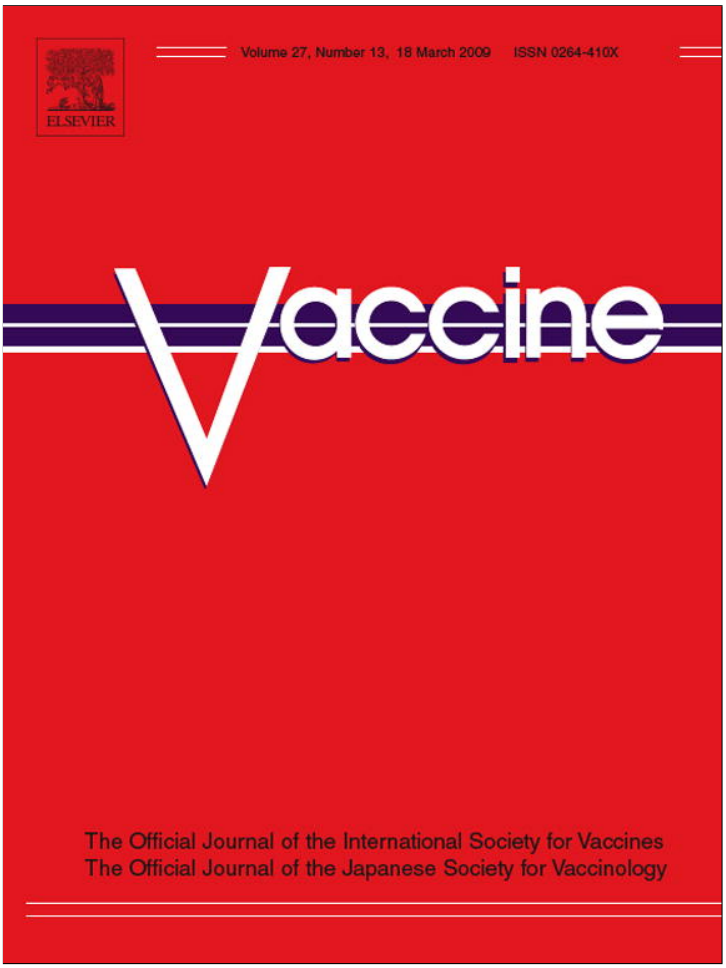

Vaccine 27 (2009) 1938–1948

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

The immunogenicity of viral haemorragic septicaemia rhabdovirus (VHSV) DNA vaccines can depend on plasmid regulatory sequences

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ARTICLE INFO

Article history:
Received 28 October 2008
Received in revised form 14 January 2009
Accepted 22 January 2009
Available online 31 January 2009

Keywords:
VHS virus
DNA vaccine
Promoter
Plasmid
Glycoprotein
Interferon
Fish
Gene expression profile

ABSTRACT

A plasmid DNA encoding the viral hemorrhagic septicaemia virus (VHSV)-G glycoprotein under the control of 5′ upstream sequences (enhancer/promoter sequence plus both non-coding 1st exon and 1st intron sequences) from carp β-actin gene (pAE6-G_{VHSV}) was compared to the vaccine plasmid usually described the gene expression is regulated by the human cytomegalovirus (CMV) immediate-early promoter (pMCV1.4-G_{VHSV}). We observed that these two plasmids produced a markedly different profile in the level and time of expression of the encoded-antigen, and this may have a direct effect upon the intensity and suitability of the *in vivo* immune response. Thus, fish genetic immunisation assays were carried out to study the immune response of both plasmids. A significantly enhanced specific-antibody response against the viral glycoprotein was found in the fish immunised with pAE6-G_{VHSV}. However, the protective efficacy against VHSV challenge conferred by both plasmids was similar. Later analysis of the transcription profile of a set of representative immune-related genes in the DNA immunized fish suggested that depending on the plasmid-related regulatory sequences controlling its expression, the plasmid might activate distinct patterns of the immune system. All together, the results from this study mainly point out that the selection of a determinate encoded-antigen/vector combination for genetic immunisation is of extraordinary importance in designing optimised DNA vaccines that, when required for inducing protective immune response, could elicit responses biased to antigen-specific antibodies or cytotoxic T cells generation.

1. Introduction

Prevention of infectious diseases through immunization is one of the greatest achievements of modern medicine [1,2]. Deoxyribonucleic acid-based immunisation (DNA vaccination) has initiated a new era of vaccine research as it offers an extremely powerful tool to develop new vaccines. DNA vaccines are the simplest embodiment of vaccines that, rather than administering the antigen itself, provide genes encoding it [3] using a plasmid DNA as vector. The inoculation of animals with these plasmid encoded-antigens either by intramuscular or intradermal route has shown to provide protection from infections stemming from an array of pathogens, which include viruses, bacteria, and parasites [4–8].

Early in the development of DNA vaccines, it became clear that maximizing the expression of the encoded-antigen, without compromising its conformation/structure, was critical for the induction of potent immune responses. In this regard, the expression vector sequences driving the expression of the foreign antigen, the so-called promoter/enhancer sequences are likely to play a crucial role. Most DNA vaccines, including those developed against west Nile virus in horses [9–11] and infectious haematopoietic necrosis rhabdovirus, IHNV in fish [12] recently licensed, rely on the immediate-early cytomegalovirus (CMV) gene enhancer/promoter sequences [3,10,13,14]. When used for gene expression, these sequences act as a very strong promoter capable of transcribing most genes assayed in a wide range of eukaryotic cells including cells from lower vertebrates such as fish [15,16]. Despite the effectiveness of this system, since CMV enhancer/promoter sequences are derived from a human pathogenic virus, the license and commercialisation of these vaccines constitutes an impairment in many countries. For example, the use of the DNA vaccine based on the IHN rhabdovirus glycoprotein G (G_{IHNV}) gene licensed in Canada has been not authorised by European Union, although the economic costs of rhabdoviruses-caused diseases, mainly the viral haemorrhagic septicaemia (VHS), to the European salmonid aquaculture industry are estimated at about 40–50 million euros per year [17], and there are neither specific agents nor other efficient vaccines for the treatment or prevention of these diseases. In this context,

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doi:10.1016/j.vaccine.2009.01.103

the search for alternative regulatory sequences that can substitute those from CMV is of definitive interest for the licensing and marketing of fish DNA vaccines that have proven to be efficient against viral diseases such as the rhabdovirosis, when other strategies for protection had failed.

Commercial plasmids with regulatory sequences of non-viral origin designed for use in mammalian cells are available, but are likely to be less efficient in fish cells. In fish, several plasmids containing regulatory sequences from fish gene promoters have been assessed for their ability to drive the expression of foreign genes in fish cell lines and tissues. The rainbow trout metallothionein-A promoter [18], the Atlantic salmon histone H3 promoter [19] and the sockeye salmon histone, metallothionein and protamine promoters [20] have been assayed as promoters for in vivo delivery without very promising results. Lower protection levels of those achieved when the expression of G_{IHNV} was driven by CMV regulatory sequences were also observed after immunising rainbow trout with plasmids including the upstream regulatory sequences of trout Mx or IRF1A genes [16]. However the optimisation of β-actin promoters seems as a good alternative, since β-actin promoters of different species have proved to be almost as effective as CMV [21].

In this context, in the current work, we have compared the immunogenicity, protective efficacy and immune-related gene transcription profile induced by the VHSV-glycoprotein G (G_{VHSV}) when expressed under the control of 5′ immediate-early CMV or carp β-actin gene sequences. For analysing the immune-related gene transcription profile we chose a set of immuno-related genes, known to be up-regulated in response to CMV-based plasmids encoding the G_{VHSV} or G_{IHNV} genes, and therefore likely to play an important role in protection [22–39].

Differences related to *in vitro and in vivo* G_{VHSV} expression, specific antibody response and immune gene induction were found indicating that G_{VHSV} immunogenicity can be different depending on the plasmid regulatory sequences. Therefore, although previous studies dealing with the immune response to DNA vaccines have never focused on the effect of regulatory sequences, our results indicate that the selection of a determined encoded-antigen/vector combination could be crucial for the development of optimised prophylactic DNA vaccines against fish viruses.

2. Materials and methods

2.1. Plasmids

The expression vectors, pMCV1.4 (Ready-Vector, Madrid, Spain) [40] and pEA6 (kindly provided by Dr. T.T. Chen, University of Connecticut, USA) [41,42], both containing SV40 terminator sequence, were used. The regulatory sequences (2577 base pairs, bp) of pAE6 vector included the carp β-actin enhancer/promoter sequence as well as both the 1st exon (a non-coding exon) and 1st intron sequence from the carp β-actin gene [43] (Gene bank accession number M24113). Likewise, pMCV1.4 vector promoter included an enhancer/promoter sequence (740 bp from the human cytomegalovirus, CMV, major immediate-early gene) and an intron sequence (~200 bp of a quimeric intron present in many commercial vectors). The pAE6-G_{VHSV} plasmid construction was obtained by subcloning the G_{VHSV} cDNA sequence from the pMCV1.4-G_{VHSV} plasmid construction [44] into vector pAE6 following standard procedures.

2.2. Fish

Rainbow trout (*Oncorhynchus mykiss*) of approximately 5–6 cm obtained from a VHSV-free commercial farm (Lillogen, Leon, Spain) were maintained in 50 l tanks at the University Miguel Hernandez (UMH) facilities at 12–14 °C with a re-circulating dechlorinated-water system and fed daily with a commercial diet (Trow, Leon, Spain). Prior to experiments, fish were acclimatised to laboratory conditions for 2 weeks.

2.3. Cell cultures and virus

The EPC (*epithelioma papulosum cyprinid*) fish cell line [45], purchased from the European collection of cell cultures (ECACC n° 93120820) was used in this work. EPC cells were maintained at 28 °C in a 5% CO_2 atmosphere with RPMI-1640 Dutch modified (Gibco, Invitrogen corporation, UK) cell culture medium containing 10% fetal calf serum (FCS, Sigma, Che. Co, St. Louis, MS), 1 mM Pyruvate (Gibco), 2 mM Glutamine (Gibco), 50 μg/ml gentamicin (Gibco) and 2 μg/ml fungizone (Gibco).

Viral haemorrhagic septicaemia virus (VHSV-07.71) isolated in France from rainbow trout, *Oncorhynchus mykiss* [46] was propagated in EPC cells at 14 °C as previously reported [47]. Supernatants from VHSV-07.71 infected EPC cell monolayers were clarified by centrifugation at 1000 × *g* for 20 min and kept in aliquots at −70 °C. Clarified supernatants were used for the experiments. The virus was tittered according to Reed and Muench [48].

2.4. Cell transfection assays

Cell transfection assays were performed as previously described [35,42,49,50]. Briefly, EPC cell monolayers, grown in culture flasks of 75 cm^2, were detached using trypsine (Sigma), washed, resuspended in culture medium supplemented with 10% of FCS and dispensed into 24-well plates at a concentration of 2.5×10^5 cells per well in a final volume of 250 μl. The following day, the pMCV1.4-G_{VHSV} or pAE6-G_{VHSV} plasmids (0.5 μg) complexed with 0.7 μl of FuGene 6 (Roche, Barcelona, Spain) were incubated for 15 min in 50 μl of RPMI-1640 containing 2 mM Cl_2Ca and then added to each well in 200 μl of culture medium with 10% of FCS. As an additional control, EPC cells were transfected with FuGene 6 without DNA following the same procedure. The plates were further incubated at 20 °C for 2 or 5 days.

2.5. Analysis of VHSV-G protein expression in transfected cells

The expression of G_{VHSV} in EPC transfected cells was analysed at both transcriptional and protein levels by quantitative real time RT-PCR (RT-qPCR) and flow cytometry, respectively.

For qRT-PCR assays, cell RNA extraction and cDNA synthesis were performed as previously described [51,52]. qPCR was carried out using the G_{VHSV} gene specific primers and probe as well as the conditions previously described [51–53]. The internal reference to normalize data was the cellular 18S rRNA.

For flow cytometry assays, transfected EPC cells were detached and resuspended in Ca^{2+}- and Mg^{2+}-free PBS and centrifuged (3500 × *g* for 15 min at 10 °C). The pellets were then incubated with the monoclonal antibody (MAb) anti-G_{VHSV} C10 [54], a conformation-dependant MAb, diluted 400-fold in Ca^{2+}- and Mg^{2+}-free PBS for 4 h at room temperature (RT). Afterwards, the cells were washed with PBS and incubated for 45 min with fluorescein-labeled rabbit anti-mouse immunoglobulin G (IgG) Ab (Sigma) diluted 200-fold in Ca^{2+}- and Mg^{2+}-free PBS. Finally, the cells were washed three times (3500 × *g* for 15 min) and resuspended in FACS buffer. Cell-associated fluorescence of 10,000 cells per sample was analysed with a Coulter Epics XL cytometry apparatus (Becton Dickinson, San Jose, CA) by using the Expo 32 software (Becton Dickinson). Background fluorescence profiles were obtained using non-transfected EPC cells. Three independent experiments, each in duplicate, were performed.

2.6. DNA immunisation protocol

DNA immunisation of fish was carried out following procedures previously described [35,49]. Briefly, trout were anaesthetized by immersion in 50 μg/ml buffered tricaine methanesulfonate (MS-222; Sigma) prior to handling and then divided into five groups. Groups were intramuscularly injected with one of the following: 50 μl of PBS (non-immunised or control fish) or 50 μl of PBS containing 1 μg of pAE6 or pCMV empty plasmids, pAE6-G_{VHSV}, or pMCV1.4-G_{VHSV} plasmids. At days 3 and 10 post-immunisation (p.i.) three fish from each group were sacrificed by overexposure to MS-222 and tissues from muscle (site of injection), head kidney and spleen removed. At days 10 and 30, blood from the caudal vein was also extracted (four fish per group) in order to study antibody production. Two independent DNA immunisation experiments were performed but samples for RT-PCR analysis of immune-related genes were only collected in the first experiment.

2.7. Challenge with VHSV

Thirty-one days after DNA immunisation, all the remaining trout in each tank (24–31 trout per group) were challenged by bath with VHSV. For this, the trout were introduced in 2 l of water containing 3×10^6 $TCID_{50}$ VHSV-07.71/ml (about four times the Lethal $Dose_{50}$) for 1 h. The tanks were then filled with fresh dechlorinated-water and the flow through the filters was restored. Mortality was recorded daily for 20 days and the relative percent survival (RPS) was calculated by the formula: RPS = [1 − (% mortality of immunized fish/% mortality of control fish)] × 100. Statistical analysis was carry out using a Student test, where $p < 0.01$.

2.8. IgM antibody response

The presence of specific antibodies against G_{VHSV} in trout sera was determined at days 10 and 30 post-vaccination through enzyme-linked immunosorbant assay (ELISA) using a G_{VHSV} recombinant fragment (aa 56-110) previously named fragment# 11 as an antigen [55,56], as previously described [57]. As a positive control a pool of sera obtained from VHSV-resistant trout (two-fold VHSV infection resistant trout) was used.

At 30 days p.i., the presence of VHSV neutralising activity in the sera of DNA immunised fish was also analysed. Briefly, 10^4 TCID50/ml of VHSV were incubated for 3 h at 14 °C with serial dilutions of serum from immunized fish. After incubation period, 10% of fresh non-immunised trout serum was added and the mixtures further incubated for 6 h. EPC cells, grown in 96-well plates, were then infected with 100 μl of the different virus/serum mixtures for 2 h at 14 °C. After washing the cells, 100 μl of fresh culture medium containing 2% FCS were added and infected cells incubated at 14 °C. Twenty-four hour after the infection, the VHSV infectivity was valuated using a immuno-staining focus assay (focus forming units, f.f.u.) as previously described [49,52,58–61].

2.9. Analysis of VHSV-G protein expression in DNA immunised-fish muscle

The levels of expression of G_{VHSV} in the skeletal muscle of DNA immunised fish were analysed at both transcriptional and protein levels by quantitative real time RT-PCR (qRT-PCR) and ELISA, respectively. Three and 10 days after immunisation, the samples of muscle tissue were taken from the site of injection and processed for qRT-PCR as indicated for transfected cells or homogenate for ELISA assays.

For determining the G_{VHSV} expression in injected trout muscle by ELISA, about 50 mg of muscle tissue (wet weight) were homogenised in 300 μl of distilled water and clarified by centrifugation as previously described [21]. Protein was adjusted to 0.1 mg/ml by using the Bradford reagent (Biorad, Madrid, Spain) and frozen at −20 °C until use. One hundred microliters of muscle homogenates (∼5 μg of protein) were dried per well of 96-well polystyrene plates (Dynatech) by incubation overnight at 37 °C to dryness. Before use, the coated plates were incubated for 1 h at room temperature with 3% dry milk in dilution buffer, washed and then incubated for 120 min at room temperature with 100 μl/well of the MAb anti-G_{VHSV} I10 [62], a non-conformation-dependant MAb, diluted 200-fold in dilution buffer. After washing with distilled water, 100 μl/well of a peroxidase-labeled goat anti-mouse IgG Ab (Sigma) were added and ELISA continued as above indicated.

2.10. RNA extraction, cDNA synthesis and PCR of immune genes

The levels of expression of different immune genes were studied through semi-quantitative RT-PCR in fish immunised with the different plasmids. The "Total RNA Isolation System" (Promega) was used for RNA extraction from the different rainbow trout tissues following manufacturer's instructions. Isolated RNAs were resuspended in diethylpyrocarbonate (DEPC)-treated water and stored at −80 °C until used. Two micrograms of RNA were used to obtain cDNA by using the Moloney murine leukaemia virus reverse transcriptase (M-MLV) (Invitrogen) according to manufacturer's instructions.

All PCR amplification reactions were performed using 0.5 μl dNTP mix (10 mM each), 0.125 μl Taq DNA polymerase (Roche, Barcelona, Spain), 2.5 μl Taq 10× buffer, 0.5 μl of each primer (20 μM) and 2.5 μl of cDNA in a final volume of 25 μl. First, a PCR with primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [63] was performed with all samples as a positive control for RT-PCR, since GAPDH is constitutively expressed in all organs using the conditions previously described [35,49,63]. These PCR products also allowed the verification that equivalent amounts of cDNA were present in the different samples and therefore amplifications of the different immune genes were comparable among samples. A negative control in which no cDNA was added was included in all PCR reactions. Primers used for gene amplification are shown in Table 1. PCRs were carried out in a GeneAmp® PCR System 2700 cycler (Applied Biosystems) and amplification conditions always consisted of a denaturing step of 94 °C for 5 min followed 30 cycles of 45 s at 94 °C, 1 min at the annealing temperatures indicated in Table 1, 45 s at 72 °C and a final extension step of 7 min at 72 °C. All samples were amplified twice to verify the results. The PCR products (10 μl) were visualized on a 2% agarose gel stained with ethidium bromide. Samples that were to be compared were always run in the same agarose gel. A 100 bp ladder was used as a size marker. Analysis of transcription for each gene was performed and calculated as relative to the GAPDH gene transcription (expression relative to GAPDH) using the formula: intensity of target gene band/intensity of its corresponding GAPDH band.

2.11. Statistics

To compare the effects of pMCV1.4-G_{VHSV} and pAE6-G_{VHSV}, all data were analysed using Student's *t*-test. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. In vitro G_{VHSV} expression by pAE6-G_{VHSV} and pMCV1.4-G_{VHSV} plasmids

First, the effect of the different regulatory sequences on the levels of expression of the G_{VHSV} gene was analysed *in vitro* after

Table 1
Genes, primer sequences and annealing temperatures used for gene expression analysis.

Gene	Nucleotide sequence (5′ → 3′)	Annealing, T^a (°C)	Reference/accession number
gapdh	F: 5′ATGTCAGACCTCTGTGTTGG3′ R: 5′TCCTCGATGCCGAAGTTGTCG3′	52	[63]
irf3	F: 5′GTCCCTCTTTAGCACAAGTC3′ R: 5′GGTGGAGCAGTTCACAAATG3′	57	[49]
irf7	F: 5′GATGCCCTGCTCAACCTG3′ R: 5′GGCTGTTGTGGTGGGAGAT3′	58	TC131054
il1β	F: 5′AGGGAGGCAGCAGCTACCACAA3′ R: 5′GGGGGCTGCCTTCTGACACAT3′	60	[99]
tnfα	F: 5′TTCGGGCAAATATTCAGTCG3′ R: 5′GCCGTCATCCTTTCTCCACT3′	60	[100]
il8	F: 5′GAATGTCAGCCAGCCTTGTC3′ R: 5′TCCAGACAAATCTCCTGACCG3′	60	[101]
ck7a	F: 5′TCTGCAGGTGTCATTAAGTTGG3′ R: 5′TCTTTGTGGTGAAAATCAGTGC3′	55	[69]
ck6	F: 5′CGAATCTGCTCTGACACTTCC3′ R: 5′TGGTGAGTTGTTGACCATTGA3′	55	[69]
ck5b	F: 5′TTTGCTGATCGTCAGATACCC3′ R: 5′GGACCATGACTGCTCTCTCTG3′	55	[69]
mx1	F: 5′ATGCCACCCTACAGGAGATGAT3′ R: 5′TAACTTCTATTACATTTACTATGCAA3′	52	[35]
mx3	F: 5′ATGCCACCCTACAGGAGATGAT3′ R: 5′CCACAGTGTACATTTAGTTG3′	52	[32]
vig1	F: 5′CAGTTCAGTGGCTTTGACGA3′ R: 5′ACAAACGCCTCAAGGTATGG3′	55	[24]
inos	F: 5′CATACGCCCCCAACAAACCAGTGC3′ R: 5′CCTCGCCTTCTCATCTCCAGTGTC3′	62	[100]
ifnγ	F: 5′GTGAGCAGAGGGTGTTGATG3′ R: 5′GATGGTAATGAACTCGGACAG3′	60	[102]
mhcII	F: 5′ATGTCGATGCCAATTGCCTTCTA3′ R: 5′TGTCTTGTCCAGTATGGCGCT3′	57	[100]
nkef	F: 5′AGGTCATTGGTGCCTCTGTC3′ R: 5′GGGCTTGATGGTGTCACTTC3′	58	AF250194
psmb9	F: 5′GGACCACCATCATTGCTATT3′ R: 5′ATAGACGTAGGAGCTTCCAGA3′	56	[103]
tcrβ	F: 5′CTCCGCTAAGGAGTGTGAAGATAG3′ R: 5′CAGGCCATAGAAGGTACTCTTAGC3′	62	[104]
cd4	F: 5′GCCACAAACAAGTACCTCAG3′ R: 5′AGGATAGTGGAGGAGGAATG3′	62	AY973028.1
migm	F: 5′CATCGAGCCGTCTCTTGAGG3′ R: 5′GCTCCAACGCCATACAGCAG3′	61	X65263
sigm	F: 5′GTACAGCACAACCAGCTATC3′ R: 5′TCTCCCTCTTGTAGGCTTTC3′	57	X65261
igt	F: 5′TCACTGCTACAGACCAGAAC3′ R: 5′AGGCACATCAGAGTCACATC3′	57	AY870265

gapdh, glyceraldehyde 3-phosphate dehydrogenase; *irf3*, interferon-regulatory factor 3; *irf7*, interferon-regulatory factor 7; *il1β*, interleukin-1 beta; *tnfα*, tumor necrosis factor alpha; *il8*, interleukin-8; *ck7a*, inducible CC chemokine 7A; *ck6*, inducible CC chemokine 6; *ck5b*, inducible CC chemokine 5B; *mx1*, protein Mx-1; *mx3*, protein Mx-3; *vig1*, VHSV-induced gene 1; *inos*, Inducible nitric oxide synthase; *ifnγ*, interferon gamma; *mhcII*, major hystocompatibility complex II; *nkef*, Natural killer cell enhancement factor-like gene; *psmb9*, immuno-proteasome subunit beta type 9; *tcrβ*, T cell receptor beta chain; *cd4*, CD4 beta chain; *migm*, immunoglobulin Mu membrane heavy chain; *sigm*, immunoglobulin Mu secreted heavy chain; *igt*, immunoglobulin Tau heavy chain

transfecting EPC cells with plasmids pAE6-G_{VHSV} or pMCV1.4-G_{VHSV}. The expression of the G_{VHSV} gene was analysed both at a transcriptional and protein level by RT-qPCR and flow cytometry, respectively. Two days after transfection, both the accumulation of G_{VHSV} transcripts (Fig. 1A) and the percentage of fluorescent cells (G_{VHSV}-surface expressing cells) (Fig. 1B, right panel) was higher in the cells transfected with pMCV1.4-G_{VHSV}. However, the fluorescence intensity of the G_{VHSV}-expressing cells was superior in the cells transfected with pAE6-G_{VHSV} (Fig. 1B, right panel) indicating that higher levels of G_{VHSV} expression per cell can be obtained with the pAE6-G_{VHSV}. At later times post-transfection (5 days), both the accumulation of G_{VHSV} transcripts (Fig. 1A) and the percentages of fluorescent cells as well as the fluorescence intensity per cell (Fig. 1B, left panel) were higher in the cells transfected with pAE6-G_{VHSV} suggesting a slower but long lasting kinetic expression of the encoded-antigen by effect of 5′ carp β-actin sequences.

3.2. *In vivo* G_{VHSV} *expression by pAE6-*G_{VHSV} *and pMCV1.4-*G_{VHSV} *plasmids*

Since the *in vivo* conditions governing the expression of the proteins/Ags encoded by the plasmids might be different from those *in*

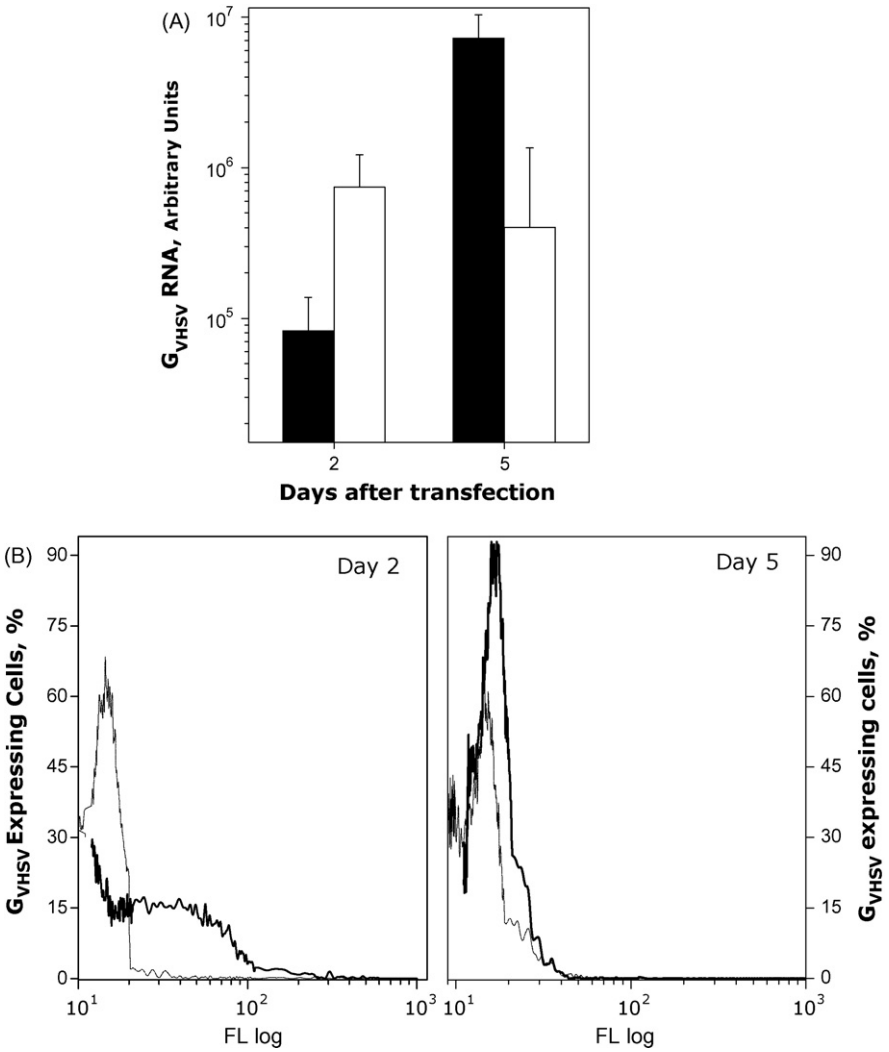

Fig. 1. Analysis of VHSV-G protein expression at transcriptional and protein level by qRT-PCR (A) and flow cytometry (B). EPC cells were grown in wells of 24-well plates and transfected with 0.5 μg per well of pAE6-G_{VHSV} or carp pMCV1.4-G_{VHSV}. The cells were then harvested for RNA isolation or flow cytometry analysis 2 or 5 days post-transfection. (A) Total RNA samples were used to obtain the cDNA using random hexamers. VHSV-G protein cDNA was amplified with specific G_{VHSV} gene primers and TaqMan probe. The internal reference to normalize data was the cellular 18S rRNA. Bars represent the average values and standard deviations from five different experiments, each performed in triplicate. Black bars, EPC cells transfected with the plasmid pAE6-G_{VHSV}; white bars, EPC cells transfected with the plasmid pMCV1.4-G_{VHSV}. (B) After harvesting, transfected cells were stained with the conformation-dependant MAb anti-G_{VHSV} C10 [54] and cell associated fluorescence estimated by flow cytometry. Solid lines, EPC cells transfected with the plasmid pAE6-G_{VHSV}; dash-dot lines, EPC cells transfected with the plasmid pMCV1.4-G_{VHSV}. The cytometry profile shows in this figure is representative of three independent experiments.

vitro [64], we also evaluated the expression of the G_{VHSV} gene in the muscle of trout intramuscularly injected with the G_{VHSV}-encoding plasmids (Fig. 2).

As in transfected cells, G_{VHSV} transcripts were detectable in the muscle of all fish injected with the G_{VHSV}-encoding plasmids, but the accumulation of G_{VHSV} transcripts was higher in the fish immunised with pAE6-G_{VHSV} at every time point measured (Fig. 2A). Because the levels of gene transcription do not always correlate with the levels of the gene encoded-protein present in the cells, the expression of the G_{VHSV} at the muscle-injection site was also determined by ELISA. The protein levels directly correlated with the transcription levels and, therefore, the highest levels of G_{VHSV} expression were found in the muscle from the fish immunised with pAE6-G_{VHSV} at day 3 post-immunization (Fig. 2B).

3.3. Specific IgM response

Having established that the G_{VHSV} gene was more efficiently expressed under the control of regulatory sequences from carp β-actin gene than when under the control of CMV regulatory sequences, the capacity of these G_{VHSV}-encoding plasmids to induce specific antibodies against G_{VHSV} was studied. For this, groups of fish were intramuscularly injected with PBS, pAE6-G_{VHSV}, pMCV1.4-G_{VHSV}, empty plasmids or with PBS. Thirty days after immunisation, all the fish injected with the pAE6-G_{VHSV} or pMCV1.4-G_{VHSV} plasmids developed specific antibodies against G_{VHSV}, whereas no specific antibodies were detected in the PBS or empty plasmids immunised groups. However, differences regarding the titres of specific G_{VHSV} Abs between the groups were observed (Fig. 3), being the titres three-fold higher in fish immunised with pAE6-G_{VHSV}.

The neutralisation capacity of the antibodies present in these sera was also assessed. None of the tested sera showed a considerable neutralising activity since the decrease of VHSV infectivity after virus neutralising was $\leq$30% (data not shown).

3.4. Protective efficiency of DNA immunization

To determine whether the higher titres of specific Abs to G_{VHSV} detected in the sera of fish immunised with pAE6-G_{VHSV} could be

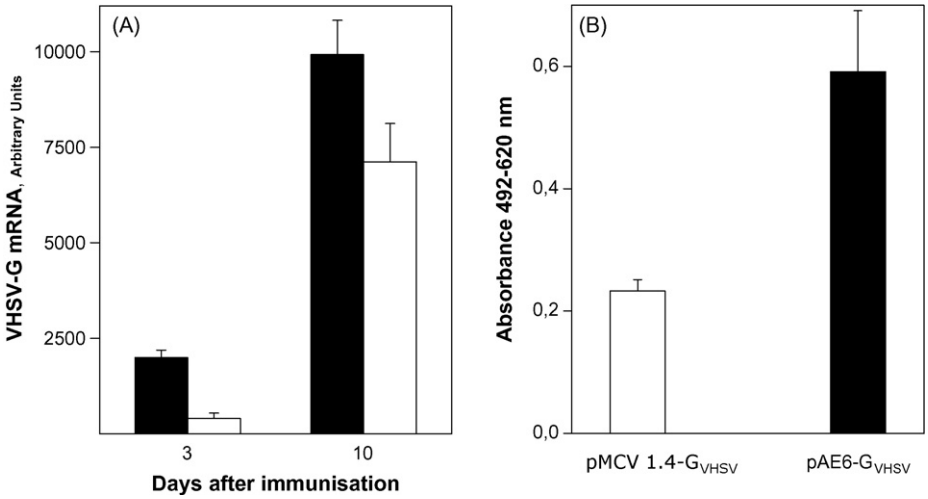

Fig. 2. VHSV-G protein expression in skeletal muscle of rainbow trout. VHSV-G expression was examined in rainbow trout injected i.m. with 1 μg of pMCV1.4-G_{VHSV} or pAE6-G_{VHSV} plasmids. After 3 or 10 days, the injected animals were sacrificed by anaesthetic overdose and muscle tissue at the injection site removed and processed. (A), Expression of G_{VHSV} transcripts in muscle tissue estimated by qRT-PCR. Total RNA extraction, cDNA synthesis and amplification were performed as indicated in Fig. 1. Bars represent the average values and standard deviations for three fish per group from two different experiments. Black bars, EPC cells transfected with the plasmid pAE6-G_{VHSV}; white bars, EPC cells transfected with the plasmid pMCV1.4-G_{VHSV}. (B), G_{VHSV} expression in muscle tissue estimated by ELISA. Three days p.i., muscle samples from immunised fish were homogenised and analysed by ELISA using a non-conformation-dependant MAb to G_{VHSV}. Absorbance readings were measured at 492 and 620 nm. Bars represent the average values and standard deviations for three fish per group from two different experiments.

correlated with the protective efficacy of immunisation, the immunised fish were challenged with VHSV 30 days post-vaccination. In this case, the level of protection conferred by both G_{VHSV}-encoding plasmids was similar (Table 2). This experiment was repeated once and the similar levels of protection were observed.

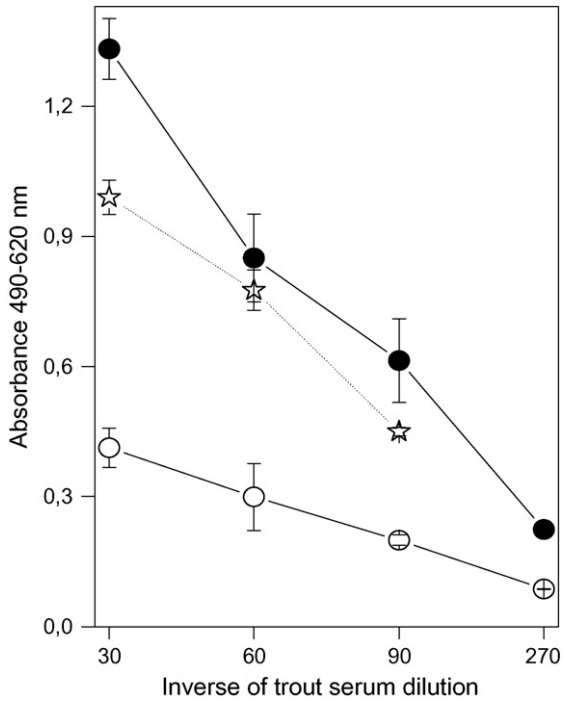

Fig. 3. Presence of anti-VHSV-G protein specific antibodies in sera from trout immunised with pAE6-G_{VHSV} or pMCV1.4-G_{VHSV}. Rainbow trout were injected i.m. with 1 μg pMCV1.4-G_{VHSV} (open circles) or pAE6-G_{VHSV} (solid circles). After 30 days, fish sera were collected and the presence of anti-VHSV-G protein specific antibodies was analysed by ELISA using a recombinant fragment of G_{VHSV} as antigen. Absorbance readings were measured at 492 and 620 nm. Bars represent the average values and standard deviations of sera from four fish per group, each assay in triplicate, from two different experiments. Stars, sera from positive control fish.

3.5. RT-PCR analysis to determine gene expression patterns after genetic immunization of fish

In order to further investigate the differences in the production of the specific IgM response to G_{VHSV} observed after the injection of plasmids pAE6-G_{VHSV} or pMCV1.4-G_{VHSV}, the levels of expression of a set of immuno-related genes was analysed through semi-quantitative RT-PCR (Table 1) in fish intramuscularly injected with either plasmid at days 3 and 10 post-vaccination in the muscle, head kidney and spleen.

Fish injected either pAE6 or pMCV1.4 empty plasmids, in some cases increased the levels of expression of immune genes when compared to the levels observed in fish injected with PBS, as seen in many previous studies [34]. However, no significant differences were observed between the levels of induction of any of the genes between the two empty plasmids. Moreover, both G_{VSHV}-containing plasmids significantly up-regulated all immune genes in all tissues studied when compared to the empty plasmids. Therefore, since the aim of this study was to compare the effects between the pAE6-G_{VHSV} or pMCV1.4-G_{VHSV} plasmids, we have expressed the results by dividing the level of expression obtained for each gene in response to pAE6-G_{VHSV} by the mean level of expression induced by pMCV1.4-G_{VHSV}. Previously, the level of expression for each gene was normalised against *gapdh*.

At the primary site of antigen delivery (skeletal muscle), the expression of most genes was induced by day 3 after immunisation in all the fish immunised with the G_{VHSV}-containing plasmids compared to the expression in fish immunised with the empty-plasmids (data not shown). However, the induction of many of them was 2–3 folds greater in the pAE6-G_{VHSV} immunised fish than in the pMCV1.4-G_{VHSV} immunised fish (Table 3). These included *irf7* (an IFN regulatory factor which itself is an IFN-stimulated gene, ISG), *vig1* (an ISG specifically induced by VHSV, which may be involved in the non-specific virus-induced synthesis of enzymatic cofactors of the nitric oxide pathway [24]), *il8* (a pro-inflammatory cytokines), *ck6* (an inducible CC chemokine related to the flounder macrophage inflammatory protein, MIP1α [34,65], *ifnγ* (an important macrophage activating Th1 cytokine [66]), *inos* (a phagocyte-related gene induced in other vertebrate species by

Table 2
Protection conferred by the plasmids pAE6-G_{VHSV} and pMCV1.4-G_{VHSV} against VHSV challenge.

Groups	No. of immunised fish	No. of challenged fish	No. of dead fish	Mortality (%)	RPS
PBS	40	30	29/30	96.66	–
pMCV1.4	40	27	23/27	85.18	11.87
pAE6	40	24	22/24	91.66	5.17
pMCV1.4-G_{VHSV}	40	30	5/30	16.66*	82.74
pAE6-G_{VHSV}	40	31	5/31	16.13*	83.31

Asterisk indicates significant difference ($p < 0.01$) in survival between immunized fish and control fish (PBS group).

the cytokine *infγ* [33,67]) and *igt* (the B-cell membrane form of IgT [33,68]). C*k7a* (an inducible CC chemokine related to the monocyte chemotactic protein (MCP) group [69]), *nkfe* (a gene potentially involved in natural killer (NK) cell activity, at least in mammals [33,70,71] and *tcrβ* chain (a T cell gene marker) genes also showed increased levels of expression in response to pAE6-G_{VHSV} but in this case the differences were non-significant (Table 3).

In the spleen, a significantly higher induction (2–3 folds) of some of these genes could be also observed in the pAE6-G_{VHSV} immunised fish by 3 days. For example, *irf7*, *mx1* (an ISG encoding an antiviral

Table 3
Comparison of the gene expression profiles between the pMCV1.4-G_{VHSV} and pAE6-G_{VHSV} immunised fish.

	Day 3 post-immunisation*			Day 10 post-immunisation*		
	Muscle	**Head Kidney**	**Spleen**	**Muscle**	**Head Kidney**	**Spleen**
Genename						
irf3	1,45±0,48	**0,65±0,13**	1,23±0,06	0,95±0,02	**0,59±0,12**	1,54±0,6
irf7	**2,03±0,53**	1,18±0,15	**1,75±0,27**	1,48±0,06	0,83±0,15	**1,72±0,07**
mx	**1,98±0,02**	1,63±0,06	**2,01±0,27**	0,99±0,07	1,28±0,05	1,39±0,07
vig1	**1,99±0,3**	1,40±0,05	1,30±0,12	0,97±0,14	**2,04±0,12**	1,58±0,09
Il1β	1,64±0,3	0,97±0,35	0,80±0,11	1,20±0,35	**0,35±0,07**	**0,24±0,08**
tnfα	0,96±0,11	**2,01±0,02**	**1,98±0,01**	**4,5±0,4,1**	**1,79±0,37**	1,59±0,07
il8	**2,16±0,57**	0,98±0,08	**2,16±0,42**	0,98±0,12	0,74±0,11	0,79±0,23
ck5b	1,44±0,09	1,40±0,02	1,28±0,06	**2,80±0,16**	1,05±0,02	1,11±0,28
ck6	**3,10±0,33**	**2,23±0,13**	1,11±0,18	**2,74±00,2**	1,58±0,57	1,03±0,04
ck7a	1,34±0,42	1,34±0,14	**1,93±0,62**	**2,04±0,11**	1,09±0,08	1,16±0,19
nkef	1,61±0,32	0,98±0,06	0,89±0,08	1,29±0,21	0,97±0,14	1,15±0,01
Ifn-γ	**3,22±0,93**	**2,92±0,33**	**1,84±0,05**	0,98±0,04	1,76±0,26	1,54±0,15
inos	**1,95±0,34**	**2,97±0,31**	**2,13±0,32**	1,12±0,08	**0,49±0,23**	**2,03±0,23**
psmb9	1,14±0,29	1,19±0,16	1,20±0,16	0,86±0,11	0,75±0,18	1,22±0,3
mhcII	0,89±0,07	1,19±0,10	1,69±0,21	0,81±0,02	0,81±0,01	1,08±0,05
tcrβ	1,32±0,19	0,99±0,09	1,57±0,09	0,98±0,07	1,04±0,34	1,16±0,15
cd4	**1,95±0,17**	**0,75±0,09**	**0,75±0,05**	1,02±0,06	0,71±0,16	**1,89±0,17**
migm	ND	0,91±0,14	0,81± 0,22	ND	1,18±0,07	0,81±0,01
sigm	1,48±0,02	1,83±0,26	1,05±0,32	0,93±0,09	0,69±0,18	1,12±0,14
igt	**1,88±0,12**	1,58±0,10	**1,99±0,25**	0,98±0,29	1,04±0,21	0,95±0,1

N.D.: not determined.
[a]The levels of expression of the different immune genes observed in fish injected with pAE6-G_{VHSV} and pMCV-G_{VHSV} were first normalized against *gapdh. Then, in order to compare the response induced by each plasmid*, the results are showed here as relative expression to pMCV1.4-G_{VHSV} immunised fish, by dividing the expression in response to pAE6-G_{VHSV} by the mean level of expression observed in response to pMCV-G_{VHSV} for each at each time point in each of the organs studied. Each value represents the mean ± S.E. of triplicate samples. Shadowed cases denote statistically significant differences between the pAE6-G_{VHSV} and pMCV-G_{VHSV} immunised fish.

factor) *il8*, *tnfα*, *ck7a* were ~2-fold up-regulated in the spleen of the pAE6-G_{VHSV} immunised fish compared to the spleen of the fish immunised with pMCV1.4-G_{VHSV} (Table 3). In addition, the transcripts of *ifnγ*, *inos* and *igt* gene were also more abundant in the spleen of these fish. By contrast, the levels of expression of the gene encoding the T-cell marker *cd4* were lower in the pAE6-G_{VHSV} immunised fish than in the pMCV1.4-G_{VHSV} immunised fish.

In the head kidney, fish immunised with pAE6-G_{VHSV} showed a significantly higher expression of the *tnfα* (a pro-inflammatory cytokines), *ck6*, *ifnγ and inos* genes than the pCVM-G_{VHSV} immunised fish (Table 3). At the same time, the levels of expression of the *irf3* (the IFN regulatory factor 3) and *cd4* genes was lower in fish injected with pAE6-G_{VHSV}.

At later times p.i. (day 10), the differences between the RNA expression patterns from pAE6-G_{VHSV} and pMCV-G_{VHSV} immunised fish were also evident as demonstrated by the up-regulation of a number of different gene categories at the injection site as well as in head kidney and spleen of the fish immunised with the pAE6-G_{VHSV} plasmid. In the muscle, the most significant expression changes were the up-regulation of the *tnfα* (4.5-fold) and the induction of all three inducible CC chemokines included in this study (2.8-fold ck5b, 2.74-fold ck6 and 2.04-fold ck7b) in the fish immunised with pAE6-G_{VHSV} (Table 3). Regarding the spleen, the expression pattern of *irf7*, *irf3*, *mx1*, *tnfα*, *ifnγ* and *inos* genes (Table 3) was similar to that observed at 3 days p.i. By contrast, the presence of transcripts of *il1β* and *cd4* genes, were either unchanged or down-regulated, respectively, in the spleen of the fish immunised with this plasmid compared to the fish immunised with the pAE6-G_{VHSV} by day 3 p.i., were now down-regulated (*il1β*) and up-regulated (*cd4*). In the case of head kidney, after 10 days p.i., higher expression levels of *tnfα*, *vig1* but lower expression of *irf3*, *inos* and *il1β* were observed in fish immunised with pAE6-G_{VHSV} compared to fish immunised with pMCV1.4-G_{VHSV} (Table 3).

4. Discussion

The viral origin of the regulatory elements present in fish DNA vaccination plasmids is an important issue to address because of safety considerations. For that reason, we have focused our current work in finding alternative regulatory sequences that are at least as effective as the promoter/enhancer regions of CMV using the genetic immunisation of fish with the gene of G_{VHSV} as a model.

For driving the G_{VHSV} gene expression we chose the pAE6, a plasmid containing a 5′ upstream sequence of the carp β-actin gene including the regulatory elements (enhancer/promoter) as well as both the 1st non-coding exon and 1st intron sequences because the successful expression of different transgenes in fish cell lines and tissues using plasmids containing the regulatory elements from carp β-actin gene had been previously reported [21,41,42,43,72] and a positive effect on the expression had been observed in transfected cells by the inclusion of intron sequences [73]. In fact, most of the commercial CMV promoter/enhancer- containing plasmids, including the CMV-based plasmid used in this study, enclosed an intron quimeric sequence.

Our *in vitro* expression assays indicated that the pAE6 plasmid might be a real alternative to the CMV-based plasmid since it offers a comparative number of G_{VHSV}-surface expressing cells, a long lasting expression and higher expression per transfected cell. These results were further reinforced by the *in vivo* expression assays showing that, the levels of G_{VHSV} expressed from pAE6 in fish muscle were higher than those expressed from pMCV1.4. This different expression profile of G_{VHSV} when linked to carp β-actin gene sequences is probably due to transcriptional and/or transcriptional-related events more than to the plasmid-copy number present in each cell, since the pAE6 is larger in comparison to pMCV1.4.

When the fish genetic immunisation assays were carried out to study the immune response induced by pAE6-G_{VHSV} and pMCV1.4-G_{VHSV} plasmids higher levels of G_{VHSV} expression obtained with pAE6-G_{VHSV} at the primary site of antigen delivery were translated into a meaningful specific IgM response in the fish immunised with this plasmid compared to the response in fish injected with pMCV1.4-G_{VHSV}. However, the immunisation with both G_{VHSV}-encoding plasmids by intramuscular injection conferred similar fish protection against VHSV (Table 2). Strikingly, although none of the sera containing anti-G_{VHSV} IgMs showed a considerable neutralising activity, the sera from immunised trout that stronger reacted by ELISA had the lower VHSV neutralising activity. These findings, as well as others previously published data [32,57,74–77], suggest that the fish humoral response induced by a recent immunisation and the generation of resistance to a challenge with VHSV are not correlated. Therefore, despite the considerable advances obtained in the last years, the trout humoral immunological response to VHSV infection remains still a matter of study. In this sense, the role that the recently identified Ig isotypes, such as the rainbow trout IgT isotype [68], may have on the protective immune response mediated by antibodies might also explain some of the current incognitos.

In an attempt to further investigate how the immune response of the pAE6-G_{VHSV} immunised fish differs from that of fish injected with pMCV1.4-G_{VHSV}, we analysed the tissue and temporal expression profile of a set of genes implicated in the innate and adaptive immunity. As expected, the magnitude of the type I IFN-related immune response correlated with the levels of G_{VHSV} expression at the injection site by day 3 p.i. (higher in fish injected with the pAE6-G_{VHSV} plasmid), confirming previous results showing that the expression of the G_{VHSV} gene in the transfected cells is directly related to the induction of IFN [78]. In the same way, viral glycoproteins from other enveloped virus including vesicular stomatitis virus (VSV) [79], the prototype virus of rhabdoviridae family, are able to trigger the type I IFN pathway [80–86]. In addition, the genes encoding pro-inflammatory cytokines (specially IL8) and CC chemokines (specially CK6), macrophage activation factors (IFNγ and iNOS), and B- (IgT) and T-cells marker (TcRβ and specially CD4) were also up-regulated in the muscle of the pAE6-G_{VHSV} immunised fish (Table 3), indicating that, among others, the infiltration and activation of immune cells processes are occurring to a greater degree in the muscle of the fish immunised with pAE6-G_{VHSV}.

Because the ability of the fish DNA vaccines to produce a systemic response is related to the magnitude of the immune response occurring at the injection site [33], the immune response developed by the fish immunised with pAE6-G_{VHSV} in the secondary tissues, specially in the spleen, was stronger as expected. Since more genes were up-regulated in the spleen than in the head kidney of the fish immunised with pAE6-G_{VHSV} compare to the fish immunised with pMCV1.4-G_{VHSV} and higher titres of specific antibodies were detected in the sera of the fish immunised with pAE6-G_{VHSV}, the spleen, the major secondary lymphoid-organ involved in antigen processing as well as the major source of B cells and MHC class II expression in trout [68], seems to play a central role in developing the specific antibody response induced by the DNA vaccines. At the same time, the higher expression levels of the *igt* gene in the spleen of the fish immunised with pAE6-G_{VHSV} compared to the fish immunised with pMCV1.4-G_{VHSV} during early stages p.i., might suggest that a subset of B cells expressing the membrane form of IgT may be involved in triggering the expression of the specific humoral immune response. Probably the stronger type I IFN-related immune response induced by the expression of the G_{VHSV} from the plasmid pAE6 at the injection could be underlying the increased presence of *igt* transcripts and specific antibody production in the fish immunised with pAE6-G_{VHSV}. It has been shown in mammals that the intensity of the type I IFN-related immune response may enhance the primary antibody responses and promote the Ig isotype switch-

ing [87–91]. However, further studies are needed to corroborate this hypothesis because of the enhancement of adaptive immunity by type I IFN is not well known in fish [92].

Regarding the signal pathway triggered by G_{VHSV} for inducing type I IFN expression, no data are availed so far. Likewise, candidate receptors for signalling by viral glycoproteins have yet to emerge [93]. However, regardless of the cellular receptor/s responsible for induction of type I IFN by G_{VHSV}, the transcription factors IRF3 and IRF7 are included among the downstream effectors of this signalling cascade since the expression of both genes was modulated by the immunisation of the fish with the G_{VHSV}-containing plasmids. This result is in consonance with recent studies showing that fish *irf3* and *irf7* genes are transcriptionally modulated by the same range of ligands (poly I:C, trout type I and II recombinant IFN and trout recombinant IL1β, etc.) [94]. Nevertheless, the expression of the *irf3* and *irf7* genes was modulate by G_{VHSV} in different way in the fish immunised with pAE6-G_{VHSV} and pMCV1.4-G_{VHSV}. Overall, the expression level of *irf3* was lower in the fish immunised with pAE6-G_{VHSV} compared to that of fish immunised with pMCV1.4-G_{VHSV}. By contrast, the expression of *irf7* gene in these fish was higher, especially in muscle and spleen. Since no differences were observed between the fish immunised with pAE6 or pCMV empty plasmids, the higher induction of IFN and, hence of the *irf7* gene, in fish immunised with pAE6-G_{VHSV}, cannot be due to the signalling cascade triggered by TLR9 recognition of the plasmid DNA containing unmethylated CpG motifs, which has IRF7 as downstream effector. In consequence, other signalling pathway, possibly acting via IRF7, could be initiated by G_{VHSV} as result of the different expression of this protein from the pAE6-G_{VHSV} at the injection site. It has been recently reported a strong induction of IRF7 triggered by the recognition of the glycoprotein G of the VSV on the cell surface by the TLR4 (directly or indirectly) culminating in the activation of type I IFN secretion [79]. Since trout *tlr4* gene has not been described so far, it is not possible to evaluate the possible occurrence of this signal pathway in trout. However, It is known that most of the teleost fish are highly resistant to LPS toxicity [95,96] and do not seem to exhibit cellular responses to LPS via TLR4 [96]. These results together with the TLR4 sensing by VSV-G glycoprotein suggest the possibility that TLR4 might be dedicated to viral sensing in LPS unresponsive species, which are known to be prey of the Rhabdoviridae [97,98] such as it was previously pointed out [79].

In summary, the immune response induced by the G_{VHSV} expressed from the pAE6 was not only stronger, but also different to that induced by the plasmid pMCV1.4-G_{VHSV} since the variations in the tissue and temporal expression profile affect a considerable number of genes but all of them. Therefore, the results of this work clearly demonstrate that the selection of a determinate regulatory sequences/encoded-antigen combination for fish genetic immunisation is of extraordinary importance. Moreover, we show that the expression vectors containing the G_{VHSV} gene linked to regulatory sequences from carp β-actin gene are very effective as vaccines in rainbow trout and represent a truly alternative to CMV plasmids.

Acknowledgements

Thanks are due to Beatriz Bonmati for technical assistance. This work was supported by the projects AGL2008-03519-C04 and Consolider ingenio 2010, CSD2007-00002 both from MEyC (Spain).

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