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EXPRESSION OF THE INTERFERON RESPONSIVE GENE IN THE COMMON CARP (*CYPRINUS CARPIO*)

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ABSTRACT

Viral haemorrhagic septicaemia (VHS) is an important rhabdo viral disease in the fish farming . Over recent years a highly effective experimental DNA vaccine to VHS has been developed. Virus-induced genes were ultimately retrieved from the subtracted cDNA library, and their differential expression was further confirmed by semi quantitative reverse transcription-PCR and Northern blot analysis. The ability of the plasmid to mediate expression of the viral G-protein on the surface of transfected cells was confirmed by specific immunostaining for the viral protein. The type 1 interferon (IFN) system is a rapid and powerful antiviral defense. Using a luciferase cell line to detect expression of IFN, it was shown that the Common Carp (*Cyprinus carpio*) Gonad cell line (CCG - p1), produces IFN when transfected with a plasmid encoding the glycoprotein of VHSV, but not with plasmid vector alone.

The results showed that the cells transfected with pcDNA3 vhs-G plasmid were expressing G protein, while the cells transfected with pcDNA3 plasmid did not show any staining. The cells transfected with the pcDNA3 vhs-G showed an increased luciferase activity of 5.4-fold compared with the control non-transfected cells . this increase was statistically significant ($p < 0.05$).

INTRODUCTION

Virus infections induce changes in the expression of host cell genes. The interferon (IFN) family consisting of alpha IFN (IFN-α), IFN-

β, IFN-ω, IFN-δ, IFN-κ, and IFN-τ, is a large group of cytokines involved in the innate immune response against various microorganisms (Altmann *et al*., 2003).

Differential cytokine production by T cells plays an important role in the outcome of the immune response (Werling *et al*., 2004). An important subdivision of effectors T cells can be made based on patterns of cytokine production and functional programmers (Chtanova *et al* ., 2001).

In infected cells, virus replication is blocked by the double-stranded RNA induced activation of antivirl proteins such as protein kinase R (PKR), 2 , 5 - oligoadenylate synthease (OAS) , and RNase, proteins responsible for inhibiting host and viral protein synthesis and degrading viral mRNA.

Genes for IFN have been cloned from a variety of mammalian, avian, and some of fish species. Viral haemorrhagic septicaemia (VHS) is an important rhabdo viral disease in the fish farming in Europe and over recent years a highly effective experimental DNA vaccine to VHS has been developed (Lorenzen *et al* ., 2002). The type 1interferon (IFN) system is a rapid and powerful antiviral defence mechanism in vertebrates. Recently, IFN genes have been identified in a number of teleost fish species (Robertsen , 2006).

The viral surface glycoprotein (G protein) gene of the respective viruses, not only stimulate the production of antibodies and protection but they also induce the expression of the interferon (IFN)-inducible anti-viral Mx gene in the muscle tissue of rainbow trout at the site of injection (McLauchlan *et al*., 2003).

Vaccinated fishes exhibit an early non-specific (heterologous) protection, which becomes specific in about 4-6 weeks post-vaccination coinciding with the appearance of serum neutralizing antibodies in some of the fish (Lorenzen *et al*., 1998).

Recombinant cytokines are valuable tools for functional studies and candidates for vaccine additives or therapeutic use in various diseases. They can also be used to generate specific antibodies to analyses the roles of different cytokines during immune responses (Werling *et al*., 2004). Furthermore, mammalian IFNs also exert additional antiviral effects by up regulating MHC class I and II molecules on the surface of both target and antigen presenting cells as well as enhancing NK cell cytotoxicity (Kim *et al* ., 2000).

Type 1 T cells produce interferon (IFN)- gamma and protect against viral pathogens, whereas type 2 cells produce cytokines such as IL-4 and IL-5 and protect against large extra cellular parasites.

MATERIALS AND METHODS

The Common Carp (*Cyprinus carpio*) leukocytes cell line has been infected with viral hemorrhagic septicemia virus (VHSV), and total RNA from infected and un-infected cells were compared post infection.

Cell line and Plasmid constructs

The transgenic Common Carp (*Cyprinus carpio*) ; gonad cell line(CCG-P1) and the plasmid construct , were performed with the cooperation of Dr Inessa A. Zelenin , Institute of Molecular Biology, Academy of Sciences, Moscow,. The gene encoding the full length VHSVG-protein expression vector pcDNA3 named pcDNA3-vhsG Cells transfected with the pcDNA3 vhs-G plasmid, Intron / exon organization. To determine the intron / exon organisation of CCF IFN, Common Carp genomic cDNA was isolated from G14D T cells and amplified by PCR using primers that flanked a region predicted to contain an intron based on a multiple alignment , primers. After confirming by automated sequencing, a single clone, designated, was identified. Cultures, plasmid are subsequently used as templates in PCR reactions using primers. PCR products from these reactions were cloned.

Transfection of CCG-P1 cells with pcDNA3-vhsG and pcDNA3 was performed and RT-PCR analysis was conducted according to the manufacturer'sinstructions. Cells transfected with pcDNA3 and untreated cells were used as positive and negative controls, respectively.

Immunological detection of G protein

The ability of the plasmid to mediate expression of the viral G - protein on the surface of transfected cells was confirmed by specific immunostaining for the viral protein (Biberfeld *et al* ., 1974).

Detection of luciferase activity

Luciferase expression by the CCG-P1 cells was measured through slight emitting activity in the presence of the luciferin substrate. Cells were trypsinised (trypsin / EDTA) to detach them from the slides and collected by centrifugation for 10 min at 800g at 4^{o}C.The cell pellet was resuspended in 100ml of Steady GL_O^{TM} luciferase substrate (Promega).

The emission of light was measured with a Victor 3 luminometer (Perkin Elmer). The integrated sum of light emitted over 10s was measured and expressed as relative light units (RLU).

Real time PCR analysis

Total RNA was extracted from the cells using the RNeasy mini Kit (Qiagen) according to the manufacturer's Animal Cell 1'protocol. The synthesis of cDNA was performed using the Taqman Kit (Applied Biosystems) at a final reaction volume of 25ml. Firstly, 9.125 ml RNA and 2.5mM oligo d (T)16 (final conc.) were mixed and denatured at 70 ^{o}C for 10min . Denaturing reactions were placed on ice and mixed with 1reverse transcriptase buffer, 5.5 mM MgCl2, 2mM dNTPs , 0.4U RNase inhibitor and reverse transcriptase. Reactions were incubated at 48 ^{o}C for 90 min and 95 ^{o}C for 5 min.

The Taqman primers and probes were designed on a splicing site in order to amplify and detecte exclusively cDNA encoding Common Carp elongation factor (ELF) or type 1interferon (IFN) and were labeled with tag 6-FAM as a marker .The sequences of primers and probes are given in Table (1).

Cells transfected with the pcDNA3vhs-G plasmid , IFN mRNA expression, were compared with the non-transfected control ones.

Statistical analysis

Results (mean SD) are pooled data from three replicate readings per sample. Data analysis was performed applying a unifactorial ANOVA test and p< 0:05 was considered significant.

RESULTS AND DISCUSSION

The VHSV G protein is a transmembrane molecule, following intramuscular injection of the DNA vaccine the extracellular domain of the G protein is expressed on the surface of transfected muscle cells (Lorenzen *et al*., 2002).VHS-G protein expression was in transfected CCG-P1cells. The cells transfected with pcDNA3 vhs-G plasmid were expressing G protein, while the cells transfected with pcDNA3 plasmid did not show any staining (Fig .1).

IFN expression luciferase activity

IFN expression in transfected CCG-P1 cells.

Cells transfected with the pcDNA3 vhs-G plasmid showed increase in their level of IFN mRNA expression, when compared with the non-transfected control cells.

VHS-G protein expression in transfected CCG-P1 cells after 48 h of transfection the cells were immunostained and the results showed that about small percentage of the cells transfected with pcDNA3 vhs-G

plasmid were expressing G protein, while the cells transfected with pcDNA3 plasmid did not show any staining .

Cells transfected with the pcDNA3 vector alone (i.e. with no VHS-G gene) and non-transfected control cells showed very low levels of luciferase activity. The cells transfected with the pcDNA3 vhs-G showed an increased luciferase activity of 5.4-fold compared with the control non-transfected cells (Fig. 2), this increase was statistically significant ($p < 0.05$).

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RT-PCR

The results of RT-PCR showed that the cells transfected with the pcDNA3vhs-G plasmid expressed IFN mRNA but non treated and control cells which transfected with the pcDNA3 Vector alone did not show any IFN mRNA expression (Fig. 3). In using a cell protection assay in RT cells, serum of rainbow trout infected with VHSV was shown to contain IFN-like factors (Dorson *et al*., 1994). Furthermore, rainbow trout blood and kidney leucocytes were able to produce IFN following incubation with active and inactivated VHSV. When leucocytes were incubated with glutaraldehyde-fixed, VHSV-infected EPC cells, IFN was detected in the supernatants and this response was blocked by a monoclonal antibody to the G protein, and to some extent also by antibodies to some of the other VHSV proteins (Rogel-Gaillard *et al*., 1993). These results indicated that viral replication was not required for induction of the IFN-like activity and suggested that the response was induced by contact of the leucocytes with viral protein expressed on the surface of the fixed infected EPC cells.

Type I IFN of fish

IFN genes have recently been cloned from zebrafish, Atlantic salmon, the Japanese pufferfish *Takifugu rubripes* (Fugu), the spotted green pufferfish *Tetraodon nigrovirides*, and channel catfish (Long *et al*., 2004).

However, in silico screening of IFN genes in the genomes of zebrafish and Fugu it was found that these fishes possess two type I IFN genes on the same chromosome (Inessa, Personal communication). Atlantic salmon was shown to possess at least two functional genes (Sasa

IFN-a1 and Sasa IFN-a2) (Charley and Laude, 1988) , although this may be a result of the tetraploidization event that occurred during the evolution of salmonids (Allendorf and Thorgaard,1984). Southern blotting suggested 2 or at most 3 IFN genes in the genome of channel catfish (Long *et al*., 2004). Surprisingly, the fish IFN genes contained 5 exons and 4 introns (Fig. 4), in contrast to the classical type I IFNs of birds and mammals, which have no introns (Charley and Laude,1988). Interestingly, the fish type I IFNs have the same exon/intron structure as the IL-10 and IFN-l gene families (Fig. 5), which suggests that the type I IFNs and IL-10 like genes are derived from a common ancestral gene (Charle *et al*., 1991). The fish IFN genes translate into putative precursor proteins containing 175 to 185 amino acids and appear to have signal peptides of 22-23 amino acids. The signal sequence of the fish IFNs showed some homology between themselves, but hardly any homology with IFN signal sequences of higher vertebrates (Charley and Laude, 1988).

The fish IFNs are similar to mammalian IFNs in size, containing 152 to 164 amino acids, which gives molecular masses of 18-19 kDa. Comparison of gene structures of fish IFNs, and human IFN-l and IL-10 values represent nucleotide numbers. Furthermore, the early protection against VHS, after intramuscular injection of rainbow trout with the VHS/ DNA vaccine pcDNA3vhsG),was correlated with enhanced expression of the Mx gene in the liver, as determined by RT-PCR (Boudinot *et al*., 1998). Similar results were obtained when rainbow trout was vaccinated with plasmids containing the G gene of IHNV, snakehead rhabdo virus (SHRV), or spring viraemia of carp virus (SVCV). All vaccines induced early protection against a lethal challenge with IHNV and high levels of Mx protein were detected in the kidney and liver (Kim *et al*., 2000).

In using the luciferase cell line ,it was found that the common carp gonad cell line, produces IFN when transfected with a plasmid encoding the glycoprotein of VHSV, but not with plasmid vector alone.

The results suggest that the expression of the G protein on the surface of the cells was important in the induction of IFN rather than the viral G-gene transcript expressed inside the transfected cells. Moreover, the results support the idea that the early protection following vaccination with the VHS DNA vaccine is related to IFN-stimulatory properties of the VHSV G protein (Altmann *et al*., 2003). This increase was statistically significant ($p<0.05$).The IFN gene expression level of cells transfected with pcDNA3 was lower than that in non-transfected cells. A CCF IFN

gene (CCF IFN-1) was identified which lacks a signal sequence and likely encodes a nonsecreted protein (Charle *et al*., 1991).

Since Southern blot analysis indicated the presence of multiple IFN bands in Common Carp genomic cDNA, attempts were done to identify additional cDNAs representing different IFN alleles or loci.

Using a luciferase cell line reporter system, it was shown that the Common Carp gonad cell line (CCGP1), produces IFN when transfected with a plasmid encoding the glycoprotein of VHSV, but not with plasmid vector alone. The ability of the plasmid to mediate expression of the viral G-protein on the surface of transfected cells was confirmed by specific immunostaining for the viral protein.

The viral surface glycoprotein (G protein) gene of the respective viruses, not only stimulate the production of antibodies and protection but they also induce the expression of the interferon (IFN)-inducible anti-viral Mx protein. Virus-induced genes were ultimately retrieved from the subtracted cDNA library, and their differential expression was further confirmed by semi quantitative reverse transcription-PCR and Northern blot analysis.

Identified a Common Carp IFN transcript, designated CCF IFN-1, from an EST library constructed using cDNA from a mixed lymphocyte culture enriched in NK-like cells (Charle *et al* ., 1991). Using a cell protection assay in RTG cells, serum of rainbow trout infected with VHSV was shown to contain IFN-like factors (Dorson *et al*., 1994).

Furthermore, rainbow trout blood and kidney leucocytes were able to produce IFN following incubation with active and inactivated VHSV. When leucocytes were incubated with glutaraldehyde - fixed, VHSV-infected EPC cells, IFN was detected in the supernatants and this response was blocked by a monoclonal antibody to the G protein, and to some extent also by antibodies to some of the other VHSV proteins (Rogel-Gaillard *et al*., 1993).

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REFERENCES

Altmann, S.M.; Mellon, M.T.; Distel, D. L. and Kim,C.H.(2003). Molecular and functional analysis of an interferon gene from the zebrafish, *Danio rerio.* J.Virol ., *77*(3): 1992-2002.

Allendorf, F.W. and Thorgaard, G. H. (1984). Tetraploidy and the evolution of salmonid fishes. In: Turner B.J, editor. Evolutionary Genetics of Fishes. New York: Plenum, 1-53 pp.

Boudinot, P.; Blanco, M.; deKinkelin, P. B. and Enmansour, A.(1998). Combined DNA immunization with the glycoprotein gene of viral haemorrhagic septicaemia virus and infectious hema topoietic necrosis virus induces double-specific protective immunity and non-specific responses in rainbow trout. J.Virol ., *49* : 297-306.

Biberfeld, P.; Biberfeld, G.; Molnar, Z. and Fagraeus, A.(1974). Fixation of cell-bound antibody in the membrane immuno fluorescence test. J. Immunol. Methods, *4*:135-148.

Charle, Y. B.; Levenant, L. and Delmas, B. (1991). Glycosylation is required for corona virus TGEV to induce an efficient production of IFNa by blood mononuclear cells. J. Immunol , *33*:435-440.

Charley, B. and Laude, H.(1988). Induction of alpha interferon by transmissible gastroenteritis corona virus : role of transmembrane glycoprotein E1. J .Virol , *62*: 8-11.

Chtanova,T.; Kemp, R.A.; Sutherland, A.P.R.; Ronchese, F. and Mackay, C.R. (2001).Gene microarrays reveal extensive differential gene expression in both CD4+ and CD8+ type 1 and type 2 T cells: J.Immunol, 167(*6*): 3057-3063.

Dorson, M.; Torhy , C. and deKinkelin, P.(1994).Viral haemorrhagic septicaemia virus multiplication and interferon production in rainbow trout and in rainbow trout x brook trouthy brids. Fish Shell & fish Immunol, *4*: 369-381.

Inessa, A. Z. Personal communication. Engelhardt Institute of Molecular Biology, Russia Moscow Academy of Sciences.

Kim, C.H.; Johnson, M. C.; Drennan, J.D.; Simon, B. E., Thomann, E. and Leong, J. C. (2000). DNA vaccines encoding viral glycoproteins induce non-specific immunity and Mx protein synthesis in fish. J. Virol *1*: 7048-7054.

Long, S.; Wilson, M.; Bengten, E.; Bryan, L.; Clem, L.W. and Miller, .W. (2004).Identification of a cDNA encoding channel catfish interferon. Dev Comp Immunol, *28* (*2*):97-111.

Lorenzen, N.; Lorenzen, E.; Einer-Jensen, K.; Heppell, J.; Wu,T. and Davis, H.(1998). Protective immunity to VHS in rainbow trout (*Oncorhynchus mykiss*, Walbaum) following DNA vaccination. Fish & Shell fish Immunol, *8* : 261-270.

Lorenzen , N.; Lorenzen, E.; Einer –Jensen, K. and LaPatra, S.(2002). DNA vaccines as a tool for analyzing the protective immune response against rhab-doviruses in rainbow trout. Fish &Shell fish Immunol,*12*:439-53.

McLauchlan, P.E.; Collet,B.; Ingerslev, E.; Secombes, C.J.; Lorenzen, N.

and Ellis, A.E. (2003).DNA vaccination against viral haemorrhagic septicaemia (VHS) in rainbow trout. Fish & Shell fish Immunol, *15*: 39-50.

Robertsen , B.(2006). The interferon system of teleost fish. Fish & Shell fish Immunol , *20*:172-191.

Rogel-Gaillard, C.; Chilmonczy, k. S. and deKinkelin, P.(1993). In vitro induction of interferon-like activity from rainbow trout leucocytes stimulated by Legated virus. Fish & Shell fish Immunol, *3*: 383-394.

Werling, D.; Hope, J.C.; Howard, C.J. and Jungi, T.W. (2004). Immunology. Differential production of cytokines, reactive oxygen and nitrogen by bovine macrophages and dendritic cells stimulated with Toll-like receptor agonists. Immunol, *111*(*1*): 41-52.