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CLONING OF A GENE OF THE DEAD BOX PROTEIN FAMILY WHICH IS SPECIFICALLY EXPRESSED IN GERM CELLS IN RATS

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The cloning of a rat cDNA, *RVLG*, which encodes a novel protein of 713 amino acid residues with a molecular mass of 77,953, is described. The putative RVLG protein shares amino acid domains that are conserved in the DEAD protein family. It is approximately 52% identical and 85% similar in amino acid sequence to the *XVLG1*, a product which we previously cloned from *Xenopus* ovaries, suggesting it is the homologue of *XVLG1*. The size of the mRNA for *RVLG* is 3.1kb and can be detected specifically in the gonads of male and female adult rats.

Recently, a family of proteins containing the conserved motif Asp-Glu-Ala-Asp, the "DEAD box" proteins, has been identified (1). It plays divergent biological roles such as germ cell formation, spermatogenesis, RNA splicing, and cell growth, etc. (1-5). From an analogous study of eukaryotic initiation factor-4A (*eIF-4A*), they are believed to posses ATP-dependent RNA helicase activity, indicating they may act to regulate genes post-transcriptionally (6-9). Of the DEAD proteins, the *vasa* gene whose maternal absence causes defects in abdomen and in germ cells in *Drosophila* was isolated and the expression pattern of the mRNA and the protein in wild-type flies were characterized (10, 11). Its transcript is expressed in both male and female germ cells. Moreover, the protein localizes to the pole plasm, which has been proved to be a germ cell determinant in *Drosophila* embryos, indicating that vasa protein is a germ cell determinant component (12, 13).

There are many germ cell determination similarities in the process of embryogenesis between insects and anuran amphibians. Determination occurs through the segregation of a specific cytoplasmic component called polar plasm in *Drosophila* and germ plasm in *Xenopus* (14-17). Under these circumstances, we isolated a vasa-like cDNA (*XVLG1*) from *Xenopus*

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laevis (18). Expressions of the mRNA and protein were restricted to germ cells. Although exactly when the first protein expression occurred was not confirmed, it was detected in primordial germ cells (PGCs) in stage 46 tadpoles, when the PGCs migrate at the dorsal mesentery. Contrary to our expectations, the protein did not localize to the germ plasm, indicating it is not a component of the germ cell determinant in *Xenopus*.

We have not concluded whether *XVLG1* is the homologue of *Drosophila vasa*, since the function of *XVLG1* has not been determined. We have, however, two hypotheses that explain the different expression of these genes. The first is that the two genes are not homologous. In fact, the DEAD proteins expressed in germ cells have been published, e.g., *PL10* (19) for mice, *ME31B* (20), and *Dbp73D* (21) for *Drosophila*. The second hypothesis is that the germ cell determination cascade is different between the two species. In order to verify these points, one of the approaches was to isolate the homologue *XVLG1* from a different species such as a mammal, in which the germ determination is generally be considered to be independent from maternal determinants (22, 23). The *XVLG1* homologue, termed *RVLG* (Rat Vasa-Like Gene), was thus cloned from rats. Following are the sequence and expression data of RVLG mRNA.

MATERIALS AND METHODS

Extraction of total RNA, selection of poly(A)$^+$ RNA, and construction of a cDNA library. Total RNA from several tissues of adult rats (Wistar-Imanishi) was isolated separately by acid guanidinium thiocyanate-phenol-chloroform method (24). Testis poly(A)$^+$ RNA was selected by oligo-(dT) chromatography (Pharmacia). A testis cDNA library was constructed using the ZAP-cDNA Synthesis Kit (Stratagene).

Amplification of DNA fragments by a polymerase chain reaction (PCR) using degenerate primers. Degenerate primers (25) were designed to fit the amino acid sequences that are conserved among DEAD protein family members (18). The amino acid sequences of the two opposing primers are MACAQTG (region I in Fig. 2) and VLDEADRM (region V). The conditions for the PCR were described in (18).

cDNA library screening and DNA sequencing. The testis ZAP cDNA library (5×10^5 independent plaques) was screened using a PCR-amplified DNA fragment as a probe (see Results). The probe was labeled using the DIG DNA Labeling Kit (Boehringer Mannheim). Hybridization and washing were carried out as described in (26). Signals were detected using the DIG Luminescent Detection Kit for Nucleic Acids (Boehringer Mannheim). Nucleotide sequences were determined using the 373A DNA sequencer and the Taq Dye Primer Sequencing Kit (A.B.I.).

Northern hybridization. Testis and liver total RNAs (20μg) were separated by electrophoresis on 1% agarose-formaldehyde gels. Following electrophoresis, the gels were rinsed in 10x SSC and transferred to Hybond-N$^+$ (Amersham) in 20x SSC. Hybridization and detection were carried out as described above.

Reverse transcriptase PCR (RTPCR). DNaseI-treated total RNAs from several tissues were used for oligodT-primed first strand cDNA synthesis (Super Script Preamplification System, B.R.L.). One set of primers were 5'-ATAATCATTTAGCACAACCT-3' (at nucleotide position 2166) and 5'-GGGAGTAAGAACAGAAGAAC-3' (at position 2535). These primers were confirmed not to amplify genomic DNA, which may be reflected in the existence of an intron(s) between the primer sites. The conditions for the reaction were 94° C for 45 seconds, 50° C for 1 minute, and 72° C for 1 minute in 25 cycles. For the control experiment, the primers for the nucleotide sequences of rat elongation factor-alpha were used in the same condition described above.

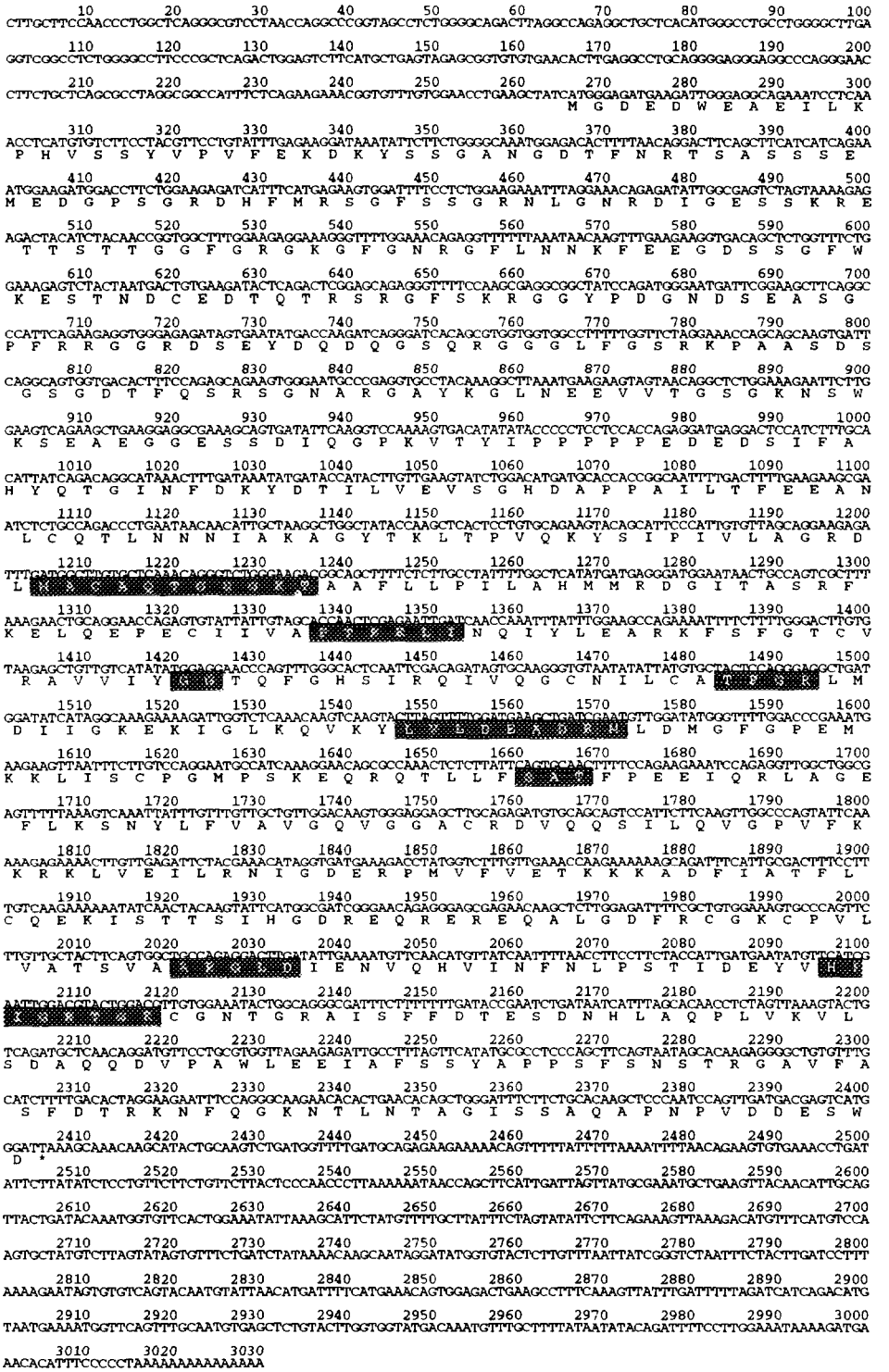


Fig. 1. Nucleotide and deduced amino acid sequences of *RVLG*. Black boxed sequences are the eight conserved regions (see Fig. 2).

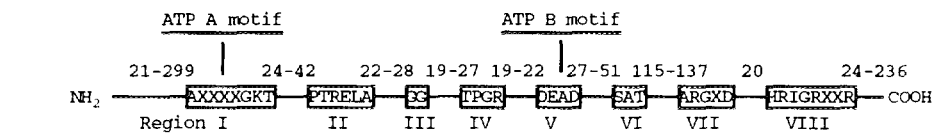
RESULTS

Cloning and sequencing *RVLG* cDNA. In order to isolate a probe for screening, we amplified the testis cDNA library by a polymerase chain reaction using degenerate primers, by which the reaction we had previously succeeded in isolating a *Xenopus* vasa-like cDNA (*XVLG1*) (18). Amplified fragments of about 400bp were cloned and sequenced to be classified into three groups: *PL10* (19), *p68* (4), and a unknown but homologous clone to *XVLG1*. We screened a testis cDNA library to isolate a full length cDNA, termed *RVLG*, using the third unknown fragment as a probe. The nucleotide and deduced amino acid sequences are listed in Fig. 1. *RVLG* encodes a protein with a molecular mass of 77, 953 and a p*I* value of 5.63. The amino acid sequence of *RVLG* was 52% identical and 85% similar to that of *XVLG1*, suggesting it is the homologue of *XVLG1*. The protein shares typical amino acid regions that are conserved among DEAD protein family members (Fig. 2).

***RVLG* mRNA expression.** *RVLG* expression was determined by a northern blotting and a RTPCR. Fig. 3 shows that the size of the *RVLG* transcript is 3.1kb (Fig. 3A), and the mRNA expression is restricted to the gonads of male and female adult rats (Fig. 3B).

DISCUSSION

There are many diversities among species in the determination processes of germ cells through embryogenesis. In *Drosophila* or *Xenopus*, determination depends on maternal factors, the germ cell determinants. However, in mammals, although totipotent cell lines (embryonic



eIF-4AI	IAQAQSGTGKT	PTRELA	GG	TPGR	VLDEADEM	SAT	ARGID	HRIGRGGR
PL10	MACAQTGSGKT	PTRELA	GG	TPGR	VLDEADRM	SAT	ARGLD	HRIGRTGR
An3	MACAQTGSGKT	PTRELA	GG	TPGR	VLDEADRM	SAT	ARGLD	HRIGRTGR
p68	VGVAQTGSGKT	PTRELA	GG	TPGR	VLDEADRM	SAT	SRGLD	HRIGRTAR
vasa	MACAQTGSGKT	PTRELA	GG	TPGR	VLDEADRM	SAT	SRGLD	HRIGRTGC
XVLG1	MACAQTGSGKT	PTRELI	GG	TPGR	VLDEADRM	SAT	ARGLD	HRIGRTGR
RVLG	MACAQTGSGKT	PTRELI	GG	TPGR	VLDEADRM	SAT	ARGLD	HRIGRTGR
PCR Primers	MACAQTG (primer A)				VLDEADRM (primer B)			

Fig. 2. A comparison of eight highly conserved amino acid regions of six DEAD protein family members and *RVLG*. Boxed sequences I to VIII show the consensus sequences of conserved regions, and the numbers between the boxes indicate the distance in amino acid residues. Underlined letters show substituted amino acids from the consensus sequences. PCR primers were designed against regions I and V.

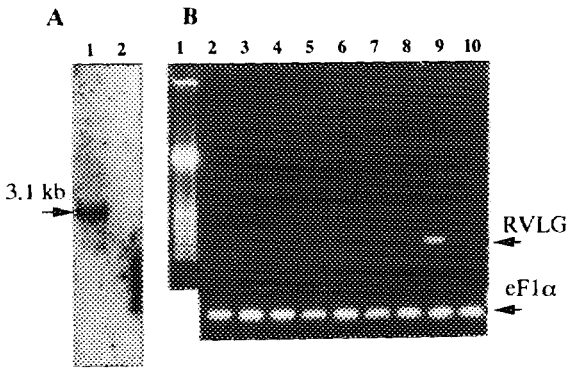


Fig. 3. Expression of *RVLG* mRNA. (A) Northern blot hybridization. Twenty micrograms of total RNA from adult testis (lane 1) and liver (lane 2) were transferred to Hybond-N. A fragment spanning the region IIX (see Fig. 2) to the poly $(A)^+$ site of RVLG was used as a probe. A single band of 3.1 kb was detected in the testis. (B) RTPCR was carried out to determine the expression level of *RVLG* mRNA. First strand cDNA libraries of different types of adult rat tissues were used as template for the PCR reaction. Primers and reaction conditions were described in the Materials and Methods section. After the reaction was complete, each ten microliter reactant was applied to 1% agarose electrophoresis gels. Specifically amplified DNA fragments of 369bp were detected only in germ cells containing testis and ovaries (lanes 9 and 10). Positive control experiments, in which primers for the rat elongation factor-alpha were used in the same conditions described above, were done. Lane 1, standard DNA size markers. Lane 2, liver. Lane 3, kidney. Lane 4, spleen. Lane 5, brain. Lane 6, skeletal muscle. Lane 7, heart. Lane 8, intestine. Lane 9, testis. Lane 10, ovary.

stem cells and embryonic germ cells) have been isolated (27, 28), it is unknown when and how determination occurs. Although we have no experimental data on the function of *RVLG*, the fact that the homologous gene to *XVLG1* that is expressed in early stage *Xenopus* PGCs, was isolated in a mammal, and, that the expression of both the mRNAs were restricted to germ cells, suggests that there may be similar mechanisms or cascades in the two species, and, that although they are thought to have different germ cell determination processes, the genes may play important roles in the reproductive systems of both *Xenopus* and rats. Similarities in the germ line cells in these species are supported by reports that nuage-like components, which can be found in all stages of *Drosophila* germ cells and are derived from germ cell determinants in oocytes (12), are found in the early stages of *Xenopus*, mice, and rats PGCs (29, 30, 31).

Further investigation will reveal the role of *RVLG* in germ cells. The mechanism of germ cell determination or differentiation in mammals will be revealed by a comparative study with an other species of *Drosophila* or *Xenopus*.

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REFERENCES

1. Linder, P., Lasko, P., Nielssen, P., Nishi, K., Schinier, J., and Slonimski, P. (1989) Nature 337, 121-122.
2. Wassarman, D. A., and Steize, J. A. (1991) Nature 349, 463-464.
3. Séraphin, B., Simon, M., Boulet, A, and Faye, G. (1989) Nature 337, 84-87.
4. Ford, M. J., Anton, I. A., and Lane, D. P. (1988) Nature 332, 736-738.
5. Hirling, H., Scheffner, M., Restle, T., and Stahl, H. (1989) Nature 339, 562-564.
6. Nielsen, P. J., McMaster, G. K., and Trachsel, H. (1985) Nuc. Acid. Res. 13, 6867-6880.
7. Nielsen, P. J., and Trashsel, H. (1988) EMBO J. 7, 2097-2105.
8. Rozen, F., Edery, I., Meerovitch, K., Dever, T. E., Merrick, W. C., and Sonenbeerg, H. (1990) Mol. Cell. Biol. 10, 1134-1144.
9. Pause, A., and Sonenberg, N. (1992) EMBO J. 11, 2643-2654.
10. Lasko, P. F., and Ashburner, M. (1988) Nature 335, 611-617.
11. Hay, B., Jan, L. Y., and Jan, Y. N. (1988) Cell 55, 577-587.
12. Hay, B., Ackerman, L., Barbel, S., Jan, L. Y., and Jan, Y. H. (1988) Development 103, 625-640.
13. Lasko, P., and Ashburner, M. Genes Dev. 4, 905-921.
14. Illmensee, K., and Mahowald, A. P. (1976) Expl Cell Res. 97, 127-140.
15. Ikenishi, K., Nakazato, S., and Okada, T. (1986) Dev. Growth and Differ. 28, 563-568.
16. Mahowald, A. P. (1992) Science 255, 1216-1217.
17. Strome, S. (1992) Nature 358, 368-369.
18. Komiya, T., Itoho, K., Ikenishi, K., and Furusawa, M. (1994) Dev. Biol. 162, 354-363.
19. Leroy, P., Alzari, P., Sassoon, D., Wolgemuth, D., and Fellous, M. (1989) Cell 57, 549-559.
20. Valoir, T. D., Tucker, M. A., Belikoff, E. J., Camp, L. A., Bolduc, C., and Beckingham, K. (1991) Proc. natl. Acad. Sci. U.S.A. 88, 2113-2117.
21. Patterson, L. F., Harvey, M., and Lasko, P. F. (1992) Nucl. Acid Res. 20, 3063-3067.
22. Johnson, M. H. (1981) Biol. Rev. 56, 463-498.
23. Tarkowski, A. K., and Wroblewska, J. (1967) J. Embryol. Exp. Morph. 18, 155-180.
24. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156-159.
25. Gould, S., Subramani, S., and Scheffler, I. E. (1989) Proc. Natl. Acad. Sci. USA 86, 1934-1938.
26. Asamino, R. M. (1986) Anal. Biochem. 152, 304-307.
27. Stevens, L. C., and Makensen, J. A. (1961) J. Natl. Cancer Inst. 27, 443-453.
28. Matsui, Y., Zsebo, K., and Hogan, B. L. M. (1992) Cell 70, 841-847.
29. Kalt, M. R. (1973) Z. Zellforsch. 138, 41-62.
30. Spiegelman, M., and Bennett, D. (1973) Embryol. exp. Morph. 30, 97-118.
31. Eddy, E. M. (1974) Anat. Rec. 178, 731-758.