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β-Catenin/Tcf-regulated transcription prior to the midblastula transition

Jing Yang[1], Change Tan[1], Rachel S. Darken[2], Paul A. Wilson[2] and Peter S. Klein[1,*]

[1]Department of Medicine (Hematology – Oncology) and Howard Hughes Medical Institute, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA
[2]Department of Cell Biology, Weill Medical College of Cornell University, 1300 York Avenue, New York, NY 10021, USA
*Author for correspondence (e-mail: pklein@mail.med.upenn.edu)

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SUMMARY

Following fertilization, the zygotic genome in many organisms is quiescent until the midblastula transition (MBT), when large-scale transcription begins. In *Xenopus* embryos, for example, transcription is believed to be repressed until the twelfth cell division. Thus, although dorsal-ventral patterning begins during the first cell cycle, little attention has been given to transcriptional regulation in pre-MBT development. We present evidence that regulated transcription begins during early cleavage stages and that the β-catenin–Tcf complex is required for the transcription of the *Xenopus* nodal genes *Xnr5* and *Xnr6* as early as the 256-cell stage. Moreover, inhibition of β-catenin/Tcf function can block dorsal development, but only if the inhibition begins early and is maintained throughout pre-MBT stages. Dorsal development can be rescued in ventralized embryos if Tcf-dependent transcription is activated prior to MBT, but activation of Tcf after MBT cannot rescue ventralized embryos, suggesting that β-catenin/Tcf-dependent transcription is required prior to MBT for dorsal-ventral patterning in *Xenopus*.

Key words: Wnt, β-catenin, Tcf, LEF, Midblastula transition, transcription, *Xenopus* embryo, Lithium

INTRODUCTION

The dorsal-ventral axis is specified during the first cell cycle in amphibians, when cortical rotation results in dorsal enrichment of β-catenin, an activator of Wnt-dependent transcription (Gerhart et al., 1989; Harland and Gerhart, 1997; Heasman, 1997). β-Catenin is required for dorsal development in vertebrates (Heasman et al., 1994; Heasman et al., 2000; Huelsken et al., 2000) and functions as a transcriptional activator when bound to DNA-binding proteins of the Tcf/Lef family (Molenaar et al., 1996). Localization of β-catenin in dorsal nuclei has been reported as early as the 16-cell stage (Larabell et al., 1997; Miller et al., 1999), yet its earliest known targets are not expressed until midblastula transition (MBT) (Brannon et al., 1997; Fan et al., 1998; Hyde and Old, 2000; Jones et al., 1995; Laurent et al., 1997; Lemaire et al., 1995; McKendry et al., 1997), when large-scale zygotic transcription begins (Newport and Kirschner, 1982a). Stabilization of β-catenin protein by a brief exposure to lithium activates dorsal gene expression and dorsal development, but only if embryos are treated prior to MBT (Fredieu et al., 1997; Hamilton et al., 2001; Kao et al., 1986; Yamaguchi and Shinagawa, 1989). The maximal effect of lithium exposure is seen at the 32- to 64-cell stage (fifth and sixth embryonic cleavage, respectively), yet the earliest expression of dorsal-specific genes such as *siamois*, *Twin* and *nodal-related-3* (*Xnr3*) is not detected until the twelfth cleavage (MBT), several hours and multiple cell cycles later. Thus, the transient exposure to lithium is somehow translated into a stable response that persists until the MBT, and cannot be generated by exposure to lithium after MBT begins, even though the embryo remains sensitive to lithium (Fredieu et al., 1997; Hamilton et al., 2001; Yamaguchi and Shinagawa, 1989). Similarly, other activators of Wnt-dependent transcription, such as ectopic Xwnt8 or an activated form of Tcf3, can induce dorsal development if presented prior to MBT, but not after the onset of MBT (Christian and Moon, 1993; Darken and Wilson, 2001; Hamilton et al., 2001; Smith and Harland, 1991).

Recently, maternal β-catenin protein was depleted in *Xenopus* embryos by the use of morpholino antisense oligonucleotides, which block translation of the endogenous mRNA (Heasman et al., 2000). When β-catenin was depleted at the four-cell stage, dorsal development was profoundly inhibited, similar to previous results depleting β-catenin in the oocyte (Heasman et al., 1994). However, when β-catenin was depleted later, at the eight-cell or 16-cell stage, dorsal development was only minimally inhibited. These observations imply that β-catenin functions in early embryos, before the MBT and at a time when zygotic transcription is widely believed to be repressed.

The MBT is an embryonic milestone that marks the transition from rapid, synchronous cell divisions and minimal zygotic transcription to loss of synchrony in cell divisions, a sudden burst of zygotic transcription and the onset of cell motility. Elegantly characterized in *Xenopus* by the landmark work of Newport and Kirschner, the MBT appears to be a general phenomenon of metazoan development. In *Xenopus*, the transcriptional apparatus is present in the pre-MBT embryo

(Newport and Kirschner, 1982b; Prioleau et al., 1994), and thus, as shown by Newport and Kirschner, the lack of zygotic gene expression appears to be because of global repression of transcription through a still uncharacterized mechanism that may in part involve regulated methylation of DNA prior to MBT (Stancheva et al., 2002). Because of this, work on pre-MBT embryonic development has focused primarily on post-transcriptional control mechanisms. Low-level transcription has been detected before MBT in *Xenopus* and *Drosophila* (Edgar and Schubiger, 1986; Kimelman et al., 1987; Nakakura et al., 1987; Shiokawa et al., 1989; Yasuda and Schubiger, 1992), but the significance of this early transcription is not known. Nevertheless, regulated transcription of specific mRNAs has not been described in vertebrate embryos and, except for *Drosophila engrailed* (discussed below) (Karr et al., 1989), little attention has been given to zygotic transcription as a regulatory mechanism in the pre-MBT embryo.

Because previous work in *Xenopus* has shown that the Wnt/β-catenin pathway must be activated prior to MBT to initiate dorsal gene expression and β-catenin functions as a transcriptional activator when it associates with Tcf, we have investigated whether β-catenin and Tcf regulate transcription prior to the MBT. We demonstrate for the first time that regulated transcription occurs in pre-MBT *Xenopus* embryos and show that β-catenin and Tcf specifically regulate pre-MBT transcription of the nodal genes *Xnr5* and *Xnr6* in dorsal blastomeres. We also provide evidence that pre-MBT β-catenin/Tcf-dependent transcription regulates dorsal-ventral patterning and dorsal gene expression.

MATERIALS AND METHODS

Embryo manipulations

Embryos were maintained at 22-23°C (Sive et al., 2000); at this temperature cell divisions occurred every 24 minutes. Injection of mRNA was according to well-described protocols (Sive et al., 2000). Injected embryos were exposed to actinomycin D (ActD, 10 μg mL^{-1}), or Dexamethasone (Dex, 10 μg mL^{-1}) added to the culture medium at various stages as described in the text. Later stages (stage seven and later) were determined by external morphology, time (assuming constant rate of cell division before MBT), and by counting the number of blastomeres in the vegetal pole.

Plasmid construction

ΔβTGR was generated by cloning dn-Xtcf3 (lacking the first 87 amino acids) into the vector CS2-hGRN, which encodes the ligand-binding domain of the human glucocorticoid receptor (residues 513-777) in pCS2. The resulting fusion encodes an *N*-terminal glucocorticoid-binding domain and a C-terminal dn-Xtcf3. The dn-Xtcf3 plasmid for this construct was provided by Alin Vonica (Vonica et al., 2000).

In vivo labeling of RNA and oligo-dT chromatography

For in vivo labeling of RNA, embryos were injected at the two-cell stage with 10 ηl of [^{32}P]UTP (10 μCi $μl^{-1}$; 3000 Ci $mmol^{-1}$) into each blastomere and cultured in medium with or without ActD (10 μg mL^{-1}) at 22-23°C. Embryos were harvested at the 128-, 512- or 2048-cell stages (corresponding to the seventh, ninth or eleventh cell cycle, respectively). Total RNA was extracted according to standard procedures; polyA+ RNA was purified using an oligo-dT kit from Qiagen. Total RNA (5 μg) and polyA+ RNA were analyzed by 1.0% agarose gel electrophoresis. Gels were then fixed in 10% methanol/10% acetic acid, dried and exposed to a phosphorimager.

RT-PCR and luciferase assay

RNA extraction and RT-PCR methods were as described previously (Deardorff et al., 1998), using 30 cycles of PCR for detection of *Siamois, Xnr3, Xnr5 and Xnr6*. Primers used were as follows: *EF1-α* (F, CAGATTGGTGCTGGATATGC, R, ACTGCCTTGATGACTCC-TAG); *Siamois* (F, AACTTTCTCCAGAACC; R, GTCAGTGTGG-TGATTC); *Xnr3* (F, ATCCAACTAACTACATCG, R, TAGTGGG-ACAAGAAGTGC); *FGFR* (F, AGTGCATCCACAGAGACC; R, ACTCAGAGCAAGAATTCGG); *ODC* (F, AATGGATTTCAGA-GACCA; R, CCAAGGCTAAAGTTGCAG); *Xnr5* (F, TCACAA-TCCTTTCACTAGGGC; R, GGAACCTCTGAAAGGAAGGC); and *Xnr6* (F, TCCAGTATGATCCATCTGTTGC; R, TTCTCGTTCCTC-TTGTGCCTT).

For the luciferase assay, 50 pg of Lef-fos plasmid [a promoter–reporter construct containing seven Lef binding sites, a minimal promoter and the luciferase open reading frame (Hsu et al., 1998)] and 25 pg of pRL-SV40 (a Renilla luciferase control for injection) were injected with mRNAs as described in Results. Embryos were harvested at the indicated stages (five embryos/group). Luciferase assay was performed with a dual luciferase assay kit (Promega), according to the manufacturer's instructions. The results are normalized to renilla luciferase activity.

RESULTS

XTcf functions in early cleavage stage embryos

Depletion of β-catenin at the four-cell stage robustly blocks dorsal development, but depletion at the 16-cell stage is far less effective (Heasman et al., 2000). This observation indicates that either β-catenin protein functions in early embryos before being depleted at the 16-cell stage, or that a small fraction of β-catenin protein somehow becomes inaccessible to the axin-APC-mediated degradation pathway. We have therefore tested whether this stage-dependent effect on dorsal development would also be observed by interfering with another component of the Wnt/β-catenin signaling pathway. Because β-catenin functionally interacts with maternally expressed members of the Tcf family, we injected mRNA encoding a dominant-negative Xtcf3 (dn-Xtcf3), which lacks the β-catenin-binding domain but retains the DNA-binding domain and thus interferes with endogenous Xtcf binding to target promoters (Molenaar et al., 1996). dn-Xtcf3 mRNA was injected on the dorsal side of *Xenopus* embryos at the four, eight or 16-cell stage and embryos were scored at the tadpole stage. To score the ventralized phenotype, we subdivided embryos into three groups based on the dorsal-anterior index (DAI) (Kao and Elinson, 1988). Embryos of group I had the strongest ventralized phenotype, lacking all dorsal and anterior structures (DAI 0; Fig. 1A). Group II embryos had an intermediate ventralized phenotype, lacking head structures, but maintaining trunk and tail or tail alone (DAI 1 and 2; Fig. 1B). Group III embryos formed head structures in addition to dorsal tissues and included both normal and mildly ventralized embryos, with cyclopic or small eyes and slightly reduced heads (DAI 3-5; Fig. 1C). We expected that injection of dn-Xtcf3 into all four dorsal blastomeres of eight or 16-cell embryos should have effects similar to injecting the two dorsal blastomeres at the four-cell stage, which give rise to the dorsal blastomeres of 8 and 16-cell embryos. Surprisingly, injecting dorsal blastomeres at the 8- or 16-cell stage showed much milder effects than injection at the four-cell stage. The

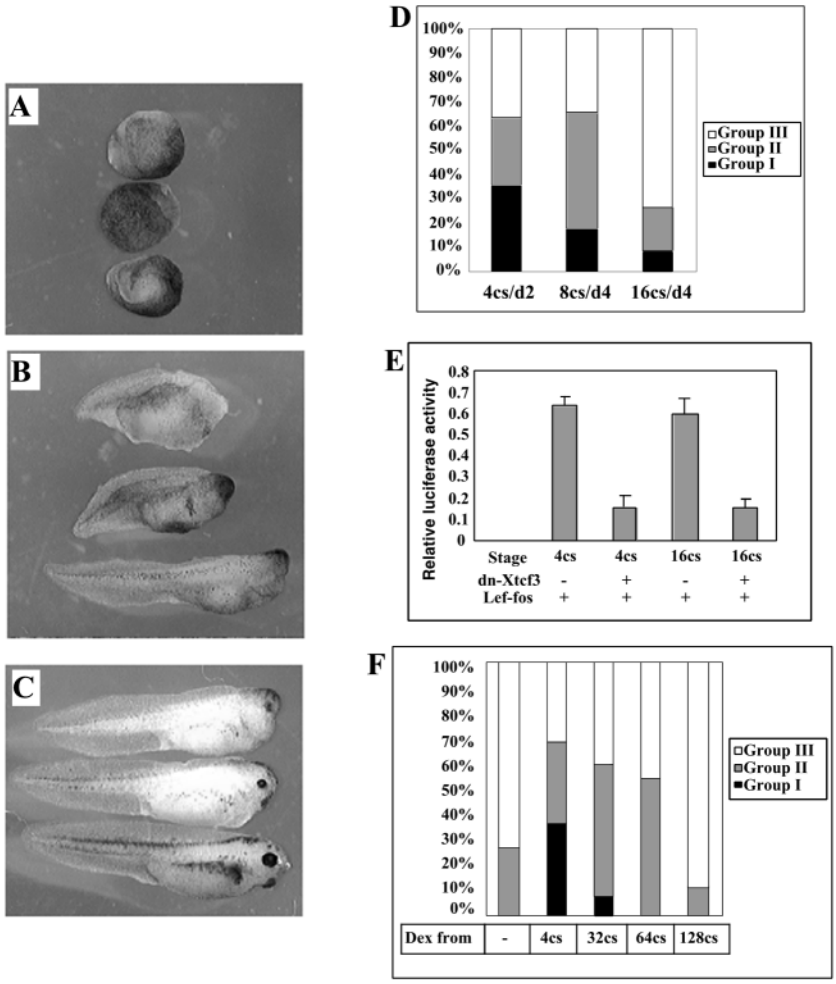

Fig. 1. Dominant-negative Xtcf3 (dn-Xtcf3) blocks endogenous dorsal axis in a stage-dependent manner. (A-C) Ventralized phenotypes caused by dn-Xcf3. (A) Group I phenotype: embryos lack all dorsal-anterior structures and fail to undergo convergent extension. (B) Group II phenotype: embryos lack head structures, but maintain trunk and tail or tail alone. (C) Group III phenotype: embryos develop dorsal and anterior structures and are either normal or have small eyes and slightly reduced heads. (D) Percentage of group I, II, III embryos caused by dn-Xtcf3 mRNA injection at the four-cell stage (4cs/d2; 500 pg for each dorsal blastomere), the eight-cell stage (8cs/d4; 250 pg for each dorsal blastomere), and 16-cell stage (16cs/d4; 250 pg for each dorsal-midline blastomere). (E) Luciferase assays for embryos injected dorsally with Lef-luciferase reporter (Lef-fos) alone (Hsu et al., 1998) or with dn-Xtcf3 at the four- or the 16-cell stage. (F) Phenotypes in embryos [as in (D)] injected with ΔβTGR (500 pg into each dorsal blastomere at the four-cell stage). Injected embryos were cultured in normal medium (untreated) or in medium containing dexamethasone (dex) from various stages (four-cell to 128-cell) until the gastrula stage. This experiment was repeated three times (with >50 embryos per group) with similar results.

percentage of completely ventralized (group I) embryos caused by dn-Xtcf3 fell from 35% (four-cell stage injection, *n*=114) to 17% (eight-cell stage injection, *n*=98) and 8% (16-cell stage injection, *n*=50; Fig. 1D).

These observations suggest that dn-Xtcf3 must be present during early cleavages in order to block dorsal development. However, injection at different stages, even separated by one cell division, could result in different distribution of injected mRNA or different levels of dn-Xtcf3 protein in embryos. To test whether similar levels of functional dn-Xtcf3 protein are synthesized when RNA is injected at the four-cell or the 16-cell stage, we co-injected dn-Xtcf3 mRNA with a Tcf/LEF luciferase reporter (Hsu et al., 1998) and found that luciferase activity (measured after the onset of MBT) was reduced to a similar extent whether RNA was injected at the four-cell or 16-cell stage (Fig. 1E). Thus, the failure to ventralize when dn-Xtcf3 was injected at the 16-cell stage is not because of reduced dn-Xtcf3 activity at MBT (Fig. 1E). Furthermore, to avoid injecting embryos at different stages, we used dn-Xtcf3 fused to the glucocorticoid receptor (ΔβTGR), which can be activated by addition of dex. RNA encoding ΔβTGR was injected into both dorsal blastomeres at the four-cell stage. Dex was then added at various stages and removed at the gastrula stage. Under these conditions, all embryos are injected in the same way, distribution of mRNA should be similar among all samples, translation of mRNA begins at the same time for all samples, and inhibition of endogenous Tcf activity depends only on the time that dex is added.

The percentage of ventralized embryos was highest when dex was added early and decreased when dex was added later (Fig. 1F). Thus, 36% of ΔβTGR-expressing embryos were completely ventralized when dex was added at the four-cell stage, whereas 32% had trunk and tail or tail only (group II phenotype), and 32% showed the group III phenotype. However, when dex was added at the 128-cell stage, none of the embryos were completely ventralized, and only 11% showed group II phenotype, whereas 89% showed complete dorsal development with either normal or slightly reduced heads (Fig. 1F). In the absence of dex, ΔβTGR-injected embryos showed a mildly ventralized phenotype, indicating that ΔβTGR has slightly leaky activity. Dex had no visible effect on uninjected embryos (not shown).

Because Tcf family members are well-characterized DNA-binding proteins that, upon interaction with β-catenin, activate transcription of specific target genes, one interpretation of these observations is that β-catenin/Tcf activates transcription during pre-MBT development. Thus dn-Xtcf3 injected at the 16-cell stage does not block dorsal development because sufficient β-catenin/Tcf-dependent transcripts accumulate before dn-Xtcf3 is introduced (see Fig. 6A). An alternative hypothesis is that the

β-catenin–Tcf complex is assembled or modified during early cleavage stages to become impervious to dn-Xtcf3, but does not activate transcription until the MBT. Because dn-Xtf3 does not bind β-catenin, and functions by competing for Tcf/LEF-binding sites in target promoters, this explanation would imply a complex involving Tcf and its DNA target sites (for details, see Tutter et al., 2001). The critical distinguishing feature of these two hypotheses is the time at which β-catenin/Tcf-dependent transcription begins. Thus, if β-catenin/Tcf transcription begins in the pre-MBT embryo, the effect of dn-Xtcf3 in cleavage-stage embryos should be sensitive to inhibition of transcription during this early time; furthermore, transcription of β-catenin/Tcf-dependent target genes prior to MBT should be observed directly and the transcriptional activity of endogenous Tcf should be required during pre-MBT stages for dorsal development. Each of these predictions was tested and the results support a role for pre-MBT β-catenin/Tcf-regulated transcription in dorsal development.

Tcf function in pre-MBT embryos is sensitive to a general inhibitor of transcription

To address more directly whether transcription occurs in early cleavage stages, we treated embryos at the two-cell stage with Actinomycin D (ActD), an inhibitor of transcription that is membrane-permeable and reversible in *Xenopus* embryos; ActD was removed at the eight- or 16-cell stage and then dn-Xtcf3 mRNA was injected. If β-catenin/Tcf-dependent transcription occurs in early cleavage stages (two-cell to eight- or 16-cell stage), this will be blocked by transient global inhibition of transcription during this period. Injection of dn-Xtcf3 at the eight- or 16-cell stage should then effectively inhibit dorsal development.

Indeed, ActD treatment strongly enhanced the ventralized phenotype caused by dn-Xtcf3 RNA injection at the eight- or 16-cell stage, with 60% (*n*=20 and 21, respectively) of embryos falling into group I, compared with 9% for embryos not treated with ActD (*n*=21 and 22, respectively; Fig. 2A). ActD alone from the two- to the 16-cell stage had little, if any, effect on embryonic development (Fig. 2A). These results were confirmed by treating ΔβTGR-injected embryos with ActD. Similar to results in Fig. 1F, only weak ventralization was observed when dex was added at the 32-cell stage; no embryos developed the group I phenotype (*n*=21), compared with 35% when dex was added at the four-cell stage for this set of embryos (*n*=23). However, if transcription was blocked by ActD from the four-cell to the 32-cell stage, and then dex was added, these embryos were strongly ventralized, with 28% of embryos in group I, 50% in group II and only 22% in group III (*n*=18; Fig. 2B). RNA-injected embryos treated with ActD, but not dex, from the four-cell to 32-cell stage did not show the group I phenotype (*n*=25; Fig. 2B). As previously reported, a morpholino antisense oligonucleotide against β-catenin caused strong ventralization when injected at the four-cell stage, but not at the eight-cell stage (Heasman et al., 2000); we found that treatment with ActD from the two-cell to the eight-cell stage enhanced ventralization caused by injection of this antisense oligonucleotide at the eight-cell stage (data not shown).

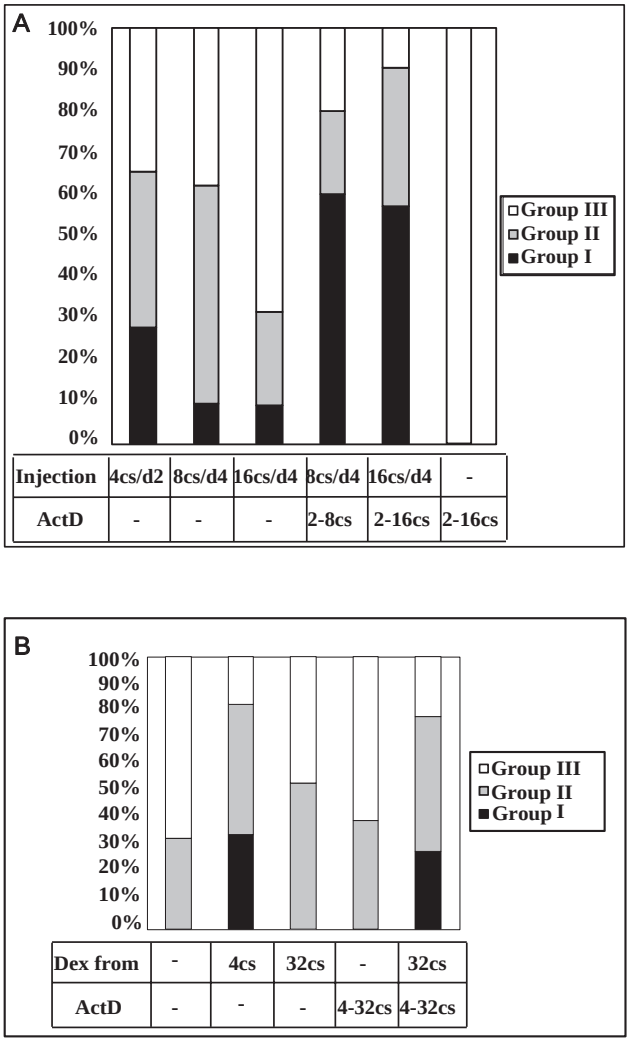

Fig. 2. Inhibition of transcription in early embryos extends sensitivity to dn-Xtcf3. (A) Embryos were exposed to actinomycin D (ActD) from the two-cell stage to either the eight-cell or 16-cell stage, and dn-Xtcf3 RNA was then injected into all four dorsal blastomeres, and phenotype was scored as in Fig. 1. (B) ΔβTGR RNA was injected into two dorsal blastomeres at the four-cell stage. Dexamethasone (dex) was then added to embryo culture medium at the four-cell or 32-cell stage. Where indicated, embryos were cultured in ActD containing medium from the four-cell stage to the 32-cell stage and then transferred to dex-containing medium until stage ten. Phenotypes were scored at tailbud stage.

Transcription of heterogeneous polyadenylated RNAs in intact embryos prior to MBT

Although it is generally believed that minimal transcription occurs prior to MBT, detection of high molecular weight RNA synthesized prior to MBT in dissociated or cleavage-arrested embryos has been reported (Kimelman et al., 1987; Nakakura et al., 1987; Shiokawa et al., 1989). However, this RNA had not been shown to be polyadenylated, a feature of most mRNAs, and had not been described in intact embryos. To confirm that polyadenylated RNA is transcribed prior to MBT in intact embryos, [^{32}P]UTP was injected into each blastomere of two-cell stage embryos, which were then cultured in the presence or absence of ActD. Total RNA was prepared from 128-cell (seventh-cell cycle), 512-cell (ninth-cell cycle), or approximately 2000-cell (eleventh-cell cycle) embryos and was analyzed by agarose gel electrophoresis and

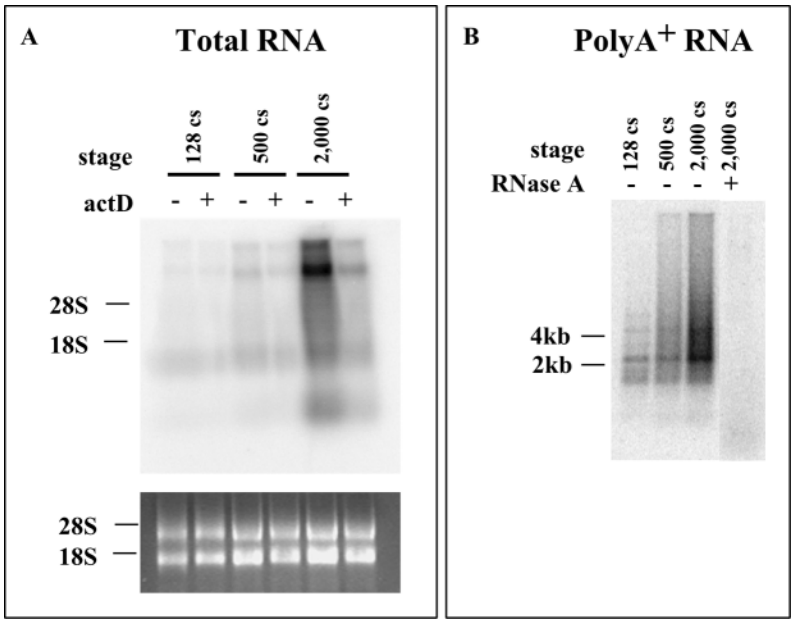

Fig. 3. Polyadenylated RNA is transcribed prior to midblastula transition (MBT) in intact embryos. (A) Total RNA transcribed in early embryos was detected by injection of α-[^{32}P]UTP in two-cell embryos, which were then cultured with or without actinomycin D (ActD). RNA was prepared from various stages prior to MBT and analyzed by electrophoresis on an agarose gel, followed by autoradiography. The upper panel is an autoradiograph showing newly synthesized RNA and the lower panel shows ethidium bromide-stained ribosomal RNA from the same samples. (B) Newly synthesized polyadenylated RNA was purified by binding to oligo-dT cellulose followed by agarose gel electrophoresis and autoradiography. An equal amount of poly-A+ RNA from the 2000-cell sample was treated with RNaseA prior to loading (+RNase). Equal amounts of polyadenylated RNA were loaded in each lane, as judged by ethidium bromide staining (not shown).

autoradiography. Newly synthesized high molecular weight RNA was readily observed as early as the 512-cell stage (the ninth-cell cycle) and increased at early stage eight, prior to MBT (Fig. 3A). ActD reduced RNA synthesis at all stages. This newly transcribed RNA included a heterogeneous population of high molecular weight polyadenylated RNA, as determined by oligo-dT affinity chromatography followed by agarose gel electrophoresis (Fig. 3B). Furthermore, this polyadenylated material was sensitive to treatment with RNaseA. Although the identity of these [^{32}P]-labeled species is unknown, (the discrete bands in Fig. 3B could represent mitochondrial mRNA), the incorporation of ^{32}P-UTP into heterogeneous high molecular weight species that are blocked by an RNA polymerase inhibitor, bind to oligo-dT, and are degraded by RNaseA suggests that this material is newly synthesized mRNA. It is also possible that mRNA is synthesized prior to the 512-cell stage but falls below the level of detection by our methods.

β-Catenin and Tcf regulate the pre-MBT transcription of nodal genes *Xnr5* and *Xnr6* in dorsal blastomeres

Several genes have been identified as targets of β-catenin/Tcf and are expressed at or soon after the MBT in *Xenopus*. These include *Siamois*, *Twin* and *nodal-related 1*, *2*, *3*, *5* and *6* (*Xnr1*, *2*, *3*, *5* and *6*) (Brannon et al., 1997; Fan et al., 1998; Hyde and Old, 2000; Jones et al., 1995; Laurent et al., 1997; Lemaire et al., 1995; McKendry et al., 1997; Takahashi et al., 2000). We therefore re-examined the expression of these genes at stages prior to MBT by RT-PCR. Consistent with published work, *Xsia* (Fig. 4A), *Xnr2* and *Xnr3* (not shown) were not detectable (after 30 cycles of PCR) prior to stage 8.5; *Xnr1* was detectable at a low level in maternal RNA but did not change during pre-MBT stages (data not shown). Weak expression of *Twin* was observed at the 1000-cell stage, but not earlier, in some experiments (data not shown). However, using the same PCR conditions, we detected *Xnr5* and *Xnr6* transcription as early as the 256-cell stage, and their expression levels continued to increase during pre-MBT stages (Fig. 4A). To address whether β-catenin/Tcf signaling is involved in the pre-MBT transcription of *Xnr5* and *Xnr6*, we either activated or inhibited β-catenin/Tcf signaling and then measured *Xnr5* and *Xnr6* mRNA levels as above. To increase β-catenin activity, mRNA encoding β-catenin was injected into two ventral blastomeres at the four-cell stage, or embryos were treated at the 32-cell stage with LiCl, which stabilizes β-catenin through inhibition of GSK-3β (Klein and Melton, 1996; Stambolic et al., 1996). To interfere with β-catenin/Tcf function, a morpholino antisense oligonucleotide against β-catenin (Heasman et al., 2000) or mRNA encoding dn-Xtcf3 (Molenaar et al., 1996) was injected into two dorsal blastomeres at the four-cell stage. Injected embryos were harvested at the 1000-cell stage (prior to MBT) and RT-PCR was performed. Expression of *Xnr5* and *Xnr6* was enhanced by ventral injection of β-catenin or exposure to LiCl (Fig. 4B, lanes 2 and 3; see also Fig. 4D, lane 6), and markedly reduced by dorsal injection of the β-catenin morpholino or dn-Xtcf3 (Fig. 4B, lanes 4 and 5). The regulation of *Xnr5* and *Xnr6* is because of new transcription, not changes in polyadenylation of the respective mRNAs, because RT-PCR results were similar with either oligo-dT primed or random primed cDNA (data not shown). Similar regulation by β-catenin/Tcf was detectable for *Xnr5* at the 500-cell stage and for *Xnr6* as early as the 256-cell stage (data not shown). Because pre-MBT *Xnr5* and *Xnr6* expression was blocked by injection of β-catenin antisense morpholino or dn-Xtcf3 mRNA specifically into dorsal blastomeres, this suggests that the pre-MBT transcription of these two targets is localized to the dorsal blastomeres; to confirm this, embryos at the 500-cell stage were dissected into dorsal and ventral halves and *Xnr5* and *Xnr6* mRNAs were detected in each half by RT-PCR. As shown in Fig. 4C, pre-MBT transcription of these two nodal genes is detected almost exclusively in dorsal blastomeres. These findings demonstrate directly and for the first time that β-catenin/Tcf activates dorsal-specific transcription during pre-MBT stages.

To test whether RNA polymerase inhibitors block pre-MBT transcription activated by β-catenin/Tcf, embryos were treated

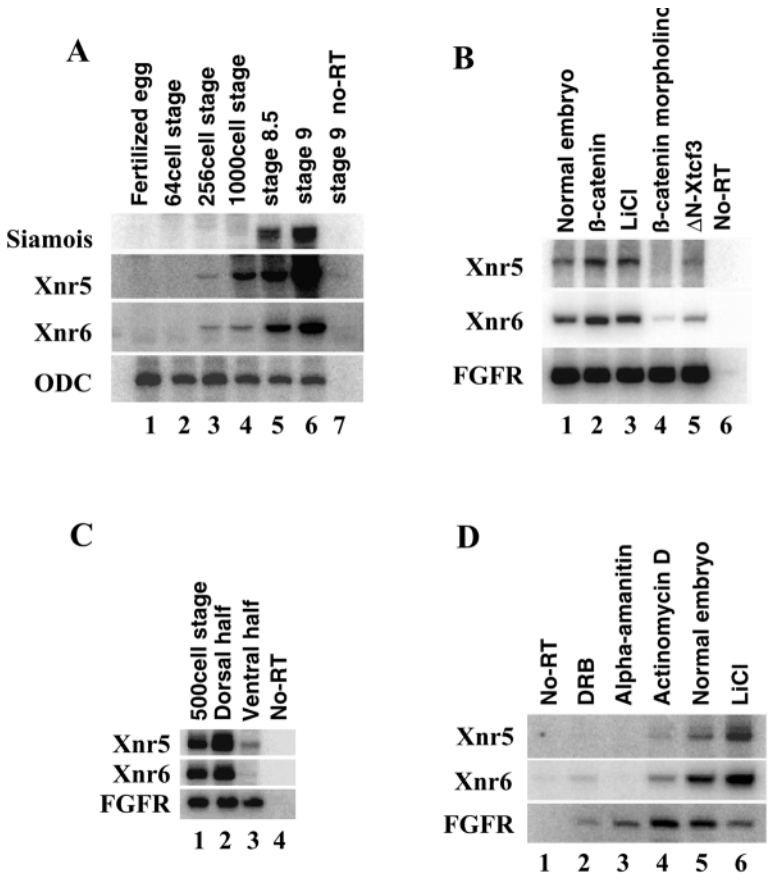

Fig. 4. Pre-midblastula transition (MBT) transcription of *Xnr5* and *Xnr6* is regulated by β-catenin and Xtcf3 in dorsal blastomeres. (A) Zygotic expression of *Xnr5* and *Xnr6* is detectable by RT-PCR as early as the 256-cell stage whereas *siamois* expression begins at the 4000-cell stage. *Ornithine decarboxylase* (*ODC*), a maternally expressed gene that does not increase significantly at MBT, was used as a loading control. 'No-RT' indicates control lacking reverse transcriptase. (B) Regulation of pre-MBT transcription by β-catenin: embryos were injected ventrally with β-catenin mRNA (500 pg), treated at the 32-cell stage with LiCl (0.3 M for 10 minutes), or injected dorsally with either dnXtcf3 mRNA (500 pg) or morpholino antisense oligonucleotide against β-catenin (10 ng). Embryos were harvested at the 1000-cell stage and analyzed by RT-PCR for *Xnr5* and *Xnr6* expression (*FGFR*: *FGF receptor* was a loading control). (C) Pre-MBT transcription of *Xnr5* and *Xnr6* is localized to dorsal blastomeres. Embryos were dissected into dorsal and ventral halves at the 500-cell stage, RNA was isolated from each half, and *Xnr5* and *Xnr6* expression was assessed by RT-PCR as in panels (A) and (B). Control whole embryo RNA is shown in lane 1. (D) RNA polymerase II-dependent pre-MBT transcription of *Xnr5* and *Xnr6*: embryos were treated with actinomycin D (ActD) from the two-cell stage, injected with the RNA polymerase II-specific inhibitors α-amanitin or 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) at the one-cell stage, or treated with LiCl as in (B). Embryos were harvested at the 1000-cell stage for analysis of *Xnr5* and *Xnr6* expression as above.

with ActD from the four-cell to the 1000-cell stage and harvested for analysis of *Xnr5* and *Xnr6* expression. ActD reduced the pre-MBT transcription of *Xnr5* and *Xnr6* (Fig. 4D, lane 4). Because ActD is a general inhibitor of transcription that functions by intercalating into double-stranded DNA, we also tested two specific inhibitors of RNA polymerase II, which function through mechanisms that are distinct from ActD. Thus, we tested 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), which specifically inhibits transcription elongation by RNA polymerase II (Chodosh et al., 1989), and α-amanitin, which binds and specifically inhibits RNA polymerase II at doses between 0.3 μg ml^{-1} and 1 μg ml^{-1} in *Xenopus* embryos (Newport and Kirschner, 1982a). Embryos were injected with DRB or α-amanitin at the one-cell stage and harvested at the 1000-cell stage. Both DRB and α-amanitin markedly reduced the pre-MBT transcription of *Xnr5* and *Xnr6*, although DRB showed a weaker activity in this assay (comparing Fig. 4D, lanes 2 to 3). As a positive control, LiCl increased the expression of *Xnr5* and *Xnr6* (Fig. 4D, lane 6; as in Fig. 4B). These observations, based on the effects of three distinct inhibitors of transcription, strongly support the conclusion that the early expression of *Xnr5* and *Xnr6* requires RNA polymerase II-dependent transcription prior to MBT.

β-Catenin-dependent transcription is required prior to MBT for dorsal development

These observations show that β-catenin-dependent transcription occurs during pre-MBT stages. To test whether β-catenin-dependent transcription is specifically required prior to MBT for dorsal development, we blocked β-catenin-dependent transcriptional activation, and then restored it at various stages prior to and after MBT. Embryos were ventralized by injection of mRNA encoding axin, a well-characterized inhibitor of β-catenin signaling that potently blocks dorsal development (Zeng et al., 1997), into both dorsal blastomeres at the four-cell stage. To restore Tcf activation, we used a hormone-inducible, activated form of Xtcf3 (TVGR), in which the β-catenin-binding site has been replaced with the VP16 transcription activation domain (Darken and Wilson, 2001). This construct was shown previously to induce dorsal axis duplication if activated at the four-cell stage, but not if activated in the gastrula stage, even though it is functional when activated at the gastrula stage (Darken and Wilson, 2001). TVGR mRNA was co-injected with axin, dex was added at various stages to restore β-catenin/Tcf-dependent transcription, and the phenotype of manipulated embryos was scored at the tadpole stage.

Activation of TVGR prior to MBT (at the four-cell or the 500-cell stage) rescues dorsal development in ventralized embryos (Fig. 5A) and also rescues pre-MBT transcription of *Xnr5* and *Xnr6* (Fig. 5C). However, activation of TVGR at MBT or later does not rescue dorsal development, even though TVGR is transcriptionally active when dex is added after MBT. Thus, 92% of axin-injected embryos were completely ventralized (n=36) and this did not change significantly when TVGR mRNA was co-injected (83% group I embryos, n=23). The addition of hormone at the four-cell stage significantly rescued the phenotype, with only 36% of embryos showing the group I phenotype (n=33). Similar results were obtained when

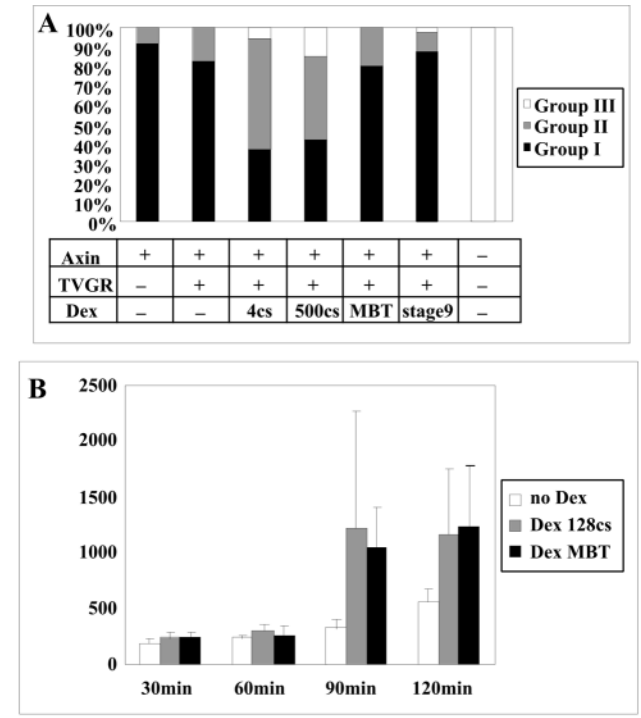

Fig. 5. Tcf-dependent transcription is required prior to midblastula transition (MBT) for dorsal development. (A) To antagonize β-catenin function and thereby block dorsal development, axin mRNA (2.5 ng) was injected into both dorsal blastomeres at the four-cell stage, as described (Zeng et al., 1997). To rescue dorsal development, hormone-inducible Xtcf3 (TVGR; 0.5 pg) was co-injected and Dexamethasone (dex) was then added at the four-cell, 500-cell stage, MBT (the 4000-cell stage), or late blastula (stage nine). Phenotypes were scored at tailbud stage. (B) Luciferase assays for embryos injected with Lef-fos (as in Fig. 1E) and TVGR (0.5 pg) into the animal pole at the two-cell stage. Injected embryos were cultured in normal medium (no dex), or dex was added at the 128-cell stage (dex, 128cs) or at MBT (dex, MBT). Embryos were harvested every 30 minutes after MBT and luciferase activity was measured. (C) Activation of Xtcf3 induces pre-MBT transcription in ventralized embryos: Axin mRNA was injected into dorsal blastomeres at the four-cell stage alone or with TVGR, as in (A). Injected embryos were cultured in normal medium (no Dex) or dex was added at the four-cell stage (Dex, 4cs). Embryos were harvested at the 1000-cell stage and analyzed for *Xnr5* and *Xnr6* expression. (EF1-α was the loading control). (D) Activation of Xtcf3 rescues expression of *Siamois* and *Xnr3* only if hormone is added before MBT, although activation after MBT can still rescue *Xnr6* transcription: injection and manipulation were performed as described in (A). Embryos were harvested at early gastrula stage (stage ten+) and analyzed by RT-PCR for *Siamois*, *Xnr3* and *Xnr6* expression (*EF1-α* was a loading control). Lane 1 represents uninjected gastrula-stage embryos.

injected embryos were exposed to hormone at the 500-cell stage, with dorsal rescue in 58% of embryos (n=33). No rescue was observed when dex was added at MBT (n=40) or at late blastula (stage 9; n=41). To address the concern that activation of TVGR by dex might be too slow to induce dorsal gene expression when it is added at the onset of MBT or later, we used the Tcf/luciferase reporter construct described in Fig. 1E to measure the time required for TVGR to respond to dex. When dex was added at the onset of MBT, a robust increase in luciferase activity was observed within 90 minutes (Fig. 5B), indicating that TVGR-regulated transcription was induced well before this time point. Interestingly, when dex was added at the 128-cell stage, elevated reporter activity was also first detected 90 minutes after MBT, similar to addition of dex at MBT, indicating that this LEF promoter construct is not active prior to MBT, similar to most zygotic genes, and that pre-MBT activation of gene expression by β-catenin is highly promoter-specific. Thus, we conclude that the activation of TVGR by dex is a rapid response.

To confirm that TVGR can activate transcription during pre-MBT stages (Fig. 5C), *Xnr5* and *Xnr6* expression was measured in ventralized embryos at the 1000-cell stage. In axin-injected embryos, expression of *Xnr5* and *Xnr6* is markedly reduced, but their expression is rescued by activation of TVGR at the four-cell stage (Fig. 5C, compare lanes 2 and 4), confirming that TVGR can activate transcription prior to MBT. (Similarly, dn-Xtcf3 inhibits pre-MBT transcription of *Xnr5* and *Xnr6*, as described above.) Furthermore, although TVGR cannot rescue dorsal development when it is activated after MBT, and does not rescue expression of *Siamois* or *Xnr3*, it can still induce transcription of selected endogenous targets when it is activated after MBT, including *engrailed* (Darken and Wilson, 2001) and *Xnr6* (Fig. 5D), confirming that the construct is active after MBT. Because TVGR can activate transcription before and after MBT but rescues dorsal development only when activated before MBT, these findings strongly support the conclusion that β-catenin/Tcf-dependent transcription prior to MBT is required for dorsal development.

DISCUSSION

Dorsal-ventral axis specification in amphibians is initiated in the first cell cycle by cortical rotation (Gerhart et al., 1989), which results in dorsal enrichment of β-catenin (Harland and Gerhart, 1997; Heasman, 1997; Larabell et al., 1997; Miller et al., 1999; Schneider et al., 1996). It is widely accepted that β-catenin, after 12 cell divisions, then activates dorsal-specific genes (such as *Siamois*, *Twin* and *Xnr3*) at MBT that specify the Spemann organizer and pattern the dorsal-ventral axis of the embryo (Harland and Gerhart, 1997; Heasman, 1997). Consistent with this, the promoters of these genes contain multiple Tcf-binding sites that mediate the activation by ectopic β-catenin. However, several lines of evidence suggest that β-catenin also functions prior to MBT. Thus, nuclear

translocation of β-catenin has been reported prior to MBT as early as the 16-cell stage (Larabell et al., 1997; Miller et al., 1999). Furthermore, lithium, an inhibitor of GSK-3β (Klein and Melton, 1996; Stambolic et al., 1996), that enhances nuclear accumulation of β-catenin (Larabell et al., 1997; Schneider et al., 1996), rescues ventralized embryos in a narrow temporal window prior to MBT (Fredieu et al., 1997; Hamilton et al., 2001; Kao et al., 1986; Yamaguchi and Shinagawa, 1989). In addition, a morpholino antisense oligonucleotide against β-catenin ventralizes embryos if injected at the four-cell stage, but not the eight-cell stage or later (Heasman et al., 2000). Finally, ectopic activation of canonical Wnt signaling induces a secondary dorsal axis only if activated prior to MBT (Christian and Moon, 1993; Darken and Wilson, 2001; Hamilton et al., 2001; Smith and Harland, 1991).

To explain these findings, we propose that the β-catenin–Tcf complex activates transcription essential for dorsal-ventral axis specification beginning in early cleavage stages of development. Once this transcription reaches a sufficient level during pre-MBT stages, dorsal development is no longer sensitive to loss of β-catenin or dn-Xtcf3 (Fig. 6A). This conclusion is supported by several observations presented here: (1) dn-Xtcf3 effectively blocks dorsal development only when present at the earliest cleavage stages (Fig. 1D,E), similar to the β-catenin morpholino results (Heasman et al., 2000). (2) Transient, global inhibition of transcription during early pre-MBT stages extends the period of sensitivity to loss of Tcf or β-catenin activity (Fig. 2, Fig. 6B). (3) β-catenin and Tcf induce pre-MBT transcription of specific target genes (Fig. 4). Interestingly, this pre-MBT transcription of *Xnr5* and *Xnr6* is localized to dorsal blastomeres. (4) Dorsal development is rescued only if Tcf-dependent transcription is restored during pre-MBT stages (Fig. 5, Fig. 6C). To our knowledge, this is the first characterization of a pathway that regulates pre-MBT transcription.

How dorsal genes such as *Siamois* are activated by β-catenin remains unclear. Our observations, as well as recently published work from other laboratories, raise the possibility that β-catenin signaling may activate post-MBT dorsal genes indirectly. Although the *Siamois* promoter contains three functional Tcf/Lef binding sites, a *Siamois* promoter construct lacking these sites (S24) is as active as the wild-type promoter in dorsal blastomeres (Brannon et al., 1997), suggesting that these Tcf binding sites are not required for the activation of *Siamois* in vivo. Furthermore, S24 activity is significantly higher than the wild-type *Siamois* promoter in ventral blastomeres, indicating these Tcf binding sites repress *Siamois* expression. Consistent with this view, depletion of maternal Xtcf3 results in ectopic expression of *Siamois* and *Xnr3* in ventral blastomeres, as well as dorsalization of embryos, suggesting Xtcf3 primarily functions as a repressor (Houston et al., 2002). Interaction between the β-catenin–Lef-1 complex and the TGF-β transducing proteins Smad3 and Smad4 has been reported to play a role in the regulation of *twin* expression (Labbe et al., 2000; Nishita et al., 2000). Whether pre-MBT expression of *Xnr5* and *Xn6* promotes this interaction, or whether Smad–LEF interactions also regulate *Siamois* transcription is not currently known. Thus, it will be interesting to analyze how β-catenin activates *Siamois* expression.

An alternative explanation for the loss of sensitivity to β-catenin depletion or dn-Xtcf3 by the 16-cell stage is that β-catenin and Tcf form a stable complex (for details, see Tutter et al., 2001) or are otherwise modified in early cleavage stages, but this complex does not activate transcription until MBT. However, we demonstrate directly that β-catenin/Tcf-dependent transcription begins long before MBT. Thus,

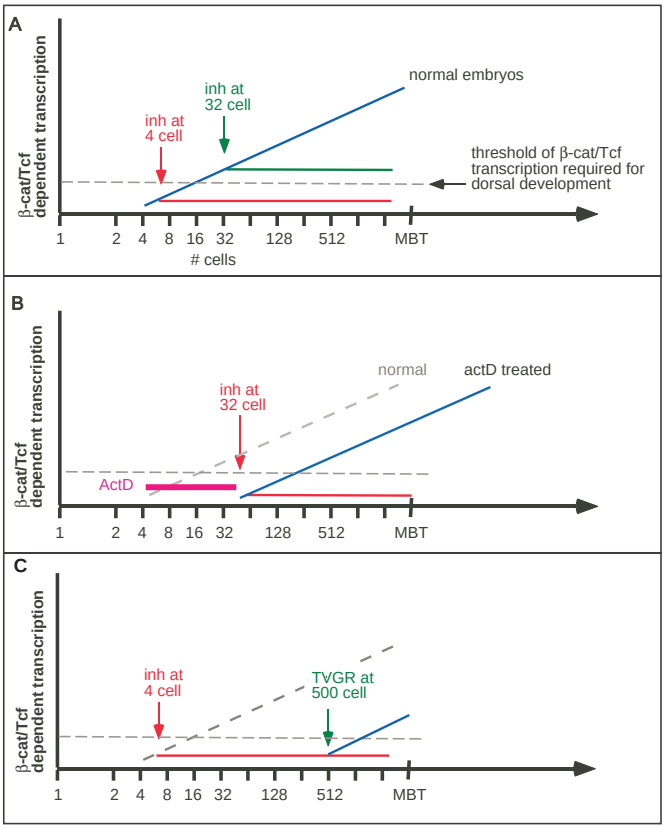

Fig. 6. Pre-midblastula transition (MBT) β-catenin/Xtcf3-dependent transcription in the regulation of dorsal development. (A) In this model, β-catenin/Xtcf-dependent transcription begins in early cleavage stages and continues throughout pre-MBT stages (blue line). Once transcription of these pre-MBT target genes reaches a critical level (horizontal dashed line), dorsal development at post-MBT stages can proceed. If β-catenin or Xtcf function is inhibited early (inh, red arrow) and inhibition is maintained throughout pre-MBT stages, dorsal development is blocked. However, if introduction of the inhibitor is delayed (inh, green arrow), then sufficient β-catenin/Xtcf-dependent transcription occurs to allow dorsal development. (B) Transient inhibition of transcription with actinomycin D (ActD) until the 32-cell stage does not disrupt dorsal development because the inhibitor is reversible and thus pre-MBT transcription resumes when ActD is removed. However, if ActD is followed directly by specific inhibition of β-catenin/Xtcf function at the 32-cell stage (inh, red arrow). at 32-cell), then dorsal development is blocked. (C) If Xtcf function is restored at the 500-cell stage (TVGR at 500 cell), then β-catenin/Xtcf-dependent transcription resumes and reaches the threshold required for dorsal development. Thus, Xtcf function at any time during pre-MBT stages appears to be sufficient for dorsal development. TVGR can also be activated post-MBT but does not rescue dorsal development under these conditions, indicating that β-catenin/Xtcf activity is required prior to MBT for dorsal development.

although β-catenin and Tcf complexes that form during pre-MBT stages may function in post-MBT stages, these complexes are clearly also functional before MBT. Furthermore, the transcriptional activity of Tcf is required prior to MBT in order to rescue dorsal development in ventralized embryos, as activation at the onset of MBT or later fails to rescue.

The midblastula transition, which is observed in diverse metazoan organisms, marks a fundamental change in embryonic cells that includes the beginning of large-scale zygotic transcription, slowing and loss of synchrony in the cell cycle, and an increase in cell motility (Newport and Kirschner, 1982a). The mechanism by which transcription is repressed prior to MBT has not been fully defined; however, the transcriptional machinery is clearly present prior to MBT, as pre-MBT *Xenopus* extracts contain active transcription factors and exogenous genes can be transcribed transiently prior to MBT (Newport and Kirschner, 1982b; Prioleau et al., 1994). Newport and Kirschner also showed that injection of plasmid DNA (pBR322) causes precocious transcription of endogenous genes, indicating that endogenous genes can be transcribed if an inhibitor(s) is titrated out by increased DNA. In addition, a plasmid containing the *c-myc* gene is transiently transcribed in pre-MBT embryos when the plasmid is pre-incubated with TATA binding protein (TBP) (Prioleau et al., 1994). These observations have led to the proposal that the repression of transcription in pre-MBT embryos is closely associated with assembly into chromatin (Newport and Kirschner, 1982b; Prioleau et al., 1994; Stancheva et al., 2002).

However, low-level pre-MBT transcription has been reported in *Xenopus* and *Drosophila* (Edgar and Schubiger, 1986; Nakakura et al., 1987; Shiokawa et al., 1989; Yasuda and Schubiger, 1992). For the most part, the importance of this early transcription has not been established; however, *engrailed* RNA begins to accumulate well before MBT in *Drosophila* and, most interestingly, loss of zygotic *engrailed* expression leads to loss of mitotic synchrony as early as the sixth cell cycle (Karr et al., 1989). These findings clearly indicate that pre-MBT transcription is also required for normal development of *Drosophila*, and is likely to be required for the development of diverse organisms.

On closer inspection, several *Xenopus* genes proposed to function in dorsal-ventral patterning show an apparent increase in expression during pre-MBT stages; in addition to *Xnr5* and *Xnr6*, these include *Msx1* (Suzuki et al., 1997) and *RGS4* (Wu et al., 2000). It is not clear why these genes are transcribed during pre-MBT stages when most zygotic genes are repressed. Pre-MBT transcription appears to be highly context-specific, as both β-catenin and VegT are required for the expression of *Xnr5* and *Xnr6* (Takahashi et al., 2000), yet not all β-catenin and VegT targets are transcribed before MBT begins; one possibility is that specific cis-elements in the promoters of pre-MBT genes recruit chromatin remodeling complexes that allow pre-MBT genes to escape the global inactivation of the genome during pre-MBT stages. Future work will include the identification of other zygotic genes transcribed prior to MBT, exploration of the mechanism that allows these genes to escape pre-MBT transcriptional repression, and characterization of the roles of pre-MBT transcription during development.

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