct-25 binds to Xvent-2 as well to Smad1 and Smad4. This observation together with the results of ChIP assays demonstrate that Oct-25 is an additional factor within the \textit{Xvent-2B} transcription complex and that it requires the interaction with other components of the BMP signaling pathway to exert its positive regulatory function.

Although Oct-25 is not the sequence orthologue to mammalian Oct-3/4, many functional properties suggest that these two POU factors are closely related. Like in fish and also in birds (35) there is no direct sequence homologue to Oct-3/4 in amphibians. In zebrafish, \textit{spiel-ohne-grenzen/Pou2} has been suggested to be the orthologue of mouse Oct-3/4 and the human \textit{Pou5f1} genes (36). Although zfPou2 is maternally expressed, it shows from blastula to early neurula stages an expression pattern that is similar to that of Oct-25. Oct-25 belongs to a \textit{Xenopus} specific subfamily of three POU domain class V factors which, in addition, comprises Oct-60 and Oct-91 (16). Oct-60 is transcribed maternally, transcripts persist during early cleavage stages
in animal cap and marginal zone cells, but are rapidly degraded during gastrulation (37, 38). Oct-25 and Oct-91 genes are activated after midblastula transition in animal cap and marginal zone cells, transcripts reach their maximum level during gastrulation and rapidly decrease during early neurula stages. We show here that Oct-25, like zebrafish Pou2 (36), persists in the posterior neuroectoderm, while Oct-91 has been reported to be localized to ventroposterior regions in early neurula stage embryos (28). It is reasonable to assume that the three Xenopus genes in combination account for the temporal and spatial patterns of zebrafish Pou2 and of mammalian Oct-3/4 genes, albeit - in contrast to the latter - they are not transcribed in primordial germ cells. Ectopic expression of Oct-91 has been shown to cause severe posterior truncations and both, Oct-91 and zebrafish Pou2, have been postulated to regulate the competence for distinct FGFs (39, 40). We here demonstrate that overexpression of Oct-25 results in posterior and anterior truncations with a lack of differentiation in neural and mesodermal tissues. This observation is supported by the lack of transcripts encoding N-CAM, α-actin and α-globin. It can be concluded that an increase of Oct-25 during early development keeps the cells in an undifferentiated state and prevents their differentiation, a role that was previously assigned to Oct-3/4 (20). How does this assumption correlate with the observed upregulation of BMP-4 and Xvent-2 after overexpression of Oct-25? For neural induction it is well known that it requires inactivation of BMP signaling or, vice versa, increased BMP-4 and Xvent-2 activity inhibit formation of neuroectoderm (41, 2). Also, formation of mesoderm derived tissues, like somites and notochord, is not possible in the presence of high BMP-4 and Xvent levels (6). Even more interesting is the observation that PV.1, another BMP-4 induced member of the Xvent family, does not only counteract neural inducers in animal caps restoring them to their original epidermal fate, but does also inhibit blood formation (42, 43). A failure of globin gene activation in activin A induced caps has also been reported for Xom, a homologue of Xvent-2 (3). Thus it is evident that
overexpression of Oct-25 leads to an increased Xvent-2 activity which, in turn, prevents premature differentiation of neuroectodermal and mesodermal tissues. This is not restricted to dorsal and lateral mesoderm, but also implies ventral mesoderm. In contrast to cultured mammalian embryonic stem cells, the inhibitory effect on differentiation in the living amphibian embryo is restricted to a short time interval, because Xvent genes are transcribed most strongly at blastula/gastrula stages and the Xvent-2 proteins are subject to regulated proteolysis already during gastrulation (7).

In summary, we here propose a novel mechanism, by which Oct-25 as a putative functional homologue of mammalian Oct-3/4 stimulates transcription of Xvent genes and that Xvent proteins for a defined and limited time period during germ layer formation prevent the cells from entering a terminal differentiation pathway. This sheds also some new light on the role of BMPs during the early determination process. Differentiation is not only suppressed by BMP mediated induction of Id (inhibitor of differentiation) proteins (44) but also by Xvent proteins. Although their relationship is not yet clear, Xvent-proteins belong to the same Nkx/Bar subfamily of homeodomain proteins as the nanog protein, a pluripotency sustaining factor in mammalian embryonic stem cells (45). Analysis of nanog in Xenopus embryos including its inhibitory effect on genes expressed in differentiating tissues will probably help to understand such a functional relationship. Involvement of POU and Nkx/Bar type homeodomain proteins in mammals and amphibia might indicate an evolutionary conserved principle in the inhibition of premature differentiation during early vertebrate embryogenesis.
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FOOTNOTES

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ABBREVIATIONS

The abbreviations used are: TGF-β, Transforming Growth Factor-β; BMP, bone morphogenetic protein; SBE, Smad binding element; BRE, BMP response element; GST, glutathione S-transferase; MT, myc tag; OAZ, Olf-1/EBF-associated zinc finger; RT, reverse transcriptase.
Figure 1: **Xvent-2B promoter.** A) Proteins that bind to the 5’flanking sequence and regulate *Xvent-2B* transcription (for details see text). B) Schematic drawing of yeast reporter constructs used for the one-hybrid screen. Three copies of the -267 to -214 region of the *Xvent-2B* gene were cloned in front of the *His* or *LacZ* gene, respectively.

Figure 2: **Localisation of Oct-25 transcripts in Xenopus laevis embryos.** Whole mount in situ hybridizations were performed with embryos of various developmental stages (24). A) stage 9, B) stage 11, C) stage 12, D-F) stage 15, E) double whole mount with Krox 20/Oct-25(BM-purple/BCIP); rhombomeres 3 and 5 are indicated. F) double whole mount with en-2/Oct-25(BM-purple/BCIP), the former staining the midbrain/hindbrain boundary. G) stage 19, H) stage 23, I) stage 27, K) stage 30, L) tip of the tail at stage 35, M) transverse section of a stage 14 embryo. no: notochord

Figure 3: **Overexpression of Oct-25.** Oct-25 RNA was injected into all four blastomeres of 4-cell stage embryos at a total amount of 150 pg (A), 300 pg (B) or 1 ng (C). Dorsal (d) and ventral (v) views of injected embryos are shown. The insert in (A) shows an uninjected control embryo.

Figure 4: **Oct-25 overexpression inhibits terminal differentiation.** Embryos were injected with 600 pg Oct-25 RNA at the 4-cell stage and grown until uninjected control embryos had reached stage 19 or stage 30, respectively. Total RNA was prepared from uninjected and injected embryos and analysed by RT-PCR for indicated transcripts. ODC (ornithine decarboxylase) was
used as internal standard, RT- (no reverse transcriptase) controls for the absence of endogeneous DNA.

Figure 5: **DNase I footprint of the Oct-25 binding site on the Xvent-2B promoter.** The -305 to -123 upstream region of the Xvent-2B gene was cloned in sense and in antisense orientation. After 5'-end labelling with [32P] the DNA fragments were submitted to DNase I footprinting using bacterially expressed Oct-25 protein. Lanes M denote the G/A chemical sequencing reactions, the next lane contains DNase I digested free DNA. The next four lanes contain the reactions with increasing amounts of Oct-25 protein (triangle). The vertical bars indicate protected regions which, at the bottom, are shown to correlate for the sense and antisense strands (horizontal bars). Note that the protected region contains two putative POU factor binding motifs, each of them containing a binding site for the POU specific (POUS) and for the homeodomain (POUH).

Figure 6: **Mutational analysis of the Oct-25 binding site.** A) The oligonucleotides containing the wild type or mutated -234 to -204 region of the Xvent-2B gene were [32P]-end labeled and annealed. wt is the wild type sequence, mu1 and mu2 are mutated at the indicated positions (nucleotides in small capitals). B) Samples were incubated with increasing concentrations of the POU/homeodomain of Oct-25 protein (black triangle) and submitted to EMSA. Note the failure of mu1 to bind the protein. C) Binding of the POU/homeodomain of Oct-25 with wt or mu2 was competed for by increasing concentrations of unlabeled wt, mu1 or mu2 targets (white triangles). Note that binding of labeled wt or mu2 is efficiently reduced by unlabeled wt and mu2, but not by mu1.
Figure 7: Oct-25 stimulates Xvent-2B transcription. The Xvent-2B upstream region (-275/+44), the 5’-deletion (-174/+44) or the -275/+44 region carrying an internal deletion (Δ-200/-32) were fused to the luciferase reporter gene. 20 pg of DNA were co-injected into both blastomeres at the two-cell stage of *Xenopus* embryos with Oct-25 RNA (250 pg), and/or BMP-4 RNA (300 pg) as indicated. Luciferase activity was measured at stage 12. The reporter activities determined in the absence of RNA were set as 100%. All values are averaged from at least three independent experiments.

Figure 8: Oct-25 is bound to the Xvent-2B promoter in vivo. Myc-tagged Oct-25 (MT-Oct-25) RNA or RNA encoding the myc-tag fused to a nuclear localization signal ((NLS-MT) were injected into all blastomeres of 4-cell stage embryos. Embryos were collected for ChIP assay when control sibling embryos had reached stage 19. The presence of the Xvent-2B promoter was detected by PCR from the DNA samples after anti-myc antibody precipitation (anti-myc), without antibody precipitation (no Ab), and the crosslinked chromatin supernatant before immunoprecipitation (input), respectively. Another ChIP control was performed in parallel under identical conditions with uninjected embryos.

Figure 9: Interaction of Oct-25 with components of the BMP signaling pathway. Oct-25, Smad1, Smad4, Xvent-2, N-terminal (ΔN) and C-terminal (ΔC) truncated versions of Xvent-2 and Oct-25, and an Oct-25 mutant lacking the POU and the homeodomain were labeled with [35S]methione by *in vitro* transcription/translation and incubated with GST or GST-fusion proteins as indicted. The pull-down reactions were washed and analyzed by 10% SDS-PAGE.
Fig. 3
Fig. 4
Fig. 5
Fig. 7
Fig. 8
Fig. 9
The POU factor Oct-25 regulates the Xvent-2B gene and counteracts terminal differentiation in Xenopus embryos

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