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Molecular cloning and expression patterns of the *Vasa* gene from *Rana nigromaculata* (Amphibia: Anura)

Rui Jia [1]; Liu-Wang Nie [1, 2]; Ning Wang [1] & Jingjing Wang [1]

[1] *College of Life Sciences, Anhui Normal University. 1 East Beijing Road, Wuhu, Anhui, 241000, China. E-mail: jiarui20021104@yahoo.com.cn;*

[2] *Corresponding author. E-mail: lwnie@mail.ahnu.edu.cn*

ABSTRACT. The *Vasa* protein is a member of the DEAD (Asp-Glu-Alu-Asp) box family of ATP-dependent RNA helicases. The *Vasa* gene is specifically expressed in germ-line cells of many metazoans and is known to play a critical role in gametogenesis and reproductive regulation. In this paper, we isolate the full length cDNA sequence of the *Vasa* gene from the frog *Rana nigromaculata* Hallowell, 1861. The open reading frame (ORF) encoding 398 amino acid residues has nine conserved motifs. According to the similarities at the amino acid sequenceÿthe phylogenetic analysis of *Vasa* gene was consistent with the evolution relationships from chordates to mammals. Furthermore, the expression pattern analysis of *RnVasa* mRNA, using the technique of Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR), showed a high level of transcripts in testis, ovary and kidney, whereas little to no signal was detected in other tissues, which suggests that it may play a role during gametogenesis.

KEYWORDS. DEAD-box; phylogenetic analysis; RT-PCR.

The *Vasa* gene encodes an ATP-dependent RNA helicase that belongs to the DEAD-box (Asp-Glu-Ala-Asp) protein family (Mochizuki *et al*. 2001), and is specifically expressed in the germ cell lineage of vertebrates (Castrillon *et al*. 2000, Tsunekawa *et al*. 2000), insects (Nakao 1999, Chang *et al*. 2002), ascidians (Fujimura & Takamura. 2000), oysters (Fabious *et al*. 2004), brachiopods (Sagawa *et al*. 2005), nematodes (Kuznicki *et al*. 2000), planarians (Mochizuki *et al*. 2001), and cnidarians (Mochizuki *et al*. 2001). In *Drosophila* Meigen, 1830, after the *Vasa* gene expression was shown to be essential for polar granule formation and germ cell development (Liang *et al*. 1994), it has been broadly used as a germ cell marker for the germline cells in a variety of organisms.

RNA helicases are enzymes that catalyze the unwinding or separation of double-stranded RNA (dsRNA) by utilizing energy derived from hydrolysis of NTP (generally ATP) (Huang & Liu 2002). In addition, recent studies also suggested that some members might be involved in dissociation of protein-RNA interactions and modulation of other RNA secondary or tertiary structures (Rossler *et al*. 2001). Besides *Vasa*, some other RNA helicases, such as *p68* (Huang & Liu 2002), *P72* (Lamm *et al*. 1996), *Abstrakt* (Schmucker *et al*. 2000), etc.have different cellular locations and biological functions. RNA helicases are present in organisms ranging from *E. coli* to human and many viruses. RNA helicases are also involved in almost every cellular process of RNA metabolism including transcription, pre-mRNA splicing, rRNA processing, mRNA transport, translation initiation, ribosomal biogenesis and RNA decay during cell development and differentiation, exhibiting RNA helicase activity (Liang *et al*. 1994). The majority of RNA helicases have at least nine highly conserved motifs including a DEAD sequence. The large "DEAD-box" proteins family comprises several subclasses (including DEAD, DEAH and DExH groups) and all the members are generally believed to have both ATPase and RNA/DNA helicase activities (Huang & Liu 2002).

Vasa is required for the formation of germ cells and abdominal segments in the fruit fly (Johnstone & Lasko 2004). *Vasa* is also required for the embryonic stem cells differentiating into primordial germ cells and spermatogonium stem cells (Geijsen *et al*. 2004, Hubner *et al*. 2003) and as a molecular marker for the diagnosis of male infertility (Guo *et al*. 2007). *Vasa* is not only required for spermatogenesis, but also for the embryonic stem cells differentiating into primordial germ cells (Hubner *et al*. 2003). It may also play a role during gamete formation, for different splice variants have been identified with different expression patterns during male and female gonad differentiation in zebrafish (Krovel & Olsen 2004). During male gametogenesis, mouse *Vasa* knockouts affect proliferation and differentiation of the premeiotic germ cells, and no sperm are formed in the developing gonad (Tanaka *et al*. 2000). In frogs, *XVLG1* (Xenopus Vasa-like gene 1) protein was reported to function not only in germline cells but also in somatic cells (Ikenishi & Tanaka 2000).

The widespread dark-spotted frog, *Rana nigromaculata* Hallowell, 1861, is a representative of Ranidae, Anura, Amphibia (Chen *et al*. 1991), and plays an important role in the ecological

balance of nature. Amphibians represent a bridge in the evolution of vertebrates from aquatic to terrestrial. They have highly specialized morphological and functional characteristics to adapt to different environments (Fei *et al*. 1999). The molecular mechanism of their reproductive biology, such as spermiogenesis and sex determination, have not been well investigated in this group. In our study, we used *R. nigromaculata* as the study object, isolated and cloned the full length of *RnVasa* cDNA, discussed its phylogenetic evolution and elucidated the expression pattern in various tissues, including gonads, by RT-PCR technique.

MATERIAL AND METHODS

Animals

Three male and six female adult dark-spotted frogs were obtained from Wuhu, Anhui, China. Various tissues including testicle, ovary, brain, kidney, liver, spleen, heart, and muscle were dissected from the adult frogs, immediately dipped in liquid nitrogen, and stored at -80°C until used for total RNA isolation.

RNA preparation and reverse transcription

Total RNA was isolated from the testis of two adult frogs of *R. nigromaculata* according to the protocol of Trizol® Reagent (Takara, USA) and DNase digestion was completed during RNA purification using the RNase Free DNase Set (Takara, USA) according to the manufacturer's instructions. Total RNA integrity was checked by gel electrophoresis and purity established by calculating the ratio of the absorbance readings at 260 nm and 280 nm. Total RNA (1 μg) was reverse transcribed using 100 U of SuperScripti III Reverse Transcriptase (Invitrogen, USA) and 50 ng of random hexamer primers (Invitrogen, USA) in a 25-μl reaction according to the manufacturer's instructions. RNA samples were tested for genomic DNA contamination by including no enzyme reverse transcription (RT) and for reagent and aerosol contamination by including two no-template controls (one closed lid tube and one open lid tube during template addition).

Isolation of *Vasa* from *R. nigromaculata*

Degenerate polymerase chain reaction

According to Mochizuki *et al*. (2001), degenerate sense and antisense primers (Tab. I) were designed and synthesized according to a nucleotide alignment of conservative motifs of *Vasa* from related species that could be obtained from the National Center Biotechnology Information (NCBI) website. One μl of cDNA (equivalent to 40 ng of total RNA) was amplified using 1 U of Taq DNA polymerase (MBI, Fermentas, USA) and the degenerate primers described in table I at a 1 μM final primer concentration, in a 20-μl reaction with 64°C as the annealing temperature according to Fujimura & Takamura (2000) and PCR products were analyzed on a 2% agarose gel containing 0.5 μg/μλ of ethidium bromide and visualized under ultraviolet illumination. Gel bands of the correct estimated size were extracted using a Gel purification Kit (Sangon Inc, China).

Table I Primers for PCR amplification and analysis.

Primers	Sequence (5′-3′)
Sense	ATGGCNTG(C/T)GCNCA(A/G)ACNG
Antisense	TATTGCCACAGCGTCCAGT
M13.F	TTGTAAAACGACGGCCAGTG
M13.R	CACACAGGAAACAGCTATGACCATG
GSP. F	ACGTTCTGAACAAGGTCCA
GSP. R	CAACCTCTTCAAGCCAAGC
UTR. F	GATTGACGATAATCGTAGGC
UTR R	TGGCTTTGGCTGTCCGTAT
EVASF	CAACAGATAGGTAGTGAACG
EVASR	TATTGCCACAGCGTCCAGT
Actin5	GGGAGTGATGGTTGGCATGG
Actin3	AGGAAGGAAGGCTGGAAGAG

Cloning, sequencing, and analysis of data

Extracted products were cloned into pGEM®T Easy Vector System (Promega, USA) and sequenced using an ABI 377 sequencer (using M13.F and M13.R primers; Tab. I). The software Sequencher version 4.2 (Gene Codes Corporation) was used for sequence analysis. Analyzed sequences were compared to the GenBank database by BLASTN analysis (http://www.ncbi.nlm.nih.gov/blast).

5′-and 3′-rapid amplification of cDNA ends (RACE)

According to Frohman *et al*. (1988), 5′- and 3′-ends of cDNA were elongated. The sequence that had similarity greater than 80% to *Vasa* (i.e., *Rana lessonae Vasa* and *Xenopus Vasa*-like gene (GenBank accession nos. Q3MSQ8 and Q91372, respectively) was used to design gene specific primers (GSP.F and GSP.R; Tab. I) for use in RACE experiments. The SMART™ RACE cDNA Amplification Kit (Takara, USA) was used to isolate a full length Vasa-like cDNA by performing 5′- and 3′-RACE. A total of 1 μg testis RNA was reverse transcribed for each of the 5′ and 3′ reactions. RACE products were analyzed by gel electrophoresis, extracted, cloned, and sequenced as described above. The full-length open reading frame (ORF) of the *R. nigromaculata Vasa* homologue, *RnVasa*, was confirmed by designing primers in the untranslated regions (UTR) (UTR.F and UTR.R; Tab. I) and amplifying a continuous product. This product was visualized, extracted, cloned, and sequenced using the same procedures.

Comparative sequence analysis

The Entrez was performed iteratively at NCBI to search all full length Vasa protein sequences. Eight Vasa proteins from different evolutionary order species, including vertebrates and invertebrates, were selected to study their alignment with the deduced RnVasa sequence with Clustal W (Version2.0) multiple alignment s program (Thompson *et al*. 1994).

Phylogenetic analysis

Following alignment, a bootstrap neighbor-joining (NJ) tree (Saitou & Nei 1987) was produced in Mega 3.1 (Kumar *et al.* 2004). The proteins used in the analysis are typical members of DEAD-box family proteins including Vasa in vertebrates and invertebrates: HsVasa; *Homo sapiens* Linnaeus, 1758 (BC088362), MoDVH: *Mus musculus* Linnaeus, 1758 (NM_010029), RnVLG: *Rattus norvegicus* (Berkenhout, 1769) (NM_001077647), GgVasa: *Gallus gallus* (Linnaeus, 1758) (NM_204708), RlVasa: *Rana lessonae* Camerano, 1882 (Q3MSQ8), XlVLG: *Xenopus laevis* Daudin, 1802 (Q91372), XtVasa: *Xenopus tropicalis* (Gray, 1864) (CAJ82187), OmVas: *Oncorhynchus mykiss* (Walbaum, 1792) (AB032566), ZfVLG*: Danio rerio* (Hamilton, 1822) (Y12007), CIDEAD1a: *Ciona intestinalis* (Linnaeus, 1767) (NM_001032421), Olvas: *Oryzias latipes* (Temminck and Schlegel, 1846) (NM_001104676), BmVLG: *Bombyx mori* Linnaeus, 1758 (NM_001043882), DmVasa: *Drosophila melanogaster* Meigen, 1830 (AAM49782), CgVLG: *Crassostrea gigas* (Thunberg, 1793) (AAR37337), TtuVas: *Tubifex tubifex* (Mueller, 1774) (AB205013), LvVLG: *Litopenaeus vannamei* (Boone, 1931) (DQ095772), TcVas: *Tribolium castaneum* (Herbst, 1797) (NM_001039431), AaVLG: *Aedes aegypti* Linnaeus, 1762 (AAY41941), CeGLH1: *Caenorhabditis elegans* Maupas, 1900 (XP_001301342), CeGLH2: *C. elegans* (EAX88412), MoPL10: *M. musculus* (NP_149068), XlAn3: *X. laevis* (NP_001095245), Hsp68: *H. sapiens* (AAB53236), Xlp68: *X. laevis* (AAH82849), ZfPL10: *D. rerio* (AAH59794), Mjpl10: *Marsupenaeus japonicus* (Bate, 1888) (ABC88642), MoeIF4A: *M. musculus* (P60843).

Reverse transcription-polymerase chain reaction analysis (RT-PCR)

To make single-stranded cDNA, 2 μg total RNA from each tissue of testis, ovary, brain, kidney, liver, spleen, heart and muscle was transcribed to 1st strand cDNA respectively by using SuperScriptTM III reverse transcriptase (Invitrogen,USA) with oligo $(dT)_{20}$ primer in a reaction volume of 20 μl. RT-PCR reaction conditions were as follows: 94°C, 4 min; 35 cycles of 95°C, 40 s; 56.5°C, 50 s; 72°C, 60 s; and 72°C for 10 min. For RT-PCR amplification, we used 1 μl of cDNA per reaction and 0.2 μM primer sets of Evasa. In order to adjust for variations in the amount of input cDNA, the housekeeping gene β-actin was used as an internal standard (Tab. I). Each PCR product was detected by electrophoresis on 1.2% agarose gel.

RESULTS

Cloning and molecular characterization of *RnVasa*

Blackspotted frog full length *RnVasa* cDNA (2,175 bp, Accession no. EU035615, sequence not shown) was isolated from the testis. First, a 918-bp fragment was obtained by degenerated PCR. It was located in the middle of the open reading frame (ORF). Then, by using RACE PCR, a 5′ region of 974 bp and a 3′ region of 822 bp were isolated. *RnVasa* cDNA is composed of 1,197 bp, including an ATG start site that predicts an ORF encoding a protein of 398 amino acids. It has a 602 bp of 5′-untranslated region (UTR) and a 358 bp 3′-untranslated region (UTR), including a typical polyadenylation signal sequence AATAAA located 34 bp upstream from the poly (A) tail. Comparison of nucleotide sequence of *RnVasa* with those of other vertebrates indicates that the *R. nigromaculata* sequence is very similar to other anurans: 94% identity to *R. lessonae (Rana)* and 84% to *X. laevis* (*Xenopus*). The deduced amino acid sequence shows 68% of identity to zebrafish, 67% to chicken and 64% to mouse or human indicating that the *Vasa* gene is conserved through the process of evolution.

The *RnVasa* shows a high similarity to other *Vasa* proteins at two-thirds of its C-terminal protein (Fig. 1, shaded). It contains all nine conserved characteristic motifs, similar to the other DEAD-box proteins. Eight of the nine motifs are absolutely conserved including the four important ones (motifs I, II, III, and IV).

The nine characteristic motifs of *Vasa* proteins are as follows: AQTGSGKTAA (I), PTRELAVQ (Ia1), GG (Ia2), TPGR (Ib), DEAD (II), SAT (III), HGD (IV), ARGLD (V), HRIGRTGR (VI). The variable residues in the motif of Ia1 are indicated by a frame (Fig. 1). Due to these characteristic motifs always present in DEAD-box proteins and now found in the deduced *Vasa* sequence, *Vasa* can be inferred to encode a putative RNA helicase of DEAD-box family. And the analysis also shows that the conserved motifs structure is almost the same as in vertebrates and invertebrates, suggesting strong conservation of the gene structure of *Vasa* during evolution.

Phylogenetic analysis

As shown in figure 2, the chosen members fell into several subgroups, such as the Vasa subgroup, p68 subgroup and PL10 subgroup, GLH subgroup,etc. Generally, the proteins of the same type cluster together, even if they belong to different species, such as Vasa subgroup, PL10 subgroup and GLH subgroup, whereas those of different types are not always close to each other, even from the same species, i.e. there is such a long distance between Vasa and p68 from *X. laevis*. Within a clade, DEAD-box proteins from closely related species often cluster, i.e. Vasas from amphibians cluster together, whereas those from distant species are often far away from each other, as in the case of Vasa from humans being distant from Vasa from silkworm. In addition, even though they are all found in amphibians, Vasa from *R. nigromaculata* is closer phylogenetically to that of its closely related species, *R. lessonae*, than to that of other frog species, *X. tropicalis* and *X. laevis*, as expected. The phylogenetic tree using the unweighted pair-group method using arithmetic averages (UPGMA) method also shows an identical branching pattern (not shown). But the topology and branch distances within each clade are not in good agreement with their expected evolutionary relationships, perhaps due to the structural divergence and functional complexity of DEAD-box proteins within each subfamily.

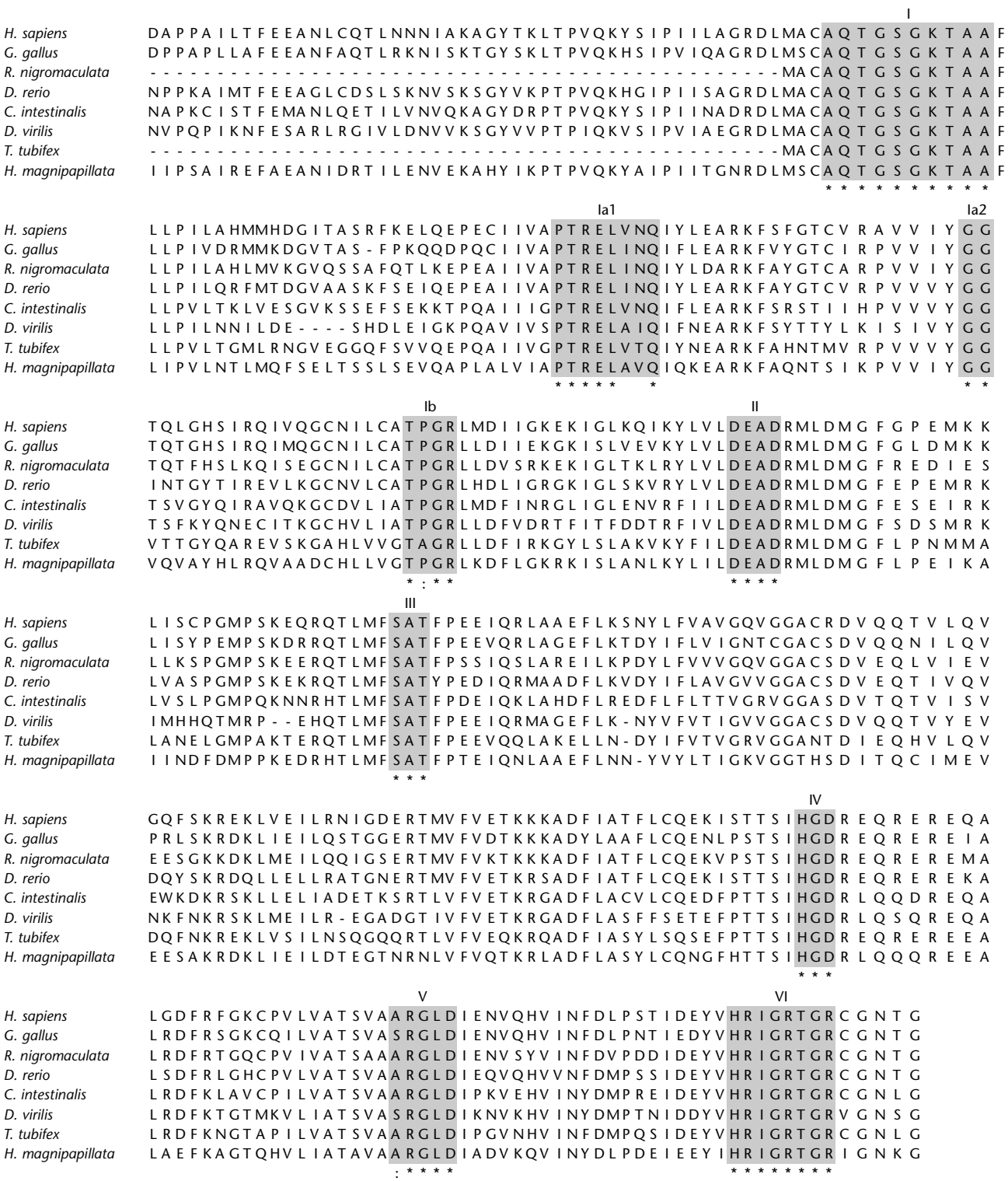

Figure 1. Alignment of the RnVasa protein sequence with the other vasa proteins included in the phylogenetic tree (Fig. 2). The characteristic motifs of Vasa proteins are shaded gray.

RT-PCR

RT-PCR was performed to study the *RnVasa* expression pattern in individual tissues. Results show that *RnVasa* is expressed in the heart, ovary, kidney, brain, and testis (Fig. 2). The level of expression, in descending order, is testis, kidney, ovary, brain, with faint expression in the heart. There is slight signal in other tissues such as spleen, muscle, pituitary and liver.

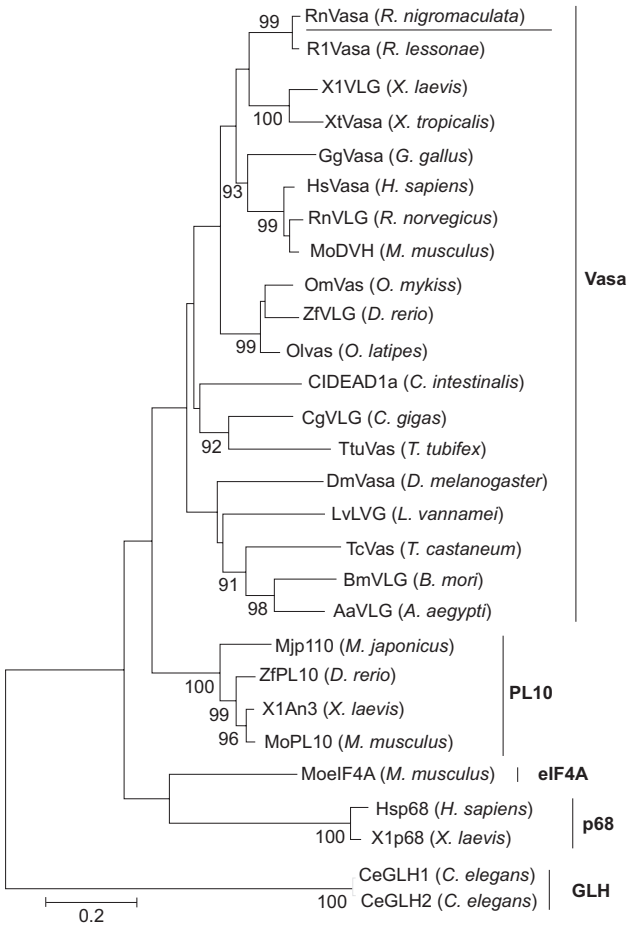

Figure 2. Phylogenetic trees of the deduced amino acid sequence of RnVasa and the complete amino acid sequences of 27 other DEAD-box proteins from different species. Numbers next to nodes are bootstrap values based on 1000 replicates, which indicate the reliability of branches in each tree. Branches with less than 90% support have been collapsed. Scale bars indicate number of substitutions per 100 amino acidresidues.

DISCUSSION

In this study, *RnVasa*, the full-length testis-derived cDNA of a putative *Vasa* from *R. nigromaculata*, was cloned, sequenced and its expression patterns were analyzed. The ORF of *RnVasa* was considerably shorter than the characterized complete coding sequences for *Vasa* in other species. Interestingly, however, the UTR at the 5′ and 3′ ends were similar in nucleotide length. This indicates that the UTRs of *RnVasa* may have similar functional roles to other characterized *Vasa* gene UTR regions (Bartfa & Orban 2003). In *RnVasa* sequence, all nine known characteristic motifs of DEAD-box protein are present in their appropriate spacing. However, the I a 1 (PTRELINQ) motif shows conservative substitution in its amino acid sequence, Isoleucine for Alanine in PTRELAxQ motif (Fig. 1). Motif II (or Walker B motif) has the amino acids DEAD, for which the family is given the name. This motif, together with motif I (or Walker A motif), the Q-motif and motif VI, is required for ATP binding and hydrolysis (Tanner *et al.* 2003). Motifs Ia and Ib, III, IV and V have been characterized less well but may be involved in interaction with RNA and in intramolecular rearrangements necessary for remodeling activity.

Repeats in the N-terminal region (commonly arginine-glycine-glycine (RGG) repeats) are also characteristic of many known Vasa proteins (Yoshizaki *et al.* 2000), It has been reported that this region is involved in the interaction with RNA (Kiledjian & Dreyfuss 1992). However, no such repeats were found in *RnVasa*. Our finding is similar for *Vasa* genes of *Danio rerio* and *Ciona intestinalis* in which no duplicated regions have been found. So RGG is not necessary for *RnVasa*, the function of this region can be realized by the spatial interaction of different conserved motifs (Cordin *et al.* 2006). There are four acidic amino acid residues in the last seven amino acid residues of RnVasa (Glutamate E and Aspartate D), that is in coincidence with the characteristic of Vasa protein sequences, which is identified by the ubiquity of several acidic amino acid in C-terminal of single strand DNA-binding protein (Fabious *et al.* 2004). The N-terminal of An3 knockout *X. laevis* has a normal ATPase activity than that of C-terminal knockout frog (Gururajan & Weeks 1997), suggesting that the comparatively conservative C-terminal sequence in DEAD-box family proteins is important to the maintenance of protein function than that of N-terminal.

The phylogenetic analysis supports that DEAD-box proteins assemble into different subgroups. It has been reported that similar DEAD-box proteins function similarly, i.e. p68, PL10 and related proteins within the same subgroup have similar biochemical properties (Rossler *et al.* 2001). Thus, it is very attractive to analyze structure, function and their correspondence of each subgroup and achieve some underlying rules.

Ikenishi & Tanaka (2000) demonstrated that *XVLG1* is expressed during early embryogenesis and might be involved in the differentiation not only of germline cells but also somatic cells. It is noteworthy that *XVLG1* was principally recognized in a juxtanuclear location of embryonic and adult germ cells and was detected transiently in the perinuclear region of somatic cells only at a certain period during development. After embryonic development, *X. laevis DMRT1* was specifically ex-

pressed in the primordial gonads at stage 52. In this study, we showed that *RnVasa* was expressed in the gonad of testis and ovary adult frogs by RT-PCR (Fig. 3). This gives us a clue to track the origin, translocation and differentiation of primordial germ cells (PGCs) of *R. nigromaculata*. It is plausible that RnVasa protein also functions in translation as the RNA helicase not only in germline cells but also in somatic cells. Therefore, it is possible that RnVasa protein functions in the translation of unknown mRNA, each of which is essential to the specification of a certain cell type.

To date, RNA of *Vasa* and its homologs have mainly been studied in germline cells of larval or adult gonads as a molecular marker of germ-line cells. Accordingly, information about the spatio-temporal distribution of RNA in embryos is scarce for most of the animals, including *Rana*. It is important to know the distribution in this animal and to compare it to others if we are to understand to what extent the mechanism of germ cell formation is conserved among animal species. We will investigate it further. Lastly, the presence of *RnVasa* in the brain opens up the possibility of an autocrine control of gonadotropin of Vasa in this tissue, and, together with the presence of *RnVasa* in the heart and other tissues, additionally suggests a role for Vasa in functions other than reproduction.

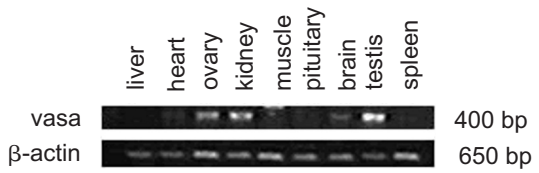

Figure 3. Tissue-specific expression of *RnVasa* in adult *Rana nigromaculata*. Total RNA was extracted from different adult tissues: liver, heart, ovary, kidney, muscle, pituitary, brain, testis and spleen. After the Dnase treatment, samples were subjected to RT-PCR. β-actin was used as a positive control.

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