

***Xenopus* VegT RNA is localized to the vegetal cortex during oogenesis and encodes a novel T-box transcription factor involved in mesodermal patterning**

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SUMMARY

An RNA localized to the vegetal cortex of *Xenopus* oocytes encodes a novel T-box protein (VegT) capable of inducing either dorsal or posterior ventral mesoderm at different times in development. VegT is a nuclear protein and its C-terminal domain can activate transcription in a yeast reporter assay, observations consistent with VegT functioning as a transcription factor. Zygotic expression is dynamic along the dorsoventral axis, with transcripts first expressed in the dorsal marginal zone. By the end of gastrulation, *VegT* is expressed exclusively in posterior ventral and lateral mesoderm and is excluded from the notochord. Later expression is confined to a subset of Rohon-Beard cells, a type of primary sensory neuron. In animal cap

assays, VegT is capable of converting prospective ectoderm into ventral lateral mesoderm. Such ectopic expression of VegT induces its own expression as well as that of *Xwnt-8* in caps, suggesting that a Wnt pathway may be involved. Mis-expression of VegT in dorsal animal blastomeres fated to contribute to brain suppresses head formation. Our results suggest that VegT is a localized transcription factor, which operates sequentially in several developmental pathways during embryogenesis, including dorsoventral and posterior patterning of mesoderm.

Key words: *Xenopus*, *VegT*, RNA, vegetal cortex, oogenesis, transcription factor, mesoderm, T-box

INTRODUCTION

The basic spatial organization of the amphibian embryo is established early in development through the sequential formation of localized centers with inductive capacities. Evidence suggests that the first such center to be elaborated is the oocyte's vegetal cortex, a region rich in cytoskeletal components and localized RNAs (reviewed by King, 1995; Elinson et al., 1993; Forristall et al., 1995). The initial event that introduces a dorsoventral axis into the radially symmetrical egg is the movement of cortex material relative to the deep ooplasm before first cleavage (reviewed by Gerhart et al., 1989). Cytoplasmic transfer and UV rescue experiments (Gimlich and Gerhart, 1984; Gimlich, 1986) indicate that dorsal determinants are specifically localized in the vegetal cortex and that cortical rotation moves and/or modifies the determinant on the future dorsal side (Vincent et al., 1986; Holowacz and Elinson, 1993; Fujisue et al., 1993). Cortical rotation sets up a vegetal dorsalizing domain, the Nieuwkoop center, which consists of a small group of dorsovegetal cells (reviewed by Gerhart et al., 1989). These cells are competent to induce overlying marginal zone cells to form the third center, Spemann's organizer, which is capable of organizing a complete second axis when grafted to the ventral side (Spemann, 1938). The organizer contains information for patterning both anteroposterior and dorsoventral mesoderm (reviewed by Dawid, 1994).

Results from dominant negative receptor experiments have strongly indicated that some members of the TGF β family such as BVg1 or activin are involved in dorsal mesoderm induction

(Hemmati-Brivanlou and Melton, 1992), while members of the FGF family and BMP4 are required for ventral mesoderm formation (Amaya et al., 1991; Graff et al., 1994). Other genes have been shown to modify the mesoderm, including *Xwnt-8*, *noggin*, *goosecoid*, *chordin* and *nodal*-related genes (reviewed by Kessler and Melton, 1994; reviewed by Harland, 1994; Jones et al., 1995). Although much information is available about mesoderm-inducing factors, little is known about how the initial signals are transduced to activate the early genes after MBT. It has been shown that activin can activate a maternal factor long before MBT (Huang et al., 1995). Recently identified Mad proteins are downstream effectors of mesoderm-inducing factors, but whether Mads directly activate immediate early genes has yet to be determined (Graff et al., 1996). Clearly, an important goal is to understand the relationship between maternal prepatterning and zygotic gene expression that results in cell type and tissue differentiation.

Although an inductive event is required for mesoderm formation (Gimlich, 1986), induction does not account for the observation that both *goosecoid* and *Xwnt-8* can be activated in the correct dorsoventral region of the embryo in the complete absence of cell-cell interactions (Lemaire and Gurdon, 1994). Lemaire and Gurdon (1994) have suggested that formation and patterning of mesoderm requires the simultaneous activation of two different pathways: an inductive pathway and a cell-autonomous pathway. The maternal factors that could participate in the later pathway are unknown.

Differential screening and expression cloning have been used to identify genes that are good candidates for involvement in

the earliest steps of embryonic patterning. Our strategy was to select maternal transcripts localized in the vegetal cortex by differential screening and then to test for patterning activity. This approach has yielded *VegT*, a new member of the T-box family whose original member was the mouse *Brachyury* gene (reviewed by Herrmann and Kispert, 1994). *Brachyury*, in both mouse and zebrafish, functions as a DNA-sequence-specific transcription factor and is important in axial development (Kispert et al., 1995). The *VegT* expression pattern is dynamic along the animal/vegetal axis during oogenesis and, later, along the dorsoventral axis in the marginal zone. Unlike the *Brachyury* members of the T-box family, *VegT* is never expressed in the notochord. We show that *VegT* is a nuclear protein and that its C-terminal region can activate transcription in a yeast reporter system. We also show that ectopic expression of *VegT* can induce *Xwnt-8* expression and converts prospective ectoderm into ventral mesoderm. Mis-expression of localized *VegT* in dorsal animal blastomeres results in the suppression of head formation. The results presented here support a role for *VegT* as a localized transcription factor that patterns mesoderm along the dorsoventral and posterior axis in *Xenopus*.

MATERIALS AND METHODS

Embryo manipulations

An adult ovary was treated with collagenase to obtain oocytes free of follicle cells. Embryos were obtained by in vitro fertilization, dejellied and cultivated in 0.1× Modified Barth Saline with Hepes (MBSH) (Gurdon, 1977). Embryos were staged according to Nieuwkoop and Faber (1967). Animal cap explants were isolated at stage 8 and cultured in isolation in 0.5× MBSH until the desired stage.

Probe preparation and differential screening

To prepare cDNA probes for differential screening, ten vegetal or animal cortices were isolated from stage VI oocytes and total cortical RNA was prepared as described previously (Elinson et al., 1993). The synthesis and PCR amplification of cortical cDNA were performed according to Brady et al. (1990) with the following modifications. RNA equivalent to three cortices was denatured at 70°C for 10 minutes and then added to reverse transcription buffer (50 mM Tris HCl [pH 8.3], 75 mM KCl, 3 mM MgCl₂, 0.25% NP-40, 10 mM DTT, containing 250 ng/ml primer-adaptor *XC1*: CTC GAG ACG CTG TCT AGA (T)₁₅, 2 U/μl RNasin [Promega], and 2.5 mM each of dATP, dGTP, dCTP and dTTP). Reverse transcription was initiated by adding 200 U of SuperScript™ II RNase H⁻ reverse transcriptase (Gibco BRL) followed by incubation at 37°C for 20 minutes. Reaction mixtures were inactivated at 70°C for 10 minutes. One third of the mixture was used in the poly(dA)-tailing reaction carried out according to the manufacturer's instructions (Boehringer). One third of the poly(dA)-tailed cDNA was brought to 100 μl in PCR buffer: 10 mM Tris-HCl [pH 9.0], 50 mM KCl, 2.5 mM MgCl₂, 100 mg/ml bovine serum albumin, 0.1% Triton X-100 and containing 0.5 mM of dATP, dGTP, dCTP, dTTP, 10 U of Taq polymerase and 5 ng of the primer-adaptor *XC-1*. PCR amplification was performed as follows: 94°C (6 minutes), 42°C (3 minutes) and 72°C (6 minutes) followed by an additional 45 cycles: 94°C (1 minute), 53°C (2 minutes) and 72°C (6 minutes plus 10 seconds extension for each cycle). The PCR products were analyzed by Southern blot hybridization. The blot was probed with α-³²P-labelled *Xcat-3*, a vegetally localized RNA, to ensure cortical RNAs were amplified in a representative manner (Elinson et al., 1993). Amplified cDNAs were labeled with [α-³²P]dCTP by random priming (Boehringer). *Vg1*, *Xcat-2* and *Xcat-3* cDNA probes were synthesized, mixed together and used to screen the oocyte cDNA library.

An amplified oocyte cDNA library (7,000 pfu) 50-fold enriched for vegetally localized mRNAs (see Mosquera et al., 1993) was plated and lifted onto triplicate filters (NEN). The filters were hybridized with the animal, vegetal and the above mixed probes, respectively. Those plaques were selected as positive clones that gave a strong signal with the vegetal cortical probe and a weak to no signal with the animal cortical and mixed probes. 35 clones were positive after the second round of screening. These clones were classified into five groups by cross-hybridization using dot blot analysis and partial DNA sequencing. Three clones of *VegT* were isolated, the longest one was 1.7 kb (clone B12). Northern blot analysis indicated that the endogenous transcript was approximately 3.0 kb in length. Additional screening of the same library failed to recover a longer clone.

5' RACE and sequencing

To obtain a full-length cDNA for *VegT*, 5' RACE was used to clone the 5' end (Frohman et al., 1988). The primer sequences were: gene-specific primer 1 (GSP1) 5'-GGA ACC ACC CAC TGA TCT GCT CCA T-3'; gene-specific primer 2 (GSP2) 5'-AAC ATG TGC AGT TCT GGA GC-3'; the anchor primer was *XC1* as shown above. 5' RACE was performed according to the manufacturer's instructions (Gibco BRL). Briefly, total RNA from stage I oocytes was reverse transcribed into cDNA using GSP1 and poly(dA)-tailed with terminal transferase. 40-cycle PCR was performed at 94°C (1 minute), 60°C (1 minute) and 72°C (2 minutes). Four independent PCRs were used to generate the correct sequence for *VegT*. DNA sequence was obtained by double-strand sequencing using the Sequenase version 2 kit (USB Biomedical). Sequence analysis was carried out using the GCG package (University of Wisconsin).

Yeast transcription reporter assay

pGBD-*VegT*, encoding a GBD-*VegT* fusion plasmid, was constructed as a chimeric DNA-binding protein. An *AocI* fragment (encoding aa from 261 to 437) from *VegT* was cloned into the *BamHI* site of pGBT9 (Fields and Song, 1989) in such an orientation that the *VegT* C-terminal open reading frame (ORF) was expressed as a translation fusion to GBD. Yeast strain Y526 (Legrain and Rosbash, 1989) was transformed with pGBD-*VegT*. Transformants were selected on SC medium lacking tryptophan. Filter and quantitative assays were performed as previously described (Preker et al., 1995).

RNA extraction and blot analysis

Total RNA was isolated from different stage oocytes, embryos and animal explants (Forristall et al., 1995). Northern blot analysis was carried out as previously detailed (Mosquera et al., 1993). Fibroblast growth factor receptor (FGFR) was used as an RNA loading control and was a gift from Jan Christian.

Plasmids and synthetic mRNAs

The entire *VegT* cDNA was subcloned into pNB40 (a gift of R. Lehmann) and designated pNB-*VegT*. A C-terminal truncated construct of *VegT* was made by deletion of the *AocI* fragment (aa. 261 to 437) from pNB40-*VegT* and was designated as pNB-*VegTΔAD*. For myc-tagged *VegT* mRNA, *VegT* cDNA was subcloned into pCS2+*MT* (a gift of D. Turner) and designated as pCS2+*MT-VegT*. pCS2+*nβgal* is also a gift from D. Turner. The *VegT*, *VegTΔAD*, *MT-VegT* and *nβgal* mRNAs were synthesized in vitro in the presence of cap analog and GTP (ratio 4:1) using the Megascript kit (Ambion) from the plasmids linearized with *NotI*, respectively. The mRNAs were dissolved in RNase-free water at concentrations indicated in the figure legends and injected at 5-10 nl in volume.

In situ hybridization, histology and immunostaining

Whole-mount in situ hybridization was done as described previously (Harland, 1991). The *VegT* in situ hybridization probe was synthesized using SP6 RNA polymerase from the plasmid B12 linearized

with *NotI*. Probes for *En2* (Hemmati-Brivanlou et al., 1991) and *Xlim-1* (Taira et al., 1994) were prepared as described previously.

Embryos and animal cap explants were fixed in MEMFA for 2 hours, embedded in paraffin wax, and 7 μ m sections stained with a combination of Feulgen, light green and orange G as described in Green et al. (1990), unless stated otherwise.

For immunostaining, myc-tagged *VegT* RNA-injected embryos were frozen and dehydrated in 100% ethanol at -80°C for 3 days. The embryos were then embedded in paraffin wax and 7 μ m sections were cut with a microtome. The slides were dewaxed and rehydrated through a series of ethanols and treated with 3 M urea for 3 minutes (Hausen and Dreyer, 1982). Positive signal was detected with c-myc monoclonal antibody 9E10 (Oncogene Science) at 10 mg/ml and goat anti-mouse secondary antibody conjugated with horseradish peroxidase (Gibco BRL). Notochord staining was performed with MZ-15 antibody (Smith and Watt, 1985). β -galactosidase staining was carried out as described (Vize and Melton, 1991).

RESULTS

Differential screening for vegetally localized cDNAs

A rare set of localized RNAs have been shown to be selectively retained in the vegetal cortex of the stage VI *Xenopus* oocyte (Weeks and Melton, 1987; Mosquera et al., 1993; Ku and Melton, 1993; Kloc et al., 1993; Elinson et al., 1993). This observation has led to a view of the vegetal cortex as a unique cytoskeletal domain, which functions to anchor specific RNAs and thus to polarize genetic information within the oocyte (Elinson et al., 1993; reviewed by King, 1995). Some of these RNAs are likely to encode proteins involved in dorsoventral patterning (Elinson et al., 1993; Holowacz and Elinson, 1993; Fujisue et al., 1993). To isolate such determinants, we constructed a cDNA library from oocyte RNA enriched for other known localized RNAs such as *Vg1* and *Xcat-2* (Mosquera et al., 1993). To screen this library, we developed a PCR-based method to generate cDNA probes from hand-isolated vegetal or animal cortices (see Materials and Methods). The library was also screened with a mixture of *Xcat-2*, *Xcat-3* and *Vg1* to eliminate them from consideration. Five independent groups of cDNAs were isolated, each enriched in vegetal cortices. Interestingly, no clone was selected that represented RNAs enriched in animal cortices, lending further support to the premise that the vegetal cortex is a specialized region for anchoring RNAs.

Whole-mount in situ hybridization analysis (Harland, 1991) confirmed that all of the clones were localized to the vegetal pole (data not shown). Their localization patterns during oogenesis are virtually identical to that of *Vg1* or *Xcat-2* and further support our finding of two major RNA localization pathways during oogenesis (Forristall et al., 1995; J. Z. and M. L. K., unpublished data). One group of three clones (B12) represented an RNA transiently expressed on the dorsal side and was selected for further characterization.

VegT cDNA encodes a new member of the T-box gene family

The longest B12 clone isolated from our library was 1.7 kb and an additional 1.0 kb of sequence was obtained by 5' RACE. The amplified DNAs were sequenced and the correct RACE fragments were used to generate the 2.7 kb cDNA sequence. The first methionine at amino acid position 57 in

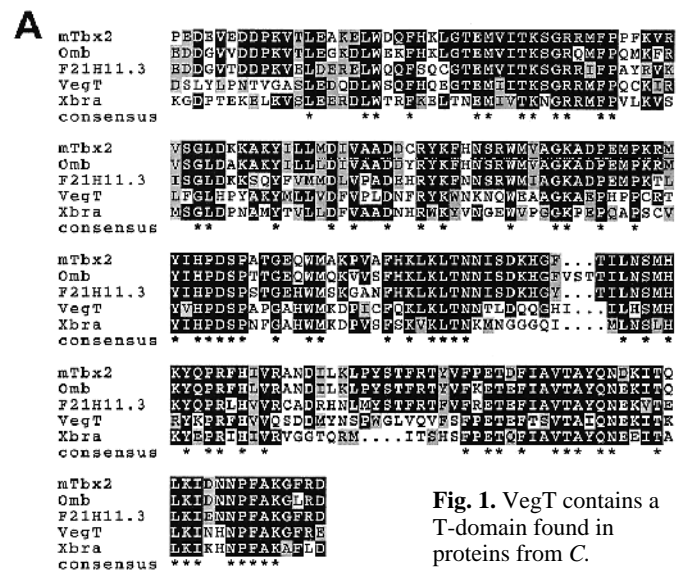
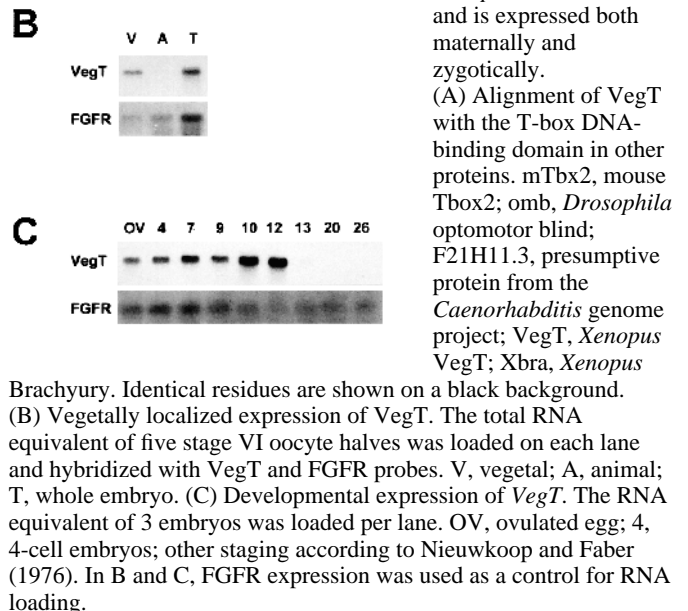


Fig. 1. VegT contains a T-domain found in proteins from *C. elegans*, *Drosophila*, *Xenopus* and mammals and is expressed both maternally and zygotically. (A) Alignment of VegT with the T-box DNA-binding domain in other proteins. mTbx2, mouse Tbx2; omb, *Drosophila* optomotor blind; F21H11.3, presumptive protein from the *Caenorhabditis* genome project; VegT, *Xenopus* VegT; Xbra, *Xenopus*



Brachyury. Identical residues are shown on a black background. (B) Vegetally localized expression of VegT. The total RNA equivalent of five stage VI oocyte halves was loaded on each lane and hybridized with VegT and FGFR probes. V, vegetal; A, animal; T, whole embryo. (C) Developmental expression of VegT. The RNA equivalent of 3 embryos was loaded per lane. OV, ovulated egg; 4, 4-cell embryos; other staging according to Nieuwkoop and Faber (1976). In B and C, FGFR expression was used as a control for RNA loading.

the longest ORF was assigned as the translational start point. The cDNA encodes a 437 aa polypeptide with a predicted molecular mass of 49.6 kDa. The cDNA includes a 1.2 kb 3' untranslated region (3'UTR) (data not shown, accession number U59483). The presence of an AAUAAA consensus polyadenylation signal suggests that the cDNA clone has a complete 3' end.

A full-length RNA transcript synthesized from the cDNA was translated in vitro and yielded a 50 kDa protein product, in agreement with the predicted size (data not shown). Comparison of the deduced amino acid sequence with a protein database revealed that it represents a previously unknown member of the T-box family. We have named the new gene *VegT*. Mouse (Herrmann et al., 1990), zebrafish (Schulte-Merker et al., 1994) and *Xenopus Brachyury* (*Xbra*) (Smith et al., 1991) members of this emerging family are essential in patterning posterior mesoderm.

A comparison of the predicted 191 amino acid N-terminal T-box domain of VegT with T-domains in other members of this family reveals a 55% identity and 72% similarity to mouse or human Tbx2 (Fig. 1A). The next closest known relatives are *Drosophila* optomotor-blind (omb), which plays a role in the development of specific neurons of the optic lobe (Pflugfelder et al., 1992), followed by F21H11.3 (function unknown; accession number U11279) in *C. elegans*. VegT is just slightly more related to these genes in other species than it is to Xbra, showing a 51% identity and 72% similarity to the Xbra T-box domain. It is unlikely that Tbx2 or omb are homologs of VegT. The similarity between T domains among VegT and other T-box proteins suggests that the T-domain in VegT may also bind to specific DNA targets.

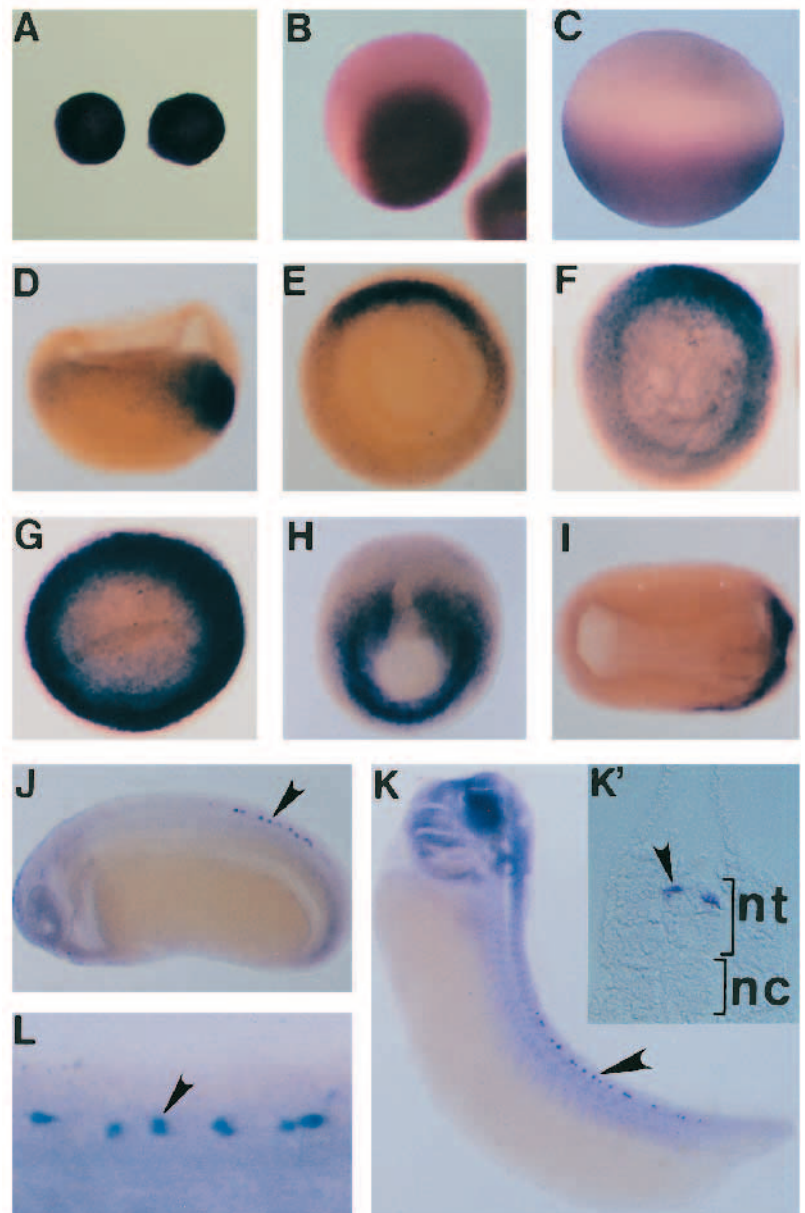
As is typical for T proteins, no homologous sequences in the databases were found outside the T-box domain. However, the C-terminal part of VegT is rich in serine and threonine residues. Computer analysis of the VegT sequence shows several putative protein kinase C and casein kinase II sites. It has been suggested that phosphorylation of serine and threonine residues would increase the net negative charge of the domain, thereby mimicking an acidic activation domain (Hunter and Karin, 1992). The potential net negative charge of this region provides an interface for interactions with other proteins, including transcriptional co-activators or components of the basal transcription complex (Roberts et al., 1993).

Fig. 2. Spatial expression of *VegT* during oogenesis and embryogenesis. Whole-mount in situ hybridization with digoxigenin-labeled *VegT* RNA probe. (A) Stage I oocytes. *VegT* is uniformly distributed. (B) Stage IV oocyte. *VegT* is localized to the vegetal hemisphere. (C) Ovulated egg. *VegT* remains in the vegetal hemisphere. (D) Stage 9.5 embryo, side view. Zygotic *VegT* is expressed on the dorsal side with significantly lower expression laterally and ventrally within the marginal zone. Animal pole is at the top. Blastocoel is somewhat collapsed. (E) Stage 9.5 embryo, vegetal pole view of the embryo in D. Staining mostly restricted to the dorsal side of the embryo. (F) Stage 10.25 embryo, vegetal pole view. Staining still mostly on the dorsal side, but an increase along the lateral and ventral sides is now apparent. (G) Stage 10.5 embryo, vegetal view. Staining in entire marginal zone. (H) Stage 12.5 embryo, posterior view. Ventral and lateral staining around the blastopore. Note the dorsal-most region is not stained. (I) Dorsal view of a cleared mid neural fold embryo (stage 16). *VegT* RNA is restricted to the posterior end. (J) Side view of a tail bud embryo (stage 23). Newly transcribed *VegT* RNA within a posterior subset of primary sensory neuron cells (arrowhead). (K) Side view of a tadpole (stage 31). Staining only within the same neurons (arrowhead) as shown in J. Staining observed at the anterior end of the embryo is non-specific. (K') Transverse section of stage shown in K. Staining is in the large primary sensory neurons found within the dorsal lateral spinal cord (arrowhead). nt, neural tube; nc, notochord. (L) High magnification of the same embryo in K.

Maternal and zygotic *VegT* mRNA is expressed along specific axes during development

Northern blot analysis confirmed that *VegT* transcripts are concentrated within the vegetal half of fully grown oocytes (Fig. 1B). A single transcript about 3.0 kb was observed. During development, maternal RNA levels remain constant from ovulated egg to late blastula. Zygotic transcripts appear to accumulate from late blastula, reaching maximum levels during gastrulation and greatly declining at the time of blastopore closure at stage 13 (Fig. 1C) (staging according to Nieuwkoop and Faber, 1967). Expression beyond neurula was not detected on this blot.

Fig. 2 shows the dynamic spatial and temporal pattern of expression of *VegT* as revealed by whole-mount in situ hybridization (Harland, 1991). *VegT* mRNA is uniformly distributed in stage I/II oocytes, and localizes to the vegetal cortex during late stage III and early stage IV in a pattern similar to that of *Vgl* (Melton, 1987; Forristall et al., 1995) (Fig. 2A,B). In the



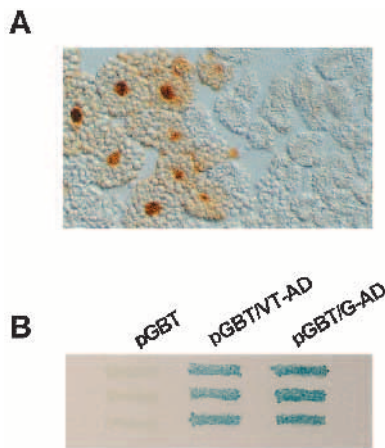


Fig. 3. VegT can function as a transcription factor. (A) Nuclear localization of myc-tagged VegT protein. Single blastomere of a 2-cell embryo was injected at the animal pole with *MT-VegT* RNA. Shown is a stage 9 embryo stained with myc antibody 9E10. Progeny of the uninjected blastomere are on the right and served as a negative control. (B) Yeast transcription reporter assay. Three colonies from each transformation group were transferred to a filter and stained with X-gal. Each construct contains the Gal4 DNA-binding domain fused with either vector alone (pGBT), VegT C-terminal 176 amino acids (pGBT/VT-AD), or the Gal4 activation domain (pGBT/G-AD).

ovulated egg, *VegT* RNA remains concentrated in the vegetal hemisphere (Fig. 2C).

Zygotic *VegT* expression is first detected at stage 9.5, where it appears in a highly restricted pattern on the dorsal side of the marginal zone at the site of the future dorsal lip (Fig. 2D,E). A vegetal view of a cleared embryo at this stage shows faint expression along the lateral and ventral sides (Fig. 2E). The strong dorsal pattern of *VegT* expression continues into the early gastrula embryo (stage 10) but with increased staining along the lateral and ventral sides (Fig. 2F). The staining pattern and its relation to the dorsal lip was confirmed using pigmented embryos where the dorsal lip is quite easily observed (data not shown). Transverse sections of the stained embryo in Fig. 2F showed that the transcripts were in the presumptive mesoderm on the ventral lateral side. On the dorsal side, both epithelial and deeper layers of the organizer express *VegT*, although staining was less intense in the epithelial cells (data not shown).

An hour later, at stage 10.5, expression is now uniformly distributed within the entire marginal zone (Fig. 2G). At stage 12.5, intense staining is now observed around the closing blastopore at the posterior end of the embryo. *VegT* expression is specifically absent from a narrow region in the dorsalmost area, which corresponds to where the notochord will form (Fig. 2H). Histological sections of this stage show that the staining is restricted to the posterior paraxial and lateral mesodermal layers and is not detected anteriorly nor in the epithelial layer (data not shown). At the mid-neural fold stage (stage 16-17), *VegT* transcripts are expressed exclusively within the deep mesoderm in the posterior region of the embryo, similar to *Xbra* (Smith et al., 1991). Importantly, the pattern of *VegT* expression differs from *Xbra* in that *VegT* transcripts are never observed in the notochord (Fig. 2I). By early tail bud (stage 23), the posterior expression of *VegT* has greatly diminished

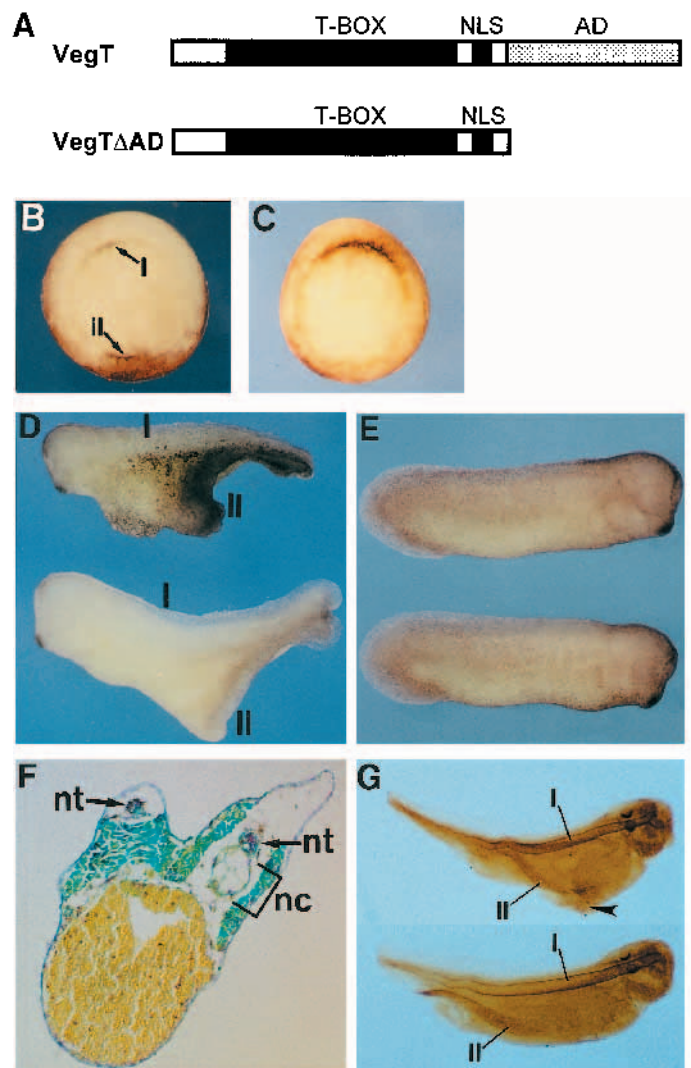


Fig. 4. Secondary axis formation after *VegT* RNA injection into vegetal/ventral blastomeres. *VegT* RNA was injected into one of the vegetal ventral blastomeres of the 8-cell embryo. (A) Diagrammatic representation of the polypeptides synthesized from the injected RNAs. On the top is the deduced full-length VegT peptide with the T-box in black, the nuclear localization signal (NLS) in dark-grey and the activation domain in light-grey. The bottom diagram is the truncated form missing the C-terminal region, the putative activation domain. (B) Stage 10.5 *VegT*-injected embryo showing the primary (I) and secondary (II) dorsal blastopore lips. (C) Control embryos (stage 10.5) injected with the truncated form *VegT*ΔAD or buffer showing only one lip. (D) *VegT*-injected embryos (stage 33) showing primary axes (I) and partial secondary axes (II). (E) Control embryos (stage 33) showing only primary axes. (F) Section of a *VegT*-injected embryo showing large blocks of muscle (in green) and a neural tube in the secondary axis. nt, neural tube; nc, notochord. (G) *VegT*-injected embryos were stained with a notochord marker (MZ-15) antibody at stage 33 and cleared. Primary (I) and secondary (II) notochords are indicated. Extra auditory vesicles (arrowhead) are present.

and is barely detected. Instead, new expression is observed in approximately 15 to 20 cells of the posterior neural tube (Fig. 2J). These cells appear to be a subset of Rohon-Beard cells, a type of primary sensory neuron, populating the embryo at this

stage. The number of cells stained remained constant at stage 31 tadpole (Fig. 2K,L) and through late tadpole stages (not shown). Sections of stage 31 show that these cells are in the dorsal-lateral region of the neural tube (Fig. 2K'). Embryos hybridized with the control sense probe were negative except for non-specific staining within cavities in the anterior region (also observed in Fig. 2K).

In summary, zygotic expression patterns of *VegT* shift from an intense dorsal expression in late blastula and early gastrula to a ventral/lateral expression at the posterior end of later stage embryos. *VegT* appears excluded from the notochord. Finally, in tailbud and tadpole stages, *VegT* is expressed exclusively in a subset of posterior Rohon-Beard neurons.

VegT can function as a transcription factor

The T protein encoded by *Brachyury* has been shown to be a tissue-specific transcription factor (Kispert et al., 1995). Analysis of the *VegT* sequence indicated a putative nuclear localization sequence from residue 250 to 256 (KRQKRKK). To determine if *VegT* could enter the nuclei of cells, we injected myc-tagged *VegT* (*MT-VegT*) into one blastomere of a 2-cell embryo. The other blastomere served as the negative control. Immunostaining with anti-myc antibody of sections taken from a blastula embryo (stage 7 or 9) revealed myc-tagged *VegT* protein only in the nuclei of the progeny of the injected blastomere (Fig. 3A). We conclude that *VegT* protein can enter and accumulate within nuclei consistent with the hypothesis that it functions as a transcription factor.

Next we asked whether the C-terminal segment of *VegT* could function as a regulatory domain in a yeast reporter assay (Fields and Song, 1989). The C-terminal 176 aa (from 261 aa to 437 aa) of *VegT* fused to a GAL-4 DNA-binding domain significantly transactivated the β -gal reporter gene in yeast (Fig. 3B). The activation activity of the chimeric *VegT* was at least 70-fold higher than that of the negative control. From these results, we conclude that *VegT* protein likely functions as a transcription factor and that the C-terminal region of the protein could regulate its transcriptional activity.

VegT-injected blastomeres can mimic a Nieuwkoop center and induce a secondary axis

The initial expression of zygotic *VegT* in the presumptive dorsal lip region suggested that *VegT* might be involved in dorsal patterning. To test whether *VegT* has dorsalizing activity, 2.5 ng of the full-length transcript was injected into both ventrovegetal blastomeres of the 8-cell embryo. The first overt indication that *VegT* had altered the fate of ventral cells was the appearance of a second invagination on the ventral side shortly after gastrulation had started (Fig. 4B). Secondary axes formed in 46% of the injected embryos ($n=301$). Secondary axis formation seems to be dose-dependent (0.7 ng resulted in 17%, $n=41$; 2.5 ng in 46%, $n=301$; 5 ng in 56%, $n=32$), although a fine dose analysis has not yet been done. Embryos injected with control RNAs encoding either a truncated *VegT* (*VegT* Δ AD, missing the presumptive regulatory domain) or a nuclear form of β -galactosidase ($n\beta$ gal), were unaffected (Fig. 4A,C,E).

Typical duplicated axes that formed are shown in Fig. 4D. Secondary axes lacked anterior structures such as head, cement gland and eyes. The formation of a separate tail in some of the secondary axes is reminiscent of the tail-organizing activity

observed in *chordin*-injected embryos (Sasai et al., 1994). Histological analysis of the secondary axis revealed massive amounts of muscle and, at lower frequencies, neural tubes (Fig. 4F). Immunostaining with an anti-notochord-specific antibody showed that 55% of the embryos ($n=24$) have positively stained cells in the secondary axis (Fig. 4G). In some cases, organized notochords can be seen (Fig. 4G). Also evident were otic vesicles in 30% ($n=24$) of the cases with or without an organized notochord, suggesting anterior development extends at least as far as the hindbrain. Injection of *n\beta*gal and *VegT* Δ AD did not result in any specific phenotype and were comparable to non-injected embryos. From this analysis, we conclude that *VegT* can trigger a partial duplicated axis, which includes hindbrain and the dorsalmost mesodermal derivative, notochord.

To determine if *VegT* were acting within an inductive pathway or whether it only altered the fate of injected cells, the lineage tracer *n\beta*-gal was co-injected on the ventral side with *VegT*. In each case, X-gal nuclear staining was found within embryonic endoderm directly underlying the unstained dorsal tissues of the secondary axes (Fig. 5A). Therefore, *VegT*-injected ventrovegetal blastomeres were able to induce a secondary axis. Histological examination of representative embryos shows that the X-gal-stained cells constitute a small domain forming a clear boundary between the descendants of the injected blastomeres and the induced tissues, suggesting a paracrine mechanism may be involved in the process (Fig. 5B). The progeny of *VegT*-injected blastomeres appear to be converted into a cell group resembling Nieuwkoop's center (Nieuwkoop, 1973), which functions to induce other blastomeres to form and organize a dorsal axis.

VegT can convert ectoderm into ventral lateral mesoderm

Although *VegT* can induce mesodermal tissues in a partial secondary dorsal axis, the question remained whether *VegT* is capable of directly inducing mesoderm. To address this question, embryos were injected at the 2-cell stage with *VegT* mRNA (0.7 ng, 2 ng, 5 ng). Animal caps were then explanted at stage 8 and cultured in isolation. All three doses of *VegT* caused the formation of mesoderm in the explants (100%, $n=126$). The convergence and extension observed at stage 11 was moderate compared to that of dorsal marginal zone explants (data not shown). Histological examination of the cultured animal caps revealed extensive mesenchyme, blood and muscle, indicating that ventral and lateral mesoderm had been formed (Fig. 6D,E). We did not observe a clear dose-dependent induction of ventral versus lateral mesoderm which has been shown for *Xbra* (O'Reilly et al., 1995). Animal caps injected with control RNAs (*VegT* Δ AD or *n\beta*-gal) formed only atypical epidermis (Fig. 6A,C). This ventral mesodermal phenotype is similar to that triggered by ventralizing factors, such as *Xwnt-8* (Christian and Moon, 1993). To understand how *VegT* may function in this pathway, we asked whether *VegT* can activate the expression of *Xwnt-8* in *VegT*-injected animal caps. The results indicate that *VegT* is capable of causing the de novo expression of *Xwnt-8* (Fig. 7). Interestingly, *VegT* can also positively regulate itself (Fig. 7). Thus, *VegT* can trigger the formation of ventral lateral mesoderm and may do this through a Wnt signalling pathway although other factors like eFGF could also be involved.

Ectopically expressed *VegT* at the animal pole blocked the completion of gastrulation

The distribution of maternal *VegT* is highly restricted to the vegetal pole of eggs (Fig. 2C). To assess the effect of mis-expression, 2 ng of *VegT* mRNA was injected into the animal pole of 2-cell embryos and the embryos were left to develop through gastrulation. The results from injecting *MT-VegT* RNA at the animal pole (Fig. 3A) had indicated that the myc-tagged protein diffused well into the vegetal hemisphere (data not shown). As a control, identical amounts of *VegT Δ AD* were also injected (Fig. 6F). The *VegT Δ AD*-injected embryos developed through blastula and started gastrulation normally compared to non-injected embryos. However, the *VegT*-injected embryos did not close their blastopores, a phenotype similar to that described for *Xbra*-injected embryos (Cunliffe and Smith, 1992) (Fig. 6G,H). Apparently invagination was not affected, but involution ceased and the embryos failed to close their blastopores.

In *VegT*-injected embryos, the blastocoel collapsed and formed a cavity sinking into the vegetal mass (Fig. 6G). Protrusions of the animal cap epithelium (Fig. 6G,H) were common and expected since mesoderm is induced in the ectoderm by contact with the endoderm (Nieuwkoop, 1973). Histological examination of these injected embryos revealed that the blastocoel was affected. In some cases, the blastocoel fluid had escaped into the vegetal mass of these embryos indicating the integrity of the enclosing epithelium had been lost (Fig. 6H). In contrast, *VegT Δ AD* or *n β -gal* RNA-injected embryos were indistinguishable from non-injected embryos (Fig. 6F). Therefore, the effects observed depended on a functional *VegT* protein and were not simply due to toxic effects. These results show that ectopic expression of *VegT* at the animal pole interferes, most likely indirectly, with normal cell-cell contact and the morphogenetic movements of gastrulation.

Misexpression of *VegT* in dorsal animal blastomeres suppresses head formation

To more accurately evaluate *VegT* mis-expression at the animal pole, 2 ng *VegT* mRNA was injected into the dorsal animal blastomeres of the 8-cell embryo whose progeny are fated to contribute largely to head mesoderm (Moody, 1987a,b; Dale and Slack, 1987). *VegT*, but not the control RNAs, suppressed head formation similar to FGF (Isaacs et al., 1994) and *Xwnt-8* (Christian and Moon, 1993). Significant numbers of tadpoles developed without cement glands and eyes (83%, $n=61$; Fig. 8). Interestingly, the *VegT*-induced secondary axis also lacked anterior head structures (Fig. 4D, 4G). It appears that *VegT* expression is involved in trunk and tail formation.

To determine more precisely the boundary of the anterior depletion, we examined *En-2* expression in such embryos by whole-mount in situ hybridization. *En-2* is mainly expressed at the midbrain-hindbrain junction (Hemmati-Brivanlou et al., 1991). In some *VegT*-injected tadpoles (Fig. 8D), *En-2* expression was missing, indicating that both forebrain and midbrain were suppressed. In other examples where the *En-2* transcripts (arrowhead) could be detected, the tadpoles lacked cement gland (open arrow) and eyes (Fig. 8D), suggesting that only the most anterior structures, such as forebrain were missing.

Since *Lim1* is required for head formation in mouse (Shawlot and Behringer, 1995), we tested whether injection of

VegT into dorsal animal blastomeres had any effect on *Xlim1* (*Lim1* homolog in *Xenopus*) expression. Whole-mount in situ hybridization revealed no differences in *Xlim1* expression between *VegT*-injected embryos and control embryos at gastrula (data not shown), suggesting that inhibition of anterior structures by *VegT* may not act through a *Xlim1* pathway.

DISCUSSION

In this report, we describe a novel approach for identifying maternally expressed genes involved in embryonic patterning. A differential screening procedure was designed to identify RNAs uniquely localized to the vegetal cortex. We targeted this region because of its demonstrated importance in dorsal axis formation (Nieuwkoop, 1973; Wylie et al., 1987; Fujisue et al., 1993) and potential significance in mesoderm induction (Thomsen and Melton, 1993; Kessler and Melton, 1995). The cDNA library screened was constructed from oocyte RNA 50-fold enriched for localized RNAs (Mosquera et al., 1993). *VegT* cDNA was one of five isolated by this approach and was selected for further study because of its dynamic and unique pattern of expression along the dorsal/ventral axis in the marginal zone.

VegT encodes a novel nuclear T-box protein that can function as a transcription factor

VegT can be divided into two domains which, either by sequence or function, identify *VegT* as a member of the growing T-box family (reviewed by Herrmann and Kispert, 1994). The N-terminal domain (46aa-242aa) shows significant homology to the T domain of other T-box genes, including *Brachyury* and *Xbra* (Fig. 1A). Evolutionarily conserved T domains in *Drosophila* (Pflugfelder et al., 1992), *Xenopus*, mouse and zebrafish have all been shown to specifically bind a 20 bp DNA sequence (T site) in vitro (reviewed by Herrmann and Kispert, 1994). Mouse *Brachyury* has further been shown to act as a transcription factor in transformed HeLa cells (Kispert et al., 1995). The transcriptional regulatory domain lies within the C-terminal domain, which bears no homology to other proteins (Kispert et al., 1995). Immediately downstream to the T domain, *VegT* was also found to have a putative nuclear localization signal. Expression of a myc-tagged *VegT* at 2-cell stage confirmed the competency of *VegT* to enter the nuclei of the developing embryo. The C-terminal domain of *VegT* is capable of activating β -galactosidase through a Gal4 DNA-binding domain in a yeast reporter assay. The activation level is not as high as in the positive control and two explanations may account for this. First, the chimeric protein may not fold into the optimal conformation to execute its activation activity. Alternatively, the C-terminal domain may contain both activation and repression motif(s) that result in a dampening of activation in yeast. In fact, *Brachyury* does appear to have activation and repression domains in its regulatory domain (Kispert et al., 1995). Taken together, the data support a role for *VegT* as a transcriptional regulator related to *Brachyury*.

Maternal *VegT* may function to prepattern the marginal zone

The dynamic and unique expression pattern of *VegT* suggests

that it has multiple functions during embryogenesis. As a putative transcription factor, *VegT* can only regulate downstream mediators of patterning, after the MBT, when zygotic transcription is initiated. The strict localization of *VegT* to one pole of the amphibian egg is intriguing and suggestive. Such localization could provide a mechanism for generating a gradient in *VegT* protein along the A/V axis that would result in different blastomeres inheriting different concentrations of the transcription factor. In addition, strict localization prevents *VegT* from being present in the animal hemisphere where it might activate or repress inappropriate genetic pathways. Lastly, *VegT*'s cortical location might allow it to act synergistically with other maternal components localized there. Interestingly, *VegT* is localized at the same time and within the same cortical domain as *Vg1* (Weeks and Melton, 1987; Forrestall et al., 1993). Processed *Vg1* protein can induce dorsal mesoderm (Thomson and Melton, 1993; Dale et al., 1993; Kessler and Melton, 1995) and it could be co-expressed with *VegT*. This maternal pattern is very different from that of *Xbra* mRNA whose low expression level is detected only by RNase protection and which appears to be uniformly distributed (Smith et al., 1991).

At MBT, *VegT* is expressed in the marginal zone along the entire dorsal/ventral axis underlying the blastocoel. Two important features of this expression pattern should be noted. Zygotic *VegT* is initially expressed in a gradient and the highest concentration is at the dorsal end. Shortly thereafter the gradient is gone and expression is uniform throughout the marginal zone, similar to *Xbra*. Later at stage 12.5, *VegT* expression is missing in the presumptive notochordal area where *Xbra* is expressed. At the end of gastrulation, *VegT* is expressed around the closing blastopore ventrally and laterally, where it persists through neurula stages. The different expression patterns of *VegT* and *Xbra* in posterior mesoderm, especially in notochord, suggests distinct roles for these two genes in mesodermal patterning.

Fig. 6. Ectopic effect of *VegT* injection on animal cap explants and whole embryos. *VegT* RNA was injected into the animal pole of both cells at the 2-cell stage. (A-E) Animal caps were explanted at blastula-stage (stage 8) and cultured to the equivalent of stage 41. (A,B) show cap morphology. (C-E) are histological sections. (A) Control animal caps form ciliated epidermis. (B) *VegT*-injected animal caps form swelled vesicles, a phenotype of ventral mesoderm. (C) Control animal caps showing atypical epidermis. (D) *VegT* RNA-injected cap showing block of muscle cells. (E) Another example of *VegT* RNA-injected cap where extensive ventral mesoderm including mesenchyme and blood were formed. (F-H) Alternatively, RNAs were injected into the animal pole at the 2-cell stage and the embryos were cultured to stage 11. (F) Stage 11.5 embryo. *VegT*- Δ AD-injected control together with *VegT*-injected sibling. Note the failure of the blastopore to close. (G) Section of stage 11.5 embryo injected with 0.7 ng *VegT* RNA showing collapse of the blastocoel. (H) Section of stage 11.5 embryo injected with 2.0 ng *VegT* RNA showing the complete loss of the blastocoel. Note the protrusive activity of the ectoderm in G and H.

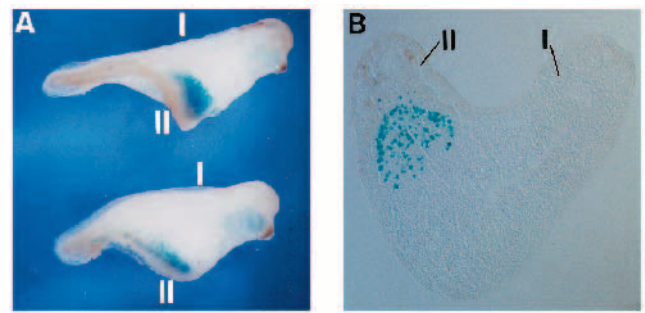
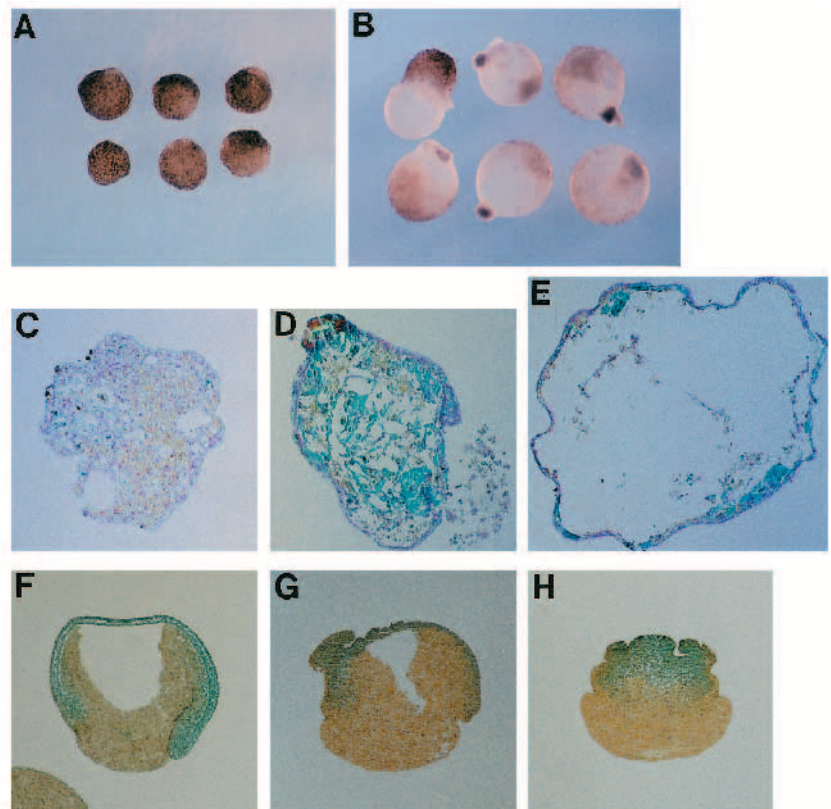


Fig. 5. Lineage tracing of *VegT*-injected blastomeres. (A) *VegT* and *nβgal* RNAs were co-injected into a single ventral-vegetal blastomere at the 8-cell stage. At stage 33, the tadpoles were stained with X-gal. Specific staining is nuclear and is present almost exclusively in the endoderm underlying the secondary axes (II). (B) Transverse section of embryo in (A). Virtually all staining is in the gut region.

Injection of *VegT* RNA into the animal pole region caused ventral mesodermal differentiation in animal caps. Consistent with this finding, we have shown that *VegT* can activate *Xwnt-8*, a ventral marker in animal caps. *Xwnt-8* is zygotically expressed in the marginal zone, but is excluded from the organizer during gastrulation (Smith and Harland, 1991). *Goosecoid*, an immediate early response gene to mesoderm induction expressed in the organizer, has been shown to repress *Xwnt-8* expression there (Christian and Moon, 1993).

Our data can be explained in the context of *VegT* activating *Xwnt-8*. First, *VegT* misexpressed in the dorsal animal pole blastomere results in the suppression of head structures, most predominantly forebrain as indicated by the midbrain/hindbrain



boundary marker *En-2*. *Xwnt-8* misexpression also results in the loss of anterior structures. Second, both *VegT* and *Xwnt-8* have ventralizing activity. Third, overexpression of *VegT* prior to the MBT in ventral vegetal blastomeres can induce secondary dorsal axes, just like ectopic *Xwnt-8* expression in these blastomeres, with one important difference (Christian and Moon, 1993). *Xwnt-8* can induce formation of a complete secondary axis (Smith and Harland, 1991; Sokol et al., 1991), while *VegT* can only cause a partial one to form (Fig. 4). Dorsal formation is unlikely to be a simple event elicited by a single molecule. The combination of induction and cell autonomous action and possible cross-talk between these two pathways suggests that a number of factors are required to orchestrate this complex process. Different molecules may have different regional and temporal roles. Molecules such as *VegT* may only specify the posterior dorsal axis, while *noggin* and unidentified maternal Wnts may act on anterior dorsal structures (Smith and Harland, 1992; Steinbeisser et al., 1993). Novel vegetally localized RNAs isolated in our laboratory may implicate additional components that may also be required to specify the dorsal axis and mesoderm and bear further investigation.

***VegT* in mesoderm formation and gastrulation**

VegT generated different cell types when expressed in different embryonic domains. Different competences of the responding cells may contribute to these observed results. Animal cap cells normally do not form mesoderm, and their ability to respond to inducing factors and cell autonomous factors, could be quite different from those cells in the marginal zone. Furthermore, *VegT* most likely co-operates with other mesoderm-inducing and/or patterning factors to lay down the final pattern of mesoderm. It has been previously demonstrated that *Xbra* causes dorsal mesodermal differentiation when co-expressed with the secreted proteins *noggin* and *Xwnt-8* (Cunliffe and Smith, 1994). None of these three molecules cause dorsal mesoderm formation when expressed alone (Cunliffe and Smith, 1994). Synergistic interaction was also observed between *Xbra* and another mesoderm-specific transcription factor, *Pintallavis* (O'Reilly et al., 1995). Taken together, these data suggest that phenotypes generated by ectopic expression of single molecules may not reflect what occurs in vivo.

It is not surprising that misexpression of *VegT* at the animal pole caused the arrest of gastrulation given that *VegT* is capable of converting ectodermal cells to mesoderm. However, the molecular basis underlying this arrest is unknown. In *VegT* RNA-injected embryos, the complete loss of the blastocoel, in some cases, indicates the loss of cell-cell adhesion (Fig. 6H). Recently, it has been suggested that *Brachyury* may act during gastrulation to alter cell surface properties as cells pass through the primitive streak (Wilson et al., 1995). Ectopically expressed *VegT* may share a similar activity with *Xbra*.

Can *VegT* act as a morphogen?

Drosophila early development is regulated by four systems all using the same basic strategy to define the anteroposterior or the dorsoventral axis (Nusslein-Volhard, 1991). In each system, an asymmetrically localized transcription factor results in a concentration gradient that defines the

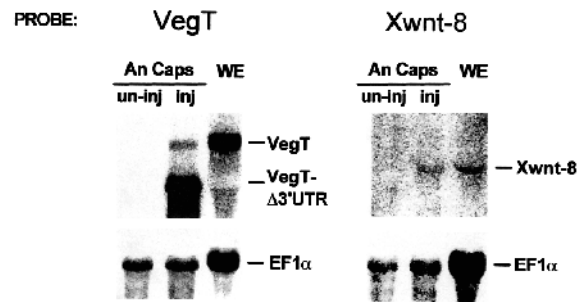


Fig. 7. *VegT* can activate expression of *Xwnt-8* and itself in animal caps. 2-cell-stage embryos were injected with 2 ng *VegTΔ3'UTR* RNAs at the animal pole. At stage 8, the animal caps were explanted and cultured until control embryos reached stage 11. Total RNA was isolated from twenty animal caps (An caps) and assayed by northern blot hybridization with *VegT* (left panel) or *Xwnt-8* (right panel) as probes. *VegT*, endogenous *VegT* RNAs; *VegTΔ3'UTR*, injected *VegT* RNA; un-inj., uninjected; inj., injected; WE, whole embryos. *EF1α* was used as an RNA loading control.

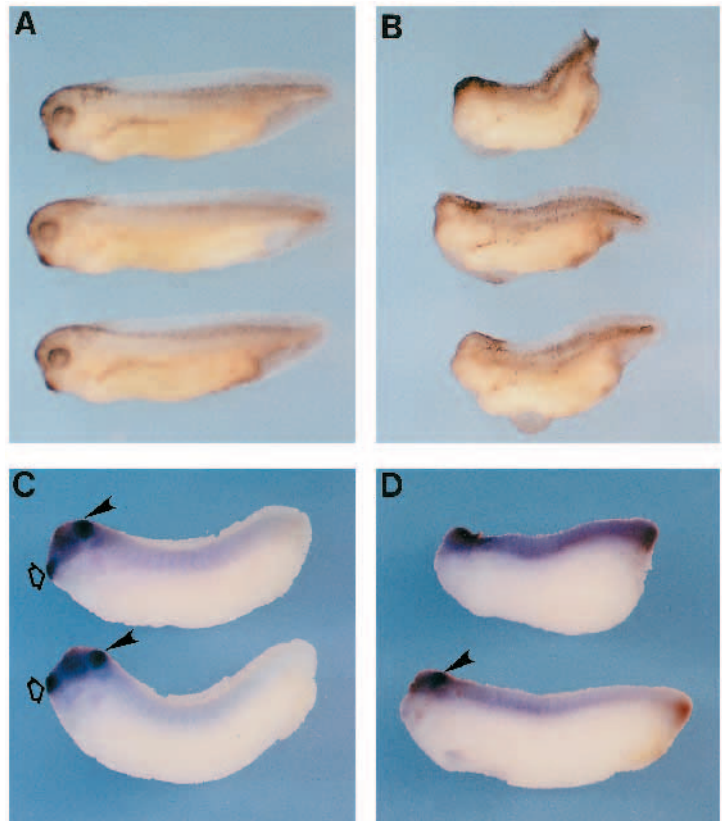


Fig. 8. Ectopic expression of *VegT* in dorsal/animal blastomeres suppresses head formation. At the 8-cell stage, 1 ng of RNA was injected into each dorsal/animal blastomere. The embryos were allowed to develop to stage 34 for morphological assay (A,B) or to stage 28 for whole-mount in situ hybridization with digoxigenin-labeled *En-2* RNA probe (C,D). (A) Control embryos injected with *VegTΔAD* or buffer. (B) Embryos injected with *VegT*. (C) Control embryos showing dark-blue staining of *En-2* transcripts (arrowheads). Darkly pigmented cement glands are also indicated (open arrows). (D) *VegT*-injected embryos showing variation of *En-2* expression. The embryo on the top shows no *En-2* expression, while the bottom one has normal expression of *En-2*, although it lacks cement gland and eyes.

spatial limits of expression of one or more zygotic genes (Nusslein-Volhard, 1991). For example, the anterior morphogen bicoid is essential for *hunchback* expression and normal head/thorax development (Driever and Nusslein-Volhard, 1988). Like the *Drosophila* syncytial blastoderm, molecular gradients may form in *Xenopus* eggs and positional information likewise be inherited by individual blastomeres. Our working hypothesis is that maternal VegT may form such a concentration gradient along the A/V axis. VegT activity along such a gradient could determine regional or cellular identity by regulating the expression of zygotic target genes in a dose-dependent way. Different levels of VegT could elicit either the activation or the repression of the same gene as has been shown for *Drosophila hunchback*. Hunchback activates *Kruppel* at high concentrations and represses it at low concentrations (Schulz and Tautz, 1994). Brachyury has both activation and repression regions in its regulatory domain (Kispert et al., 1995) and this may also be important for VegT regulatory activity as well. The suggestion that VegT may act as a morphogen may appear to contradict our observation that VegT is likely a dose-independent agent in mesoderm formation. However, given its multiple expression patterns during early embryogenesis, VegT may possess other yet unidentified activities, which may also be important in patterning.

After MBT, with the induction of organizer-specific genes like *goosecoid*, *Xwnt-8* is downregulated (Christian and Moon, 1993) and *VegT* is subsequently downregulated in this region. The evidence suggests that *Xwnt-8* is affected rapidly and *VegT* secondarily so. *Xwnt-8* may be required for the maintenance of VegT in a similar fashion as has been described for the Xbra and FGF regulatory loop (Isaacs et al., 1994; Schulte-Merker and Smith, 1995). In related experiments, we have also seen that VegT can autoregulate and turns itself on when expressed in animal caps (Fig. 7). Misexpression of *Xwnt-8* in the presumptive region of the notochord results in embryos missing forebrain and notochord, indicating that it is important to downregulate *Xwnt-8* in the organizer (Christian and Moon, 1993). If VegT turns *Xwnt-8* on in vivo as it does in animal caps, then it follows that VegT must also be eliminated from the presumptive notochordal region. Such a prediction is consistent with our observations as noted. It will be important to determine the immediate downstream factors of VegT and whether mesoderm-inducing factors can modulate VegT's function at a post-translational level.

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Note added in proof

An allele to *VegT*, *Xombi*, has been isolated and characterized by Kevin Lustig and Marc Kirschner (this issue).