Molecular cloning and expression in *Escherichia coli* of the glycoprotein gene of VHS virus, and immunization of rainbow trout with the recombinant protein

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The gene encoding the envelope glycoprotein of a recent Danish isolate of a salmonid rhabdovirus, viral haemorrhagic septicaemia virus (VHSV) has been cloned and sequenced at the cDNA level. When compared with the deduced sequence of a French isolate of VHSV, it was noted that there were 13 amino acid substitutions in the Danish virus. Amino acid homologies with the glycoprotein of a North American salmonid rhabdovirus (infectious haematopoietic necrosis virus) indicate a high degree of structural similarity between the two fish rhabdovirus glycoproteins. Results from partial enzymatic deglycosylation of the viral protein indicate that all four NXT/S sites found in the sequence are N-glycosylated in the virus. The glycoprotein, without the N-terminal leader sequence and C-terminal hydrophobic anchor segment, was expressed in *Escherichia coli* as a factor Xa protease-cleavable fusion protein. The purified and renatured viral part of the recombinant protein was able to elicit VHSV-specific antibodies and neutralizing antibody activity in serum when injected into rainbow trout.

Introduction

Viral haemorrhagic septicaemia virus (VHSV) is an enveloped negative strand RNA virus belonging to the Rhabdoviridae. VHS is one of the most devastating diseases in European rainbow trout farms and although vaccination of rainbow trout against VHS is possible it has not yet been successful beyond the experimental stage (for review, see Kinkelin, 1988). A vaccine developed by recombinant protein technology may overcome the economic and epidemiological safety problems associated with traditional vaccine preparations based on killed or attenuated virus, respectively. Development of such a vaccine will require identification and characterization of viral antigens that are able to induce a protective immune response in the fish.

Neutralizing antibodies are one of the immunological factors that are believed to play a role in immunity to rhabdovirus infections in general (Celis, 1990; Kelley et al., 1972). Neutralizing and protective monoclonal antibodies to the viral glycoprotein (G protein) of VHSV have been described earlier (Lorenzen et al., 1990) and the G protein is therefore a potential candidate for a recombinant vaccine.

This report describes cloning, sequencing and expression in *Escherichia coli* of the gene encoding this protein of a Danish isolate of VHSV. The amino acid sequence deduced is compared with the sequence of a French isolate of VHSV (Thiry, 1991) and with the sequence of the G protein of the North American salmonid rhabdovirus, infectious haematopoietic necrosis virus (IHNV) (Koener et al., 1987). The ability of the recombinant protein to induce formation of specific antibodies was tested in trout as well as in rabbits.

Methods

Molecular biology methods not described in detail here were performed according to Maniatis et al. (1982).

**Virus and cells.** A recent field isolate of VHSV serologically related to reference strain F1 (Jensen, 1965; VHSV DK-3592B) was inoculated into blue gill fry (BF2) cell cultures (Wolf et al., 1966) at a multiplicity of approximately 10 and RNA was harvested 24 h later.

**Synthesis of cDNA and cloning in λgt 10 and λgt 11 vectors.** Total RNA was isolated from infected cells by the acid–phenol–guanidinium thiocyanate procedure (Chomczynski & Sacchi, 1987). Polyadenylated RNA was purified on a polyuridylic acid-coated membrane (Amersham). Five µg of poly(A)⁺ RNA was used as template for cDNA synthesis (Pharmacia, cDNA kit), primed with random hexamer

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The nucleotide sequence data reported have been submitted to the EMBL database and assigned the accession number X66134.

oligodeoxynucleotides. The cDNA product was cut with EcoRI and cloned into the bacteriophage vectors λgt 10 and λgt 11.

Isolation and DNA sequencing of the cDNA corresponding to the G protein gene. The λgt 11 cDNA library was packed in vitro (Gigacpack, Stratagene) and plated on E. coli Y 1090 (ρE, M') cells in NZYM-top agarose with 50 μg ampicillin/ml. After incubation at 37 °C for 5 h small plaques were visible and the top agarose was overlaid with sheets of dry nitrocellulose BA85 membrane filters (Schleicher & Schuell), previously impregnated with IPTG in a 10 mM solution as previously described by Mierendorf et al. (1987). Incubation was continued overnight and the filters were subsequently incubated for 1 h in 0.15 M-PBS pH 7.2, containing 5% BSA. The filters were screened for plaques containing recombinant G protein by immunostaining with the monoclonal antibody MAb IP1H3 (Lorenzen et al., 1988). Plaques appearing positive were purified and λ DNA was isolated from mini-preparations. The λgt 11 inserts were subcloned into pUC9 for further analysis by nucleotide sequencing, using the Sequenase version 2.0 sequencing kit, essentially according to the supplier's description (United States Biochemical Corporation). Selected inserts were labelled with [3P] and used as hybridization probes for Northern blot analysis and screening of λgt 10 libraries.

Construction of recombinant plasmid for expression. A 1287 bp DNA fragment (VHS rG-1) housing the central part of the VHSV G protein gene, i.e. encoding the reading frame from Ser7 to Asn448 (Fig. 1), was amplified following the protocol of Sakai et al. (1988). This was accomplished by amplification in an ABACUS thermocycler (Hybaid), using 5 µg pUC9 harbouring a full length 16-kbp cDNA insert as template, one unit Taq DNA polymerase (HT Biotechnology), and 80 pmol of each of the two primers: 5' CGTCCTGGATCCATCGAGGGTAGGAGCACCACACCACAGATC 3' and 5' CGACCGGAGCCAGGCATGGCGGCCGAA GCCCGAAGGAAGATGGCGATG 3'.

The DNA fragment VHS rG-1 was excised with BamHI and HindIII (Boehringer), isolated after agarose gel electrophoresis, and ligated into the expression vector pLclIMLC (Nagai & Thogersen, 1987). This plasmid was modified from the expression vector pLclMLC (Nagai & Thogersen, 1987) by insertion of an oligonucleotide encoding six histidine residues following the expression-amplifying part of myosin light chain (MLC). At the 5' end, the VHS rG-1 nucleotide sequence was fused to a nucleotide sequence encoding the recognition amino acid sequence for the restriction protease factor Xa (Hle-Glu-Gly-Arg).

The resulting plasmid for expression of the VHS rG-1 gene in E. coli, pCMHV-G-1, is shown in Fig. 3(a).

Expression of the recombinant VHSV G protein (VHS rGp-1) in E. coli. E. coli QY 13 cells (F + lacI, trp, θ, Bb) bio-256 N + e857 ΔH Sm recA supplied by Dr. S. Brenner), containing a λ prophage with a temperature-sensitive mutation in the cl repressor gene and thus encoding a temperature-sensitive repressor protein, were transformed with pCMHV-G-1 and plated on NZYM agar containing 50 μg ampicillin/ml, and were grown overnight at 30 °C. Protein expression was induced in replica colonies by increasing the temperature to 42 °C for 3 h. A colony corresponding to a good producer of the MLCh2-vHS rGp-1 fusion protein, as judged by immunoblotting with MAb IP1H3 (Lorenzen et al., 1988), was selected from the 30 °C plate and inoculated into yeast–tryptone (2 x YT) medium at 30 °C with 50 μg ampicillin/ml and 0.25 ml/l antifoam (Sigma) for batch fermentation. As the exponentially growing culture reached an optical density of 1 at 600 nm, the temperature was raised to 42 °C. Fermentation was continued for 16 h and the cells were subsequently harvested by centrifugation.

Purification and folding of the recombinant protein. Inclusion bodies containing the VHS rGp-1 fusion protein were isolated, washed with detergent and solubilized overnight at 4 °C in 6 M-guanidinium chloride, 100 mM-DDT and 50 mM-Tris–HCl pH 8. The fusion protein was transferred into buffer A at pH 8 (8 M-urea, 1 M-NaCl, 50 mM-sodium phosphate) by gel filtration on Sephadex G-25 (Pharmacia) and then single-step-purified by affinity column chromatography on Ni2+-saturated nitrotetrazotetracyclic acid agarose (Ni–NTA column; Hochuli et al., 1988). The fusion protein loaded on the column in buffer A at pH 8 remained adsorbed to the column during washing in buffer A at pH 7 and was eluted with buffer A at pH 6.

The denatured protein was again reduced and thiol groups were fully blocked in a reaction with an excess of pyridyl glutathionyl disulphide before subjecting the protein to disulphide exchange under mildly oxidative and non-denaturing conditions (T. L. Holtet, unpublished procedure). The viral part of the fusion protein, rGp-1, was released by incubation of the soluble reoxidized fusion protein with factor Xa.

Immunization of trout and rabbits. Precipitated recombinant protein was resuspended in 50 mM-Tris–HCl, 2.5 mM-EDTA, 0.3 M-NaCl pH 7.5 at a concentration of 5 to 10 μg/ml. Four rainbow trout (Oncorhynchus mykiss) and two New Zealand white rabbits (Oryctolagus cuniculus) were each given two intraperitoneal injections of 0.5 ml of recombinant protein mixed with Freund's complete adjuvant with an interval of 5 weeks. A booster injection of diluted (10-1) protein without adjuvant was given 4 weeks later. Blood samples were taken 10 days after the booster injection. In the case of the rabbits, preimmune sera taken before the first injection served as negative controls and, in the case of the fish, four animals injected with adjuvant and buffer without VHS rGp-1 protein in parallel with those receiving the protein were used as controls.

Serological and electrophoretic analysis. Whole E. coli cell extracts were analysed for VHS rGp-1 by immunoblotting using MAb IP1H3 (Lorenzen et al., 1988) or serum from a trout surviving challenge with VHSV following immunization with natural G protein purified from virus particles (Lorenzen et al., 1993) for detection.

Sera from immunized animals were analysed for antibodies to VHSV in immunofluorescence tests (Jorgensen et al., 1991), plaque neutralization tests (PNT; Olsen & Jorgensen, 1986), and immunoblotting (Lorenzen et al., 1990, 1993). SDS-PAGE in 13% polyacrylamide gels followed the principles given by Laemmli (1970).

Deglycosylation of native G protein. Purified virus particles were treated with endoglycosidase F/N-glycosidase F (Boehringer Mannheim) as described by Lorenzen et al. (1990) with the modification that the enzyme treatment was limited to 3 h at room temperature and SDS was omitted from the reaction buffer when partial deglycosylation was desired.

Results

Cloning and sequence analysis of the VHSV G protein gene

By immunoscreening 106 plaques plated from the randomly primed λgt 11 cDNA library, using the monoclonal antibody MAb IP1H3 (Lorenzen et al., 1988), a clone carrying a cDNA insert of 850 bp was isolated. Northern blot analysis of RNA isolated from purified VHSV, VHSV-infected BF2 cells, and non-infected BF2 cells, with the 850 bp cDNA insert as probe, showed that the insert hybridized to viral genomic RNA and to an RNA band of approximately 1600 nucleotides present only in VHSV-infected cells (data not shown). A DNA fragment derived from the 5' end of the 850 bp cDNA insert was used as a hybridization probe to screen 5 x 104 plaques from the randomly
Recombinant glycoprotein of VHS virus

Fig. 1. Comparison of the amino acid sequences of the VHSV G protein (isolate DK-3592B) and IHNV G (Koener et al., 1987). Identical amino acid residues are indicated by vertical bars. Single amino acid substitutions between the DK-3592B and the French 07-71 (Thiry, 1991) sequences are indicated below the VHSV G sequence. Potential sites for N-glycosylation are marked with asterisks (*). The amino- and carboxy-terminal residues of the expressed protein VHS rGp-1 are indicated.

Production of recombinant G protein (VHS rGp-1)

The DNA segment of the VHSV G protein gene encoding Ser17 to Asn445 (VHS rG-1) selected for expression in E. coli does not include the nucleotide sequences encoding the putative signal peptide and the sequence from the putative transmembrane domain in order to reduce aggregation of the VHS rGp-1 protein due to hydrophobic interactions.

After expression of the protein, bacterial cells were analysed by SDS-PAGE. The fusion protein appeared as a distinct band in the molecular range of 60K (Fig. 3 b). The relative amount of recombinant protein, as analysed by SDS-PAGE, was greatly increased following separation of inclusion bodies from whole cell lysate. The inclusion body protein was soluble only under reducing conditions in a denaturing buffer. Affinity chromatography on the Ni-NTA column further increased the purity, although minor contaminating bands could still
Fig. 3. Expression of the recombinant VHSV G protein in E. coli. (a) Map of the expression plasmid pCMHVG-1. The non-viral part of the fusion protein consists of N-terminal fragments of λ cII and MLC followed by six histidine residues and a factor X~ cleavage site. (b) VHS rGp-1 protein preparations analysed by SDS-PAGE. Purified VHSV particles (lane 1); total protein of induced E. coli cells carrying the expression plasmid without insert (lane 2) or with the VHS rGp-1 insert (lane 3); washed inclusion protein (lane 4); purified recombinant protein after cleavage with factor X~, lane 5 (reduced) and lane 7 (unreduced). Factor X~ used for proteolytic cleavage (lane 6). MLCH~VHS rGp-1 fusion protein: fP.

Fig. 4. Identification of fusion protein (fP) in immunoblotting by antibodies specific for the VHSV G protein. Purified VHSV particles (lane 1); total protein of induced E. coli cells carrying the expression plasmid without insert (lane 2) or with the VHS rGp-1 insert (lane 3). (a) The membrane was stained with MAb IP1H3. (b) Staining with serum from a trout surviving challenge with virus following immunization with purified native VHSV G protein. (c) Staining with colloidal gold for total protein.

be detected. By carefully controlling the disulphide exchange reaction it was possible to prevent precipitation of the fusion protein when transferred to non-denaturing buffer conditions. Some intermolecular disulphide bonds were formed during this process since the protein was unable to enter 13% polyacrylamide gels except under reducing conditions (Fig. 3b). Repeated freezing and thawing resulted in the formation of precipitating aggregates. Additionally, when the purified fusion protein was cleaved with factor X~, the viral moiety as well as some uncleaved fusion protein precipitated. The molecular mass shifted from approx. 60K for the fusion protein to approx. 45K (Fig. 5) for the VHS rGp-1 protein (calculated mass 48K) upon cleavage with factor X~.

Serological analysis

In immunoblotting, the recombinant protein was specifically recognized by MAb IP1H3, and also by trout serum from a fish surviving challenge with VHSV following immunization with purified natural G protein (Fig. 4). Apart from the recombinant fusion protein a minor band was recognized by the trout serum (Fig. 4b) but not by the MAb (Fig. 4a). This band presumably represents a fusion protein degradation product that did not contain the epitope recognized by the MAb. Three of four rainbow trout injected with the VHS rGp-1 protein, but none of the control fish, were seropositive in PNT with normal trout serum as the complement source. The
Table 1. Serological analysis of animals injected with recombinant G protein

<table>
<thead>
<tr>
<th>Injected substance</th>
<th>Serum*</th>
<th>50% PNT†</th>
<th>IF‡</th>
<th>IB</th>
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<tbody>
<tr>
<td>Recombinant G</td>
<td>T1</td>
<td>1280</td>
<td>++</td>
<td>VHSV G</td>
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<tr>
<td></td>
<td>T2</td>
<td>&lt;160</td>
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<td></td>
<td>T3</td>
<td>1280</td>
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<td>T4</td>
<td>2560</td>
<td>+</td>
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<td></td>
<td>R1</td>
<td>&lt;40</td>
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<td></td>
<td>R2</td>
<td>&lt;40</td>
<td>+</td>
<td>VHSV G</td>
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<tr>
<td>Buffer</td>
<td>T5-T8</td>
<td>&lt;160</td>
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<td>--</td>
</tr>
<tr>
<td>None</td>
<td>R1-R2</td>
<td>&lt;40</td>
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* T refers to sera from trout, R to sera from rabbits.
† Mean of two determinations. Trout sera were not analysed in dilutions below 160⁻¹ and rabbit sera not below 40⁻¹ owing to background reactivities and cytotoxic serum components.
‡ Immunofluorescence reactivity (IF) was evaluated as either negative (−) or positive on a scale from + (weak) to +++ (strong).

Fig. 5. Double immunofluorescence staining of VHSV-infected CHSE (Fryer et al., 1965) cells. Staining was performed with a 250⁻² dilution of a serum from a trout (T1) immunized with the VHS rGp-1 protein (a) and with MAb IP5B11 recognizing VHSV N protein (b) (Lorenzen et al., 1988). Bar marker represents 10 μm.

Discussion

With a future recombinant vaccine in mind, one aim of the present study was to investigate whether a recombinant form of the VHSV glycoprotein produced in E. coli would be immunogenic in rainbow trout. Also, a major goal was knowledge of sequence differences between the G protein of the Danish VHSV isolate used in this study (DK-3592B) and the previously cloned G protein of the French isolate 07-71 (Thiry, 1991; Thiry et al., 1991), in relation to structural predictions and protective epitopes described for the G protein of the related IHNV (Koener et al., 1987; Xu et al., 1991).

The VHSV glycoprotein gene as reported here contains 1521 nucleotides corresponding to a predicted protein of 507 amino acids and a calculated Mr of 57K, including the presumed N-terminal signal peptide. This is in agreement with the published size of 55K for the
deglycosylated viral protein (Lorenzen et al., 1990). Thirteen amino acid substitutions noted in the Danish isolate on comparison with the French 07-71 isolate were all, except for Lys91 changed to Arg91 in the 07-71 sequence, confined to the C-terminal portion of the G protein from Ala8 to Ile462 (Fig. 1).

The VHSV G protein and the IHNV G protein amino acid sequences are 38% identical, which is nearly twice the degree of similarity between the VHSV G and the G proteins of rabies and vesicular stomatitis virus (not shown), indicating a closer evolutionary relationship between VHSV and IHNV than VHSV and mammalian rhabdoviruses. Also, certain conserved features of the deduced amino acid sequence of the two salmonid rhabdovirus G proteins indicate the existence of structural and functional homologies beyond general rhabdovirus similarities. Among the 16 cysteine residues, 15 occur in identical positions (Fig. 1). Owing to their ability to form intramolecular disulphide bonds, cysteine residues are important in preserving the secondary and tertiary structure of proteins (Matsumura et al., 1989). Hence, conservation of the sequence positioning of cysteine residues strongly indicates that the three-dimensional structures of the two viral G proteins are very similar. This is supported by the presence of seven stretches of five to nine identical amino acids in a row in the extracellular part of the protein and also by the fact that none of the differences between the Danish VHSV DK-3295B and French (F) 07-71 isolates are located at positions where homology to IHNV occurs, except at His459 (DK)/Asn459 (F). The amino acid sequence similarity is also high between the hydrophobic regions in the termini of the VHSV and the IHNV G proteins (Fig. 1). As suggested by Koener et al. (1987), the differences from mammalian rhabdoviruses in these regions may indicate specialization for the fish cell membrane.

Although the VHSV and IHNV G proteins share only one site for N-glycosylation, i.e. at Asn438, the overall pattern of potential N-glycosylation sites (one site in the amino-terminal part, no sites in the middle, and three sites in the carboxy-terminal part) of the protein is shared (Fig. 1). As the presence of oligosaccharide side-chains may influence protein structure and function (Sjöblom et al., 1987; Vidal et al., 1989) this similarity represents a further indication of a conserved structure in the glycoproteins of the two salmonid rhabdoviruses.

When more fish rhabdovirus G protein genes have been sequenced, it will be interesting to analyse whether the described similarities between VHSV and IHNV are due only to an evolutionarily close relationship between the two viruses or whether a general structural conservation of fish rhabdovirus G proteins also exists.

Partial enzymatic deglycosylation of the natural VHSV glycoprotein resulted in a four-step degradation pattern (Fig. 2) which, as suggested by Elder & Alexander (1982), could be taken as evidence for the presence of four oligosaccharide side-chains. This indicates that the potential sites for N-glycosylation in the VHSV G protein are all used. Whether the G protein of the French VHSV isolate 07-71 contains five oligosaccharide chains corresponding to the five predicted glycosylation sites remains to be investigated.

Previous reported attempts to produce immunogenic forms of the rabies virus G protein in E. coli have been unsuccessful (Malek et al., 1984). One reason could be that no attempts to renature the recombinant proteins were made.

When expressed in E. coli eukaryotic proteins are not glycosylated. If the carbohydrate side-chains play an important role in the immunological properties of the protein, a future recombinant subunit vaccine will have to be produced in a eukaryotic expression system. Successful vaccinations, however, against IHNV with a prokaryotic recombinant G fusion protein have been reported recently. Bath immunization with lysates of whole E. coli cells containing inclusion bodies of recombinant forms of IHNV G polypeptides derived from
the C-terminal half of the protein was shown to protect trout against challenge with IHNV (Gilmore et al., 1988; Xu et al., 1991). Neither the immunological factors involved nor the specificity of the protection were investigated.

On the basis of the similarity and the described conservation of structurally important characteristics between the IHNV G and VHSV G proteins it might be expected that similar results could be obtained with a recombinant form of the VHSV G protein. However, attempts to induce antibody formation as well as to protect rainbow trout against VHSV by immunization with bacterial cell lysates or inclusion bodies containing a truncated VHSV G fusion protein, essentially consisting of MLC and amino acids 225 to 507 of the VHSV G protein have been unsuccessful (N. Lorenzen et al., unpublished). In their successful immunizations, Gilmore et al. (1988) and Xu et al. (1991) used even more truncated forms of the IHNV G protein, expressed as a TrpE fusion protein. Selection of the relevant domain of the G protein and perhaps a proper non-viral moiety in the fusion protein thus may be crucial for the immunogenicity of the inclusion proteins.

Xu et al. (1991) have mapped two linear epitopes, 2F and 136J, to the C-terminal half of the IHNV G protein within the part of the protein giving the highest protection against IHNV when expressed as a recombinant fusion protein. In the present study we found the highest degree of heterogeneity between the Danish 3592B and the French 07-71 VHSV isolates in the corresponding region. Whether or not this will have any implications for the ability of a future recombinant G protein vaccine to protect against both isolates of VHSV needs to be clarified by future challenge experiments.

In the present study the identity of the recombinant protein containing amino acids 17 to 445 of the VHSV G protein was verified by the G-specific MAb IP1H3 and by serum from a trout immunized with purified natural G protein. By immunization with a purified and refolded form of the recombinant protein, neutralizing activity was induced in three of four trout. The neutralizing ability was complement-dependent, as is normally observed for trout sera (Dorson & Torchy, 1979; Olesen & Jørgensen, 1986), but the antibody activity also appeared sensitive to heating. That this thermodurable activity was in fact related to antibody reactivity in the trout sera was shown by the ability of a MAb to trout Ig (MAb 4C10; Thuvdander et al., 1990) to inhibit the neutralizing ability (not shown). The trout antibodies involved in the neutralization process in the present case may thus be of rather low affinity and their reactivity more influenced by heat denaturation. VHSV-specific antibodies could be detected by indirect immunofluorescence and/or immunoblotting in two of the fish sera as well as in one of the rabbit sera (Table 1). No cross-reactivity with IHNV was detected as analysed by immunofluorescence or neutralization (not shown). The recombinant protein is thus immunogenic in both species and as only small (µg) amounts of protein were used for immunization, a stronger and more uniform response is probably obtainable by increasing the amount of antigen injected.

Qualitative improvements of the recombinant protein may also increase the immunogenicity. Formation of intermolecular disulphide bonds as well as the problems concerning precipitation of the partly refolded recombinant protein described here indicate incomplete protein refolding. Renaturation after purification under denaturing conditions is probably an essential factor for immunogenicity in trout, since trout antibodies to the VHSV G protein have often been observed to be directed against epitopes of a discontinuous character, e.g. epitopes dependent on intramolecular disulphide bonds (Lorenzen et al., 1993).

Further experiments concerning protein renaturation as well as vaccination experiments are required before it can be decided whether development of a prokaryotic recombinant vaccine against VHSV in rainbow trout is a realistic objective. Nevertheless, the results presented here represent an important point in demonstrating that a recombinant protein produced in E. coli can induce a specific immune response to the VHSV G protein in rainbow trout.

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