Transcription factors Mix1 and VegT, relocalization of *vegt* mRNA, and conserved endoderm and dorsal specification in frogs

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Protein expression of the transcription factor genes *mix1* and *vegt* characterized the presumptive endoderm in embryos of the frogs *Engystomops randi*, *Epipedobates machalilla*, *Gastrotheca riobambae*, and *Eleutherodactylus coqui*, as in *Xenopus laevis* embryos. Protein VegT was detected in the animal hemisphere of the early blastula in all frogs, and only the animal pole was VegT-negative. This finding stimulated a *vegt* mRNA analysis in *X. laevis* eggs and embryos. *vegt* mRNA was detected in the animal region of *X. laevis* eggs and early embryos, in agreement with the VegT localization observed in the analyzed frogs. Moreover, a dorso-animal relocalization of *vegt* mRNA occurred in the egg at fertilization. Thus, the comparative analysis indicated that *vegt* may participate in dorsal development besides its known roles in endoderm development, and germ-layer specification. Zygotic *vegt* (*zvegt*) mRNA was detected as a minor isoform besides the major maternal (*mvegt*) isoform of the *X. laevis* egg. In addition, α-amanitin–insensitive *vegt* transcripts were detected around vegetal nuclei of the blastula. Thus, accumulation of *vegt* mRNA around vegetal nuclei was caused by relocalization rather than new mRNA synthesis. The localization of *vegt* mRNA around vegetal nuclei may contribute to the identity of vegetal blastomeres. These and previously reportedly localization features of *vegt* mRNA and protein derive from the master role of *vegt* in the development of frogs. The comparative analysis indicated that the strategies for endoderm, and dorsal specification, involving *vegt* and *mix1*, have been evolutionary conserved in frogs.

mvegt | zvegt | germ plasm | blastula | gastrula

The *Xenopus laevis* transcription factor T-box gene, *vegt* (1), encodes two transcripts, maternal and so-called "zygotic" ones (*mvegt* and *zvegt* mRNAs, respectively), which are transcribed from different promoters (2, 3). *vegt* is also known as *antipodean* (4), *xombi* (5), and *brat* (6). *mvegt* specifies the germ layers and dorso–ventral patterning of the mesoderm (1, 7). It is expressed at high levels during oogenesis, and the transcripts become anchored to the vegetal cortex of the full grown oocyte. During cleavage, *mvegt* mRNA is found in the vegetal hemisphere (1, 4–6). The protein, mVegT, becomes distributed to the vegetal hemisphere of cleaving embryos and disappears at the beginning of gastrulation (2). *mvegt* mRNA was found only in amphibians and not in other vertebrates (3, 8). *mvegt* is an inducer of the endoderm and a major regulator for specification of the germ layers in *X. laevis* (7, 9). *zvegt* is expressed in the dorsal marginal zone of the mid-blastula followed by expression in the marginal zone of the gastrula, except for the notochord region (3). *zvegt* plays an important role in paraxial mesoderm formation (3, 10–12). *zvegt* is the orthologous gene of zebrafish *spadetail*/*Tbx16* and chick *bx6L*/*Tbx6* (3).

The transcription factor genes *vegt* and *mix1* provide molecular identity to the *X. laevis* endoderm. The signals of mVegT and Wnt trigger the differentiation of the mesoderm and endoderm by up-regulation of nodal expression (xnr5 and xnr6) in the vegetal region of the blastula (11, 12). Nodal induces expression of *mix1*. *mix1* is involved in formation of the endoderm and repression of the posterior mesoderm (13). *mix1* encodes a paired-like homeodomain transcription factor expressed ubiquitously in the endoderm and mesoderm (14). Its expression is detected in the mesendoderm shortly after the mid-blastula transition and expression peaks in the presumptive endoderm and mesoderm of the *X. laevis* gastrula (14, 15). *mix1* has not been sequenced in frogs other than *Xenopus*/*Silurana*.

The *vegt* ORF has been sequenced in the amphibians *E. coqui* (*Ec_vegt*), *Rana pipiens* (*Rp_vegt*), and *Ambystoma mexicanum* (*Am_vegt*) (16–18) and partially sequenced in *Epipedobates machalilla* (*Cm_vegt*), *Pipa pipa* (*Pp_vegt*), and *Gastrotheca riobambae* (*Gr_vegt*) (8). A high degree of *vegt* conservation was detected in these anurans (8). Moreover, when *Ec_vegt* or *Rp_vegt* mRNAs were injected into two-cell embryos of *X. laevis*, endodermal, mesodermal, and organizer genes were induced (16). These findings demonstrated that the function of *vegt* as meso-endodermal determinant is conserved in frogs (16). The RNA localizations and functions of *vegt* orthologs in germ-layer specification may be synapomorphies for anuran amphibians (16).

We analyzed the expression of the proteins VegT and Mix1 (the same as Mix.1, Mixl1) in embryos of four Neotropical frogs in comparison with *X. laevis* to characterize endoderm specification in frogs with diverse developmental strategies (Fig. S1*A*) (19). The analyzed frogs were the foam-nesting túngara frog *Engystomops randi* (Leptodactylidae), the poison-arrow frog *E. machalilla* (Dendrobatidae), the marsupial frog *G. riobambae* (Hemiphractidae), the frog without tadpoles *E. coqui* (*Eleutherodactylidae:* Terrarana), and the aquatic frog *X. laevis* (Pipidae) (20). The localization of *vegt* and *mix1* mRNAs in *X. laevis* embryos were reexamined for comparison. We found conserved patterns of

Significance

The transcription factors VegT and Mix1 are markers of the future gut (presumptive endoderm) of the frog *Xenopus laevis*. VegT and Mix1 proteins localized to the embryo vegetal hemisphere, providing an equivalent identity to the presumptive endoderm in the five frog species that were analyzed. Moreover, VegT protein localized to the animal hemisphere in embryos of all frogs. Similarly, *vegt* transcripts relocalized to the dorso-animal region of the egg after fertilization in *X. laevis*, suggesting that *vegt* may participate in dorsal development. In addition, *vegt* transcripts relocalized around vegetal nuclei of *X. laevis* embryos. This relocalization may provide endodermal identity to the large vegetal cells. The results suggest conservation of the developmental strategies for endoderm and dorsal specification in frogs.

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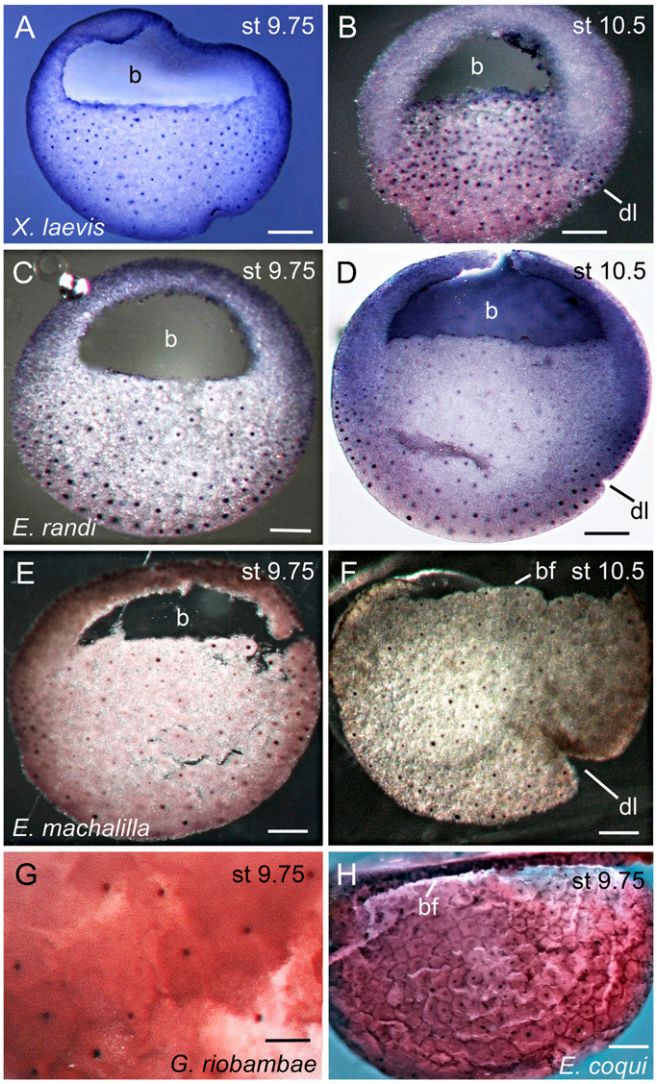

Fig. 1. Immune localization of Mix1 in frog embryos. The animal hemisphere is oriented toward the top. Darkly stained Mix1-positive nuclei were detected in the vegetal hemisphere in embryos of all species. (*A*, *C*, *E*, *G*, and *H*) Blastula sections and a bisected blastula in *H*. (*B*, *D*, and *F*) Sagittal sections of gastrulae with the dorsal side oriented to the right. (*A* and *B*) *X. laevis* embryos. (*C* and *D*) *E. randi* embryos. (*E* and *F*) *E. machalilla* embryos. (*G*) Vegetal cells of a *G. riobambae* stage 9.75 blastula. (*H*) Bisected blastula of *E. coqui* stage 9.75. The blastocoel roof collapsed during fixation. Cleavage and gastrula embryos of *G. riobambae* and *E. coqui* were not analyzed. b, blastocoel; bf, blastocoel floor; dl, dorsal blastopore lip. (Scale bars: *A*, *B*, *E*, and *F*, 200 μm; *C* and *D*, 150 μm; *G*, 100 μm; *H*, 400 μm.)

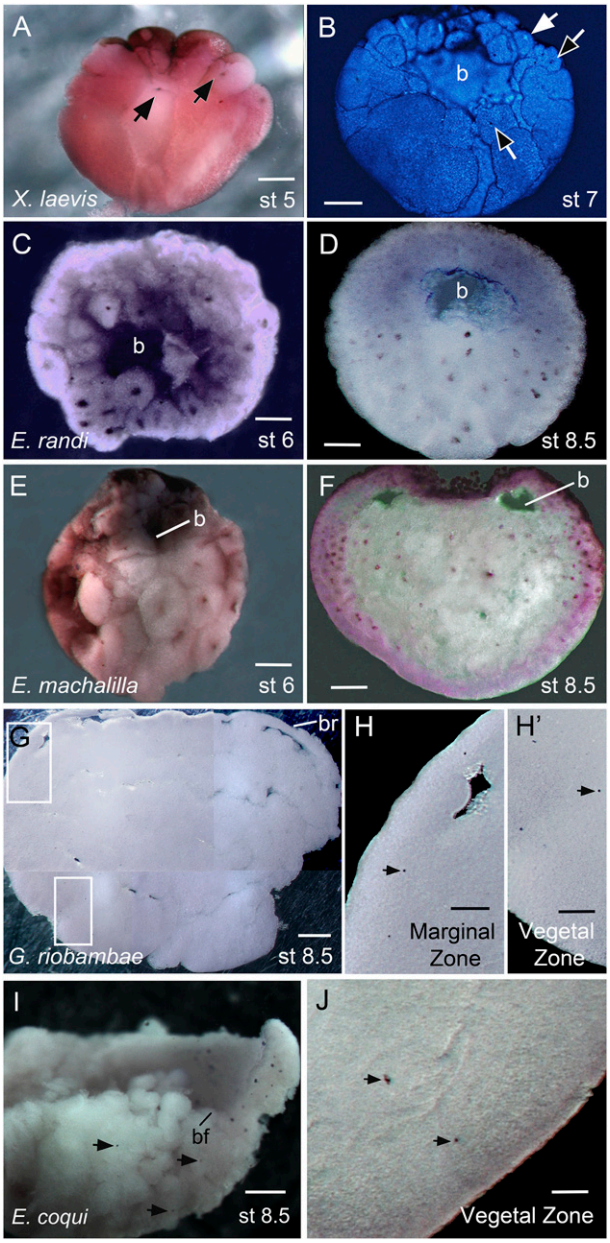

Fig. 2. Immune localization of VegT in frog embryos. The animal hemisphere is oriented toward the top. Darkly stained VegT-positive nuclei were detected in the animal and vegetal hemispheres in the embryos of all species and only the uppermost area of the animal pole was VegT-negative. (*A* and *B*) Embryos of *X. laevis*. (*A*) Parasagittal section of a st 5 embryo. VegT-positive nuclei were observed in animal and vegetal cells. (*B*) VegT immune localization and Hoechst 33258 fluorescence of a bisected blastula. The dark color of VegT-positive reaction quenched Hoechst 33258 nuclear fluorescence in animal and vegetal cells, whereas VegT-negative nuclei were brightly fluorescent. (*C* and *D*) *E. randi* embryos. (*E* and *F*) Embryos of *E. machalilla*. The blastocoel roof collapsed during embryo fixation in *F*. (*G*–*H*′) An embryo of *G. riobambae*. (*G*) Panoramic view of a st-8.5 embryo section. The figure is a composite of multiple images. Areas of the marginal and vegetal regions are boxed and shown at higher magnification in *H* and *H*′ to show VegT-positive nuclei. (*H*) Marginal zone. (*H*′) Vegetal region. (*I* and *J*) *E. coqui* embryos. (*I*) Bisected blastula. VegT-positive nuclei were observed in the marginal zone and vegetal region. (*J*) Vegetal region from a st-8.5 embryo at higher magnification. VegT-positive nuclei are visible. Black arrows in *A*, *B*, *H*, *H*′, *I*, and *J* point to VegT-positive nuclei. White arrow in *B* points to a VegT-negative nucleus. b, blastocoel; bf, blastocoel floor; br, blastocoel roof. (Scale bars: *A*, *B*, and *F*, 200 μm; *C* and *D*, 150 μm; *E*, 250 μm; *G* and *I*, 300 μm; *H*, *H*′, and *J*, 100 μm.)

endoderm and dorsal specification in frogs that belong into different families and have diverse reproductive strategies.

Results

Antibodies and Immune Localization of Mix1 and VegT. We used rabbit polyclonal antibodies (abs) against Mix1 and VegT from *X. laevis* (15) to detect these proteins in embryos of four additional frog species. The Mix1 polyclonal ab was raised against the C-terminal region of *X. laevis* Mix1 (amino acids 156–377; accession no. NP_001081294) (15). This amino acid region is outside the homeodomain (15). In addition, anti-Mix1 is specific for Mix1 and it does not cross-react with the paralogous protein Mixer (15). This epitope region is conserved in *Xenopus tropicalis* (75% amino acid identity).

The polyclonal antibody Anti-VegT was raised against the C-terminal region of VegT (amino acids 237–363 of mVegT; accession no. NP_001081665) and recognizes the common region of mVegT and zVegT (15). This epitope sequence is well conserved in frogs (8). These abs were generated by using the GST gene fusion system and were affinity purified (15). Because the amino acid sequence of VegT is highly conserved in frogs (Fig. S1 *B* and *C*), this ab likely cross-reacted with VegT of other frog embryos.

Anti-Mix1 and anti-VegT recognized the in vitro-synthesized *X. laevis* Mix1 and VegT proteins with migration sizes of 42.24 and 51.79 kDa, respectively. The abs cross-reacted with embryos of other frogs and the immune localization signals were restricted to cell nuclei (Figs. 1 and 2). Specificity of these abs was tested by immune detection without the primary abs. The results were negative in embryos of all species. The nuclear identity of the Mix1 and VegT signals were confirmed by double staining with Hoechst 33258 or DAPI in the embryos of various species. The immune color reaction quenched the nuclear fluorescence of Hoechst 33258 or DAPI, as shown for VegT in *X. laevis* embryos (Fig. 2*B* and Fig. S2 *A* and *A′*). Double staining was not done in the embryos of *E. coqui* after anti-Mix1 treatment. Immune blot analyses with both abs were reported for *X. laevis* embryos (15).

Comparison of Mix1 Localization. We analyzed the expression of *mix1* mRNA in *X. laevis* embryos to provide a comparison for Mix1 localization in the embryos of various frogs. *mix1* mRNA was first detected in the dorsal marginal zone of the *X. laevis* blastula [stage (st) 8.5]. In st 9 embryos, *mix1* mRNA was weakly detected in the vegetal hemisphere. The *mix1* expression became strong in the presumptive endoderm of the late blastula and gastrula (Fig. S3) (14, 15).

In embryos of all five frog species, Mix1-positive nuclei were detected in the vegetal hemisphere of the late blastula, st 9.75 (Fig. 1 *A*, *C*, *E*, *G*, and *H*). In gastrulae of *X. laevis*, *E. randi* and *E. machalilla*, Mix1-positive nuclei were detected in the central core of the vegetal hemisphere, whereas the marginal zone was Mix1-negative (Fig. 1 *B*, *D*, and *F*). Mix1 was not detected in cleavage stage embryos of *E. randi* and *E. machalilla*. The results of Mix1 immune localization in the embryos of various frogs were in agreement with the *mix1* mRNA localization in *X. laevis* embryos (Fig. 1 and Fig. S3).

Comparison of VegT Localization. VegT-positive nuclei were detected at cleavage and blastula stages of *X. laevis* and other frogs (Fig. 2 and Fig. S2 *A* and *A′*). However, anti-VegT did not recognize VegT-positive nuclei in the gastrula of the various frogs. This result may be due to masking of the epitope, because *vegt* mRNA was detected by whole-mount in situ hybridization in the marginal zone of *X. laevis* embryos (Fig. 3 *L–N*). Moreover, immune precipitation followed by Western blotting detected the VegT protein in the *X. laevis* gastrula (15). Therefore, nuclear staining with Anti-VegT in cleavage to blastula stages probably demonstrates the nuclear localization of VegT in embryos of these frogs (Fig. 2).

VegT-positive nuclei were detected in animal and vegetal hemispheres, excluding the uppermost area of the animal pole, in *X. laevis* cleavage (st 5), and in blastula (st 6) embryos of all frogs, shown for *X. laevis*, *E. randi*, and *E. machalilla* (Fig. 2 *A–C* and *E*).

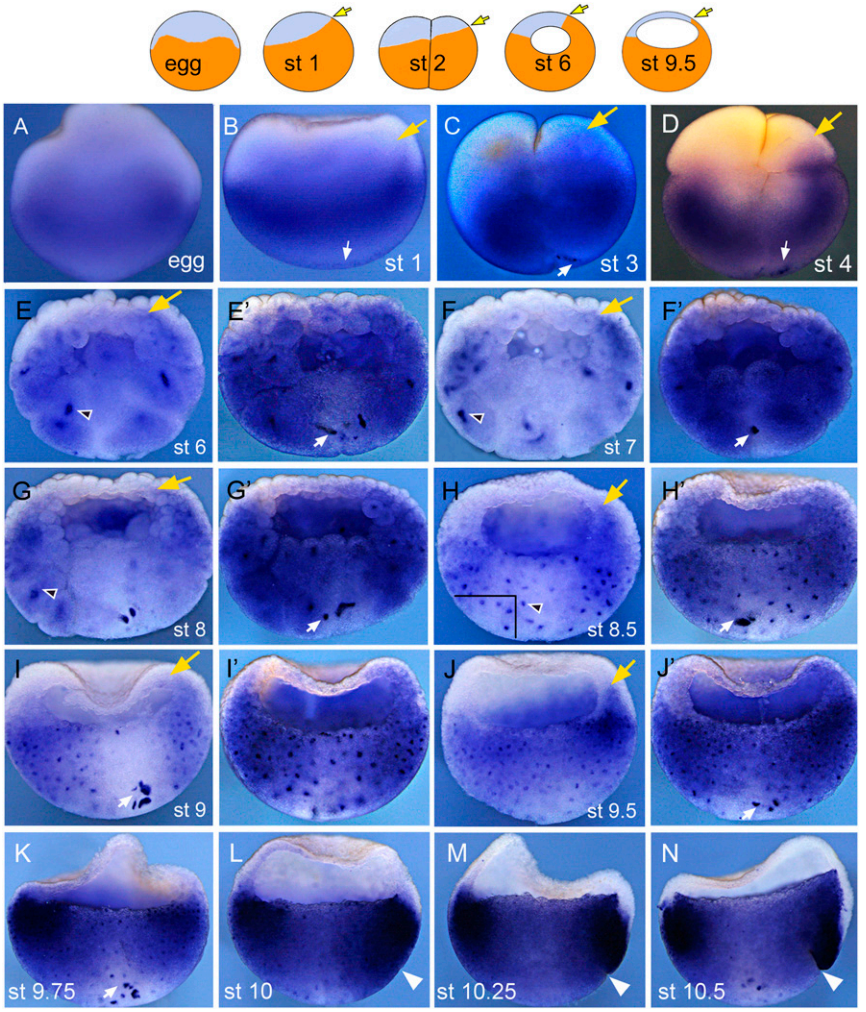

Fig. 3. *vegt* mRNA localization in *X. laevis* embryos. The animal hemisphere is oriented toward the top. In all embryos, dorsal is oriented to the right. Embryos were dorsally hemibisected before in situ hybridization. The row of diagrams summarizes the *vegt* expression of the unfertilized egg and st 1–9.5 embryos. The animal hemisphere of the egg and the blastocoel roof (shown in gray) were *vegt*-negative and the vegetal region (in orange) was *vegt*-positive. (*A*) The unfertilized egg. *vegt* mRNA was found in the vegetal hemisphere with a lower concentration toward the animal region. (*B*) The egg 1 h after fertilization. *vegt* mRNA was detected in the vegetal hemisphere with a displacement toward the dorsal-animal side. In addition, *vegt* transcripts were detected in irregular areas located at the vegetal pole and colocalized with the germ plasm. Germ plasm and mitochondria localization are shown in Fig. S4 *E–G*. (*C*) The four-cell embryo. (*D*) The eight-cell embryo. (*E–K*) Blastulae. (*L–N*) Gastulae. (*E–J*) Lightly stained half–blastulae after *vegt* in situ hybridization. *vegt* mRNA localized mainly around the nuclei of vegetal cells. The boxed area in *H* is shown at higher magnification in Fig. S2*B*. (*E′–J′*) Darkly stained half-blastulae after in situ hybridization. The dark color of the embryos partially masked the *vegt* mRNA signal around nuclei in *E′–G′* in comparison with the lightly stained half blastulae in *E–G*. Yellow arrows indicate the dorso–anterior limit of *vegt* mRNA localization. White arrows at the vegetal region mark the accumulation of *vegt* transcripts in the germ plasm. Black arrowheads point to the accumulation of *vegt* transcripts in the central region of vegetal blastomeres. White arrowheads indicate the position of the dorsal blastopore lip.

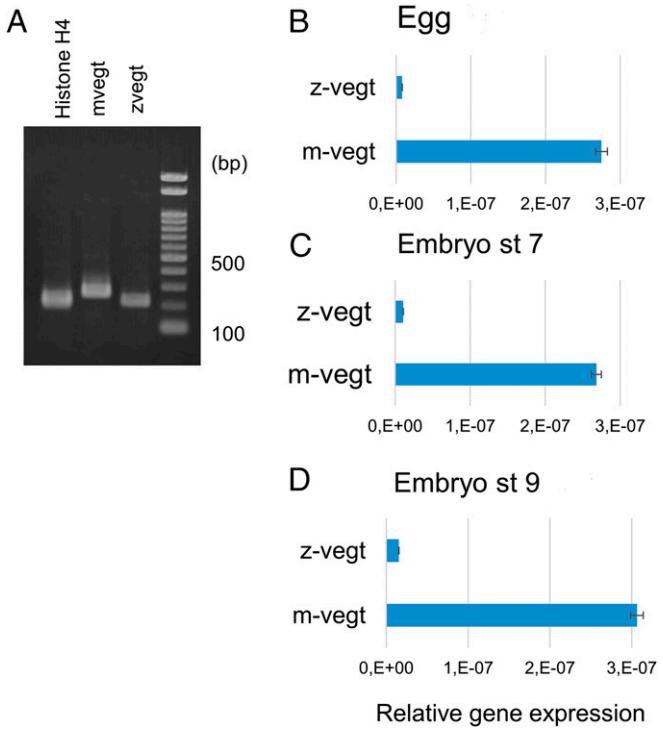

Fig. 4. *vegt* mRNA isoforms of the *X. laevis* unfertilized egg and embryos. (*A*) *vegt* mRNA isoforms of the unfertilized egg amplified by RT-PCR and separated in an agarose gel. (*B–D*) Relative expression of *mvegt* and *zvegt* by RT-qPCR and ΔΔCt analysis normalized by histone H4 expression. (*B*) Unfertilized egg. (*C*) Embryo st 7. (*D*) Early st 9 embryo. The ratios of *zvegt* mRNA to *mvegt* mRNA were 2.8% in *B*, 4.0% in *C*, and 5.2% in *D*. The efficiency of *mvegt* and *zvegt* primer sets is almost the same. The results are the means of three independent experiments.

However, at st 8.5, VegT-positive nuclei were restricted to the marginal zone and vegetal hemisphere of the analyzed frogs (Fig. 2 *D* and *F–J*), and the blastocoel roof was VegT-negative (Fig. 2 *D*, *F*, and *G*). Epiboly may expand the VegT-negative animal pole. Thus, it may contribute to make the blastocoel *vegt*-negative, whereas *vegt*-positive cells become restricted to the vegetal hemisphere. The role of VegT in specifying the presumptive endoderm, or in excluding the ectoderm, may be evolutionarily conserved among these diverse frog species.

***vegt* mRNA in *X. laevis* Embryos.** The unexpected finding of Vegt-positive cells in the animal hemisphere of the various frog species (Fig. 2 *A–C* and *E*) stimulated our analysis of *vegt* mRNA localization in *X. laevis* embryos.

zvegt-type mRNA is a minor maternal component of the egg. *zvegt* mRNA has been detected by RT-PCR as zygotic transcripts, starting in the midblastula of *X. laevis* (3). However, by the use of RT-PCR and quantitative RT-PCR (RT-qPCR) methods, we detected *zvegt* mRNA in unfertilized eggs and cleavage to blastula embryos, although *mvegt* mRNA was the major isoform (Fig. 4 *A–D*). The *zvegt* transcripts, detected at cleavage to blastula stages, are likely maternal in origin, because those mRNAs were α-amanitin–insensitive as described below.

Vegetal-animal localization of* vegt *mRNA. *vegt* transcripts were localized in the vegetal and animal hemispheres of unfertilized eggs as reported by ref. 1, fertilized eggs, and in cleavage to late blastula embryos, (Fig. 3 *A–K*), with essentially no *vegt* mRNA in the animal pole area. Moreover, the blastocoel roof was *vegt*-negative in the gastrula (Fig. 3 *L–N*) as reported by ref. 1.

Dorsoventral relocalization of* vegt *mRNA. In the unfertilized egg, no asymmetric distribution of *vegt* transcripts was detected by in situ hybridization of hemisections done through any midplane (Fig. 3*A*). However, a dorsal relocalization of *vegt* transcripts toward the animal pole was observed from 1 h after fertilization (st 1) to the late blastula (st 9) (indicated by yellow arrows in Fig. 3 *B–J*). The dorsoventral relocalization of *vegt* mRNA was quantitated by RT-qPCR in animal blastomeres of eight-cell stage embryos (Fig. 5). The dorsal relocalization of *vegt* mRNA may be caused by cortical rotation upon fertilization. The result suggests that VegT might have a role in the embryo dorso-animal region.

vegt mRNA relocalization around vegetal nuclei. We observed an apparent subcellular relocalization of *vegt* mRNA in vegetal cells from cleavage to blastula stages. *vegt* mRNA was distributed in the entire cytoplasm of vegetal cells at stages 2 and 3 (Fig. 3 *C* and *D*). However, at advanced cleavage and in the blastula, *vegt* transcripts were detected in the central region of each blastomere (Fig. 3 *E–J′*). The *vegt* mRNA relocalization was clearly observed in lightly stained hemisections (Fig. 3 *E–J*), leading to an apparent reduction of staining in the central core of the vegetal hemisphere. *vegt* mRNA was localized in the cytoplasm, around nuclei of vegetal blastomeres. Moreover, the vegetal nuclei were *vegt*-negative, as revealed by double staining with DAPI (Fig. S2 *B* and *C*). The localization of *vegt* transcripts around nuclei was not due to zygotic transcription because the staining was α-amanitin–insensitive (Fig. 6), as described below. The colocalization of *vegt* mRNA with mitochondria in the germ plasm (21) stimulated our analysis of mitochondria localization in the vegetal blastomeres. Both *vegt* mRNA and mitochondria were concentrated around nuclei of cleavage stage embryos (Fig. 3 *E–J* and Fig. S4 *A–D*).

Embryonic* vegt *transcription as assayed by α-amanitin treatment. The localization of *vegt* mRNA around cell nuclei in vegetal cells raised the question of *vegt* embryonic transcription. However, neither *zvegt*- nor *mvegt*-type mRNA increased significantly from the egg to stages 7–9, suggesting no strong transcription of new embryonic mRNA for *zvegt*, even at stage 9 (Fig. 4 *B–D*). To examine the possibility of early embryonic transcription, we blocked transcription in each blastomere at the two-cell stage by injection of α-amanitin (100 pg per cell) and looked for the presence of mRNA at stages 7 and 9. This α-amanitin dose almost completely inhibited the expression of *mix1* and *Xnr5* at st 9 (Fig. 6 *E–I*). In contrast, α-amanitin did not reduce the *mvegt* mRNA level and reduced the *zvegt* level only modestly (Fig. 6 *C*

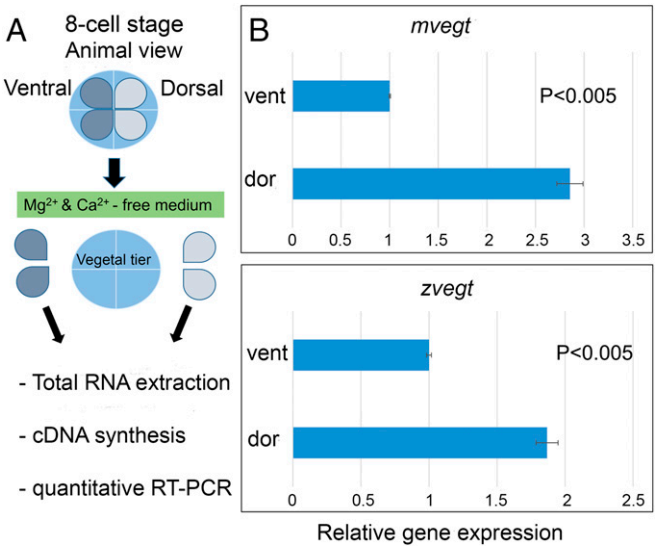

Fig. 5. Dorsoventral relocalization of *vegt* mRNA in animal blastomeres of eight-cell stage embryos (st 4) of *X. laevis*. (*A*) Outline of the methods. (*B*) Quantitation of *mvegt* and *zvegt* expression by RT-qPCR in animal blastomeres, normalized by the expression of histone H4. The data show the results of three independent experiments. dor, dorsal; P, statistics *P* value; vent, ventral.

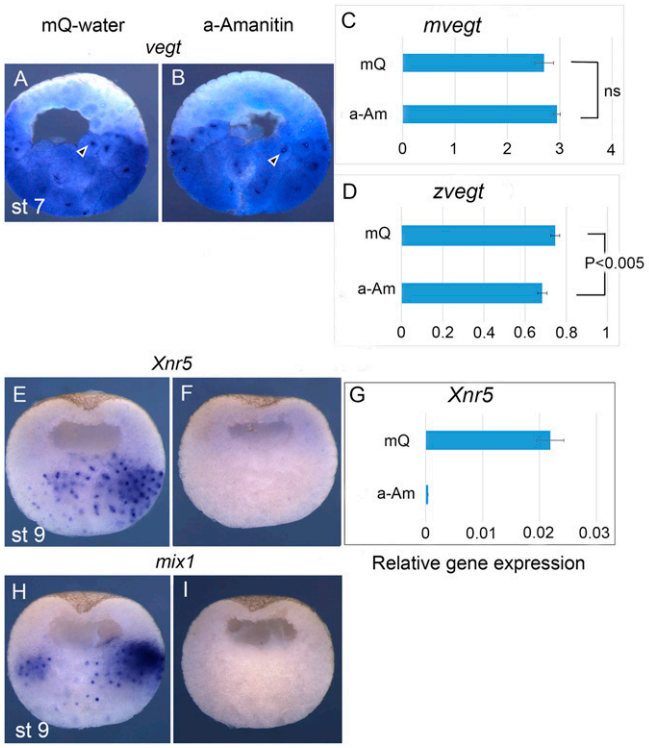

Fig. 6. Effect of α-amanitin on the expression of *vegt*, *mix1*, and *Xnr5* mRNA in *X. laevis* embryos. milliQ-water or α-amanitin (100 pg per cell) were injected into each blastomere at the two-cell stage followed by in situ hybridization of hemi-bisected embryos or by RT-qPCR at the indicated stages. (*A*, *E*, and *H*) In situ hybridization of embryos treated with milliQ-water. (*B*, *F*, and *I*) In situ hybridization of embryos treated with α-amanitin. (*C*, *D*, and *G*) Relative gene expressions of *mvegt*, *zvegt*, and *Xnr5* detected by RT-qPCR normalized by histone H4 expression. (*A*–*D*) *vegt* mRNA expression of st-7 embryos. (*A* and *B*) *vegt* mRNA in situ hybridization. (*C*) *mvegt* relative gene expression. (*D*) *zvegt* relative gene expression. (*E*–*G*) *Xnr5* mRNA expression in st-9 embryos. (*E* and *F*) *Xnr5* mRNA in situ hybridization. (*G*) *Xnr5* relative gene expression. (*H* and *I*) *mix1* mRNA expression of st-9 embryos detected by in situ hybridization. The data show the results of three independent experiments. Black arrowheads in *A* and *B* point to the accumulation of *vegt* transcripts around nuclei of vegetal blastomeres. a-Am, α-amanitin; mQ, milliQ water; ns, nonsignificant; P, statistics *P* value.

and *D*). Moreover, no gross change in the mRNA accumulation around nuclei was detected after α-amanitin treatment by whole-mount in situ hybridization (Fig. 6 *A* and *B*). Taken together, the data suggest that there is a minor maternal component of *zvegt*-type mRNA, and that *vegt* transcripts accumulated around vegetal nuclei by relocalization of maternal transcripts.

Localization of* vegt *mRNA in the germ plasm. *vegt* transcripts were detected in islands of the vegetal pole, considered to be the germ plasm, from stages 1–10.5 (Fig. 3 *B*–*N* and Fig. S5 *A*–*C*), as reported (21, 22). The germ plasm was identified by its enrichment in mitochondria, besides the localization of *vegt* transcripts and protein (Fig. S4 *E*–*G*).

Localization of* vegt *mRNA in the marginal zone. *vegt* transcripts were detected in the marginal zone of the late blastula to gastrula stages (st 9.5–10.5). The marginal zone had a qualitatively higher concentration of *vegt* transcripts in comparison with the central core of the vegetal hemisphere (Fig. 3 *J*–*N*), as reported (1, 4–6).

The *vegt* mRNA in situ signal became smooth and intense in the late blastula and gastrula (Fig. 3 *K*–*N*). Moreover, differential accumulation of *vegt* transcripts around nuclei was no longer detected, likely due to the small cell size, and the strong transcription of *zvegt* in the gastrula. Expression of *zvegt* in the gastrula apparently displayed the general behavior observed in *mix1* and other transcription factors, with expression in nuclei at early stages, and then throughout the embryo at more advanced stages of development (Fig. 3 *K*–*N* and Fig. S3 *D*–*F*).

Discussion

VegT, Mix1, and the Presumptive Endoderm. Frogs share a conserved gene expression pattern in the presumptive endoderm indicated by expression of VegT and Mix1 in the vegetal hemisphere of early embryos (Figs. 1 and 2). Moreover, the orthologous genes *Ec_vegt* and *Rp_vegt* can induce expression of *mix1* in *X. laevis* embryo assays (16). Thus, the function of *vegt* and *mix1* in frog endoderm specification is conserved. However, the endoderm of *E. coqui* is modified and includes the definitive endoderm and the nutritional endoderm. The nutritional endoderm derives from the vegetal-core cells and has no function other than nutrition (23). It allows development of the frog, without intermediate tadpoles (23). However, the presumptive nutritional endoderm expresses endodermal genes, including *Ec_vegt* (18). In oocytes, *Ec_vegt* transcripts localize predominantly to the animal hemisphere. However, in 16-cell embryos, *Ec_vegt* transcripts were detected in equal proportions in animal and vegetal blastomeres (18). In addition, the presumptive nutritional endoderm expresses *EcSox 17*, *activin b*, *derriere*, *Ecsmad2*, and the proteins Smad2, Smad4 (23–25), VegT, and Mix1 (this report). Starting in the gastrula, transcriptional repression occurs only in the nutritional endoderm (24, 25). Thus, the two types of *E. coqui* endoderm can develop from the common presumptive endoderm that expresses conserved endodermal genes.

Dorsal Localization of *vegt* mRNA and Protein. The animal localization of VegT in the early blastula of *X. laevis* and other frogs indicated a likely role of VegT in dorsal development (Fig. 2). To complement these results, we analyzed the localization of *vegt* transcripts in *X. laevis* eggs. In the unfertilized egg, *vegt* transcripts were observed in the vegetal and animal hemispheres. Moreover, after fertilization, *vegt* mRNA partially relocalized toward the dorsal-animal region (Fig. 3*B*). The dorsal localization of *vegt* mRNA may likely associate with dorsal axis specification, as *mvegt* and wnt/β-catenin determine the organizer and trigger dorsal development (12). The animal expression of *vegt* may contribute with induction of *gsc* in the Blastula Chordin- and Noggin-expressing Center (BCNE) in the dorso-animal region of the late blastula, because *gsc* can be activated by VegT (15). The role of *vegt* in dorsal development of other frogs is indicated by the induction of *gsc* and *chordin* in *X. laevis* embryo assays by the orthologous genes *Ec_vegt* and *Rp_vegt* (16). However, VegT reportedly represses the BCNE-expressing genes *chordin* and *noggin* in *X. laevis* (26). The comparison indicates that the activity of *vegt* in the animal region of early embryos is a conserved feature of frog development. However, the role of *vegt* in dorso-animal development remains to be clarified.

Localizations of *vegt* in *X. laevis* Embryos. We confirmed previously demonstrated localizations and found other localizations of *vegt* transcripts in *X. laevis* embryos. Our observations included the dorsal relocalization of *vegt* mRNA at fertilization, the discovery of *zvegt* mRNA as a minor maternal *vegt* isoform of the egg, and the accumulation of *vegt* transcripts around vegetal nuclei. Accumulation of *vegt* mRNA around vegetal nuclei resulted from relocalization of maternal transcripts instead of new transcription. Differential localization of mRNA in the cytoplasm is a post-transcriptional mechanism that allows refinement of gene expression during development (27, 28). Thus, relocalization of *vegt* mRNA around nuclei may contribute to give endodermal identity to vegetal blastomeres. The complex pattern of *vegt* mRNA localization in *X. laevis*, and of VegT protein localizations in embryos of other frogs, gives evidence of the evolutionary conserved roles of *vegt* as a master gene that guides early development in frogs.

Materials and Methods

Frogs and Embryos. The localities for frog collections in Ecuador, frog maintenance, and embryo culture were described (20, 29, 30). Frog collection

was authorized by the Ministry of the Environment of Ecuador, authorization 016-IC-FAU-DNBAP-MA. Embryos of *Eleutherodactylus coqui* and *X. laevis* came from laboratory maintained animals at Duquesne University and at the University of Tokyo, respectively. Gastrulae were staged according to normal tables of stages (20, 29–31). The Faculty of Exact and Natural Sciences of the Pontificia Universidad Católica del Ecuador approved procedures for the maintenance and handling of frogs and embryos. The Animal Care and Use Committees of the University of Tokyo approved all experiments with *X. laevis*.

Antibodies and Immunostaining. The rabbit polyclonal abs anti-VegT and anti-Mix1 for *X. laevis* were described previously (15). Anti-VegT and anti-Mix1 were used for whole-mount immune detection at 1:70 and 1:100 dilutions, respectively. The secondary ab was sheep anti-rabbit IgG conjugated to alkaline phosphatase at a 1:500 dilution (Boehringer Mannheim). Immune detection in whole mounts was done as described (30). The large embryos of *G. riobambae* and *E. coqui* were bisected before immune detection. Sections of 50–100 μm of immune-stained embryos were done with a Vibratome 1000 (Technical Products International), and some sections were counterstained with Hoechst 33258 (Sigma-Aldrich) to detect cell nuclei as described (30). Immune detection of mitochondria around nuclei and in the germ plasm with the mitochondrial marker ab14730 (Abcam) was done as indicated in *SI Materials and Methods* and Fig. S4).

Whole-Mount in Situ Hybridization. Whole-mount in situ hybridization of *X. laevis* embryos with digoxigenin-labeled antisense RNA probes was done according to ref. 32. Hemi sections through the dorsoventral midline were done with a razor blade before hybridization (15). In one half-embryo, the color reaction was stopped shortly after the onset of color development. In the other half-embryo, the color reaction was allowed to continue. Digoxigenin-labeled *vegt* and *mix1* RNA antisense probes were transcribed from PCR templates given in pCS2+VegT (1) and pBluescript SK(+)Mix.1 (14). Some embryos were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) to detect cell nuclei.

RNA Extraction, RT-PCR, and RT-qPCR Analyses. Total RNA was extracted by using TRIzol Reagent (Life Technologies). After DNase treatment, cDNA was synthesized by using a standard protocol. qPCR was performed by using SYBR Green. *vegt* primer sets (2) were added to nucleotides for qPCR (Table S1). PCR amplicons and primer sets were determined by using genome data of Xenbase (www.xenbase.org/entry). RT-PCR products were separated in agarose gels. The relative expression levels of the genes, analyzed by RT-qPCR, were calculated after normalization with the expression level of histone H4. The primer sets for RT-qPCR are given in Table S1. *vegt* mRNA was quantitated by RT-qPCR in isolated animal blastomeres of eight-cell stage embryos. The procedures are outlined in Fig. 5. Student's *t* test was used to separate means. The values reported were the mean of three independent experiments.

α-Amanitin Treatment. Embryos were dejellied, and α-amanitin (100 pg per cell) was injected into each blastomere at the two-cell stage. Control embryos were injected with milli-Q water. We collected embryos at stages 4, 7, and 9 (early) for whole-mount in situ hybridization and RT-PCR. Embryos injected with α-amanitin developed until st 9 and died at gastrulation because of blocking of zygotic transcription by α-amanitin.

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