

Expression of *vox* and *vent* mRNAs and encoded proteins in zebrafish embryos

Elena S. Pshennikova¹, Maria B. Tereshina², Anna S. Voronina¹

¹A.N. Bach Institute of Biochemistry, Research Center of Biotechnology RAS, Moscow, Russia; ²Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry RAS, Moscow, Russia

Contributions: (I) Conception and design: ES Pshennikova, AS Voronina; (II) Administrative support: None; (III) Provision of study material or patients: None; (IV) Collection and assembly of data: All authors; (V) Data analysis and interpretation: All authors; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Correspondence to: Anna S. Voronina. A.N. Bach Institute of Biochemistry, Research Center of Biotechnology RAS, Moscow 119071, Russia. Email: voronina_a@mail.ru.

Abstract: In *Danio rerio* (zebrafish), members of the *vent* gene-family (*vox/vega1*, *vent/vega2*) are considered as ventralizing factors. We investigated not only the expression of their mRNAs by *in situ* hybridization at different stages of embryonic development, but also the spatial distribution of the encoded proteins by whole-mount immunostaining. We showed *vox* mRNA to be available in embryos since early cleavage and later on. *Vent* mRNA appeared after zygotic genome activation only. The *vox* and *vent* proteins were revealed at stage of eight blastomeres. At blastula and gastrula the *vox* and *vent* protein staining areas completely overlapped those of the mRNAs. They were expressed uniformly throughout the embryo except for a small region of clearing on the dorsal side. From the bud stage throughout somitogenesis, the *vox* and *vent* proteins staining progressively covered the embryos except for dorsal side: at the bud stage it resembled that of mRNA and at the beginning of somitogenesis it was clearly seen along the axis structures. At the pharyngula period stages the proteins were located in neural crest zone, but their mRNAs appeared to be in the tail tips. Thus during embryogenesis, the spatial distributions of a protein and its mRNA may not always quite coincide. We observed such mismatches in embryos at the cleavage stage and in the pharyngula period.

Keywords: Zebrafish embryos; *vox*; *vent*; mRNA patterns; protein patterns; RNA isolation from fixed embryos

Received: 27 March 2017; Accepted: 24 May 2017; Published: 30 June 2017.

doi: 10.21037/sci.2017.06.05

View this article at: <http://dx.doi.org/10.21037/sci.2017.06.05>

Introduction

The vertebrate *vent*-family transcription factors play a crucial role in the establishment of the dorsoventral axis in embryos, by repressing organizer genes. Two zebrafish homeobox genes, *vox/vega1* and *vent/vega2*, encode transcriptional repressors implicated in patterning of the dorsoventral axis (1-6). The *vox* also has an important function in regulation of ventral endoderm development and the separation of endoderm from mesoderm and ectoderm (7). Recently it was shown in medaka fish embryos (8), that *vox* was expressed in the developing neural tube similarly to central nervous system (CNS) markers. Thus the *vent* family genes, in addition to their

fundamental role in body axis formation, may be involved in regionalization of vertebrate CNS.

A presence or absence of the specific mRNA in the cell is commonly considered as a presence or absence of the corresponding protein. However, there are lots of evidences that mRNA may be associated with inactive non-translated ribonucleoprotein complexes (9) and once synthesized protein may live long. Previously we have shown (10) that *Xvent-2* mRNA in tails of *Xenopus* embryos was not active at the early tail bud stages. Although the *Xvent-2* mRNA in *Xenopus* and *vox* mRNA in zebrafish embryos were detected in the tips of the tails, the *Xvent-2* and *vox* proteins were revealed in other regions of the tails—along

axial structures and around somites. In this study we for the first time compared the patterns of *vent*-family mRNAs and the encoded proteins in zebrafish embryos at early stages, before and after zygotic genes activation.

Methods

Embryo stages

Zebrafish embryos developmental stages were identified according to tables (11).

Isolation of RNA and RT-PCR analysis

Zebrafish embryos at stages 1–4 blastomeres were fixed with MEMFA solution (0.1 M MOPS pH 7.4; 2 mM EGTA; 2 mM MgSO₄; formaldehyde 3.7%) for 6 hours at room temperature and 12 hours at 4 °C. Then they were transferred gradually into 70% ethanol and stored at –20 °C. RNA isolation was performed as in (12). The rehydrated embryos were washed sequentially with 2 M glycine containing 5 mM EDTA pH 8.0 and the buffer for proteinase K (10 mM Tris pH 8.0, 5 mM EDTA, 50 mM NaCl). To the last wash solution 1 mg of proteinase K and 30 mkg of glycogen were added. After incubation at 42 °C for 1 h, the sample was supplemented with 1 ml of the solution containing 40 mM Tris pH 7.5, 4% SDS, 0.4 M NaCl, and 10 mM EDTA and incubated for an additional 30 min at 42 °C and 30 min at 80 °C. Then the sample was transferred into a tube, and a standard phenol-chloroform deproteinization was carried on (13). After DNase treatment the standard phenol-chloroform deproteinization was performed again and the isolated RNA was used in the reverse transcription reaction with the primer 5' TTCAGTAGTAATGATGTCTGGGCG 3'. The PCR was performed with this primer and the second primer 5' GAAATCATGGTGAAGAAGCTTTTCC 3'. The cDNA product was supposed to correspond to a full-length coding region of *vox* mRNA—736bp. The product of RT-PCR was analyzed by electrophoresis in 1.5% agarose gel.

Whole-mount in situ hybridization

Whole-mount *in situ* hybridization was carried out by a standard procedure (14). Plasmids pCS2+ carrying *vent* cDNA and pBS-SK carrying *vox* cDNA were used for the synthesis of digoxigenin-RNA by T3 RNA polymerase (Roche, Fermentas). The accession number for *vox* is AF255045 and for *vent* is AF255044. Plasmids were controlled by sequencing.

Microm HM 525 (Thermo Scientific) was employed for 18 μm cryosectioning.

Production of antibodies

Rabbit polyclonal antibodies against zebrafish *vox* and *vent* were produced by Almbion Company (Russia) using pvlvdvqepkktrphvpc and skfsvewlsqsfhdqekc peptides, respectively. Both antibodies were purified by affinity chromatography and conjugated with horseradish peroxidase. The same conjugate for rabbit polyclonal antibodies against human immunoglobulin (Imtek, Russia) was used in control experiments.

Western-blot analysis

The dechorionized embryos were homogenized in sample buffer and analyzed in Laemmli PAAG electrophoresis. Western-blot analysis was performed with single appropriate HRP-conjugated antibodies using SuperSignal Western Blot Enhancer Kit (Thermo Scientific, USA) according the manufacturer recommendations.

Whole-mount immunostaining

Embryos were primarily fixed in MEMFA solution, following procedures were described in (10).

Results and discussion

Expression of vox and vent mRNAs and the proteins before zygotic genes activation

To know a spatio-temporal distribution of *vox* and *vent* mRNAs in zebrafish embryos, we performed the whole-mount *in situ* hybridization at different stages of embryonic development. We have shown, and somewhat reaffirmed the results of our previously published work (10), that the *vox* mRNA could be clearly revealed in embryos since the stage of 8–16 blastomeres [Figure 1A (a-c)]. Cryosectioning indicated that the cell nucleus were not stained and the *vox* mRNA staining was seen in cytoplasm, apparently associated with endoplasmic reticulum [Figure 1A (c)]. This result contradicts with the data, that the *vox* expression was activated immediately after midblastula transition (15). In contrast, the *vent* mRNA in our study was not revealed at early cleavage stages [Figure 1A (d, e)] like in (15). The whole-mount immunostaining revealed both *vox* and *vent* proteins at the stage of 8-16 blastomeres (Figure 1B).

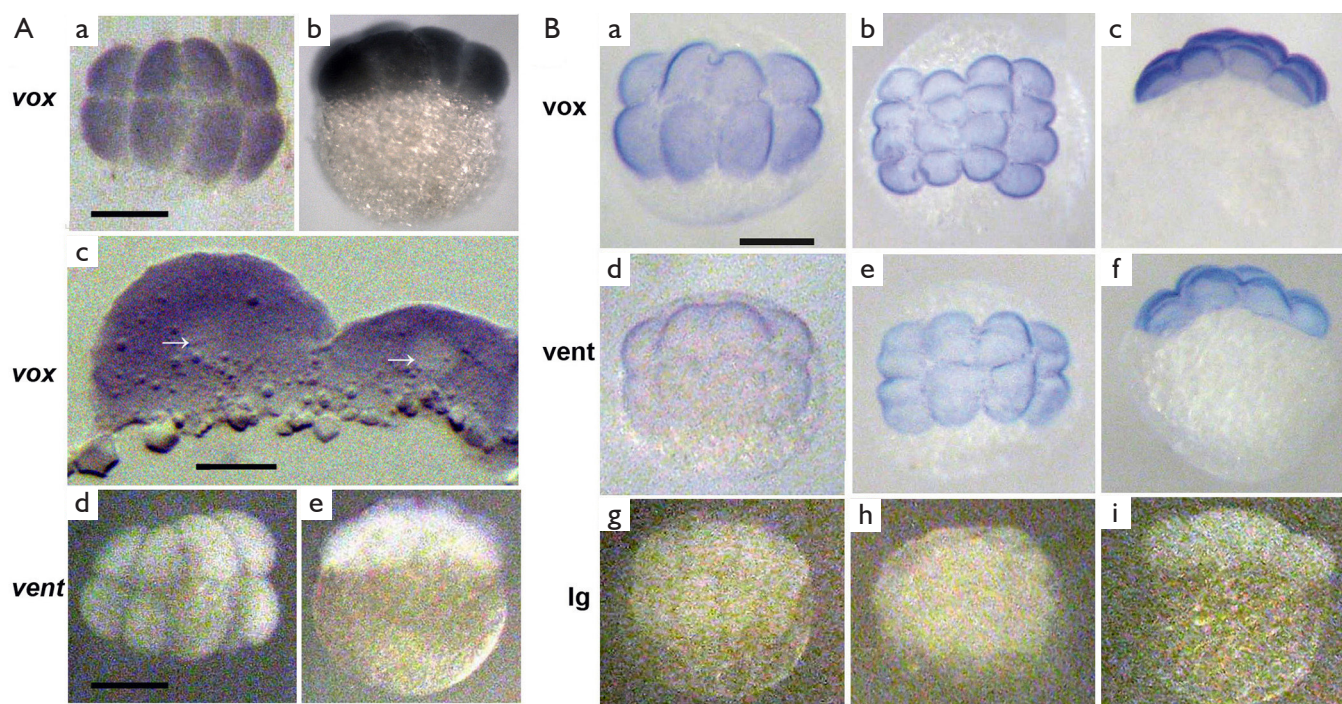


Figure 1 Expression of *vox* and *vent* mRNAs (A) and their proteins (B) at the stage of 8–16 blastomeres. [A (a-e)] *In situ* hybridization, [A (c)] vertical cryosection of blastomeres of the embryo at the [Figure 1A (b)]. Arrows indicate the nuclei; [B (a-i)] immunostaining of proteins. Ig—immunoglobulin staining (control). Scale bar 250 μ m except for 50 μ m in [A (c)].

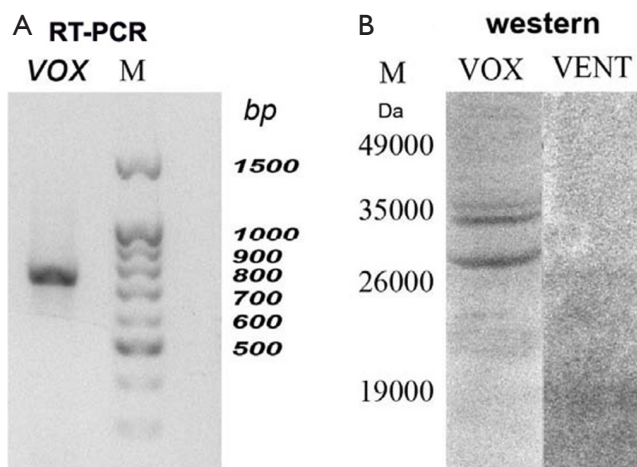


Figure 2 Analysis of RNA and proteins by the electrophoresis. (A) RT-PCR of *vox* mRNA at stage of 1–4 blastomeres. (B) Western-blot with anti-*vox* and anti-*vent* antibodies at stage of 8 blastomeres. M: length (bp) and molecular mass (Da) markers.

For validation, we had isolated the RNA from the earliest embryos (1–4 blastomeres) and performed the RT-PCR (Figure 2A). It is seen that the full-length coding *vox* mRNA

was present in embryos. We supposed that by the TRIZOL method, as in (15) the RNA could not be extracted perfectly, but it was successfully extracted after proteinase treatment. Western blot analysis had also revealed *vox* and *vent* proteins at these stages (Figure 2B). It means that while the *vox* protein could be synthesized on the prestored transcripts or be prestored maternally, the *vent* protein was exclusively prestored maternally. The content of the *vent* protein in the embryos is very low, so the Western blot detected weak bands of about 19 kDa. The *vox* showed quite good two bands. Such duplets were also described for some other proteins, for example (16), probably due to different extend of phosphorylation. Figure 2B also demonstrates an absence of cross-reaction with antibodies against *vox* and *vent*.

Expression of vox and vent mRNAs and the proteins after zygotic genes activation, at blastula, gastrula and segmentation periods

At the sphere stage the *vox* mRNA was expressed uniformly throughout the embryo except for a small region of clearing on the dorsal side [Figure 3A (a, b)]. The *vox* expression, by the shield stage, was heavy at the margin and cleared from

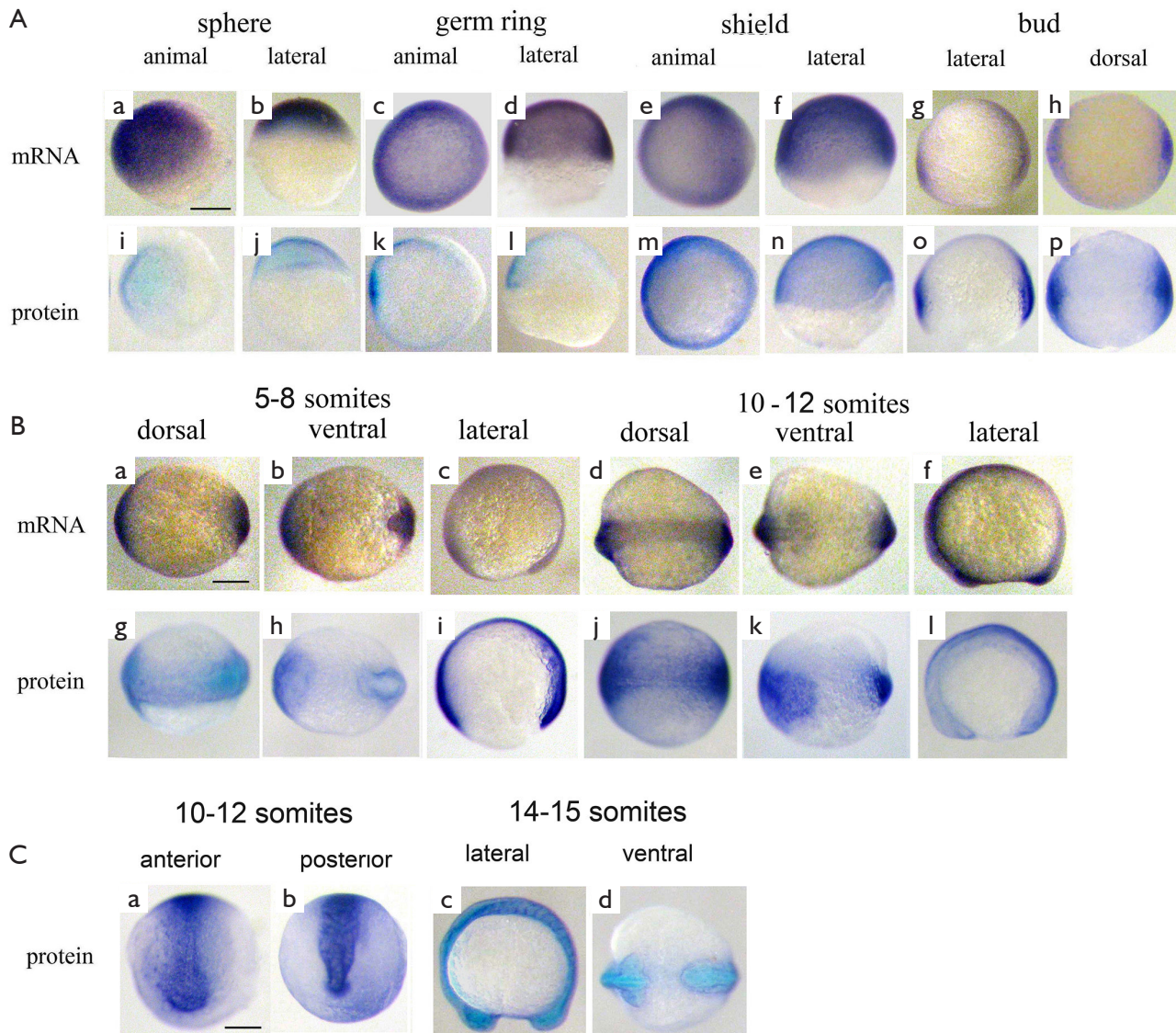


Figure 3 The *vox* mRNA expression pattern, compared with that of *vox* protein, during development of zebrafish embryos. *Figure 3A* (a-h) and *Figure 3B* (a-f)—mRNA staining; *Figure 3A* (i-p), *Figure 3B* (g-l) and *Figure 3C* (a-d)—protein staining. The stages of development and views are indicated over images. Scale bar 250 μ m.

a region on the dorsal side [*Figure 3A* (c-f)], corresponding to the organizer and neurectoderm. From the bud stage throughout somitogenesis, *vox* was expressed in the tailbud [*Figure 3A* (g, h); *Figure 3B* (a-f)], similar to other genes involved in the specification of ventral tissues (17,18). Up to the shield stage the *vox* protein staining progressively covered the embryos except for dorsal side [*Figure 3A* (i-p)], and resembled that of the mRNA. At the beginning of somitogenesis *vox* was clearly seen along the axis structures [*Figure 3B* (g-l) and *Figure 3C* (a-d)], while

the mRNA at these stages was still concentrated in rostral and tail regions [*Figure 3B* (a-f)]. At the subsequent stages, the *vox* mRNA staining appeared along the dorsal side and the protein area covered that of mRNA and adjacent part of the yolk syncytial layer [*Figure 3B* (j-l)]. Earlier it was mentioned in literature that both *vega 1* (*vox*) and *vega 2* (*vent*) were also expressed within the yolk syncytial layer underlying the *vega1* and *vega2* expressing blastomeres (1,2).

The *vent* mRNA was first expressed at the sphere

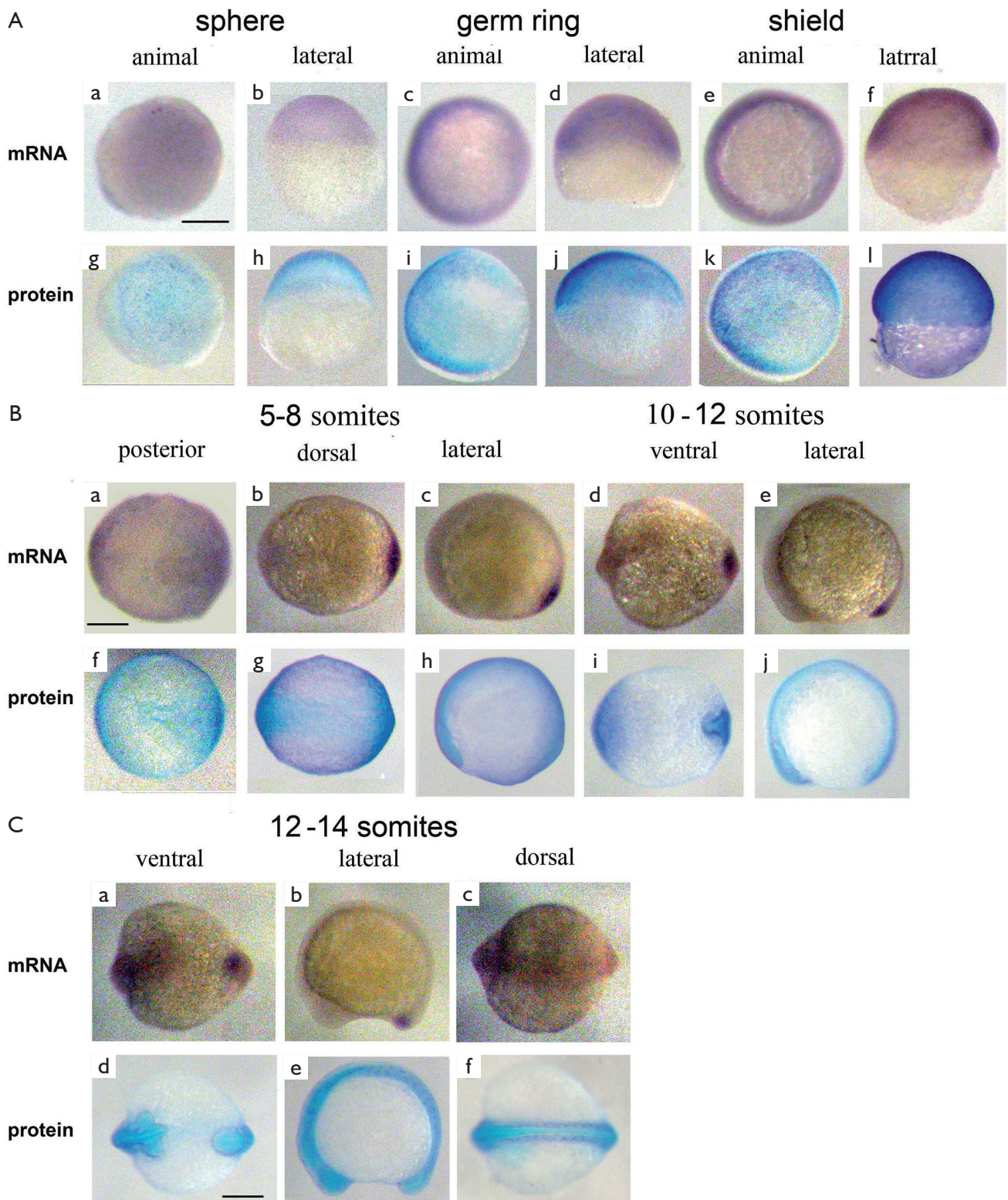


Figure 4 The *vent* mRNA expression pattern, compared with that of vent protein, during development of zebrafish embryos. *Figure 4A* (a-f), *Figure 4B* (a-e) and *Figure 4C* (a-c)—mRNA staining; *Figure 4A* (g-l), *Figure 4B* (f-j) and *Figure 4C* (d-f)—protein staining. The stages of development and views are indicated over images. Scale bar 250 μ m.

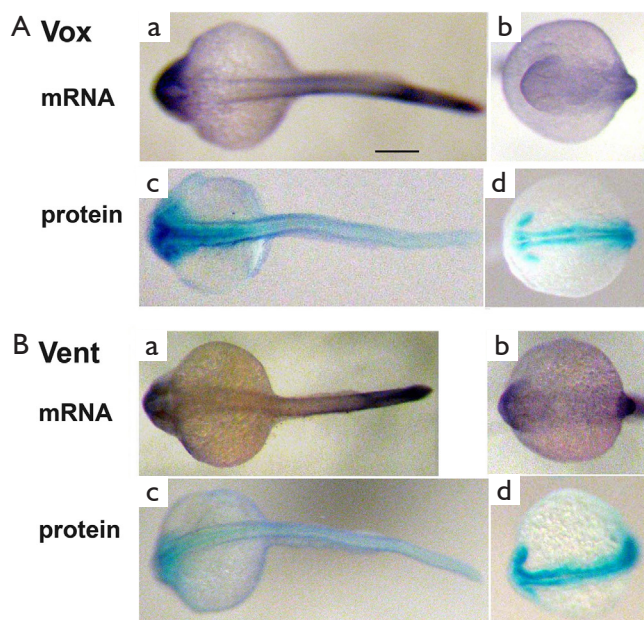


Figure 5 *Vox* and *vent* mRNA and protein expression at the pharyngula period in zebrafish development. *Figure 5A* (a, b) and *Figure 5B* (a, b)—mRNA staining; *Figure 5A* (c, d) and *Figure 5B* (c, d)—protein staining. (A) *vox*; (B) *vent*; dorsal and dorso-anterior views.

stage [*Figure 4A* (a, b)], developing into a half-ring at the blastoderm margin, which became stronger by the shield stage. The *vent* expression at the shield stage [*Figure 4A* (c-f)] was confined to the margin, extending less far dorsally than the *vox*. Herewith the *vent* was mainly expressed posteriorly [*Figure 4B* (a-e); *Figure 4C* (a-c)] while the *vox* expression was extending from the tailbud to the rostral areas. The vent protein staining, since the sphere stage and up to the stage of bud [*Figure 4A* (g-l)], almost covered the mRNA staining area. From the bud stage throughout the somitogenesis, the *vent* mRNA staining was distinctly concentrated in the tail region while the protein staining was more extensive [*Figure 4B* (f-j); *Figure 4C* (d-f)].

Expression of vox and vent mRNAs and the proteins at the pharyngula period

The expression of *vox* mRNA and the protein at segmentation period was described in our previous work (19). At the pharyngula period stages the *vox* mRNA staining was concentrated mostly in rostral and distal tail areas, while the *vox* protein staining was revealed in the head region of the embryos and was absent from the tail

tips (*Figure 5A*). The *vent* mRNA and the protein patterns slightly reminded those of the *vox* at these stages (*Figure 5B*). The *vox* and *vent* proteins were seen alongside the neural tube where neural crest cells were thought to be located, and also in presumptive eyes. Probably, the *vox* and *vent* regulate the activity of genes of the neural crest cells. This is consistent with our data on *Xenopus* embryos tails and zebrafish embryos at segmentation period (19).

Conclusions

Our results show that the spatial distributions of a protein and its mRNA may not always quite coincide during zebrafish embryogenesis. We observed such mismatches in embryos at the cleavage stage and in the pharyngula period. We propose that the cells, where a synthesis of a certain transcription factor had occurred, might migrate away from their native area.

Acknowledgements

We would like to thank Dr. D Onichtchouk for zebrafish embryos and plasmids kindly donated.

Funding: This study was supported by grant Russian Foundation for Basic Research No. 14-54 12008.

Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: All animal manipulations complied to European directive 2010/63, under the control of the veterinary services of Paris (authorization #75-419).

References

1. Kawahara A, Wilm T, Solnica-Krezel L, et al. Antagonistic role of *vega1* and *bozozok/dharma* homeobox genes in organizer formation. *Proc Natl Acad Sci U S A* 2000;97:12121-6.
2. Kawahara A, Wilm T, Solnica-Krezel L, et al. Functional interaction of *vega2* and gooseoid homeobox genes in zebrafish. *Genesis* 2000;28:58-67.
3. Melby AE, Beach C, Mullins M, et al. Patterning the early zebrafish by the opposing actions of *bozozok* and *vox/vent*. *Dev Biol* 2000;224:275-85.
4. Imai Y, Gates MA, Melby AE, et al. The homeobox genes

- vox and vent are redundant repressors of dorsal fates in zebrafish. *Development* 2001;128:2407-20.
5. Ramel MC, Lekven AC. Repression of the vertebrate organizer by Wnt8 is mediated by Vent and Vox. *Development* 2004;131:3991-4000.
 6. Belting HG, Wendik B, Lunde K, et al. Pou5f1 contributes to dorsoventral patterning by positive regulation of vox and modulation of fgf8a expression. *Dev Biol* 2011;356:323-36.
 7. Zhao J, Lambert G, Meijer AH, et al. The transcription factor Vox represses endoderm development by interacting with Casanova and Pou2. *Development* 2013;140:1090-9.
 8. Fabian P, Pantzartzi CN, Kozmikova I, et al. vox homeobox gene: a novel regulator of midbrain-hindbrain boundary development in medaka fish? *Dev Genes Evol* 2016;226:99-107.
 9. Voronina AS, Pshennikova ES. mRNPs: From informosomes to stress granules. *Mol Biol (Mosk)* 2010;44:591-600.
 10. Voronina A, Pshennikova E. The Vox mRNA and protein expression in zebrafish Pou5f3 MZspg mutant embryos. *Stem Cell Investig* 2016;3:79.
 11. Kimmel CB, Ballard WW, Kimmel SR, et al. Stages of embryonic development of the zebrafish. *Dev Dyn* 1995;203:253-310.
 12. Voronina AS, Pshennikova ES. RNA isolation from the ribonucleoproteins fixed with formaldehyde. *Prikl Biokhim Mikrobiol* 2008;44:241-5.
 13. Sambrook J, Fritsch EF, Maniatis T. *Molecular cloning: a laboratory manual*. Volume 2. Cold Spring Harbor Laboratory Press, 1989.
 14. Lehmann R, Tautz D. In situ hybridization to RNA. *Methods Cell Biol* 1994;44:575-98.
 15. Gilardelli CN, Pozzoli O, Sordino P, et al. Functional and hierarchical interactions among zebrafish vox/vent homeobox genes. *Dev Dyn* 2004;230:494-508.
 16. Lippok B, Song S, Driever W. Pou5f1 protein expression and posttranslational modification during early zebrafish development. *Dev Dyn* 2014;243:468-77.
 17. Connors SA, Trout J, Ekker M, et al. The role of tolloid/mini fin in dorsoventral pattern formation of the zebrafish embryo. *Development* 1999;126:3119-30.
 18. Joly JS, Joly C, Schulte-Merker S, et al. The ventral and posterior expression of the zebrafish homeobox gene eve1 is perturbed in dorsalized and mutant embryos. *Development* 1993;119:1261-75.
 19. Pshennikova ES, Voronina AS. The proteins of Vent-family and their mRNAs are located in different areas of the tails of Zebrafish and Xenopus embryos. *Int J Biochem Cell Biol* 2016;79:388-92.

doi: 10.21037/sci.2017.06.05

Cite this article as: Pshennikova ES, Tereshina MB, Voronina AS. Expression of vox and vent mRNAs and encoded proteins in zebrafish embryos. *Stem Cell Investig* 2017;4:60.