Visualization of Primordial Germ Cells in Transgenic Rainbow Trout Carrying Green Fluorescent Protein Gene Driven by *vasa* Promoter.

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SUMMARY: To develop a cell-mediated gene transfer technique in fish, a cell line that can differentiate into the germ cell lineage is needed. In this study, we developed techniques to identify, visualize, and isolate live primordial germ cells (PGCs) for *in vitro* culture and later for gene transfer work. In many animals, the *vasa* gene products have been found in the germ cell lineage, and we have previously identified and characterized its homologue (*RtVLG*) in rainbow trout. Whole mount *in situ* hybridization of trout embryos at various developmental stages revealed that transcripts were localized to PGCs, suggesting that *RtVLG* transcription can be used as a marker for PGCs. To identify PGCs *in vivo*, we constructed a plasmid containing the green fluorescent protein (GFP) gene driven by the *RtVLG* regulatory regions (*pvasa-GFP*), microinjected it into fertilized eggs, and raised mature fish. The F1 generation of these transgenic trout showed green fluorescence only in PGCs in all developmental stages. This expression pattern was consistent with that of the endogenous *RtVLG* gene. To obtain PGCs for *in vitro* culture, the genital ridges were excised from transgenic embryos, dissociated by trypsin, and a flow cytometer was used to sort them into GFP-positive and GFP-negative fractions. RT-PCR analysis revealed that *RtVLG* was expressed only in GFP-positive cells. These results confirm that the isolated GFP-positive cells are PGCs. We are presently attempting to establish cell lines for further work.

KEY WORDS: primordial germ cell, *vasa*, GFP, transgenic fish, flow cytometry

INTRODUCTION

A cell-mediated gene transfer technique would make it possible to modify endogenous gene sequences via homologous recombination. This technique is called gene targeting and will provide an especially powerful tool for breeding, for basic studies of gene expression, and for understanding the function of gene products. The cell-mediated gene transfer method follows three major steps (Fig. 1): I) the establishment of stem cell lines which can differentiate into the germ cell lineage; II) the introduction of a foreign gene into cultured cells, and selection of stably transformed cells using markers such as drug resistance; and III) the conversion of the transformed cells to individual fish via germ-line chimera fish production. The

germ-line chimera is produced by transplanting the transformed cells into developing embryos. The fish that hatch consist of 2 types of cells - those derived from the host egg, and those from the transformed cells. If the transplanted cells can differentiate into the germ cell lineage, the gonads of chimeric fish can contain gametes from the transplanted cells. This means that if eggs and sperm are both from the transplanted cells, the resulting fish will be derived completely from the transformed cells.

In mice, cells containing a genome modified by homologous recombination can be selected *in vitro* by using marker genes²⁾. If the parent cells are totipotent cells, such as embryonic stem (ES) cells, the genetic modifications will appear in the germ cell lineage in chimeric individuals³⁾. In mice, ES

cell lines that can differentiate into the germ cell lineage have been established⁴⁾ but in fish, the lack of totipotent cell lines or a cell line that can

Fig. 1 Schematic representation of the strategy to produce transgenic fish using "cell-mediated gene transfer". White and gray cells show non-transformed and transformed cells, respectively. Black and gray fishes (or gametes) represent fishes (or gametes) derived from the host eggs and transplanted cultured cells carrying foreign genes, respectively¹⁾.

differentiate into germ cell lineage is a major obstacle to achieving the cell-mediated gene transfer. Although there have been many attempts to establish ES-like cells from early blastomeres^{5,6)}, germ line-competent ES cell lines have not yet been established. Therefore, we have chosen the primordial germ cell (PGC), a precursor of germ cell lineage, as the initial material for *in vitro* cell culture used for the cell-mediated gene transfer. This is because the most important characteristic required for the cells used is the ability to differentiate into the germ cell lineage.

In order to establish a cell line from PGCs, techniques to identify and isolate live PGCs are necessary. In fish, however, PGCs cannot be distinguished by morphological characteristics in live embryos, and a convenient molecular marker has not yet been reported. *Vasa* gene products have been found in the germ cells of a wide range of animals including zebrafish^{7,8,9,10)}. Therefore, in this study, isolation and characterization of a rainbow trout (*Oncorhynchus mykiss*) *vasa* cDNA was carried out as a first step to examine the applicability of *vasa* transcripts as a marker of PGCs. Then, we have cloned and characterized the rainbow trout *vasa*-like gene (*RtVLG*) regulatory regions, and produced transgenic trout carrying a green fluorescence protein (GFP) reporter gene driven by the *RtVLG* regulatory regions in order to visualize live PGCs *in vivo*. Finally, a method to isolate live primordial germ cells was established.

ISOLATION AND CHARACTERIZATION OF RAINBOW TROUT VASA-LIKE GENE cDNA¹¹⁾

Cloning of *vasa* cDNA was performed by degenerate- and RACE-PCR. The predicted amino acid sequence contained eight consensus sequences for the DEAD protein family and five arginine-glycine-glycine repeats, a common character of *Vasa* homologues¹²⁾. Overall the amino acid sequence was 79.2% similar to the *Vasa* protein of *Drosophila*¹³⁾. Whole mount *in situ* hybridization of embryos (14-days post fertilization, eyed stage -24 days; and hatching stage -32 days) revealed that transcripts were localized to the putative PGCs (Fig. 2). In adult rainbow trout, both ovaries and testes contained large amounts of *vasa* transcripts. Reverse transcription-PCR analysis of unfertilized eggs showed that *vasa* is a maternal factor. Although we

have not determined whether *vasa* functions as a germ cell determinant, its restricted expression in the germ cell lineage suggested that it can be used as a marker for PGCs. This marker will make it possible to identify the PGCs or presumptive PGCs in early trout embryos whose germ cells can not be distinguished by morphological characteristics.

Fig. 2 *In situ* localization of *RtVLG* transcripts in an eyed-stage embryo. Whole mount *in situ* hybridization was performed with an anti-sense DIG-labeled cRNA probe. *RtVLG* transcripts were localized in PGCs¹¹⁾.

PRODUCTION OF TRANSGENIC RAINBOW TROUT CARRYING A GFP GENE DRIVEN BY THE *RtVLG* PROMOTER¹⁴⁾

Since the *vasa* transcript is restricted to the germ cell lineage, its regulatory regions should be activated only in PGCs. Therefore, we produced transgenic trout carrying GFP gene driven by the *RtVLG* regulatory regions (*pvasa*-GFP, Fig.3) in order to

Fig.3 Structure of *pvasa*-GFP. White and black boxes represent untranslated regions and amino acid coding regions, respectively.

Fig.4 Fluorescence picture of the trunk region from an F1 transgenic embryo carrying *pvasa*-GFP. All PGCs showed green fluorescence.

visualize 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, we amplified a 4.7-kb DNA fragment containing 3.3 kb of the 5'-flanking region, 130 bp of the first exon, 1.3 kb of the first intron, and a part of the second exon including the start codon. The 3'-vectorette-PCR amplified 1.5 kb of the 3'-flanking region. The plasmid DNA for microinjection, designated as *pvasa*-GFP, was constructed using the 4.7 -kb 5'-fragment, the enhanced green fluorescent protein (EGFP) gene, the 3'-untranslated region (0.6 kb) derived from *RtVLG* cDNA¹¹⁾ and 1.5 -kb of the 3'-flanking region. This construct was microinjected into the cytoplasm of fertilized eggs. In the transgenic trout, cells showing green fluorescence were first observed at the mid-blastula stage (2.5 days after fertilization), but expression was not restricted to a particular location, or to a particular cell type. However, at the hatching stage, 69.2% of the transgenic embryos showed GFP gene expression only in PGCs, an expression pattern consistent with that obtained by whole-mount *in situ* hybridization with an antisense *RtVLG* cRNA probe. The F1 and F2 embryos produced by mating wild-type females and transgenic males showed green fluorescence only in PGCs during all developmental stages (Fig.4). An immunohistochemical study of hatching-stage transgenic trout revealed that the

GFP-expressing cells are located in the genital ridges and are relatively large, which is consistent with their being PGCs¹⁵). Thus, expression of the GFP transgene was consistent with that of the endogenous *RtVLG* gene. These results suggest that the 5'- and 3'-flanking regions and the first intron had enough *cis*-elements for PGC-specific expression.

ISOLATION OF LIVE PGCs USING A FLOW CYTOMETER

GFP-labeled PGCs were purified using a flow-cytometer. The genital ridges were excised from hatching embryos using green fluorescence as an indicator. The isolated genital ridges were dissociated by 0.5% trypsin and sorted into GFP-positive cells and GFP-negative cells. RT-PCR analysis with *RtVLG*-specific primers confirmed that *RtVLG* was expressed only in GFP-positive cells, confirming that they were PGCs.

In this study, we developed techniques to visualize and isolate live PGCs and now culturing these for gene transfer experiments. We have previously developed a method to transplant blastomeres into early embryos¹⁶), and are currently adapting this for transfer of PGCs into embryos, to obtain functional gametes derived from isolated PGCs.

ACKNOWLEDGEMENTS

This study was supported by grants from the Ministry of Agriculture, Forestry and Fisheries (Bio Design Project, BDP-01-IV-2-8) and Japan Society for the Promotion of Science (JSPS-RFTF97L00902).

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