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The *Xenopus* homologue of *Bicaudal-C* is a localized maternal mRNA that can induce endoderm formation

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SUMMARY

In *Xenopus*, zygotic transcription starts 6 hours after fertilization at the midblastula transition and therefore the first steps in embryonic development are regulated by maternally inherited proteins and mRNAs. While animal-vegetal polarity is already present in the oocyte, the dorsoventral axis is only established upon fertilization by the entry of the sperm and the subsequent rotation of the egg cortex. In a screen for maternal mRNAs whose stability is regulated by this cortical rotation, we isolated the *Xenopus* homologue of the *Drosophila* gene *Bicaudal-C* (*xBic-C*). It encodes a putative RNA-binding molecule expressed maternally and localized predominantly to the vegetal half of the egg. Upon fertilization and cortical rotation, *xBic-C* mRNA is displaced together with the heavy yolk towards the future dorsal side of the embryo. In UV-ventralized embryos, *xBic-C* is polyadenylated less than in untreated embryos that undergo cortical rotation. Overexpression of *xBic-C* by injection of synthetic mRNA in whole embryos or in ectodermal explants leads to ectopic endoderm formation. This endoderm-inducing activity is dependent on the presence of the RNA-binding domain of the protein. In contrast to the two other known maternally encoded endoderm inducers, Vg1 and VegT, *xBic-C* ectopic expression leads specifically to endoderm formation in the absence of mesoderm induction.

Key words: Bicaudal, Egg determinant, Endoderm, Induction, Polyadenylation, VegT, Vg1, *Xenopus*

INTRODUCTION

One of the earliest events in embryonic patterning is the establishment of polarity and germ layer specificity. While the principal signaling events in the determination and early patterning of the neuroectoderm and mesoderm are starting to be understood, the establishment of the third germ layer, the endoderm, has been less intensively studied. In vertebrates, endoderm forms the lining of the gut, the pancreas, the liver, the gall bladder, the respiratory system and the derivatives of the pharyngeal pouches (for recent reviews see Gannon and Wright, 1999; Wells and Melton, 1999). In the past years, a variety of genes specifically involved in the determination and patterning of the endodermal germ layer have been isolated and studied using both ectopic expression in *Xenopus* embryos and mutational analysis in mouse and zebrafish. Due to these efforts, details of pathways involving genes like *HNF-3β*, *GATA4*, *GATA5*, *GATA6*, *Sox17α*, *Soxβ*, *Mixer* and other members of the *Mix* homeobox gene family are starting to emerge (Laverriere et al., 1994; Hudson et al., 1997; Dufort et al., 1998; Henry and Melton, 1998; Alexander et al., 1999; Reiter et al., 1999).

In *Xenopus*, the endoderm arises from the yolk-rich cells of the vegetal hemisphere (Dale and Slack, 1987). As in other vertebrates, these cells are not initially committed to the endodermal lineage (Minsuk and Keller, 1997; Zhang et al., 1998) and only after the onset of zygotic transcription at stage 10 is the commitment irreversible (Snape et al., 1987; Wylie et al., 1987). In zebrafish, this segregation occurs even later in development and uncommitted mesendodermal cell precursors can still be found at 40% epiboly (Warga and Nüsslein-Volhard, 1999). These differences in the timing of commitment also affect the specificity of expression of early endodermal genes. For example, the homeobox gene *Mixer* in *Xenopus* is only expressed in the future endodermal cells (Henry and Melton, 1998), whereas its zebrafish homologue is expressed in both endodermal and mesodermal progenitors (Alexander and Stainier, 1999). The molecular mechanisms underlying the developmental choice between endodermal and mesodermal fates remain largely unknown.

Culture of vegetal explants with and without cell dissociation has suggested that both cell autonomous and non-cell autonomous mechanisms are involved in the activation of the first endodermal-specific genes (Yasuo and Lemaire, 1999). Data from *Xenopus* and zebrafish point to an involvement of Nodal-related molecules in this process (Gamer and Wright, 1995; Henry et al., 1996; Alexander et al., 1999; Alexander and Stainier, 1999; Clements et al., 1999; Osada and Wright, 1999; Yasuo and Lemaire, 1999). However, Nodal-related molecules are involved in the formation of both mesoderm and endoderm, suggesting the existence of additional factors required to specifically generate the endodermal germ layer.

In *Xenopus*, two early endodermal determinants, *VegT* and *Vg1*, have been isolated to date. Both are encoded by maternal messages localized to the vegetal half of the oocyte. *Vg1*, like the zygotic Nodal-related molecules, is a member of the TGFβ superfamily and has been shown by gain- and loss-of-function analyses to function in the formation of dorsal mesoderm and endoderm (Henry et al., 1996; Joseph and Melton, 1998). *VegT*, is a transcription factor of the T-box class (Lustig et al., 1996; Zhang and King, 1996; Stennard et al., 1996; Horb and Thomsen, 1997). While overexpression of this cDNA leads to formation of both mesoderm and endoderm, initial loss-of-function analyses suggested that the maternal pool of this gene was only required for endoderm formation (Zhang et al., 1998). However, using improved antisense oligonucleotides this finding has recently been extended with the demonstration that depletion of *VegT* abolishes both endoderm and mesoderm induction (Kofron et al., 1999). Thus, like the zygotically expressed Nodal-related molecules, the maternal factors *Vg1* and *VegT* regulate the induction of both mesoderm and endoderm. It seems possible that additional unidentified maternal factors may exist to confer specificity for endodermal differentiation.

In addition to participating in the determination of the endodermal germ layer, the vegetal pole of the *Xenopus* embryo is also patterned along the dorsoventral axis (Henry et al., 1996). Dorsal development in amphibians is initiated by fertilization and cortical rotation, which displaces the heavy yolk towards the future dorsal side of the embryo (Nieuwkoop, 1977; Harland and Gerhart, 1997; Heasman, 1997). Recently, it was shown that cortical rotation can affect RNA stability and polyadenylation in the case of *Xwnt-11* (Schroeder et al., 1999). In an experiment designed to isolate mRNAs encoding dorsal determinants, we treated embryos with ultraviolet (UV) light to prevent cortical and a differential cDNA screen was carried out before the onset of zygotic transcription. The goal was to identify maternal mRNAs present in wild-type eggs but decreased in UV-treated embryos.

In this study, we present one of the genes identified, the amphibian homologue of the *Drosophila* gene *Bicaudal-C* (*Bic-C*), which provides an additional player in endoderm determination. *Drosophila Bic-C* is an RNA-binding molecule that functions as a repressor of translation in *Drosophila* embryos (Saffman et al., 1998). *Xenopus Bic-C* is a maternal mRNA that accumulates as a gradient from the vegetal to the animal pole. Upon fertilization and cortical rotation, *xBic-C* mRNA moves with the heavy yolk to the prospective dorsal side of the embryo and undergoes polyadenylation, which is inhibited by UV treatment. Overexpression of synthetic RNA showed that *xBic-C* specifically induces dorsal endoderm, both in whole embryos as well as in ectodermal explants. Mutational analysis of *xBic-C* cDNA revealed that the RNA-binding domain was required for this activity and that a construct in which this domain is deleted can function as a dominant-negative version, inhibiting endoderm formation by Vg1 in animal cap explants.

MATERIALS AND METHODS

Differential screening

To isolate genes regulated by egg cortical rotation, we screened for maternal mRNAs destabilized by UV irradiation. Poly(A)$^+$ RNA from normal or UV-treated pre-midblastula embryos (6 hours after fertilization at 20°C) was isolated. These mRNAs were then used to generate two differential probes by representational difference analysis (RDA, Braun et al., 1995): UV-treated subtracted with untreated embryos (UV-wt) and untreated subtracted with UV-treated embryos (wt-UV). Since this procedure involves poly(A) mRNA isolation, not only absolute differences in mRNA levels, but also mRNAs containing a longer poly(A) tail are selected. Both probes were then labeled and used in a differential screen of duplicate filters of an unfertilized egg library (Gont et al., 1993; Bouwmeester et al., 1996). Plaques positive for the wt-UV probe and negative for the UV-wt probe were analyzed further. Phages were converted into plasmid DNA according to manufacturer's instructions (predigested λZAP II/*Eco*RI cloning kit, Stratagene), sequenced from the 5′-end and analyzed by in situ hybridization.

Plasmid construction and RNA synthesis

To generate pCS2-*xBic-C-ΔKH* (deletion of amino acids 18-424), two fragments of the wild-type cDNA (one cut with *Bam*HI, digested with Mung Bean Nuclease and re-cut with *Eco*RI, the other cut with *Acc*I, filled in with Klenow and re-cut with *Xho*I) were ligated into pCS2 cut with *Eco*RI and *Xho*I. For pCS2-*xBic-C-ΔSAM* (deletion of amino acids 835-963), the xBic-C ORF was terminated at the *Pst*I site. All of the constructs were found to produce stable protein products in reticulocyte lysate assays (Promega). To generate synthetic mRNAs, the plasmids pCS2-*xBic-C*, pCS2-*xBic-C-ΔKH*, pCS2-*xBic-C-ΔSAM*, pCS2-*A-Vg1* and pCS2-*A-Xnr-1* were linearized with *Not*I and transcribed with SP6 RNA polymerase as described (Piccolo et al., 1999).

Embryo manipulations

Xenopus embryos obtained by in vitro fertilization were maintained in 0.1× modified Barth medium and staged according to Nieuwkoop and Faber (1994). RNA injections were performed at the 4- or 8-cell stage. Ectodermal explants were cut at stage 9, cultured in 0.5× MMR saline until sibling embryos reached the required stage. In situ hybridization was performed on whole embryos, hemisections or on paraplast sections as described (Lemaire and Gurdon, 1994; Belo et al., 1997; http://www.lifesci.ucla.edu/hhmi/derobertis/index.html).

RT-PCR analysis

Embryos and explants were processed for RT-PCR analysis as described (Sasai et al., 1995). The following primer sets were used: *α-actin*, *α-globin*, *Brachyury* (*XBra*), *EF1α*, *NCAM* and *Ornithine decarboxylase* (*ODC*) (Agius et al., 2000), *cerberus* (Bouwmeester et al., 1996), *IFABP* (Henry et al., 1996), *Mixer* (Henry and Melton, 1998), *HNF1β* and *Sox17β* (Hudson et al., 1997), *endodermin* (Edd) and *XlHBox8* (Sasai et al., 1996), *Otx*-2 (Sasai et al., 1995). New primer sets were used for *xBic-C*: 255 bp, forward 5′-AAAACTG-GAGGGAAAGGAAT-3′, reverse 5′-CAATCTCTTGCTGCTGG-AAT-3′, 25 cycles and *cytokeratin*: 217 bp, forward 5′-CACCAG-AACACAGAGTAC-3′, reverse 5′-CAACCTTCCCATCAACCA-3′, 20 cycles.

RESULTS

Isolation of *Xenopus Bicaudal-C*

In *Xenopus laevis*, the first hours of development until the mid-blastula transition (MBT) occur in the absence of de novo transcription and rely completely on maternally stored mRNAs and proteins (Newport and Kirschner, 1982). However, these early events can still be influenced after fertilization by treatments like UV irradiation or exposure to LiCl. Making the assumption that these treatments may not only affect the embryos at the protein but also at the mRNA level, we undertook a differential screen for maternal messages whose

stability might be changed by the ventralizing effect of UV irradiation.

Using this approach, numerous previously characterized cDNAs, including the maternal regulators *XLPOU-60* (Whitfield et al., 1995) and *β-catenin* (Wylie et al., 1996), were isolated. Among the novel cDNAs that we identified, was the *Xenopus* homologue of the *Drosophila* gene *Bicaudal-C*. We analyzed this gene in more detail since it was known from genetic studies in *Drosophila* that it plays an important role in early embryonic patterning. Mutations of *Bic-C* results in embryos exhibiting a double abdomen phenotype, which results from the premature translation of *oskar* mRNA in the anterior of the *Drosophila* egg (Mohler and Wieschaus, 1986; Mahone et al., 1995; Saffman et al., 1998).

To confirm that *xBic-C* mRNA was indeed differentially regulated before MBT, we performed RT-PCR analysis on stage 8 embryos treated with UV or LiCl, or left untreated. The cDNA was prepared in two different ways, using either hexamer primers for total RNA or oligo(dT) for poly(A)$^+$ mRNA. As seen in Fig. 1A,B, *xBic-C* total mRNA levels (indicated by dN_6) were not changed by any of the treatments, but oligo(dT)-primed cDNA *xBic-C* mRNA was significantly decreased in the UV-treated sample. These results suggest that we isolated *xBic-C* in our screen because UV irradiation decreased its polyadenylation. The phenomenon is not general, since other maternal messages such as *Xenopus dishevelled* (*xDsh*) and *glycogen synthase-3* (*xGSK-3)* were unaffected (Fig. 1B and data not shown). These results are in agreement with the recent report that UV irradiation interferes with polyadenylation of maternal *Xwnt-11* mRNA (Schroeder et al., 1999).

xBic-C is homologous to *Drosophila Bicaudal-C*

The 3300 nucleotides of a *xBic-C* clone were sequenced in both strands (GenBank accession number AF224746) and found to encode the protein shown in Fig. 2A. Comparison of xBic-C to its *Drosophila* homologue (Mahone et al., 1995) showed an overall identity of 36%. As shown in Fig. 2B, regions of high amino acid conservation lie within the five N-terminal RNA-binding modules known as KH (for RNA-binding protein K Homology) domains (Burd and Dreyfuss, 1994; Adinolfi et al., 1999), and the C-terminal SAM (for sterile alpha motif) domain (Schultz et al., 1997). SAM domains are involved in protein-protein interactions with other SAM domains (Stapleton et al., 1999; Thanos et al., 1999). In addition, a conserved tyrosine is believed to mediate interactions with SH-2 domain-containing proteins (Stein et al., 1996, Fig. 2B). The Serine/Glycine-rich region in the central part of the protein did not show significant conservation. Besides these previously described domains (Mahone et al., 1995), we observed a potential protein degradation motif (PEST sequence) at the N terminus of the molecule. Searching the databases, we also

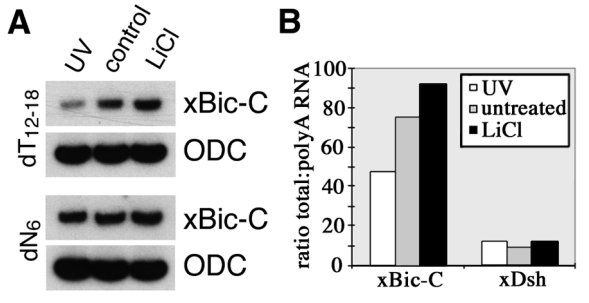

Fig. 1. Differential polyadenylation of *xBic-C* mRNA in UV-treated embryos. (A) RT-PCR analysis of the expression of *xBic-C* in stage 8 embryos ventralized by UV irradiation, untreated or dorsalized with LiCl, using either hexamer (dN6) or oligo(dT) (dT12-18) primers for the RT reaction to distinguish between polyadenylated and non-polyadenylated mRNA. *ODC* serves as loading control.
(B) Quantitation of the experiment in A by a Phosphorimager showing the level of polyadenylation. Note that while *xBic-C* is increasingly polyadenylated depending on progressive dorsalization, another maternal message, *xDsh*, is not regulated by this mechanism.

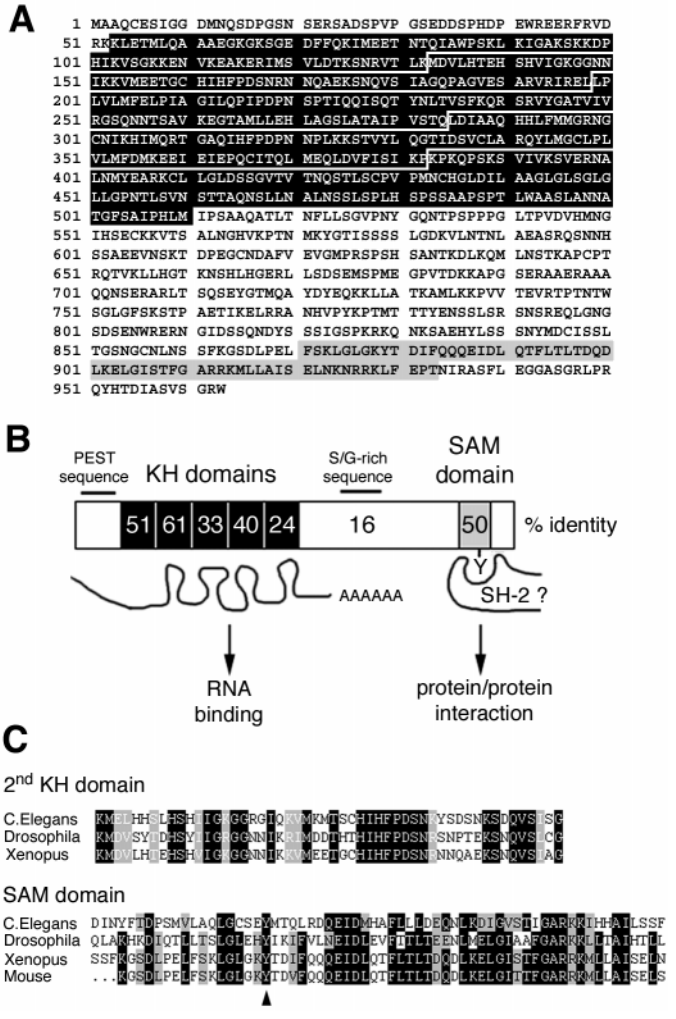

Fig. 2. Structure of xBic-C protein. (A) Primary structure deduced from *xBic-C* c-DNA; the 5KH domains are indicated in black and the SAM domain in gray. (B) Schematic representation of the structure of xBic-C showing a conserved PEST sequence, the KH domains, the S/G-rich sequence and the SAM interaction domain potentially involved in protein-protein interaction. Percentage of identity between *Xenopus* and *Drosophila* are indicated. (C) Sequence alignment of the second KH and the SAM domain of xBic-C with a genomic clone encoding the Bic-C homologue of *C. elegans* (accession number Z68337), the *Drosophila* (accession number U15928). In the case of the SAM domain, a mouse Bic-C EST (accession number AA276596) was analyzed as well. The arrowhead indicates the conserved tyrosine which upon phosphorylation has been proposed to lead to interaction with SH-2 domains.

identified a mouse EST and a genomic clone of *C. elegans* with sequence similarity to *xBic-C*. All *Bic-C* homologues share a strong conservation in the RNA-binding and SAM domains (Fig. 2C). In summary, screening for maternal mRNAs that were more abundant in wild-type than in UV-treated poly(A)$^+$ mRNA before MBT, we identified a homologue of the *Drosophila Bicaudal-C* RNA-binding protein.

Expression of *xBic-C*

Expression analysis using in situ hybridization showed that *xBic-C* is a maternal mRNA enriched in the vegetal half of the oocyte (Fig. 3A-C). Localization to the vegetal pole follows a time course similar to *Vg1* and *VegT* (Forristall et al., 1995; Lustig et al., 1996; Zhang and King, 1996; Stennard et al., 1996; Horb and Thomsen, 1997). In stage II oocytes, *xBic-C* is present uniformly in the cytoplasm; at stage III, it is concentrated towards the vegetal pole and finally clearly accumulates in the vegetal cortex by stage IV and V (Fig. 3A). However, unlike *VegT* or *Vg1*, *xBic-C* mRNA is not exclusively vegetal and can be detected by in situ hybridization on histological sections throughout the oocyte cytoplasm, forming a vegetal-to-animal gradient (Fig. 3B); sense mRNA controls were devoid of staining as in Fig. 4C below.

After fertilization, maternal *xBic-C* mRNA persists in the yolky endodermal cells until MBT (Fig. 3D,E). At MBT, maternal *xBic-C* mRNA is abundant in the large endodermal cells (Fig. 3E). By the gastrula stage, this endodermal core is found in the center of the embryo (Fig. 3F) in a distribution consistent with the vegetal rotation movements proposed recently by Winklbauer and Schürfeld (1999). At stage 12, an independent zygotic expression domain appears in the late dorsal blastopore lip (Fig. 3G,H). This expression continues in the midline of the neural tube and finally regresses to the tip of the tail (Fig. 3I,J and data not shown). Sections at stage 30 showed expression in the floorplate (Fig. 3K). At tailbud stage, a third independent expression domain was detected in the pronephros and pronephric duct (Fig. 3J,K). RT-PCR analysis confirmed the strong maternal component of *xBic-C* mRNA and its expression throughout development (Fig. 3L). In the remainder of this study, we focus on the early expression of *xBic-C* and its role in endoderm formation.

Interestingly, close analysis by whole-mount in situ hybridization of regularly cleaving 4-cell stage embryos with

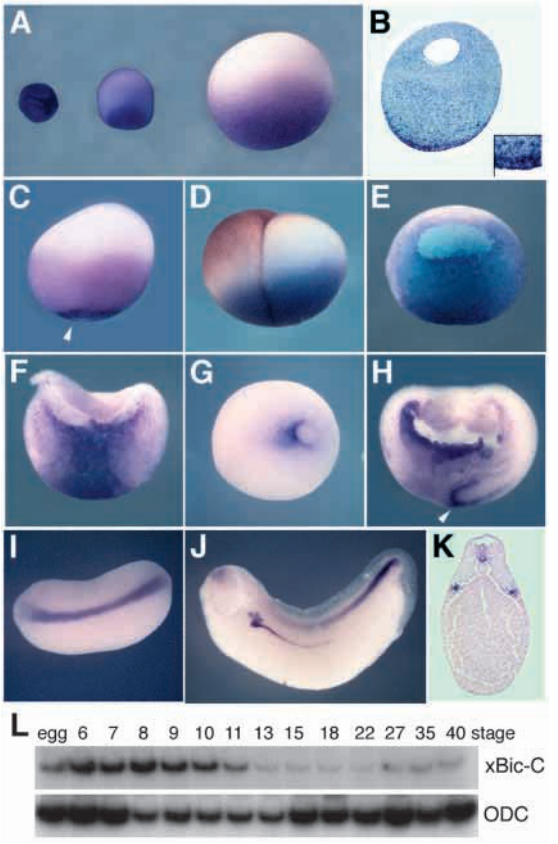

Fig. 3. Expression analysis of *x-Bic-C*. (A-K) Localization of *xBic-C* mRNA analyzed by in situ hybridization. (A) Albino oocytes at stages II, III and V. (B) In situ hybridization of a stage V oocyte section, inset shows a magnification of the punctate staining in the oocyte vegetal cortex. Low levels of *xBic-C* transcripts are found in the animal cytoplasm, but not in the germinal vesicle nucleus. (C) Double in situ hybridization showing vegetal expression of *xBic-C* and of the germ plasm marker *Xcat-2* (Forristall et al., 1995); the arrowhead indicates *Xcat-2*. Although both genes are localized to the vegetal pole, their distribution is clearly different. (D) Regularly cleaving embryos with a strong dorsoventral polarity at the 4-cell stage. Note the dorsal (light blastomeres) displacement of *xBic-C* mRNA upon cortical rotation. (E,F) Hemisections of stage 8 and 10.5 embryos on a plane perpendicular to the dorsoventral axis. (G,H) Stage 12 hybridized as a whole mount and as a hemisection; note zygotic expression in the dorsal lip (arrowhead). (I) Stage 24, dorsal view; (J) stage 30, lateral view; staining is seen in the pronephros, the pronephric duct and the posterior spinal cord. (K) Transverse section of a stage 30 embryo showing the staining in the pronephros anlage as well as in the floorplate. (L) RT-PCR analysis of the expression of *xBic-C* at different developmental stages using *ODC* as loading control.

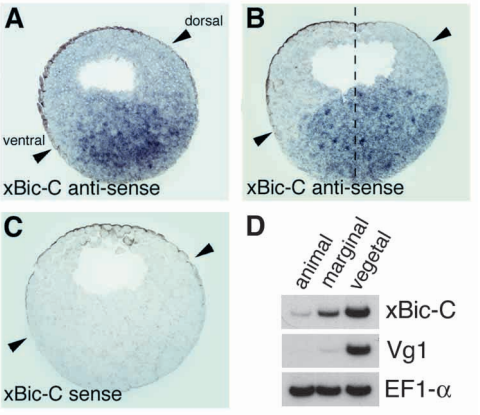

Fig. 4. *xBic-C* mRNA is displaced towards the dorsal side of the embryo by midblastula. In situ hybridization was performed on sagittal sections of embryos with strong dorsal/ventral polarity at stage 8. (A,B) *xBic-C* antisense probe, two different embryos are shown. (C) *xBic-C* sense probe. The arrowheads mark the rotation of the pigment indicating the future dorsoventral axis. While no signal for the *xBic-C* sense control could be detected, some staining in the animal region was present in the case of *xBic-C*. Note that the *xBic-C* signal is stronger in dorsal endoderm than in ventral endoderm. (D) RT-PCR analysis of stage 8 embryos dissected into animal, marginal and vegetal thirds showing that in contrast to *Vg1*, low levels of *xBic-C* mRNA can be detected in the animal pole; EF1-α shows equal loading of the RNA.

prominent dorsoventral polarity showed that upon fertilization and cortical rotation *xBic-C* mRNA is displaced along with the heavy yolk (Nieuwkoop, 1977) to the future dorsal side of the embryo (Fig. 3D, *n*=25). To confirm this asymmetric distribution, embryos were sectioned sagittally at mid-blastula along the dorsoventral axis and hybridized with *xBic-C* (Fig. 4A-C). Once again, *xBic-C* mRNA was shifted towards the dorsal half of the embryo (Fig. 4A,B). As in the case of oocytes, *xBic-C* mRNA was weakly detectable throughout the embryo, even in the animal cap (compare Fig. 4A to C). The presence of *xBic-C* mRNA at low levels in the animal cap region was further documented by RT-PCR analyses using dissected animal, marginal and vegetal thirds of 6 hour (pre-MBT) *Xenopus* embryos (Fig. 4D). We conclude that *xBic-C* is a novel vegetally enriched maternal transcript that is displaced together with the heavy yolk towards the dorsal side after fertilization.

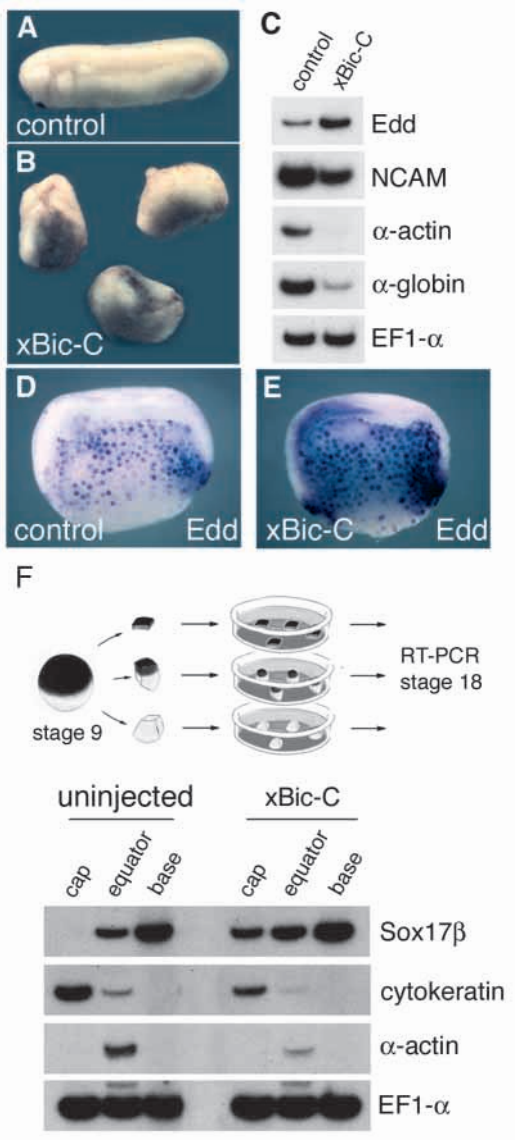

Fig. 5. Overexpression of *xBic-C* mRNA leads to excessive endoderm formation. Synthetic RNA for *xBic-C* (160 pg) was injected marginally into each of the 4 blastomeres of 4-cell-stage embryos. (A,B) Phenotypic appearance at stage 26; injected embryos did not undergo proper gastrulation movements. (C) RT-PCR analysis of similar embryos at stage 26 showed elevated levels of *endodermin* (*Edd*) expression, but a decrease in the mesodermal markers *α-actin* and *α-globin*. *NCAM* was less decreased. (D,E) In situ hybridization of *Edd* on hemisections of embryos injected as described above and harvested at stage 10.5. (F) Control and *xBic-C-injected* embryos were dissected at blastula stage (stage 9) into animal cap, equatorial and vegetal region. Explants were kept in culture until sibling embryos reached stage 18 and then analyzed by RT-PCR using *Sox17β* as a marker for endoderm, *cytokeratin* for epidermis and *α-actin* for mesoderm. Note that upon overexpression of *xBic-C* the endodermal germ layer extends into the animal cap. *EF1α* indicates equal loading of RNA.

xBic-C induces dorsal endoderm

To test *xBic-C* function during *Xenopus* development, we overexpressed its product. Synthetic mRNA encoding *xBic-C* was injected into the marginal region of all four blastomeres at the 4-cell stage and embryos allowed to develop until stage 26. As seen in Fig. 5B, ectopic expression of *xBic-C* severely impaired normal development. The embryos did not gastrulate properly, epiboly failed and the blastopore did not close. RT-PCR analyses of these injected embryos (Fig. 5C) showed that mesodermal markers (*α-actin* and *α-globin*) and the pan-neural marker *NCAM* were reduced or absent, whereas *endodermin* (*Edd*), a marker of endoderm differentiation (Sasai et al., 1996), was increased. This upregulation of *Edd* could be detected by in situ hybridization already by the early gastrula stage (Fig. 5D,E). In addition, vegetal injections of *xBic-C* increased the expression of the endodermal markers *Mix.1* and

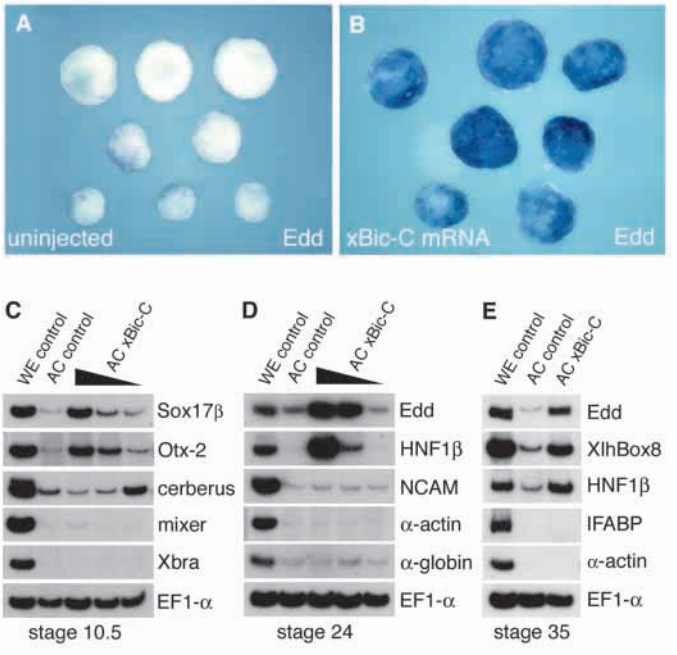

Fig. 6. Ectodermal explants expressing *xBic-C* form dorsal endoderm. Either 160 pg (B,E) or three two-fold dilutions of *xBic-C* (160, 80, 40 pg; C,D) of *xBic-C* mRNA were injected into each animal blastomeres at the 4-cell stage, dissected at stage 9 and analyzed by in situ hybridization at stage 35 with *Edd* (A,B, 160 pg injected) or by RT-PCR at stage 10.5 (C), stage 24 (D) and stage 35 (E). Injections of *xBic-C* mRNA caused the induction of dorsal endodermal markers, but not of the induction of the posterior (small intestine) marker *IFABP*.

Sox17β by about 2-fold but did not appear to change the endogenous levels of *Mixer* mRNA (data not shown).

Microinjection of *xBic-C* mRNA leads to allocation of additional embryonic cells to endodermal fates, as illustrated by the following experiment. Injected and control embryos were dissected before the onset of gastrulation (stage 9) into animal cap, equatorial and vegetal fragments, which were then cultured until sibling embryos reached stage 18 and analyzed by RT-PCR (Fig. 5F). Ectopic endoderm differentiation marked by *Sox17β* (Hudson et al., 1997) was observed following radial *xBic-C* injection in all three types of explants, including the animal cap, while markers for mesodermal (*α-actin*) and epidermal (*cytokeratin*) fates were reduced (Fig. 5F).

To characterize further endoderm induction by *xBic-C*, mRNA was injected into the 4 animal blastomeres of 8-cell-stage embryos and ectodermal explants analyzed at stages 10.5, 24 and 35. Expression of the pan-endodermal marker *Edd* was readily detected at stage 35 by in situ hybridization in *xBic-C*-injected animal caps (Fig. 6A,B). In RT-PCR assays, overexpression of *xBic-C* resulted in specific upregulation of endodermal marker genes in the animal explants. Neither mesodermal markers like *Xbra*, *α-actin* and *α-globin* nor the neural marker *NCAM* could be detected at any stage (Fig. 6C-E). Interestingly, not all endodermal marker genes were activated equally. At stage 10.5, expression of *xBic-C* induced *Sox17β, Otx-2* and *cerberus*, but not the early endodermal marker *Mixer* (Fig. 6C). The lack of *Mixer* induction strengthens the view that multiple pathways for endoderm formation may exist (Henry et al., 1996; Henry and Melton, 1998; Alexander et al., 1999). At stage 24, the dorsal endodermal marker *HNF1β* (Demartis et al., 1994) and the pan-endodermal marker *Edd* were induced (Fig. 6D). From stage 35 on, a broader spectrum of endodermal markers is available and permitted us to distinguish between dorsal and ventral endoderm (Fig. 6E). *xBic-C* mRNA induced *XlHBox-8* (Wright et al., 1989), a marker for dorsal endoderm, but not the posterior (small intestine) endodermal marker *IFABP* (Shi and Hayes, 1994). This preference for dorsal endoderm formation is noteworthy, since it correlates with the early dorsoventral asymmetry of maternal *xBic-C* mRNA after cortical rotation (Figs 3D, 4A,B).

Dominant-negative *xBic-C* interferes with endoderm formation in animal cap explants

We next tested whether one could interfere with the wild-type function of *xBic-C* by generating a dominant-negative version of the molecule. It has been shown in *Drosophila* that the KH domains are essential for the function of Bicaudal-C (Mahone et al., 1995). Although, in the fruit fly, mutations in the SAM domain have not been recovered, the strong conservation of this motif in the different organisms (Fig. 2C) suggested that this region should also be of functional importance. We therefore generated two constructs (Fig. 7A), one lacking the RNA-binding domains (*xBic-C-ΔKH*) and another lacking the putative protein interaction domain (*xBic-C-ΔSAM*). These mutants were then tested in animal cap explants for their ability to induce endoderm (Fig. 7B). Microinjected *xBic-C-ΔKH* was not able to induce *Edd*. However, *xBic-C-ΔSAM* was a strong endodermal inducer (Fig. 7B, lane 5) indicating that the function of the SAM domain is dispensable for endoderm induction. To find out whether the *xBic-C-ΔKH* mutant form could dominantly interfere with wild-type xBic-C protein, both mRNAs were injected alone or in combination into animal caps (Fig. 7C). The *ΔKH* version of *xBic-C* blocked *Edd* induction by the wild-type protein (Fig. 7C, lane 5). This suggested that *xBic-C-ΔKH* may function as a dominant-negative version of the protein.

Finally, we tested whether *xBic-C-ΔKH* in animal cap assays also interfered with endoderm induction by other molecules. We used *A-Vg1*, an Activin βB pre-pro-region fused to the mature part of Vg1, which permits efficient processing of Vg1

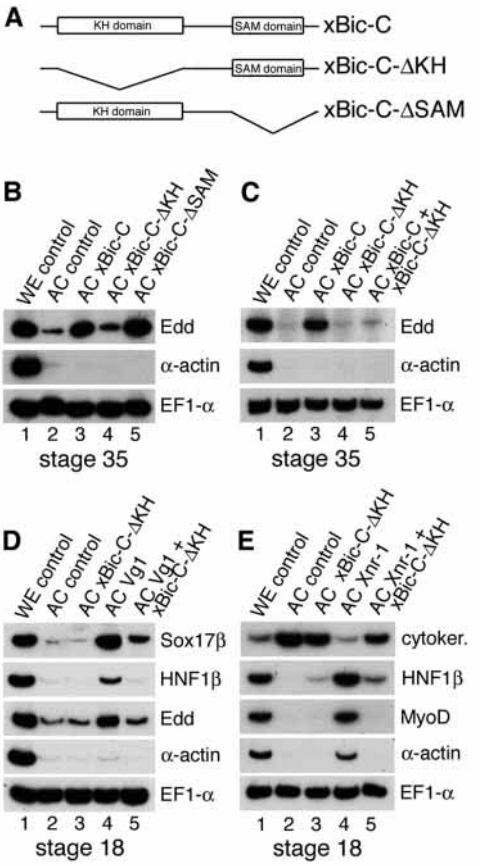

Fig. 7. A mutated version of *xBic-C* lacking the RNA-binding domain counteracts the activity of wild-type *xBic-C* and *A-Vg1* in animal cap explants. (A) Schematic representation of the constructs. (B) 8-cell embryos were injected into each animal blastomere with 160 pg *xBic-C*, *xBic-C-ΔKH* or *xBic-C-ΔSAM*, dissected at stage 9 and analyzed at stage 35 by RT-PCR for endoderm induction, marked by *Edd* transcripts. (C) *xBic-C* (320 pg), and *xBic-C-ΔKH* (1.2 ng) were injected either alone or in combination into animal blastomeres and analyzed as outlined in B. (D) *A-Vg1* (50 pg), *xBic-C-ΔKH* (300 pg), and *xBic-C-ΔKH* together with *A-Vg1* mRNA were injected into each animal blastomere at the 8-cell stage and the ectodermal explants analyzed at stage 18 for the endodermal marker genes *Sox17β*, *HNF1β* and *Edd*, as well as for the mesodermal *α-actin*. (E) Ectodermal explants injected into each animal blastomere at the 8-cell stage with *A-Xnr-1* (50 pg), *xBic-C-ΔKH* (300 pg) and *xBic-C-ΔKH* together with *A-Xnr-1* mRNA were analyzed by RT-PCR at stage 18 for the ectodermal marker *cytokeratin* (*cytoker.*), the endodermal marker *HNF1β* and the mesodermal markers *MyoD* and *α-actin*. *EF1α* serves as a loading control.

(Thomsen and Melton, 1993). Injection of a high amount of *A-Vg1* mRNA (50 pg) into all 4 animal blastomeres caused the induction of endoderm in the absence of mesoderm, as indicated by the upregulation of *HNF1β*, *Edd* and *Sox17β,* and the absence of α-*actin* at stage 18 (Fig. 7D, lane 4). Co-injection of *xBic-C-ΔKH* inhibited the endodermal-inducing activity of *A-Vg1* mRNA (Fig. 7D, lane 5). This suggests that maternal *xBic-C* is required for endoderm formation in this experimental assay. Endogenous *xBic-C* presumably functions in the embryo in a pathway either downstream or in parallel to *Vg1*.

To test whether *xBic-C-ΔKH* diverts animal cap cells towards an epidermal cell fate at the expense of endoderm and mesoderm, we analyzed animal caps injected with *A-Xnr-1* mRNA at a concentration that gives rise to endoderm (marked by *HNF1β*) and mesoderm (marked by *α-actin* and *MyoD*). As shown in Fig. 7E, lanes 4 and 5, *xBic-C-ΔKH* inhibited the formation of endoderm and mesoderm, restoring epidermal differentiation (marked by *cytokeratin*). Thus, whereas wild-type *xBic-C* mRNA promotes endoderm formation in animal caps (Fig. 6), the putative dominant-negative *xBic-C-ΔKH* had the opposite effect, causing endomesoderm to differentiate into epidermis.

It should be stated that attempts at preventing or decreasing endoderm differentiation in intact microinjected *Xenopus* embryos by overexpression of *xBic-C-ΔKH* mRNA gave inconclusive results. Using the same experimental design as in Fig. 5F, we failed to observe reduction of endoderm or vegetal displacement of mesoderm (not shown). In addition, vegetal explants injected with *xBic-C-ΔKH* failed to express mesodermal markers (*α-actin* and *α-globin*) and to elongate as is known to occur in endoderm-depleted embryos (Zhang et al., 1998). Since the levels of maternal *xBic-C* mRNA are very high in the vegetal pole and much lower in the animal pole, we assume that the endogenous *xBic-C* product can be competed more effectively in animal cap explants by the dominant-negative construct. We also note that the *xBic-C-ΔSAM* construct has a strong endoderm-inducing activity despite lacking a functional SAM protein interaction domain (Fig. 7B, lane 5). This construct could provide a useful reagent for in vitro differentiation of pluripotent stem cells towards an endodermal pathway.

DISCUSSION

Bicaudal-C in *Drosophila* and *Xenopus*

In this study, we describe the isolation and functional characterization of the *Xenopus* homologue of *Bicaudal-C* (*xBic-C*). In *Drosophila*, *Bic-C* is required for follicle cell migration and anteroposterior patterning of the oocyte. It was originally isolated as one of several maternal-effect mutants causing striking double-abdomen phenotypes (Bull, 1966; Nüsslein-Volhard, 1977; Mohler and Wieschaus, 1986; Schupbach and Wieschaus, 1991). Further analysis demonstrated that the double-abdomen phenotype is caused by the ectopic and premature translation of *oskar* mRNA in the anterior of the *Drosophila* oocyte, which in turn leads to the mislocalization of the posterior determinant *nanos* in the anterior (Mahone et al., 1995; Saffman et al., 1998). The original *Drosophila bicaudal* mutation (Bull, 1966; Nüsslein-Volhard, 1977) has recently been shown to encode for β-NAC protein, a subunit of the Nascent polypeptide Associated Complex, that affects directly the localization and translation of *nanos* (Markesich et al., 2000). Two other members of the *Bicaudal* group, *Bicaudal-D* and *egalitarian*, encode molecules involved in the transport of maternal messages from the nurse cells to the posterior of the oocyte via microtubule motors (Suter et al., 1989; Theurkauf et al., 1993; Mach and Lehmann, 1997).

Bic-C functions as a repressor of the translation of *oskar* mRNA (Mahone et al., 1995; Saffman et al., 1998). Similarly, a number of KH domain molecules from *C. elegans* have been shown to bind to their target RNA and repress their translation (Jan et al., 1999; Saccomanno et al., 1999). The functional importance for the KH RNA-binding motif is also underscored by mutations in other genes like *FMR-1* (Fragile-X syndrome) and *quaking*, in which mutations within the KH domain lead to severe developmental patterning defects (Siomi et al., 1994; Ebersole et al., 1996; Zorn and Krieg, 1997). In the case of *Drosophila Bic-C*, the allelic series supports the notion that disruption of protein-RNA interactions are the basis of the phenotype. The two strongest alleles either delete two of the five KH RNA-binding domains or introduce a point mutation in the third KH domain changing the affinity of Bic-C for RNA binding (Saffman et al., 1998).

As shown here, the deletion of all five KH domains blocks the ability of *xBic-C* to induce endoderm. Interestingly, the other main motif found in Bic-C, the SAM domain, is strongly conserved in all organisms analyzed arguing for an important function. Our ΔKH mutant has a dominant-negative effect that is presumably mediated by the SAM domain (Fig. 7A). However, the other mutant described here, lacking the SAM domain but having an intact RNA-binding domains, had an endoderm-inducing activity comparable to the wild-type protein (and in some experiments, not shown, even greater) when overexpressed in animal caps. This suggest that the *ΔSAM* construct may still be capable of repressing translation of target mRNAs in *Xenopus* embryos.

A screen for maternal mRNAs stabilized by cortical rotation before MBT

In *Xenopus*, the first 6 to 7 hours of development rely completely on maternally stored mRNAs and proteins. By the end of this period, the main decisions concerning the allocation of cells to germ layers have been already achieved (Heasman et al., 1989; Heasman, 1997). During this period, the timely activation of early gene products must depend entirely on post-transcriptional control, since there is no transcription until midblastula transition (Newport and Kirschner, 1982). A good example is provided by VegT, which has abundant maternal transcripts. Only small amounts of protein are detected in the oocyte, and *VegT* translation takes place after fertilization and peaks at early gastrula stages (Stennard et al., 1999). While the mechanism of gene silencing in this particular case is still unknown, numerous studies point towards cytoplasmic polyadenylation as an important control mechanism (for review see Richter, 1999). Polyadenylation may not only affect general gene activation, but may also provide regional information. A recent report has shown that *Xwnt-11* is specifically polyadenylated and translated in the dorsal side of the embryo (Schroeder et al., 1999). This is in agreement with

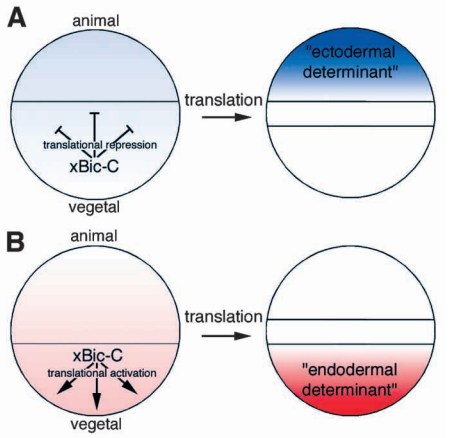

Fig. 8. Hypothetical models for the action of xBic-C RNA-binding molecule as a translational repressor of an ectodermal determinant (A) or as a translational activator of an endodermal determinant (B) – see text for details.

our study, in which we screened for cDNAs whose stability might be decreased in UV-ventralized pre-MBT embryos. The differential screening was done using poly(A)$^+$ RNA, resulting in the isolation of mRNAs in which the polyadenylation level was changed by the cortical rotation of the zygote. In addition to *xBic-C* other cDNAs were isolated, suggesting that other mRNAs will be regulated through this mechanism. The most abundant cDNA isolated encodes the POU-domain molecule *XLPOU-60*, whose state of polyadenylation is precisely regulated during the early cleavage stages (Whitfield et al., 1995). Preliminary data indicate that *XLPOU*-60 polyadenylation, like *Xwnt-11* and *xBic-C* is decreased by UV (unpublished results).

xBic-C and endoderm formation

In contrast to the *Drosophila* mutations, gain-of-function analysis in *Xenopus* did not affect early anteroposterior patterning. This could reflect temporal differences in the activity of the gene in *Drosophila* and *Xenopus*. The bicaudal embryos in *Drosophila* are caused by the absence of *Bic-C* during oocyte maturation and the resulting defective transport of maternal mRNAs from the nurse cells to the posterior pole of the oocyte (Mahone et al., 1995; Saffman et al., 1998). This period in development is not readily accessible in amphibians and synthesis of maternal mRNA molecules occurs in the oocyte itself. Furthermore, the analysis of the *Bic-C* maternal-effect locus in *Drosophila* is based on the use of heterozygous eggs, since the mutations are so severe that homozygous oocytes fail to complete oogenesis. Therefore, it is not known whether *Drosophila* embryos would exhibit an endodermal phenotype in the absence of maternal *Bic-C* product. However, in situ analysis has shown a transient expression of *Bic-C* in early cleavage *Drosophila* embryos with a somewhat higher concentration at the posterior pole (Mahone et al., 1995). Interestingly, the earliest genes identified in *Drosophila* shown to be involved in endoderm formation, *forkhead* and *HNF4-(D)*, are also localized to the posterior pole as early as the blastoderm stage (Weigel et al., 1989; Zhong et al., 1993).

In *Xenopus* embryos injected radially into each of the four blastomeres, the embryo develops with an increased proportion of endodermal cells. This phenotype occurs at the expense of both mesodermal and ectodermal cell fates (Fig. 5). The relationship between *xBic-C* and the inhibition of mesoderm formation may be secondary to the expansion of the endoderm. Although marked inhibition of late markers such as *α-actin* and *α-globin* was observed, the early pan-mesodermal marker *Xbra* was only slightly inhibited in injected embryos (data not shown). The putative dominant-negative *xBic-C-ΔKH* was able to inhibit the differentiation of both endoderm and mesoderm by *Xnr-1* mRNA in animal caps (Fig. 7E). Wild-type *xBic-C* mRNA causes endodermal differentiation in animal caps, whereas *xBic-C-ΔKH* has the opposite effect, restoring epidermal differentiation in these cells.

xBic-C as a putative regulator of an ectodermal determinant

How does *xBic-C* function in endodermal patterning of the embryo? Based on the findings in *Drosophila*, *Bic-C* most likely is an RNA-binding protein involved in the post-transcriptional regulation. Since so far no target RNA for *Bic-C* has been characterized, two models can be envisioned, as depicted in Fig. 8.

First, *xBic-C* might act as a translational repressor inhibiting genes in the vegetal half of the embryo and thereby preventing their activity. Target genes may include animal pole determinants, perhaps in the form an ectodermal (epidermal) determinant whose RNA could be uniformly expressed or enriched in the animal pole (Fig. 8A). The existence of such a maternal ectodermal-determining factor has been proposed by Zhang et al. (1998). It was found that embryos depleted for maternally stored *VegT* mRNA not only lack the endodermal germ layer, but also expand the ectoderm into more vegetal regions (Zhang et al., 1998; Kofron et al., 1999). Further support for this model comes from the analysis of *Bix4*, a direct target gene of *VegT*. Promoter analysis has identified a binding site for an as yet unknown repressor that excludes Bix4 from the animal pole, suggesting the existence of an animal-to-vegetal gradient that counteracts the activity of endodermal inducers (Casey et al., 1999). *xBic-C* could be required to establish an effective animal-to-vegetal gradient of this proposed ectodermal determinant protein. In *Xenopus*, most mRNAs enriched in the animal hemisphere are not strictly localized to the animal region, but frequently only about four times enriched compared to the vegetal hemisphere (Bashirullah et al., 1998; King et al., 1999; Mowry and Cote, 1999). The existence of a vegetal translational inhibitor that would sharpen the morphogen gradient is a very attractive scenario.

As a second hypothesis, *xBic-C* could act as a translational activator promoting translation of endoderm-specific genes in the vegetal pole (Fig. 8B). Since *xBic-C* is maternally deposited in endoderm, its target RNA could be maternally encoded as well. So far only two maternal messages, *VegT* and *Vg1*, have been shown to affect endoderm development. We did not test directly whether exogenous xBic-C increases the translation of either of these or other mRNAs. Alternatively, *xBic-C* could act on early zygotic genes expressed in the endoderm after MBT (Yasuo and Lemaire, 1999; Agius et al., 2000).

However, the observation that loss-of-function of *Drosophila Bic-C* increases *oskar* translation makes the

repressor scenario a more attractive one. Distinguishing between the two models will require the isolation of the endogenous mRNA targets to which *xBic-C* binds in vivo. The precise role of *xBic-C* in endoderm formation in vivo remains so far unresolved. A *xBic-C* mutant lacking the RNA-binding domain (*xBic-C-ΔKH*) prevented endoderm induction in animal cap assays but not in intact embryos. Loss-of-function analysis of *xBic-C* will have to wait for the interference with the protein function during oogenesis or for the depletion of the maternal mRNA pool using antisense oligonucleotide technology (Heasman et al., 1991).

In conclusion, *xBic-C* introduces a new player in germ layer specification during early *Xenopus* development. It is encoded by a maternal mRNA that is preferentially localized in the vegetal pole, although transcripts can be found at lower levels in the animal cytoplasm. The putative RNA-binding protein may influence endoderm formation by regulating the translation of maternal determinants of germ layer specification. Other maternally encoded endodermal determinants have been identified in *Xenopus*. Unlike *Vg1* and *VegT*, overexpression of *xBic-C* does not induce mesoderm induction in any assay. In sum, *xBic-C* provides a novel maternal determinant that promotes formation of the endodermal germ layer during early embryonic development.

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