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Concentrations of TATA box-binding protein (TBP)-type genes affect chordamesodermal gene expression

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ABSTRACT The TATA box-binding protein (TBP) is an essential component of transcription initiation complexes of all three eukaryotic RNA polymerases. Recent knockdown studies revealed that basic transcription factors are essential not only for gene transcription but also for regulating specific gene expression. However, the mechanism of and the effect by regulation of TBP expression are unknown during early embryogenesis. Here we show that the alteration of concentration of each TBP-type gene affected mutually one another's expression, suggesting that an optimal ratio of concentrations of TBP-type genes induce expression of specific genes.

KEY WORDS: *TBP, TBP2, TLF, gene expression, Xenopus laevis*

Introduction

The TATA box-binding protein (TBP) is an essential component of transcription initiation complexes of all three eukaryotic RNA polymerases, and is highly conserved among yeast, plants, invertebrates and vertebrates (Cormack and Struhl. 1992, Roeder 1996, Davidson 2003). In vertebrates, three *TBP*-type genes, *TBP*, *TLF/TRF2* and *TBP2/TRF3* have been isolated and studied (Veenstra *et al.*, 2000, Jallow *et al.*, 2004, Dantonel *et al.*, 1999). TBP2 has a highly conserved core domain at the C-terminus, which binds to the TATA box-binding domain, similar to TBP (Jallow *et al.*, 2004, Bartfai *et al.*, 2004). The core domain of TBP-like factor (TLF) is notably different from that of TBP, and although it interacts with TFIIA and TFIIB, TLF does not bind to the canonical TATA box-binding domain (Bartfai *et al.*, 2004, Rabenstein *et al.,* 1999, Teichmann *et al.*, 1999, Moore *et al.*, 1999). Recent studies demonstrated that TBP-type genes are not just essential for gene expression but can also regulate specific gene expression (Veenstra *et al.*, 2000, Jallow *et al.*, 2004, Bartfai *et al.*, 2004). However, those results derived from knockdown experiments, experiments with various concentrations of TBP-type genes including their over-expressions are not performed yet. Here we show that concentrations of TBP-type genes affect the regulation of gene expression during embryogenesis.

Results

It has been previously reported that injection of the antisence oligonucleotide of *TBP* into 1-cell stage embryo arrested development before complete of gastrulation but not affected expression of *Xbra* at late gastrula stage (st.13) (Veenstra *et al.*, 2000). On the other hand, *Xbra* is required for normal gastrulation movements (Wilson *et al.*, 1995, Conlon and Smith, 1999). We considered that injection at 1-cell stage would not let the antisence oligonucleotide of *TBP* spread well to the future *Xbra*-expressing region. To reconfirm whether a knockdown of *TBP* affects expression of *Xbra*, we injected antisense morpholino oligonucleotide of *TBP* (*TBP*-MO) into the future marginal zone region of all blastomeres of the 4-cell embryo. Injection of *TBP*-MO (10 ng/blatomere) decreased expression of *Xbra* (Fig. 1A, right lane) in contrast to previous report (Veenstra *et al.*, 2000). This result suggested that the antisense oligonucleotide would not spread throughout embryo, and the injected region is very important to study the function of its gene. Moreover, we found over-expression (700 pg/blasomere) of *TBP* also reduced *Xbra* expression (Fig. 1A, left lane). Taken together, these results suggested that *TBP* would affect early embryogenesis.

Over-expression of *TBP* targeted to the future marginal zone region of dorsal blastomeres of the 4-cell embryo interfered with head formation and shortened the dorsal axis, like UV-irradiated embryos (Mise and Wakahara, 1994, Medina *et al.*, 1997) (Fig. 1C). Ventral over-expression of *TBP* had no effect on embryogen-

Abbreviations used in this paper: MO, morpholino; TBP, TATA binding protein; TLF, TBP-like factor.

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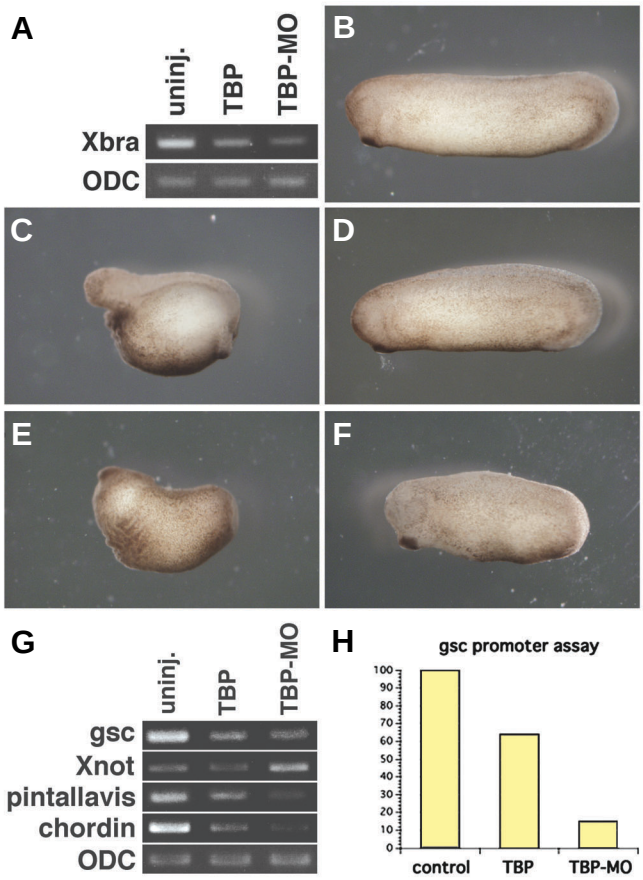
esis (Fig. 1D). In addition, dorsal injection of *TBP*-MO interfered with axis formation (Fig. 1E), while ventral injection of *TBP*-MO had no significant effect on axis formation (Fig. 1F). These results suggested that *TBP* might play an important role in axis formation at the dorsal side, in contrast to previous report that the antisense oligonucleotide of *TBP* interfered with complete of gastrulation (Veenstra *et al.*, 2000). Since many chordamesodermal genes are related to axis formation (Cho *et al.*, 1991, Sasai *et al.*, 1994, Yasuo and Lemaire, 2001, O'Reilly *et al.*, 1995), we next tested whether *TBP* reduce expressions of dorsal genes, such as *goosecoid*, *pintallavis*, *chordin* and *Xnot*. Dorsal over-expression of *TBP* reduced expression of *goosecoid*, *pintallavis* and *chordin* (Fig. 1G, center lane). Dorsal injection of *TBP*-MO also decreased expression of *goosecoid*, *pintallavis* and *chordin* (Fig. 1G, right lane). Furthermore, the promoter of *goosecoid* included a TATA box-binding site, and its promoter assay also demonstrates that both over-expression of *TBP* and injection of *TBP*-MO decreased transcription of *goosecoid* (Fig. 1H). However expression of *Xnot*



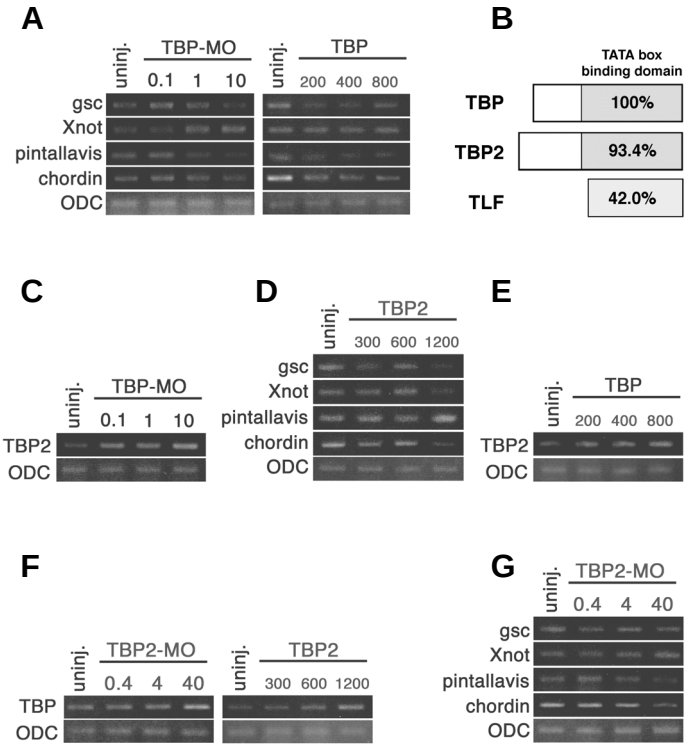


Fig. 1. Over-expression and repletion of TBP inhibits axis formation. (A) *Both over-expression of* TBP *and high-dose injection of* TBP-*MO into the future marginal zone region of all blastomeres of the 4-cell embryo reduced expression of* Xbra. **(B)** *Control embryo (st. 30).* **(C)** *Over-expression of* TBP *(700 pg/blastomere) into the future marginal zone region of dorsal blastomeres of the 4-cell embryo interfered with axis formation, including a short axis and absence of most of the head.* **(D)** *Over-expression into the ventral blastomeres of the 4-cell embryo had no effect on embryogenesis.* **(E)** *High-dose injection of* TBP-*MO (10 ng/blastomere) into the marginal zone region of dorsal blastomeres of 4-cell embryo also interfered with axis formation.* **(F)** *High-dose injection of* TBP-*MO into the ventral blastomeres of the 4-cell embryo had no significant effect on embryogenesis.* **(G)** *Over-expression of* TBP *reduced expression of chordamesodermal genes (center lane) at gastrula stage (st. 10). High-dose injection of* TBP-*MO reduced expression of all marker genes except for* Xnot, *which was increased by injection of* TBP-*MO.* **(H)** *Results of a luciferase assay using the reporter gene containing the promoter of* goosecoid *are shown. The luciferase activity was decreased by both over-expression of* TBP *and injection of* TBP-*MO at stage 10.*

Fig. 2. Optimal concentrations of TBP and TBP2 induced chordamesodermal gene expression at gastrula stage. (A) *Effects on gene expression by the various concentrations of* TBP *at stage 10. Injection of* TBP-*MO (0.1 ng-10 ng) and over-expression of* TBP *mRNA (200 pg-800 pg) is indicated in left and right panels, respectively. The low-dose injection of* TBP-*MO (0.1 ng/blastomere) increased slightly expression of* goosecoid, pintallavis *and* chordin, *but over-expression of* TBP *reduced expression of* goosecoid, pintallavis *and* chordin. *Expression of* Xnot *increased gradually depending on the dose of* TBP-*MO, and was not altered by any dose of* TBP. **(B)** *Schematic of TBP-type genes. The percentage of identical amino acids in TATA binding domain is indicated in the shaded box.* **(C)** *Injection of* TBP-*MO increased expression of* TBP2, *depending on the dose of* TBP-*MO.* **(D)** *Expression of chordamesodermal genes was increased with middle-dose (*goosecoid, Xnot *and* chordin*) or high-dose (*pintallavis*) of over-expression of* TBP2. **(E)** *Over-expression of* TBP *also increased expression of* TBP2, *depending on the dose of* TBP. **(F)** *Both injection of* TBP2-*MO (left) and over-expression of* TBP2 *(right) increased expression of* TBP, *depending on the dose of both* TBP2-*MO and* TBP2. **(G)** *Expression of some chordamesodermal genes was increased with middle-dose (*goosecoid *and* pintallavis*) or high-dose (*Xnot*) of* TBP2-*MO. Expression of* chordin *was not altered or decreased by* TBP2-MO.

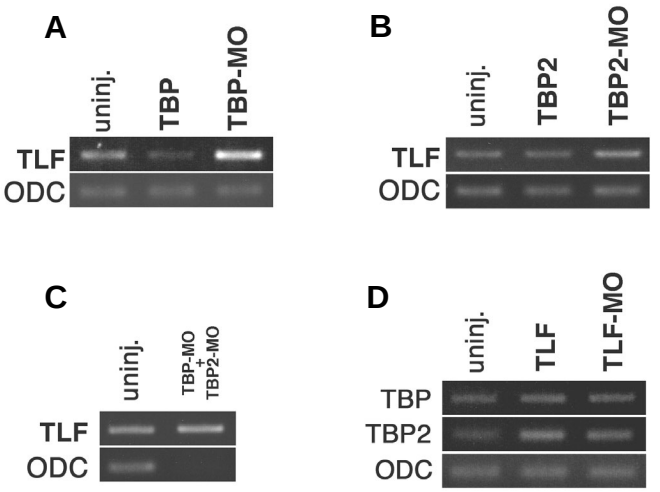


Fig. 3. The concentration of each *TBP*-type gene affected mutually one another's expression at gastrula stage. (A) *Expression of* TLF *was reduced by high-dose of* TBP *over-expression, but increased by high-dose of injection of* TBP-*MO.* **(B)** *Expression of* TLF *was not altered by high-dose of* TBP2 *over-expression, but increased by high-dose of injection of* TBP2-*MO.* **(C)** *Double injection of* TBP-*MO and* TBP2-*MO decreased expression of the internal maker,* ODC. *However expression of TLF was not affected by double injection.* **(D)** *Both high-dose over-expression of* TLF *and injection of* TLF-*MO increased slightly both expressions of* TBP *and* TBP2.

was not altered by *TBP* over-expression and induced by injection of *TBP*-MO (Fig. 1G). We therefore hypothesize that *TBP* may have very different, concentration-dependent functions. To confirm that the effect of *TBP* on chordamesoderm gene expression is concentration-dependent, we injected various doses of *TBP* mRNA and *TBP*-MO in dorsal blastomeres of the 4-cell embryo. We found that over-expression of *TBP* at all concentrations tested reduced expression of *goosecoid*, *pintallavis* and *chordin*, while expression of the same genes was increased with a low-dose (0.1 ng/blastomere) injection of *TBP*-MO (Fig. 2A). Expression of *Xnot* was increased with depending on the dose of *TBP*-MO, and showed the same level with increasing doses of *TBP* over-expression (Fig. 2A). These results indicate that an appropriate low-concentration of *TBP* increases expression of each chordamesodermal gene. Furthermore, we proposed that another TBP-type gene is required for transcription with low-concentration of *TBP*. In *Xenopus* development, TBP2 has a highly conserved TATA box-binding domain similar to TBP (Fig. 2B), and *TBP2* can partly substitute for *TBP* function (Jallow *et al.*, 2004). Therefore we considered that *TBP2* could replace or affect the function of *TBP* for regulating expression of each specific gene. First, we tested whether expression of *TBP2* is altered with low-concentrations of *TBP*. Expression of *TBP2* wsa increased depending on the dose of *TBP*-MO (Fig. 2C). And expression of *goosecoid*, *Xnot* and *chordin* was increased at a middle-dose (600 ng/blastomere) of *TBP2*, and a high-dose (1200 ng/blastomere) of *TBP2* increased *pintallavis* expression (Fig. 2D). These results confirmed that *TBP2* could partly substitute for *TBP* function for several chordamesodermal gene expressions. Moreover, over-expression of *TBP* also increased expression of *TBP2* (Fig. 2E). This result suggested that *TBP2* not only replaced the function of *TBP* in the presence of low-concentration of *TBP,* but also increased its concentration to maintain a specific equilibrium between *TBP* and *TBP2* concentrations in the high-concentration of *TBP*. On the other hand, expression of *TBP* was also increased by a high-dose injection of *TBP2*-MO (Fig. 2F, left) and over-expression of *TBP2* (Fig. 2F, right). Expression of *goosecoid*, *Xnot* and *pintallavis* was increased at an appropriate dose of *TBP2*-MO (Fig. 2G). Expression of *chordin* was not altered or decreased by the injection of *TBP2*-MO (Fig. 2G). These results suggested that both *TBP* and *TBP2* control each other's expression depending on the concentrations of the two, and that specific chordamesodermal genes are regulated by an optimal combination of *TBP* and *TBP2* concentrations.

The other vertebrate *TBP*-type gene, TLF, has no highly conserved TATA box-binding domain (Fig. 2B), and has distinct functions from TBP (Veenstra *et al.*, 2000, Rabenstein *et al.*, 1999, Teichmann *et al.*, 1999). We found over-expression of *TBP* reduced *TLF* expression (Fig. 3A, center lane), but over-expres-

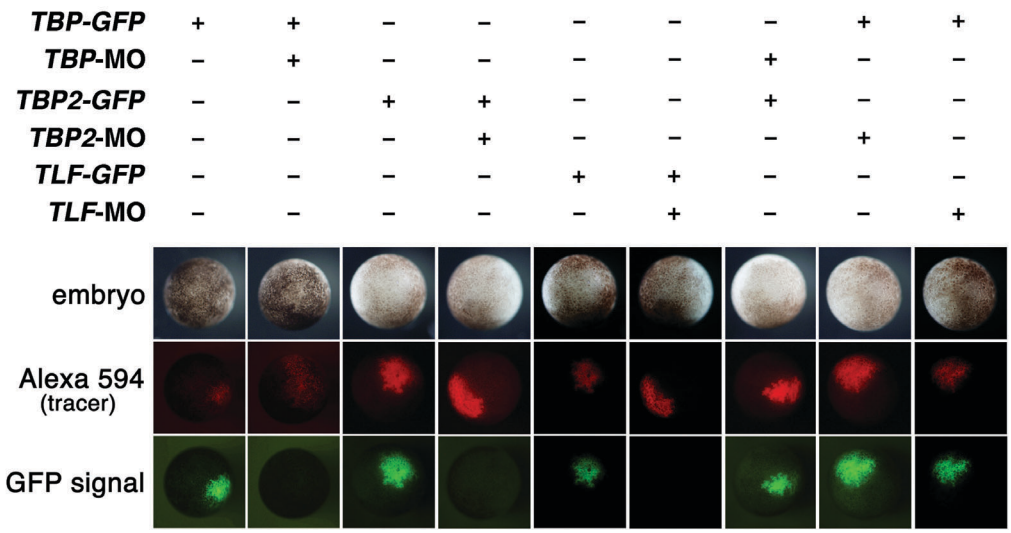


Fig. 4. The confirmation of the specificity of each morpholino. TBP-*MO,* TBP2-*MO and* TLF-*MO reduced specifically translation of* GFP *containing sequences targeted with* TBP-*MO,* TBP2-*MO and* TLF-*MO at the 5'-region, respectively.* GFP-TBP *(250pg),* GFP-TBP2 *(250pg) or* GFP-TLF *(250pg) with Alexa594-dextran (300pg) was injected in the animal pole of 4-cell embryo. The fluorescent signals were observed at stage 10. Pictures on top panels indicate the injected embryos. Pictures on center panels indicate tracer signals with Alexa594-dextran. Pictures on bottom panels indicate GFP signals. Each MO reduced translation of each corresponding* GFP *construct, but did not reduce translation of the* GFP *construct containing different targeting sequence.*

sion of *TBP2* had no such effect (Fig. 3B, center lane). High-dose injections of both *TBP*-MO and *TBP2*-MO increased expression of *TLF* (Fig. 3A, B, right lanes). Furthermore, even though expression of the internal marker gene, *ODC*, was markedly decreased by double injection of *TBP*-MO and *TBP2*-MO, expression of *TLF* was not decreased (Fig. 3C). Both over-expression of *TLF* and injection of *TLF*-MO also slightly increased expression of *TBP* and *TBP2* (Fig. 3D). Taken together, these results indicated that the expression of each of the three TBP-type genes was affected mutually by expression of the other two to regulate specific gene expression in early embryogenesis**.**

Discussion

Although TBP2 binds to the TATA box-binding domain and partly replaces the function of TBP (Jallow *et al.*, 2004), different gene promoters selectively recruit TBP and TBP2 (Yang *et al.*, 2006). Although TLF dose not bind to the canonical TATA box-binding domain, TLF and TBP compete for TFIIA and probably other general transcription factors (Chong *et al.*, 2005). Moreover, other molecules like negative cofactor 2 (NC2), PIAS and SAGA also associated with TBP-type genes and contribute to the regulation of specific gene expression (Cang *et al.*, 2002, Prigge and Schmidt, 2006, Sermwittayawong and Tan, 2006, Veenstra and Wolffe, 2001). Taken together, these studies indicate how functionally TBP-type genes regulate specific gene expression. However our present data indicate that TBP-type genes regulate mutually each other expression, suggesting that alteration of concentration of TBP-type genes themselves reflects regulating of expression of specific genes in early embryogenesis. Unknown upstream genes of TBP-type genes would play an important role for regulating such concentrations of them.

Materials and Methods

mRNA and Morpholinos

The coding sites of TBP (BC08819), TBP2 (AY753184) and TLF (AJ238441) were subcloned into modified pCS2+ vectors. The mRNAs were synthesized using T7 RNA polymerase. Morpholinos (MOs) for *TBP* (5'-tgctgttgttttgatccatgttgtg-3'), *TBP2* (5'-gaagactctccatccattttgaggg-3') and *TLF* (5'-tgccacatcactgtcagcatccatc-3') were obtained from GENE TOOLS, LLC. To confirm the specificity of each MO, we made *GFP-TBP*, *GFP-TBP2* and *GFP-TLF* constructs that contained 5' sequences that could be targeted by *TBP*-MO, *TBP2*-MO and *TLF*-MO respectively: the sequence of 5'-region of *GFP- TBP* cDNA was
5'-cacaacATGgatcaaaacaacagcagtaaaggagaagaactttt-3';
that of *GFP-TBP2* cDNA was
5'-ccctcaaaATGgatggagagtcttcgagtaaaggagaagaacttt-3';
that of *GFP-TLF* cDNA was
5'-gATGgatgctgacagtgatgtggcaagtaaaggagaagaactttt-3'
(targeted sequences are underlined; ATG is first the methionine). (See Fig. 4)

RT-PCR

For RT-PCR assay, the following primers were used: the forward 5'-ttgacacaacatggatcaaa-3' and reverse 5'-ctatgttctgtagctgagg-3' for *TBP*; the forward 5'-cttagaccaatgtgatgctg-3' and reverse 5'-tgaatcttgggtagcatctc-3' for *TBP2*; the forward 5'- agccatctcgatggatgctg-3' and reverse 5'-tcacaaaatagtttttctgc-3' for *TLF*; the forward 5'-gcaggcacccaacaagatgat-3' and reverse 5'-ccagattcggggtgcagagt-3' for *pintallavis*; 5'-attgactccatcctctccag-3' and reverse 5'-ttaggctcctacagttccac-3' for *Xnot*. Other primers are previously described in Suzawa *et al.*, (2007). We performed RT-PCR experiments more than three times to get results. *ODC* was used as a loading control.

Luciferase assay

The pOLuc plasmid containing the promoter region of goosecoid (80pg) (Watabe *et al.*, 1995) was co-injected with *TBP* (700pg) or *TBP*-MO (10ng) in the animal pole of 2-cell embryos. The supernatant of homogenized embryos at stage 10 was assayed using Dual-Luciferase Reporter Assay System (Promega).

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