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Short Contribution

Germ cell-specific expression of green fluorescent protein in transgenic rainbow trout under control of the rainbow trout *vasa*-like gene promoter

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ABSTRACT A technique to identify and isolate live fish primordial germ cells (PGCs) has not been established, in spite of the importance of purified germ cells for molecular and cellular studies. In rainbow trout, the distribution of *vasa* transcripts is restricted to the germ cell lineage, making this transcript a useful indicator of PGCs. Therefore, in this study, we cloned and characterized the rainbow trout *vasa*-like gene (*RtVLG*) regulatory regions and produced transgenic trout carrying the green fluorescent protein (GFP) gene driven by the *RtVLG* regulatory regions (p*vasa*-GFP) in order to identify live PGCs *in vivo*. In transgenic trout carrying the p*vasa*-GFP construct, cells showing green fluorescence were first observed at the mid-blastula stage; however, no cell-type-specific expression was observed at this stage. At the eyed stage, about 30% of the transgenic embryos showed specific GFP expression in PGCs, and at the hatching stage, about 70% of the transgenic embryos did so. An immunohistochemical study of hatching stage embryos revealed that the GFP-expressing cells are located in genital ridges. This transgenic trout, having visualizable PGCs, will make it possible to isolate live PGCs for *in vitro* studies and to study the ontogeny of PGCs including sex differentiation in live embryos.

KEY WORDS: *primordial germ cell, vasa, trout, transgenic, GFP*

Modifications of endogenous genes via homologous recombination (gene targeting) in fishes will provide an especially powerful tool for both genetic breeding and basic studies of gene expression and for understanding the function of gene products. In mice, cells containing a gene that has been modified by homologous recombination can be selected effectively using positive and negative selections (Thomas, 1991). If the host cells are pluripotent, such as embryonic stem (ES) cells, these genetic modifications can then be transferred to the germ line in chimeric individuals. However, fully-pluripotent ES cell lines have been established from limited strains of mice (Flechon, 1997) and the lack of fully-pluripotent cell lines or a cell line that has the ability to differentiate into germ cells is a major obstacle to achieving gene targeting in fishes. Although many attempts have been made (Wakamatsu *et al*., 1994; Sun *et al*., 1995; Hyodo *et al*., 1998; Hong *et al*., 1998), fish ES-like cells that can contribute to the germ line have not yet been established.

Embryonic germ (EG) cells that are derived from primordial germ cells (PGCs) can be an alternative to the ES cells for use in gene targeting. If the EG cells are transferred into mouse blastocysts, they can differentiate to all lineages, including the germ line (Donovan *et al*., 1997). Therefore, one way of establishing fish cell lines that have the ability to differentiate into germ cells is to establish EG cells using PGCs. As a first step in establishing a fish cell line derived from PGCs, techniques to identify and isolate live PGCs are indispensable. In mammals, cell surface antigen-specific antibodies are used to label the PGCs and these labeled PGCs can be isolated by the fluorescence-activated cell sorting (FACS) method (McCarrey *et al*., 1987). In addition, MacGregor *et al*. (1995) produced a knock-in mouse carrying a bacterial *lac*Z gene in the tissue non-specific alkaline phosphatase gene locus that is activated only in PGCs during embryogenesis. This PGC-specific *lac*Z gene expression was used as an indicator to purify PGCs by FACS (Abe *et al*., 1996). Also, Yoshimizu *et al*. (1999) produced a transgenic mouse carrying GFP driven by the Oct-4 gene promoter, which is specifically active in PGCs. In fishes, however, a convenient marker for identification and isolation of live PGCs has not been reported.

vasa gene products have been found in the germ cells of a wide range of animals (Ikenishi, 1998) including zebrafish (Olsen *et al*., 1997; Yoon *et al*., 1997; Braat *et al*., 1999; Weidinger *et al*., 1999).

Abbreviations used in this paper: bp, base pair(s); cDNA, DNA complementary to RNA; EG cell, embryonic germ cell; ES cell, embryonic stem cell; FACS, fluorescence-activated cell sorting; GFP, green fluorescent protein; kb, kilo base(s); PCR, polymerase chain reaction; PGCs, primordial germ cells; RtVLG, raibow trout *vasa*-like gene.

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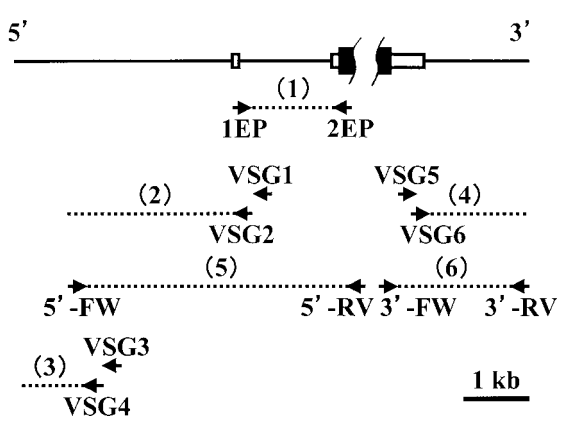

Fig. 1. Partial structure of the *RtVLG* gene and strategy for cloning the flanking regions. An initial fragment (1) was amplified using primers 1EP and 2EP. Then two consecutive vectorette-PCRs were performed to obtain the 5'-flanking region. For the first vectorette-PCR, primers VSG1 and VSG2 were used and amplified a 2.5 kb fragment (2). For the second vectorette-PCR, primers VSG3 and VSG4 were used and amplified a 1.2 kb fragment (3), sharing a 0.4 kb-overlap region with fragment 2. Primers VSG5 and VSG6 were used to amplify the 3'-flanking region (4) by vectorette-PCR. To combine fragment (1) and (2), PCR was performed using primers 5'-FW and 5'-RV. To obtain a fragment (6) containing the 3' untranslated region and the flanking region, primers 3'-FW and 3'-RV were used. To amplify the entire 5'-region, containing 3.3 kb of the 5'-flanking region, 130 bp of the first exon , 1.3 kb of the first intron, and part of the second exon (36 bp), gene SOEing was carried out using fragments (3) and (5). White and black boxes represent untranslated regions and amino acid coding regions, respectively.

In rainbow trout, the distribution of *vasa* transcripts is also restricted to the germ cell lineage, making this transcript useful for the identification of PGCs during embryogenesis (Yoshizaki *et al.*, 2000). In this study, we have cloned and characterized the rainbow trout (*Oncorhynchus mykiss*) *vasa*-like gene (*RtVLG*) regulatory regions, and produced transgenic trout carrying a GFP gene driven by the *RtVLG* regulatory regions in order to identify live PGCs *in vivo*.

Using PCR with primers specific for the first and second exons and two consecutive vectorette-PCRs for the 5'-flanking region, a 4.7 kb DNA fragment was amplified. Partial sequence analysis revealed that this fragment contained 3.3 kb of the 5'-flanking region, 130 bp of the first exon that contains only the 5'-untranslated region, 1.3 kb of the first intron, and a part of the second exon including the start codon (Fig.1). The 3'-vectorette-PCR amplified 1.5 kb of the 3'-flanking region (Fig.1). The plasmid DNA for microinjection, designated as p*vasa*-GFP, was constructed using the 4.7 kb 5'-fragment, the EGFP gene, the 3'-untranslated region derived from *RtVLG* cDNA (Yoshizaki *et al.*, 2000) and a 1.5 kb 3'-flanking region (Fig. 2). In the transgenic trout carrying the p*vasa*-GFP construct, cells showing green fluorescence were first observed at the mid-blastula stage (2.5 days after fertilization). However, the spatial locations of the fluorescent cells were inconsistent and no particular cell type-specific expression was observed. At the eyed stage, 27.6% of the transgenic embryos showed specific signals in PGCs (Fig. 3), 31.0% of the embryos showed ectopic GFP gene expression in addition to its expression in PGCs, and the remaining embryos showed only ectopic GFP gene expression. At the hatching stage, 69.2% of the transgenic embryos showed GFP gene expression only in PGCs and this GFP expression pattern was consistent with the staining pattern obtained by whole-mount *in situ* hybridization with an antisense *RtVLG* cRNA probe (Yoshizaki *et al.*, 2000). About 8% of the hatching stage embryos showed ectopic GFP gene expression in addition to its expression in PGCs, and the remaining embryos showed only ectopic gene expression (Table 1). Thus, PGCs-specific GFP expression was observed more frequently in the later developmental stages. The number of GFP-positive PGCs ranged between 1 and 15 in both the eyed- and the hatching-stages. An immunohistochemical study of the hatching-stage transgenic trout revealed that the GFP-expressing cells are located in the genital ridges and are relatively larger than the other cells (Fig. 4), which is consistent with their being PGCs (Patino and Takashima, 1995). Thus, this expression pattern of the GFP transgene was consistent with that of the endogenous *RtVLG* gene.

Fig. 2. Structure of the p*vasa*-GFP construct. White and black boxes represent the untranslated region and the amino acid coding region, respectively. The shaded box indicates the EGFP gene.

These results suggest that the 5'- and 3'-flanking regions and the first intron used in this study had enough *cis*-elements for PGCs-specific *RtVLG* expression. These transgenic trout, having visualizable PGCs will make it possible to isolate PGCs for *in vitro* studies by the FACS method. In addition, these transgenic trout can be a good model to study the ontogeny of PGCs during embryogenesis.

TABLE 1

EXPRESSION OF GFP IN TROUT EMBRYOS CARRYING THE P*VASA*-GFP CONSTRUCT

Stage of Embryos	Nos. of total Transgenic embryos observed	Nos. embryos with GFP expression only in PGCs	Nos. embryos with GFP expression in PGCs and others	Nos. embryos with ectopic GFP expression
St.15 (70 somites)	21	0	12 (57.1%)	9 (42.9%)
St.20 (eyed)	29	8 (27.6%)	9 (31.0%)	12 (41.4%)
St.23 (hatch)	26	18 (69.2%)	2 (7.7%)	6 (23.1%)

In this study, the ectopic expression of GFP was observed especially in the earlier stages. A similar ectopic expression of a transgene was reported in a transient expression system in zebrafish with the GATA-1 gene promoter (Long *et al.*, 1997). The reason why the ectopic foreign gene expression was observed in the early embryos is still unclear. In zebrafish, however, foreign genes microinjected into the cytoplasm of fertilized eggs remained in the unmethylated form during early embryogenesis and they started to be methylated at the shield stage and the degree of methylation gradually increased until the hatching-stage (Collas, 1998). It has been reported that DNA methylation plays an important role in turning off the transcription of genes whose activity is not required in a particular cell type (Kass *et al.*, 1997). Therefore, one possible explanation of the ectopic expression of the GFP gene in this study is that p*vasa*-GFP was not methylated appropriately during early embryogenesis, causing the loss of tissue specificity of the *RtVLG* promoter. In addition to this interpretation, the position effect (Bonnerot *et al.*, 1990) may be another important factor causing such an ectopic gene expression.

In this study, the number of GFP-positive cells was between 1 and 15. This small number of GFP-positive cells was probably caused by the mosaicism of the foreign gene in the host embryo. In transgenic rainbow trout produced by the same method used in this study, the percentage of foreign gene-positive cells was around 2%-30% (Yoshiura and Yoshizaki, unpublished data). The number of PGCs at the eyed-stage was predicted to be between 45 and 60 in rainbow trout embryos (Moore, 1937; Yoshizaki *et al.*, 2000). Therefore, 2-30% of 45-60 cells is about 1-18 cells and this number is in good agreement with the GFP positive-cell number observed in this study.

In conclusion, we have developed a technique to visualize and identify PGCs in live trout embryos. This technique should be a powerful tool for isolating live PGCs by dissection and enzymatic dissociation of embryos. In addition, these transgenic trout showing fluorescence in PGCs will allow us to analyze the ontogeny of PGCs, including the migration pattern of PGCs in embryos, the change in the number of PGCs during development, and the process of sex differentiation. We are raising the transgenic trout carrying p*vasa*-GFP for use as brood-stock for mass-production of F1 transgenic trout that have green fluorescence-labeled PGCs. Furthermore, we are presently attempting to establish techniques for isolating PGCs by FACS and for establishing cell lines derived from trout PGCs.

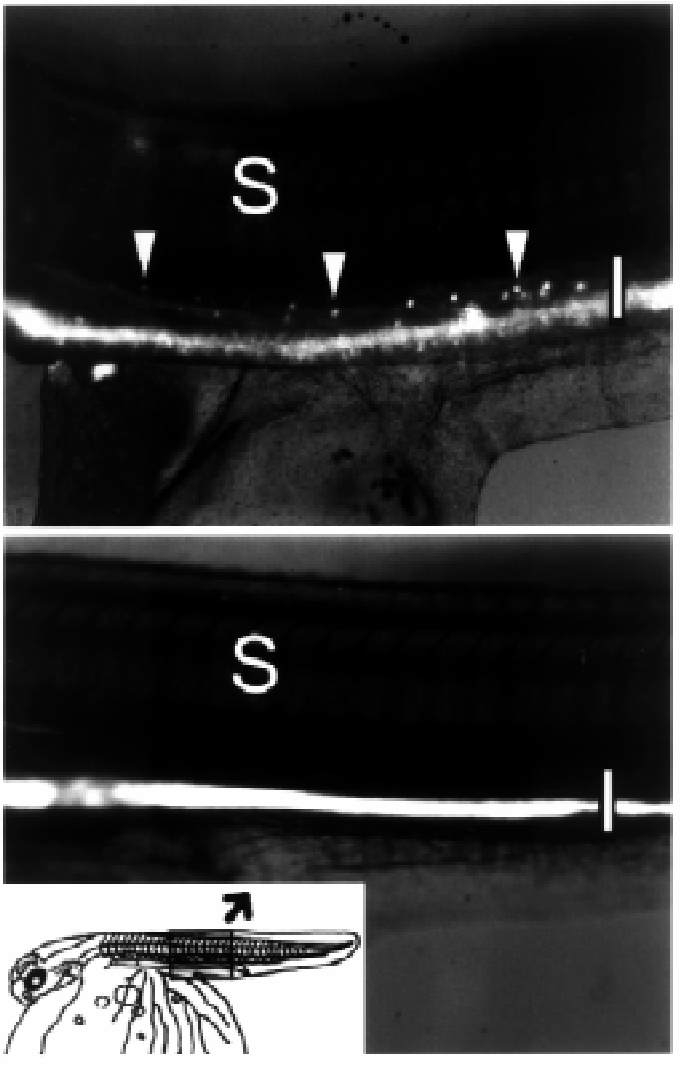

Fig. 3. Fluorescence pictures of the trunk region from a trout embryo carrying the p*vasa*-GFP construct (top) and a non-transgenic control (bottom). The GFP fluorescence is restricted to the PGCs (arrowheads). (I) indicates intestine showing auto-fluorescence. (S) indicates somite. The schematic drawing in the box shows the location of the fluorescence picture.

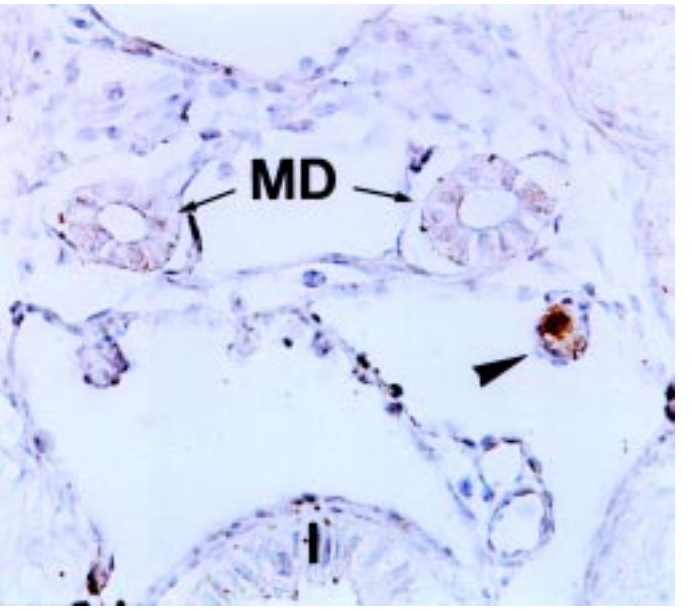

Fig. 4. Immunohistochemical identification of GFP-expressing cells in a hatching-stage embryo with a GFP-specific antibody. GFP was specifically expressed in PGCs located in a genital ridge (arrowhead). I: intestine. MD: mesonephric duct.

Experimental Procedures

Total DNA was extracted from rainbow trout liver using the proteinase K-SDS method (Yoshizaki *et al.*, 1991). To amplify the first intron, PCR was conducted using primers specific for the first exon (1EP; 5'-TGGGTTAGCTAGAAGACCGCTAGGT-3') and the second exon (2EP; 5'-CCTCATCCCAGTTGTCCATGATCTACA-3') based on the *RtVLG* cDNA sequence (Yoshizaki *et al.*, 2000) (Fig.1) with *LA Taq* (Takara Shuzo, Kyoto, Japan) according to the manufacturer's recommendations. For amplification of the 5'-flanking region, two consecutive vectorette-PCRs were carried out by the method of Siebert *et al.* (1995). For the first vectorette-PCR, primers specific for *RtVLG*, VSG1 (5'-CCTATGGCGCTAATTACCTAGCCAACA-3') and VSG2 (5'-ACCTAGCGGTCTTCTAGCTAACCCA-3') were designed using the sequence of the first intron after it was amplified by PCR (Fig. 1). VSG1 and VSG2 were used as the primary and nested primers, respectively. For the second vectorette-PCR, the primary primer (VSG3; 5'-TCATCTTCCACTGTACGCTGACGATTGGTCTA-3') and the nested primer (VSG4; 5'-CTACATTAACACCAAACCGGACACCCTCTCT-3') were designed according to the nucleotide sequence of the fragment amplified by the first vectorette-PCR (Fig.1). To obtain the 3'-flanking region, vectorette-PCR was conducted using VSG5 (5'-GACCTAACTGTGGTTCTGTTAACCA-GTCTGA-3') and VSG6 (5'-TGAGTTGACCCACGTTTTGGATCGCTTGTT-GT-3') as the primary and nested primers, respectively. These sequences are located in the 3'-untranslated region of *RtVLG* cDNA (Yoshizaki *et al.*, 2000). The amplified fragments were subcloned and sequenced as described by Yoshizaki *et al.* (2000).

Preparation of the p*vasa*-GFP construct

The fragment (DNA fragment No. 5 in Fig. 1) containing the 5'-flanking region obtained by the first vectorette-PCR, the first exon, the first intron, and a part of the second exon containing the start codon, were re-amplified using 5'-FW (5'-TGACAACATGACCAAGCGCCCGCAGAT) and 5'-RV (5'-

TGTGGTAACCTCATCCCAGTTGTCCATGA-3') as the forward and reverse primers, respectively, and *LA Taq* according to the manufacturer's recommendations. 5'-FW is located at the 5'-end of the first vectorette-PCR product (DNA fragment No. 2 in Fig.1) and 5'-RV is located at the junction of the 5'-untranslated region and amino acid coding region. This fragment and the second vectorette-PCR-amplified fragment (DNA fragment No. 3 in Fig. 1) were spliced together using the gene SOEing (splicing by overlap extension) method described by Vallejo *et al.* (1995). The fragment (DNA fragment No.6 in Fig. 1) containing the 3'-untranslated region and the 3'-flanking region were re-amplified using 3'-FW (5'-CTTCAGAGAGATGGGG-CAAGTCATCTA-3') and 3'-RV (5'-ACCATTCAAATGCAGACAGAGCTA-TGGATGTA-3') as the forward and reverse primers, respectively. 3'-FW is located at the end of amino acid-coding region (Yoshizaki *et al.*, 2000) and 3'-RV is located at the 3'-end of the vectorette-PCR product (DNA fragment No. 4 in Fig.1). The 5'-fragment of *RtVLG*, the coding region of EGFP (Clontech Laboratories, Inc., Palo Alto, CA, U.S.A.), and the 3'-fragment of *RtVLG* were spliced together using the gene SOEing method (Vallejo *et al.*, 1995) and were cloned into the pGEM-T Easy vector (Promega, Madison, WI, U.S.A.) yielding p*vasa*-GFP (Fig.2).

Production of transgenic trout and GFP observation

Two nl of p*vasa*-GFP solution (50 ng/µl in 10 mM Tris (pH 8.0), 0.1 mM EDTA) was microinjected into the blastodisc of fertilized eggs as described by Yoshizaki *et al.* (1991). Microinjected eggs were incubated at 10ºC. The distributions of GFP-positive cells in trout embryos were examined under fluorescence microscopy at the 70-somite-, eyed-, and hatching stages (stages15, 20, and 23, respectively), (Ballard, 1973) according to the method of Takeuchi *et al.* (1999).

Immunohistochemistry

Identification of the precise location and morphology of GFP-positive cells was aided by an immunohistochemical method employing an antibody specific to GFP. Tissues from transgenic and non-transgenic embryos at the hatching-stage were fixed for 24 hr in 10% formalin in phosphate buffered saline (PBS) (pH 7.4), dehydrated, and embedded in paraffin. For the immunohistochemical localization of GFP, 5-µm sections were mounted on 3-aminopropyltriethoxy silane (APS)-coated slides (Matsunami Glass, Tokyo, Japan), deparaffinized, and rehydrated.The immunoreactions were performed using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, U.S.A), and visualized using a 3,3'-diaminobenzidine (DAB) substrate kit (Vector Laboratories) following the manufacturer's protocol.Anti-GFP (F. Hoffmann-La Roche Ltd Basel, Switzerland) was used at a final dilution of 1:500 in PBS as the primary antiserum. Subsequently, the slides were washed in distilled water, and counterstained with hematoxylin.

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