Vaccine 26 (2008) 6620–6629

Contents lists available at ScienceDirect

Vaccine

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

In vitro search for alternative promoters to the human immediate early cytomegalovirus (IE-CMV) to express the G gene of viral haemorrhagic septicemia virus (VHSV) in fish epithelial cells

S. Ruiz [a], C. Tafalla [c], A. Cuesta [c], A. Estepa [b], J.M. Coll [a,*]

[a] *INIA, SGIT, Dept Biotecnología, Crt. Coruña Km 7, Madrid, Spain*
[b] *IBMC Universidad Miguel Hernández, 03202 Elche (Alicante), Spain*
[c] *INIA, CISA Valdeolmos Madrid 28130, Madrid, Spain*

ARTICLE INFO

Article history:
Received 6 May 2008
Received in revised form 6 September 2008
Accepted 15 September 2008
Available online 7 October 2008

Keywords:
VHSV
Viral haemorrhagic septicemia viruses
G-protein
Fish DNA vaccines
Epithelial vaccines
Promoters
Transcription binding sites

ABSTRACT

Present DNA vaccines against fish rhabdoviruses require intramuscular injection (fish-to-fish vaccination) of their G-protein gene under the control of the human immediate early cytomegalovirus (IE-CMV) promoter, while immersion delivery (mass DNA vaccination), for instance, by using fish epithelial-specific promoters, would be more practical for aquaculture. To find fish epithelial-specific promoters alternative to the IE-CMV, a comparative study of the effectiveness of different fish promoters constitutively expressing the G gene of the viral haemorrhagic septicemia virus (VHSV) in the *epithelial papulosum cyprini* (EPC) cell line was performed. The study included MCV1.4 (an alternative IE-CMV promoter version), AE6 (a version of the carp β-actin promoter), long terminal repeats (LTR) of zebrafish or walleye retroviruses, trout Mx1, carp myosin-heavy-chain and flatfish pleurocidin promoters and salmonid *sleeping beauty* (SB)/medaka Tol2 transposon repeats. The G-protein expression in transfected EPC cells was studied by estimating the number of cells expressing the G-protein in their membrane and the average expression level per cell. In addition, in an attempt to reduce their sizes, some regions of the MCV1.4 and AE6 promoters were deleted and expression levels compared to those observed for full-length promoters. Since both zebrafish LTR and carp AE6 promoters were the most effective regulatory sequences for expressing the VHSV G-protein in EPC cells, these sequences might be candidates for new DNA vaccine vectors for fish epithelial tissues avoiding the IE-CMV promoter. Furthermore, known transcription factor binding sites (TFBS) common to most of the fish G-expressing promoters, might enable the future design of fully synthetic or hybrid promoters with improved efficacy of VHSV G-protein expression in epithelial fish cells.

1. Introduction

Fish rhabdoviruses such as infectious haematopoietic necrosis virus (IHNV) and viral haemorrhagic septicemia virus (VHSV) constitute one of the main threats to the development of the worldwide aquaculture industry. Present DNA vaccines against fish rhabdoviruses, including, the one against IHNV licensed in Canada for use in salmonids in 2005 [1], require the intramuscular delivery by injection (fish-to-fish DNA vaccination) of a plasmid coding for their highly immunogenic protein G gene placed under the control of the human immediate early cytomegalovirus promoter (IE-CMV) [1–3]. However, both immersion delivery to obtain *in vivo* transfection throughout fish epithelial tissues (mass DNA vaccination) and alternative fish promoters to the human IE-CMV (safer constructs) will be more practical for aquaculture [4].

Rhabdoviral infections begin under fish epidermal mucus [5,6] and specifically in the fin bases [6], thus suggesting that the dermal-epithelial fish tissue is the main target of natural entrance for rhabdoviruses and, possibly, the best fish body site for immunization. Corroborating these observations, mucosal immunity implicating both intraepithelial lymphocyte T markers (CD8, CD4, CD28, CD3ϵ, TCRξ, TCRγ, TCRβ) [7] and their T cell receptor (TCR) repertoir modifications have been demonstrated in both infected and DNA vaccinated trout [7], further underlining the importance of fish body surfaces in generating immune defenses against rhabdoviral fish infections [8] and immunization. Therefore, DNA vaccination through the epithelial cells present in the fish body surface, such as that achieved through immersion DNA vaccination with ultrasounds reported earlier by our group [9] and later corroborated by others [10], could be an improved system to achieve a practical immunization protocol for fish rhabdoviruses. Because

* Corresponding author. Tel.: +34 1 3476850; fax: +34 1 3572293.
E-mail address: juliocoll@inia.es (J.M. Coll).

0264-410X/$ – see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2008.09.048

eucaryotic gene transcription is tissue-specific, epithelial-specific sequences in new promoters will be required to increase exogenous gene expression on epithelial tissues. Therefore, a search for alternative regulatory sequences or promoters that are, at least, as effective as IE-CMV and could be active when delivered to epithelial cells by immersion has been undertaken in this work. Moreover, it is believed that the substitution of the human IE-CMV promoter by a non-human analog would increase the security of these vaccines and it constitutes a highly convenient step for their commercialization in regions highly concerned with safety, as it occurs in Europe.

Because the *epithelial papulosum cyprini* (EPC) cell line is readily transfected [11–14], we have used EPC cells as a model to study fish epithelial expression of the VHSV G-protein. Up to a certain limit, the more the amount of G gene was provided in DNA vaccination, the best fish protection was obtained by using immersion vaccination [9] and/or injection vaccination [15,16], therefore optimal G gene expression might improve immunization.

Among the possible alternative fish promoters, we have selected: the inverted terminal repeats (itr) of the *sleeping beauty* (SB) transposon, reconstructed from salmon and trout inactive copies and improved for use in human cells [17,18]; the internal inverted repeats (iir) from the Tol2 transposon of medaka (*Oryzias latipes*) [19,20]; the long terminal repeats (LTR) from two fish retroviruses, the endogenous from zebrafish (ZFERV) [21] (LTRz) and the epithelial Walleye Dermal Sarcoma virus (WDSV) from *Stizostection vitreum* [22–24] (LTRw); the promoter of the pleurocidins from *Pleuronectes platessa* (antimicrobial short cationic peptides from the mucus of flatfish) [25]; the promoter from the trout Mx1 (interferon inducible proteins) [26]; the 5′ regulatory region of the carp myosin-heavy-chain gene, characterized and used to drive the expression of heterologous proteins[27,28]; and the AE6 version of the β-actin promoter [29], characterized from several fish like carp and tilapia [30] and widely used to express foreign proteins in fish [31–33].

To compare the activity of the different promoter containing plasmids, the VHSV G-protein expression was quantified by estimating both the number of cells expressing the G-protein in their membrane and the average expression level per cell. Furthermore, partial deletions were performed in the IE-CMV (MCV1.4 version) and carp β-actin (AE6 version) promoters to explore the possibility to decrease their size or to detect important enhancer sequences. The results suggested that zebrafish LTR and carp AE6 promoters might be used as improved substitutes for the IE-CMV promoter.

Since eucaryotic gene transcription depends on tissue-specific trans-acting transcription factors (TF) recognizing cis-acting DNA transcription factor binding sites (TFBS) [34,35], for an exogenous gene to be constitutively expressed in epithelial cells, TFBS sequences for epithelial-specific TF need to be present in the promoter under which control the gene would be placed. However, few epithelial specific TF/TFBS have been described in vertebrates and less so in fish. Therefore, as a first step to identify some of the required TFBS for the exogenous expression of the G gene of VHSV in epithelial cells, we studied the presence of known TFBS (TRANSFAC data base) in the higher VHSV G-protein expressing promoters. The results showed that some common TFBS could be found in the higher fish promoters, data which might be used to design new synthetic promoters or combinations of some of their sequences [12,34,36,37], such as the *SB* itr and MCV1.4 (tested in this work), to improve G-protein expression in epithelial fish cells.

2. Materials and methods

2.1. Plasmids and promoters used

The pMCV1.4 plasmid (Ready Vector, Madrid, Spain) was used as the DNA backbone (1.9 kbp) for most of the constructs employed. The MCV1.4 promoter of the pMCV1.4 plasmid is a larger immediate early cytomegalovirus (IE-CMV) promoter of 994 bp (Table 1) which includes a synthetic human globin–immunoglobulin chain intron [14]. The plasmid pMCV1.4-G coding for the G-protein of viral haemorrhagic septicemia (VHSV) strain VHSV-0771, isolated

Table 1
Summary of some properties of the promoters used in this work.

Promoter name in bold underlined		Gene Bank accession number of the promoter	Position of promoter in their plasmid constructs	Promoter size (bp)	Position of the TSS	References
Same DNA backbone						
MCV1.4	*	–	23–1016	994	743	[40]
IE-CMV						[63]
iirL	*	D84375	181–543	363	?	[41]
Tol2 transposon						
iirR	*	D84375	74–489	416	381	[41]
Tol2 transposon						
Mx	*	AF310254	1–674	674	614	[26]
trout Mx1 protein						
Ple	*	–	1–588	588	490	[25]
flatfish pleurocidin						
LTRz	*	AF503912	1–749	749	514	[21]
zebrafish retrovirus						
LTRw	*	AF033822	1–644	644	469	[64]
walleye retrovirus						[23]
itrL	*	–	1–293	293	238	[17]
SB transposon						
Different DNA backbones						
T2 MCV1.4	+	–	115–1521	1407	1156	[17]
SB transposon/IE-CMV						
FG2	+	Z37999	1–1046	1046	918	[27]
Carp myosin						
AE	+	M24113	1–2577	2577	1177	[65]
carp β-actin						[29]

Bold and underlined, the names of the promoters. TSS, transcription start site. *The pPle, pLTRz, pLTRw, piirL, piirR, pitrL and pMx plasmids were constructed by removing the MCV1.4 promoter from the pMCV1.4-G plasmid and ligating each of the promoters, therefore all of them contain the same DNA backbone. +The pT2MCV1.4-G, pFG2-G and pAE6-G plasmids contain different DNA backbones (see Section 2). The CRE promoter of the pCRE plasmid of Clontech (294 bp) has the TSS at position 234.

in France from rainbow trout (*Oncorhynchus mykiss*) [38], was constructed as previously described [39,40].

The iirL (left) and iirR (right) potential promoter sequences contained the internal inverted repeats (iir) from the Tol2 of medaka (*O. latipes*) transposon [19,20,41]. The itrL (left) potential promoter sequence included the left inverted terminal repeat (itr) of the *sleeping beauty* transposon version 2 (T2) [42], reconstructed from salmon and trout inactive transposon copies [17,18]. The Mx1 promoter corresponds to the previously described promoter of these trout interferon-inducible proteins [26]. The pleurocidin (Ple) promoter contained the 5′ UTR regulatory sequences described for the pleurocidin gene (antimicrobial cationic peptides of 21–25 amino acids from the mucus of flatfish) of *Pleuronectes platessa,* a flatfish species [25]. The LTRz and LTRw contained the long terminal repeats (LTR) from two fish retroviruses, the endogenous retrovirus of the thymus of the zebrafish *Danio rerio* (ZFERV) [21] and the Walleye Dermal Sarcoma virus (WDSV) of the epithelia of *Stizostection vitreum* [22–24]. All the above mentioned potential regulatory sequences and described promoters were chemically synthesized and flanked with NsiI and HindIII restriction enzymes sites (BioS&T, Montreal, Canada). After removing the MCV1.4 promoter of the pMCV1.4-G plasmid by digestion with the same restriction enzymes, the synthetic promoter sequences were inserted into the pMCV1.4 DNA backbone.

The FG2 promoter was derived from the 5′ regulatory region of the carp myosin-heavy-chain gene as described [27,28], kindly provided by Dr. Goldspink (University of London, England). The AE6 (β-actin) promoter was isolated and characterized from several fish like tilapia and carp [30]. It has been widely used to express foreign proteins in fish [31,32]. The pAE6-G containing the carp β-actin promoter (−1177 bp, exon 1 and intron 1) was obtained as described before [32].The β-galactosidase (β-gal) gene under the control of carp β-actin promoter versions described previously, 4.7 kbp carp βAP (−3413 bp, exon 1 and intron 1) and 1.5 kbp carp βAP (−143 bp, exon 1 and intron 1) and tilapia β-actin promoter of 1.7 kbp [30], were kindly provided by Dr. McLean (University of Southampton, England).

The pCRE-G plasmid was constructed by excising the G gene from the pMCV1.4-G and ligating it to the commercially available pCRE plasmid (Clontech, Palo Alto, CA, USA), containing multiple cAMP inducible transcription CRE binding sites in mammalian cells.

DNA backbones were defined from the TGA of the G gene to the starting position of the promoter. Four different DNA backbones were used to make all the constructs. Constructs having the pMCV1.4 DNA backbone contained the kanamycin resistance gene, the SV40 polyadenylation signal and the colE1 plasmid replication origin (1.9 kbp) and corresponded to the constructs: pMCV1.4-G, pMx-G, piirL-G, piirR-G, pLTRz-G, pLTRw-G, pPle-G and pitrL-G. Constructs having the FG2, the AE6, CRE and the hybrid T2MCV1.4 promoters, have DNA backbones that contained the ampicillin resistance gene, the SV40 polyadenylation signal and the colE1 plasmid replication origin (4.0, 3.7, 3.2 and 3.5 kbp, respectively) and corresponded to the constructs: pFG2-G, pAE6-G, pCRE and the hybrid pT2MCV1.4-G.

2.2. Construction of hybrid T2MCV1.4 and MCV1.4AE6 promoters

To construct the hybrid T2MCV1.4 promoter for the pT2MCV1.4-β-gal plasmid (the β-gal gene flanked by both itr of the *SB* transposon), the MCV1.4-β-gal expression cassette was excised from the pMCV1.4-β-gal plasmid (Ready Vector, Madrid, Spain) and ligated into the multiple cloning site of pT2/HB [42] (kindly provided by Dr. P. Hackett, University of Minnesota: http://biosci.cbs.umn.edu/labs/perry). To construct the hybrid T2MCV1.4 promoter for the pT2MCV1.4-G plasmid (the G gene flanked by both itr of the *SB* transposon), the MCV1.4-G gene expression cassette was excised from the pMCV1.4-G and ligated into the multiple cloning site of pT2/HB, following standard procedures.

To obtain the hybrid MCV1.4AE6 promoter to construct the pMCV1.4AE6-β-gal plasmid (see later), the MCV1.4 was excised from the pMCV1.4 plasmid with BsrGI and BanI and inserted into the pAE6-β-gal plasmid at the BsrGI site. The pAE6-β-gal plasmid was obtained before from the pMCV1.4-β-gal by KpnI, XbaI digestion of the β-gal gene and insertion into the pAE6-GFP [32] digested with the same enzymes.

2.3. Amplification and characterization of the plasmid constructs

Escherichia coli DH5alpha (Invitrogen, Barcelona, Spain) were transformed by electroporation with each of the plasmid constructs obtained. Large amounts of plasmid were prepared from *E.* coli pellets by using a modification of the Wizard plus Megaprep DNA purification system (Promega, Madison, USA). The concentration of the DNA was then estimated by fluorescence after SYBR binding (Sigma Che. Co., St. Louis, MI), by diluting the initial solution of SYBR 500-fold in 5 M NaCl, 100 mM $NaPO_4H_2$, pH 7 and measuring fluorescence at 485/535 nm (linearity from 5 to 200 μg DNA/ml). Possible contamination with proteins was estimated with the help of nanodrop ND1000 spectrophotometry measurements (Nanodrop Technologies Inc., Wilmington, DE, USA). Plasmid solutions were adjusted to 0.5–1 mg/ml and kept frozen until used.

2.4. Partial deletions of the MCV1.4 and AE6 promoters

Partial deletions of the MCV1.4 promoter were obtained in the pMCV1.4-pac plasmid carrying the puromicin resistance gene to facilitate their expression assays. To obtain the pMCV1.4-pac construct, the pac resistance gene was amplified from the plasmid pGEM-pac [43] by using the oligos 5′GACGGAGAATTCATGACCGAGTACAAG3′ (EcoRI site underlined) and 5′CTCAAGGGATCCTCAGGCACCGGGCTT3′ (BamHI site underlined). Plasmid pMCV1.4 linearized with EcoRI and BamHI was ligated with the PCR amplified pac gene to obtain the pMCV1.4-pac.The pMCV1.4-pac plasmid (nucleotide positions with respect to the transcription start between parentheses) was digested with NsiI(−742)/BsrGI(−640), NsiI(−742)/AseI(−577), NsiI(−742)/NdeI (−350), NsiI(−742)/SnaBI(−245), BclII(+68)/KpnI(+211) or NsiI(−742)/KpnI(+211).

Partial deletions of the carp AE6 promoter were obtained in the pAE6-β-gal plasmid carrying the β-galactosidase reporter gene to facilitate their expression assays. The pAE6-β-gal plasmid (nucleotide positions with respect to the transcription start between parentheses) was digested with BsrGI(−1021)/AflII(−529), AflII(−529)/StuI(−190), StuI(−190)/SalI(+69), SalI(+69)/BsAI(+219), BsAI(+219)/BglII(+947), BglII (+947)/KpnI(+1360) or BsrGI(−1021)/KpnI(+1360). The constructs containing different versions of the carp β-actin 5′ UTRs such as the 4.7 kbp βAP and 1.5 kbp βAP from carp and 1.7 kbp βAP from tilapia [30] were kindly provided by Dr. McLean (Univ. Southampton, England).

The digested plasmids were separated from the deleted fragments by low melting point agarose electrophoresis and extracted from the agarose with the SNAP kit by following manufacturer instructions (Invitrogen). Then the digested plasmids were blunt ended with the Klenow enzyme, religated with T4 DNA ligase and electroporated into *E. coli* DH5alpha. About 10 antibiotic resistant colonies were investigated to select those containing the deleted plasmid. Positive candidates were confirmed by digestion with selected restriction enzymes and the confirmed plasmids were amplified as described above.

2.5. Transfection of EPC cells

Epithelioma papulosum cyprini (EPC) cells [44] were grown in 25 cm^2 flasks at 28 °C in RPMI Dutch modified cell culture medium buffered with 20 mM HEPES (Flow) and supplemented with 10% fetal calf serum, 1 mM piruvate, 2 mM glutamine, 50 μg/ml of gentamicin and 2.5 μg/ml of fungizone. For the EPC cell transfections, 10 μl of plasmids at different concentrations (2-fold serially diluted in sterile double distilled water starting with 5000 ng/ml) were pipetted in each of the wells of 96-well plates and incubated during 30 min with 25 μl of RPMI medium (Flow) containing 1 μl of Fugene 6 (Roche) or TransIT LT1 (Mirus) transfection agents per well. EPC cell monolayers were then trypsinised (0.05% trypsin, 50 mM EDTA in PBS), counted with an hemocytometer, adjusted to 500,000 cells per ml and 100 μl pipetted into the plasmid + transfection agent containing wells. The cell cultures were incubated at 20 °C during 2 days, since these were the optimal conditions for the G-protein to be expressed as previously determined [11,13,14].

2.6. Production of anti-G monoclonal antibodies (MAbs) C10, I16 and 3F1A12

To obtain a reliable source of anti-G antibodies (Abs) throughout the experiments, the C10 [45,46], I16 (INRA, unpublished) and 3F1A12 (Aarhus, unpublished) were obtained from INRA (Dr. M. Bremont) and Denmark Centre of Aarhus (Dr. N. Lorenzen), respectively. At least 500 ml of hybridoma culture supernatants were obtained, characterized and concentrated-purified by affinity chromatography on protein A. The I16 was mapped in between amino acids 139 and 153 by using a G pepscan as previously described [47,48].

2.7. Estimation of the levels of protein expression of the VHSV G gene by flow cytometry in the membrane of transfected EPC cells

Transfected EPC cell monolayers were incubated with 100-fold diluted (RPMI 10% FCS medium) mix of purified-concentrated anti-G MAbs C10 + I16 + 3F1A12 during 1 h at 20 °C with occasional gentle agitation. After careful washing with RPMI 10% FCS medium, the monolayers were incubated with 200-fold diluted (RPMI 10% FCS medium) rabbit anti-mouse Fab'2 IgG-FITC conjugate (Nordic, Tilburg, The Netherlands) during 30 min at 20 °C. After washing with RPMI 10% FCS medium, the cell monolayers were detached by using FACS buffer consisting in PBS (100 mM Na_2HPO_4, 27 mM KCl, 17 mM KH_2PO_4, 1.3 M NaCl, pH 7.4), 0.1% bovine serum albumin, 0.01% Na_3N, 50 mM EDTA. Cells were transferred to tubes containing FACS buffer and 1% formaldehyde (400 μl/tube). Duplicate transfections with 250 ng per well of pMCV1.4-G (~25% of fluorescent cells) were included in each experiment to correct for variations between experiments (normalization). The number of cells expressing the G-protein in the membrane of transfected EPC cells was estimated in 5000 cells by using a Beckton-Dickinson (San José, CA) FACScan apparatus by using the program LYSYSII 1.0, filter FL1 (514–545 nm, green). Because the yield of cells and the absolute intensity of fluorescence after the staining procedure were variable experiment to experiment and that could not be measured comparatively by using any other reporter gene we used the fluorescence of pMCV1.4/pMCV1.4-G transfected EPC cells as a mean to standardize the results. Fluorescence histograms (log scale of arbitrary units) versus number of cells showed two peaks of fluorescence separated by a threshold value of significative FL1 fluorescence above the background fluorescence obtained with pMCV1.4 transfected EPC cells between 20 and 30 arbitrary fluorescence units depending on the experiment (Fig. 1). The percentage of the fluorescent cells were calculated by the formula, number of fluorescent cells above the threshold/total number of cells × 100. The average intensity of fluorescence above the threshold was estimated manually (Fig. 1). The results are expressed as means and standard deviations from three different independent experiments. An stadistically differentiation using the two population independent *t*-test with a significant level of 0.05 (Origin 6.1, Microcal Software, Inc. Northampton, MA, USA) was used to compare the G expression data for each promoter with the background data obtained with the empty pMCV1.4 plasmid (or no plasmid) at 125 and 250 ng per well. According to that data, promoters were divided in stadistically significantly different (higher promoters) and stadistically non-different from background promoters.

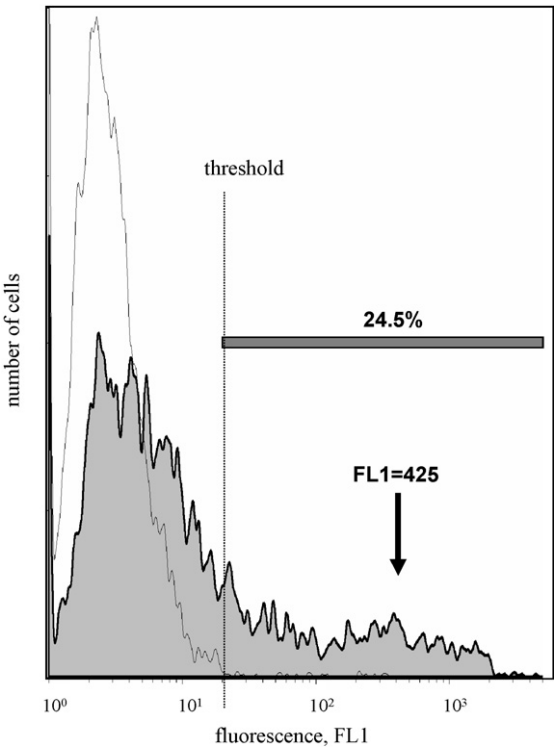

Fig. 1. Example of calculations of percentage of fluorescence (FL1) and average fluorescence from the flow cytometry results. EPC cells were transfected with pMCV1.4-G and pMCV1.4. The number of cells expressing the VHSV G-protein in their membranes was estimated by flow cytometry after staining with a mixture of anti-G Mabs (C10 + I16 + 3F1A12). The percentage of the fluorescent cells were calculated by the formula, number of fluorescent cells above the threshold at FL1 = 20/total number of cells × 100 (24.5% in the figure). The average intensity of fluorescence above the threshold was estimated manually (FL1 = 425 arbitrary units in the figure). Similar results to those obtained with pMCV1.4 were obtained when transfecting the EPC cells with pMCV1.4-Gt (Gt, G-protein sequence without their transmembrane region to inhibit their presence in the membrane) (data not shown). An stadistically differentiation using the two population independent *t*-test with a significant level of 0.05 (Origin 6.1, Microcal Software, Inc. Northampton, MA, USA) was used to compare the G expression data for each promoter with the data obtained with the empty pMCV1.4 plasmid (or no plasmid) at 125 and 250 ng per well. According to that data, promoters were divided in significantly higher and non-different promoters. Wide line and shadowed profile, log of fluorescence versus number of cells obtained in EPC cells transfected with pMCV1.4-G. Similar results were obtained for the promoters classified as higher expression. Thin line, log of fluorescence versus number of cells obtained in EPC cells transfected with pMCV1.4. Significantly not different results were obtained for the promoters classified as non-different from background expression.

2.8. Number of puromycin-resistant colonies after transfection of EPC cells with deleted pMCV1.4-pac constructs

1 and 3 days after transfection, the EPC cell monolayers were changed to medium containing 20 μg/ml of puromycin. The num-

ber of puromycin-resistant colonies were counted 8 days after transfection and remained constant for ~2 weeks. Results were expressed by the formula, number of cells obtained after transfection with deleted plasmids in the presence of puromicin/number of cells obtained after transfection with the pMCV1.4-pac plasmid in the presence of puromicin × 100. Experiments were performed at 500, 250, 125, 62 and 31 ng of plasmids per well in duplicates. Maximal activities were obtained at 500–250 ng/well. The mean of the maximal activities and their standard deviations from two independent experiments were calculated.

2.9. β-Galactosidase activity after transfection of EPC cells with deleted pAE-β-gal constructs

To assay for β-gal activity in the deleted pAE-β-gal, the Gal-screen gene assay system (Tropix, Bedford, MA, USA) was used. Briefly, after removing the cell culture medium, 50 μl/well of 0.025% Triton X-100 were added to lyse the cell monolayers and the plates agitated during 5 min. Then, 1 μl of substrate and 25 μl of lysis enhancer buffer mixture were added per well, incubated during 60 min at room temperature and counted during 1 s (counts per second, cps) in a Genios apparatus (Tecan, Salzburg, Austria). Results were expressed by the formula, maximal cps of deleted plasmids/cps of pAE6-β-gal ×100. Experiments were performed at 500, 250, 125, 62 and 31 ng of plasmids per well in duplicates. Maximal activities were obtained at 500–250 ng/well. The mean of the maximal activities and standard deviations from two independent experiments were calculated.

2.10. Determination of the transcription start sites (TSSs) in the different promoters

To be analysed, the sequences of the promoters were obtained from their corresponding plasmid constructs from the first cloned base up to the ATG of the VHSV G-protein. TSSs, required to calculate relative locations of the transcription factor binding sites (TFBS) were then taken from the references of the promoters (Table 1). TSS locations of each promoter were corroborated by localizing TATAA boxes at their 3′-end and adding 25 bp more (iirR and itrL). Except for iirL, the rest of the promoters have a consensus TATAA box sequence located around their appropriate 3′-end location. Additionally, basal promoter consensus sequences (±50 bp around TSSs) were searched by Clone Manager Software vs9 (Cary, NC, USA) for CTGT, AGC, CTTC, CCAAT, BRE (SSRCGCC), Inr (YYANWYY), GC box (GGGCGGG) and DPE (RGWYV) motifs, according to Lee [49] and the promoter elements page (http://www.epd.isb-sib.ch/promoter_elements).

2.11. Analysis of potential transcription factor binding sites (TFBS)

The Transcription Element Search Software TESS (http://www.cbil.upenn.edu/cgi-bin/tess/tess) was used to search the TRANSFAC database for known putative TFBS sequences in the promoter sequences. The output from TESS consists of Microsoft Excel lists of TFBS that match the input sequence, describing the position, length, matching estimations as log-likehood scores (La) and other statistical measurements. Significant threshold recommended by the program for La values was 12 (equivalent to six perfect matches as obtained by assigning La = 2 per nucleotide and a series of lower values for imperfect matches). Only TFBS having La > 12 were analysed to prevent noise signals and false positives. Qualitative results were compared in Excel with the help of a home-made macro that searched for the presence of each TFBS name of each promoter in the other promoters. The TESS Excel lists were also analysed with the Origin 6.1 software (Northamptom, MA, USA, http://www.OriginLab.com) to obtain a relation between the number of potential TFNS and their La score and expressed as the variation in the number of TFBS per 1000 bp with La values.

3. Results

3.1. Percentage of EPC cells expressing the VHSV G-protein and expression levels per cell after transfection of plasmids expressing the G gene under the control of different promoters

EPC cells were transfected with each of the different promoter-containing plasmids controlling G-protein expression (Table 1) and the expression of the VHSV G-protein in their membranes was estimated by flow cytometry after staining with a mixture of anti-G MAbs (Fig. 1). Fig. 2A shows that the highest number of fluorescent EPC cells (membrane G-protein expressing cells) above the threshold of non-transfected or pMCV1.4 transfected EPC cells was obtained with pMCV1.4-G (30.1%), followed by pLTRz-G (26.4%), pAE6-G (24.3%), pLTRw-G (16.9%) and pMx-G (11.8%). All those G-expression percentage means were significantly higher than the mean obtained with pMCV1.4 at the 0.05 level (*t*-test) at 125 and 250 ng of DNA per well. Those will be called the higher G-expressing promoters or higher promoters. Transfection with the plasmids pPle-G, piirL-G and pitrL-G produced ~10% of fluorescent cells while transfection with the pFG2-G and piirR-G produced <5% of fluorescent cells. Those means were not significantly different from the mean obtained with pMCV1.4 at the 0.05 level (*t*-test) at 125

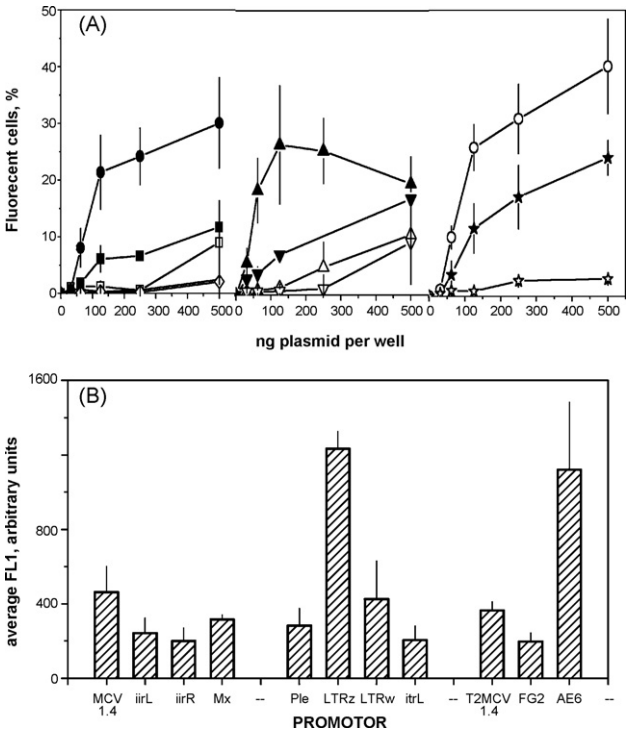

Fig. 2. Percentage (A) and average intensity (B) of fluorescence in EPC cells after their transfection with the G gene of VHSV under the control of different promoters. The different plasmids coding the VHSV G-protein under the control of different promoters (Table 1) were transfected to EPC cells. Duplicate transfections with 250 ng per well of pMCV1.4-G (~25% of fluorescent cells) were included in each independent different experiment to correct for variations between experiments (normalization). The results are expressed as means and standard deviations from three different experiments. Same pMCV1.4 DNA backbone constructs, except for the promoters: –, pMCV1.4; ●, pMCV1.4-G; ■, pMx-G; □, piirL-G; ◊, piirR-G; ▲, pLTRz-G; ▼, pLTRw-G; △, pPle-G; ▽, pitrL-G. Different DNA backbone constructs: ◯, pT2MCV1.4-G; ☆, pFG2-G; ★, pAE6-G.

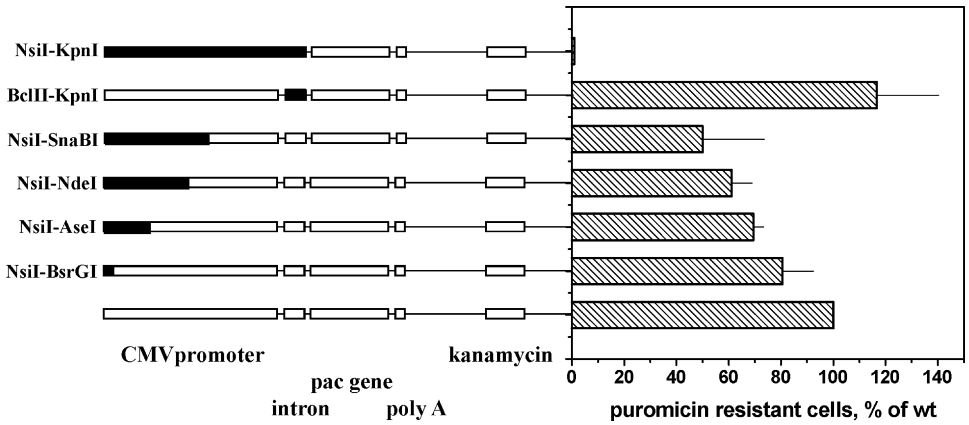

Fig. 3. Comparative expression in EPC cells transfected with pMCV1.4-pac deletions. The deletions were obtained in the pMCV1.4-pac construct by using two unique restriction enzyme digestions, blunt ending and religation. The constructs were transfected into the EPC cells, next day 20 μg/ml of puromicin were added to the EPC cell monolayers and 8 days later EPC survivor colonies were counted. Results were expressed by the formula, number of colonies obtained after transfection with deleted plasmids in the presence of puromicin/number of colonies obtained after transfection with the pMCV1.4pac plasmid in the presence of puromicin × 100. The mean and standard deviations from two independent experiments was represented. Deletions are represented as black horizontal rectangles in between the two restriction enzymes (the plasmids have been represented as lineal for clarity).

and 250 ng pf DNA per well. Those will be called the non-different from the background mean or non-different promoters.

Fig. 2B shows an estimation of the average fluorescence intensities of VHSV-G expressing cells for each of the plasmids tested in the same experiments referred above. Highest fluorescent intensities were found for pLTRz-G and pAE-G (3–5-fold higher than the rest of the constructs) and then for pMCV1.4-G and pLTRw-G (∼2-fold higher).

3.2. Hybrid promoters

The results commented above demonstrated that the left itr of *SB* had very low promoter activity (pitrL-G plasmid construct). However, since cis-enhancing properties have been recently reported for both the left and right itr of the *SB* transposon in mammalian cells [50], MCV1.4-gene cassettes were flanked by both *SB* itr to test their possible influence. As a first step, the pT2MCV1.4-β-gal plasmid was constructed and assayed, demonstrating a ∼30% increase in the activity when compared to the pMCV1.4-β-gal plasmid. Therefore, we constructed the pT2MCV1.4-G plasmid, assayed it for G-expression and found the highest percentage of cells expressing membrane G-protein obtained in this work (38.2%) (Fig. 2A). The observed slight enhancer effect with respect to pMCV1.4-G (30.1%), might be due to either incorporation of the pT2MCV1.4-G construct to the genome by the effect of endogenous *SB* transposase and/or to enhancer effects of any of the two flanking itr, similar to those recently reported in the above mentioned work.

Because, hybrid promoters having both CMV and β-actin sequences had been successfully used to increase activity in mammals [51,52] and those promoters were among the higher promoters to express the G gene in fish epithelial cells, the pMCV1.4AE6 β-gal plasmid was constructed and assayed. However, no significant increase in activity could be demonstrated in EPC fish cells compared to pMCV1.4-β-gal or to pAE6-β-gal plasmids (data not shown) and therefore no further experiments were attempted with this hybrid promoter.

3.3. Expression in transfected EPC cells with partially deleted MCV1.4 and AE6 promoters

The truncation of the MCV1.4 and AE6 promoters was made to explore the possibility to reduce any of their sizes (specially the AE6) and to study the possible relative importance of some of their main transcription factor binding sites (TFBS). The two were chosen among the higher promoters because they were the longest and have enough single-cut restriction enzymes to be truncated in several parts.

Fig. 3 shows that progressive deletions of the distal part of the MCV1.4 promoter up to position −245 reduced to 50.0% their activity, while removal of the intron (BclII/KpnI) resulted in an small increase to 116% in their activity. As a control, deletion of most of the promoter and intron (NsiI/KpnI) reduced the activity to a residual 1%.

Fig. 4A shows that deletions of the distal part of the AE6 promoter to position −190 reduced their activity to 68.1 or 87.7% (BsrGI/AflII or AflII/StuI, respectively), while deletions in the intron reduced their activity to 31.2, 39.2 or 19.9% (BglII/KpnI, BsaAI/BglII or SalI/BsaAI, respectively). The deletion StuI/SalI reduced the activity to 4.1% because of the removal of the basal promoter (TATAA box and transcription start). As a control, deletion of most of the promoter and intron (BsrGI/KpnI) also reduced their activity to a residual 5.8%.

Because the large size (2.5 kbp) and the complexity (5′ sequences, exon and intron) of the carp AE6 promoter, longer and shorter available versions of this promoter were also tested. Fig. 4B shows that longer or shorter 5′ sequences of the β-actin promoter did not increased the activity of the AE6 promoter while short constructs from similar promoter from tilapia did not have any significant activity on EPC cells.

3.4. Analysis of potential transcription factor binding sites (TFBS)

Comparative analysis of the potentially significant TFBS (La > 12) showed the presence of AP-1 and POU1F1a in most of them but more abundantly on the higher promoters (total number of 12 compared to a total of 25 sites, respectively) (Table 2). Also, the B factor/TBP and HiNF-A sites were found in all of the higher fish promoters and the AP-3(2), TCF-4E and RUSH-1alpha were found in, at least 3 of the higher fish promoters, while all those TFBS were absent from the MCV1.4 promoter. Except the AP-1 and the TCF-4E, the above mentioned TFBS were not present or scarcely present in the promoters that had expression levels stadistically non-different from background (non-different promoters) (Table 2). In addition, WT1 I-KTS (T00900), Sp1 (T00759), CTF (T00174), PEA3 (T00685),

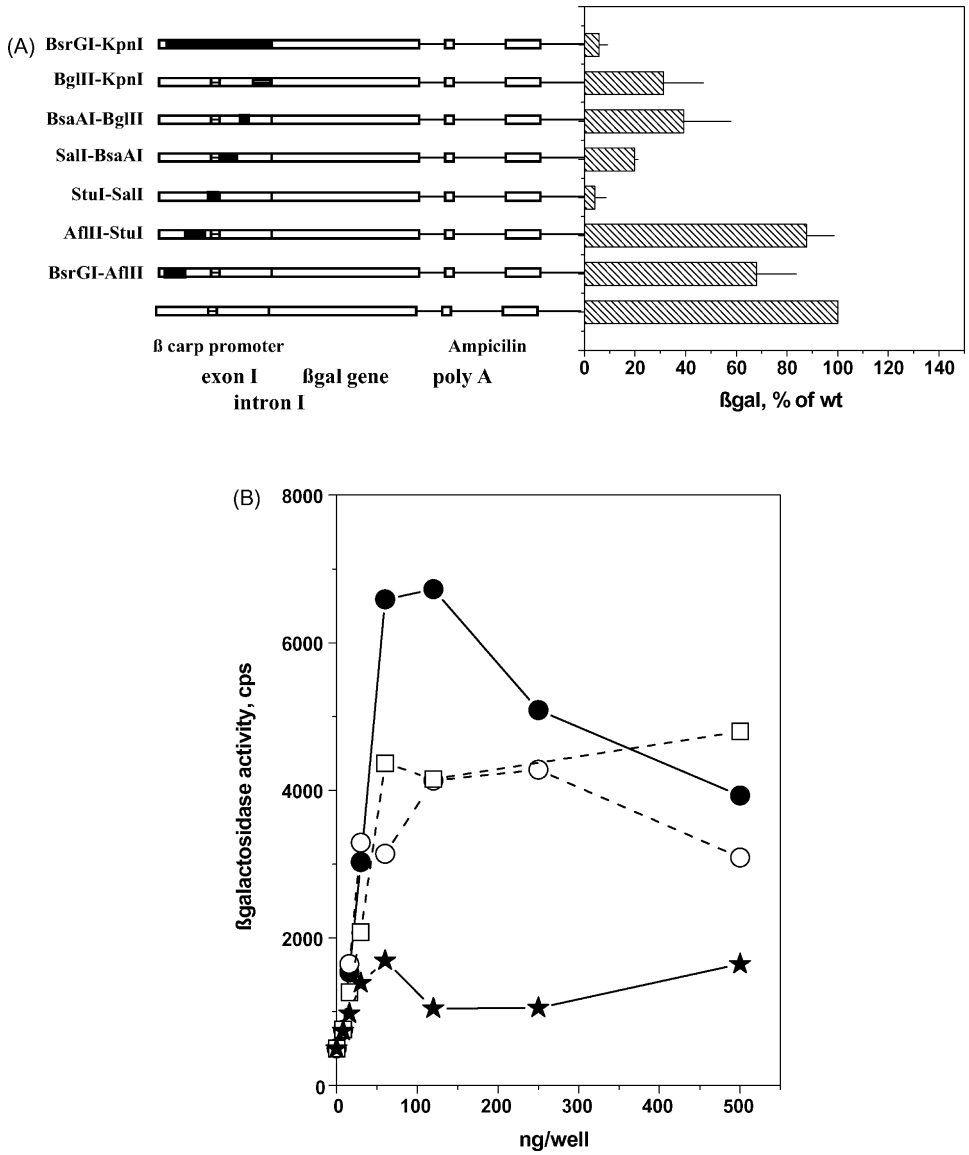

Fig. 4. Comparative expression in EPC cells transfected with different pAE6-gal: deletions (A) and versions (B). (A) The deletions were obtained in the pAE6-β-gal construct by using two consecutive unique restriction enzyme digestions, blunt ending and religation. The constructs were transfected into the EPC cells, next day the EPC cell monolayers were assayed for β-gal expression. Results were expressed by the formula, cps of deleted plasmids/cps of pAE6-β-gal ×100. The mean and standard deviations from two independent experiments was represented. Deletions are represented as black horizontal rectangles in between the two restriction enzymes (the plasmids have been represented as lineal for clarity). (B) EPC cells were transfected with several promoter versions of carp β-actin. ●, pAE6 (−1177 bp, intron 1 and exon 1). ○, 4.7 kbp carp βAP (−3413 bp, exon 1 and intron 1). □, 1.5 kbp carp βAP (−143 bp, exon 1 and intron 1). ⋆, 1.7 kbp tilapia βAP [30]. 1 day later, β-galactosidase expression was estimated by the TROPIX assay. One experiment of three is represented.

Elk-1 (T00250), C/EBPalpha (T00104), p300 ((T01427) and MCBF (T00499) were present in at least two of the higher fish promoters (AE6, LTRz, LTRw and Mx) (data not shown).

Fig. 5 shows an scheme with the relative positions of each of the La > 12 TFBS present in at least three of the higher promoters. The −170 to −300 bp region contains AP-1 (4 sites, three different promoters), AP-3 (3 sites, two different promoters) and POU1F1a (2 sites, two different fish promoters) while the −340 to −425 bp region contains POU1F1a (3 sites, three different fish promoters) and AP-1 (two different promoters).

The analysis of the TFBS data recovered in Excel files classified by their different La values showed that the MCV1.4 promoter has the highest density of TFBS (152.1 TFBS/1000 bp at La = 12) while the AE6, LTRz, LTRw, Mx promoters or the G gene had 3–4-fold lower TFBS densities (39.2, 33.4, 35.6, 65.2 or 32.2 TFBS/1000 bp at La = 12, respectively) (data not shown).

Because the CRE (inducible by cAMP) La < 12 TFBSs were abundant in the MCV1.4 and AE6 promoters (not shown), the pCRE-G construct, containing seven CRE TFBS sites, was also tested for *in vitro* VHSV G-protein expression. However, only a maximun of ∼10% of fluorescence EPC cells (not significatively different from the pMCV1.4) could be obtained after transfection of EPC cells with pCRE (data not shown). On the other hand, the CRE promoter did not shown any of the TFBS La > 12 common to the higher performing promoters (Table 2).

Comparative analysis of the La > 12 TFBS of AE6 and LTRz promoters (fish promoters inducing the highest fluorescent intensities per cell), showed that of the 25 TFBS present in LTRz, 20 were common to the AE6. Thus, AP-1, POU1F1a, B factor/TBP, HiNF-A, and AP-3 were common to both promoters with 2 sites for each (Table 2). Further common TFBS include core-BF (00000), CP-2 (T00152), Elk-1 (T00250), TBP (T00794) and

Table 2
Summary of the numbers of La > 12 TFBS appearing in three or more of the higher promoters: MCV1.4, AE6, LTRz, LTRw and Mx.

TRANSFAC accession number	TFBS name	Higher promoters:					Non-different promoters:					
		MCV1.4	Fish higher promoters				Fish non-different promoters					CRE
			AE6	LTR z	LTR w	Mx	iirL	iirR	Ple	itrL	FG2	
Total number	–	118	94	25	42	24	10	27	22	9	30	45
T00029	AP-1	5	3	2	1	2	1	3	1	0	1	0
T00691	POU1F1a	1	7	2	1	1	1	2	0	0	3	0
T01882	unc-86 (POU4F2)	1	3	0	2	0	0	0	1	0	0	0
T00627	NIT2	1	2	0	0	1	0	0	0	1	0	0
T02277	HNF-3	1	1	0	1	0	0	0	0	0	0	0
T00061	B factor/TBP	0	2	2	1	1	0	1	0	0	0	0
T00360	HiNF-A	0	3	2	1	1	0	0	2	0	0	0
T00039	AP-3(2)	0	3	2	1	0	0	0	0	0	0	0
T02878	TCF-4E	0	6	0	2	1	2	2	1	0	1	0
T04162	RUSH-1alpha	0	1	0	1	1	1	0	0	0	1	0

Total number and number of occurrences of La > 12 TFBS appearing in, at least, three different higher promoters (MCV1.4, AE6, LTRz, LTRw, Mx) and their corresponding number of occurrences in the non-different promoters (iirL, iirR, Ple, itrL, FG2, CRE).

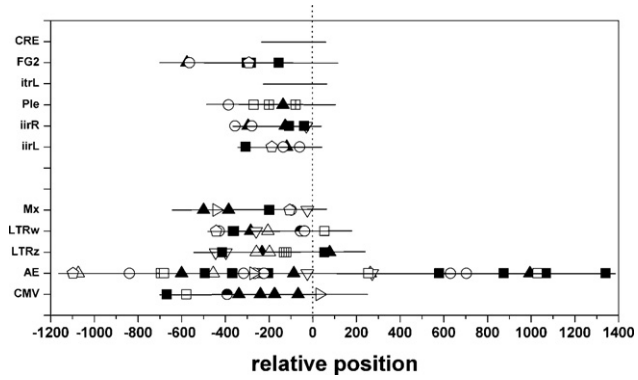

Fig. 5. Relative positions of the TFBS of MCV1.4, AE6, LTRz, LTRw, Mx and their presence in the non-different promoters. The sequences of the higher promoters (MCV1.4, AE6, LTRz, LTRw, Mx) were searched for the presence of TFBS by using the TESS software (http://www.cbil.upenn.edu/cgi-bin/tess/tess). The TFBS data corresponding to each promoter were recovered in Excel files, filtered by La > 12 and compared among the higher promoter sequences (MCV1.4, AE6, LTRz, LTRw and Mx). Only those TFBS present in at least three of the higher promoters were searched in the non-different promoters (iirL, iirR, Ple, itrL, FG2 and CRE) and represented in the figure. ▲, AP-1; △, AP-3(2); ▽, B factor/TBP; ▷, NIT2; ■, HiNF-A; ■, POU1F1a; □, unc-86; ◓, HNF-3; ○,TCF-4E. ⬠, RUSH-1a.

WT1l-K (T00900) with 1 site present in each promoter (data not shown).

Comparative analysis of the most active truncated MCV1.4 (−245 to +211) and AE6 (−191 to +1397, intron 1) sequences, showed that common La > 12 TFBS were: AP-1 (−239, −176, −174, −67 in MCV1.4 and 994 in AE6), Sp1 (−79 in MCV1.4 and 835 in AE6), NIT2 (31 in MCV1.4 and 616 in AE6) and TII (31 in MCV1.4 and 1299 in AE6).

The two truncated regions (−742 to −244 and −245 to +211) of MCV1.4 which showed each ∼50% of its activity, have similar TFBS densities (148.6 and 159.2 TFBS/1000 bp at La = 12, respectively), while the two truncated regions of AE6 which showed lower and higher activities (−1177 to −190 and −191 to +1397, respectively), have similar TFBS densities (43.6 and 36.5 TFBS/1000 bp at La = 12, respectively) (data not shown).

4. Discussion

The best promoters to express the VHSV G-protein in fish epithelial cells were selected among eleven promoters (10 of fish origin), by estimating both the number G-expressing cells and the average G-expression level per cell. The MCV1.4 (an IE-CMV version), AE6 (a carp β-actin version), LTRz, LTRw and Mx were the higher G-expressing promoters since they showed the highest percentages of cells expressing G-protein in their membranes compared to empty MCV1.4 and/or itr from *sleeping beauty* (*SB*) or iir from medaka Tol2 transposons, myosin, and/or pleurocidin promoters. Furthermore, AE6 and LTRz promoters reached a ∼3–5-fold higher average G-protein expression per cell than the rest of the tested promoters.

Comparative analysis of the potential TFBS common to the higher G-expressing promoters showed that while AP-1 and POU1F1a were found in most of the promoters assayed, they were ∼2-fold more abundant only in the higher promoters (Table 2 and Fig. 5). The AP-1 site was functional in LTRw [23], described as the predominant cis-acting regulatory element in the CMV promoter [53] and found in epithelial skin [54] and keratinocytes [55]. The POU1F1 site is a transcription factor known to be expressed in the pituitary gland to control expression of the growth hormone [56]. All POU (Pit-Oct-Unc) domain proteins belong to a subfamily of homeobox proteins that consist of a divergent POU domain and other unique domains characteristic of each member of the subfamily. In humans, AP-1 sites interact with major epithelial POU Oct1, Skn1a/I and Oct6 proteins [57], however no implications of POU1F1a or unc-86 (another POU member, POU4F2, found in 2 of the fish higher promoters) have been described yet in epithelial cells from any specie. The B factor/TBP and HiNF-A sites were also found in all the higher fish promoters and the AP-3(2), TCF-4E and RUSH-1alpha were found in, at least, 3 of the higher fish promoters, while they all were absent in the MCV1.4 promoter, suggesting those sites are fish epithelial-specific. Although the presence in the non-different promoters of binding sequences reducing rather than enhancing transcription cannot be ruled out with the present data, progress is being made about how to use the available information on *de novo* design of synthetic promoters.

A surprisingly high VHSV G-expression per cell, similar to that of AE6 (one of the versions of the carp β-actin promoter), was found when the G gene was placed under the control of the LTRz from the ZFERV endogenous zebrafish retrovirus [21]. According to all these *in vitro* data, the pAE6 and/or LTRz promoters might well be fish substitutes for the human IE-CMV (MCV1.4 version) promoter for *in vivo* fish vaccination. Furthermore, the smaller size of LTRz (749bp) compared to the AE6 (2577bp), makes the LTRz promoter easier to handle and with ∼3-fold more specific activity than AE6 and therefore a better potential candidate to be tested for salmonid DNA vaccines. However, since AE6 and LTRz have been both obtained from cyprinid fish species (carp and zebrafish, respectively) and assayed in a cyprinid cell line (EPC), it is possible that they would

not be so active when assayed *in vivo* in salmonid epithelia despite TFBS being tissue-specific rather than specie-specific. Successful epithelial vaccination of salmonids might best require homologous LTR retrovirus (not yet known) or β-actin promoters (not yet isolated). In addition, *in vivo* experimentation would be needed to further assess these alternatives. On the other hand, comparative analysis of TFBS between AE6 and LTRz, showed that of the 25 La > 12 TFBS reported by the TESS program for LTRz, 20 (80%) were common to both promoters. AP-1 (2 sites), POU1F1a (2 sites), B factor/TBP (2 sites), HiNF-A (2 sites), AP-3 (2 sites) and core-BF, CP-2, DI, WT1l-KTS and Elk-1 (1 site each) were also common. Elk-1 has been described as a basal epithelial-specific keratinocyte protein interacting with AP-1 [57,58].

To corroborate some of the above mentioned observations and to explore the possibilities to downsize the AE6 promoter (to facilitate construct manipulations and/or to increase its specific activity), partial deletions of the MCV1.4 and AE6 promoters were also obtained and studied by using constructs with gene reporters (easier for expression assays). Analysis of MCV1.4 deletions, showed that 50% of the MCV1.4 activity was dependent on the −245 to +211 region (most probably, on the −245 to 1 region, due to the apparent negative effect of the intron on fish cells). A similar region was shown to be rich in common AP-1 and POU1F1a TFBS sites by comparing all the higher promoters (Fig. 5). On the other hand, both the gene reporter results from AE6 deletion studies (Fig. 4A) and earlier carp β-actin constructs by others (Fig. 4B), showed that the exon 1-intron 1 of carp β-actin promoter from 1 to 1400 bp TSS downstream sequences was responsible for ∼70% of their activity in contrast to only 10–20% of the TSS upstream sequences. Downsizing the AE6 promoter, will probably require further studies including testing their intron 1 as a possible unique upstream sequence.

Despite the abundance of TFBS in both MCV1.4 and AE6 promoters (118 and 94 La > 12 TFBS, respectively), common TFBS were restricted to AP-1, Sp1, NIT2 and TII, thus showing that to express the VHSV G-protein many different combinations of TFBS are indeed possible.

Evidence has been gathered that suggest AE6 and/or LTRz promoters might be good substitutes for MCV1.4 (IE-CMV) since they offer a comparative number of cells expressing the VHSV G- protein and a higher expression per transfected cell *in vitro*. It remains to be studied whether any of the AE6 or LTRz promoters could be useful for fish DNA vaccines delivered throughout epithelial cells, since evidence for the specific TFBS functionality *in vivo* is out of the scope of the present paper. Thus, some of the future experiments, for instance by using only the higher promoters in an appropriated fish host (zebrafish), are now required. However, such an *in vivo* system should be first optimized (fish specie, route, dosages, etc.). Adverse immunologic reactions could conceivably result from the continuous expression of a species-foreign protein on epithelial surfaces. However, this is a common potential problem for any other DNA vaccine and has yet to be proven in every case as it was in fish intramuscularly injected with an anti-IHNV DNA vaccine [59].

Because transcription seems to be dependent on cooperative binding of a number of tissue-specific TFs to a basal promoter, previous approaches to design new promoters have been to combine DNA sequence modules recognized by individual TF by taking into account their relative locations to TSS [35,60,61]. Similar procedures could be used to design *de novo* promoters for fish vaccination purposes taking into account the data reported here. On the other hand, a 3–4-fold higher TFBS density was shown in the MCV1.4 promoter than in the rest of the promoters tested (number of TESS-identified TFBSs: ∼152.1 TFBS per 1000 bp), something that might be related to its wide tissue-specificity and/or high activity. Although TFBS density alone does not differentiate activity of promoters, as it was concluded earlier [62], it might be a complementary criteria to define new synthetic promoters. Further analysis and new combinations of the potential TFBS identified here for the higher performing fish epithelial promoters might be useful to design new improved synthetic promoters for fish DNA vaccination by immersion.

Acknowledgements

The technical collaboration of L. Alte is greatly appreciated. Thanks are due to Dr. P. Hackett (University of Minnesota) for the generous gift of pT2/HB and pT2/SVneo, to Dr. Michel Bremont (INRA, France) for the C10 and I16 MAbs and to Dr. Niels Lorenzen (Veterinær Instituttet, Aarhus, Denmark) for the 3F1A12 MAb, to Dr. McLean (Univ. Southampton, England) for the 4.7CBAβ and 1.5CBAβ from carp and 1.7 TBAβ from tilapia β-actin promoters and to Dr. Goldspink (University of London, England) for the pFG2 construct. This work was supported by Spanish CICYT projects AGL05-00339-ACU, AGL08-03519-CO4 and CONSOLIDER INGENIO 2010 2007-00002.

References

[1] Salonius K, Simard N, Harland R, Ulmer JB. The road to licensure of a DNA vaccine. Current Opinion in Investigational Drugs 2007;8:635–41.
[2] Anderson ED, Mourich DV, Leong JC. Gene expression in rainbow trout (*Onchorynchus mykiss*) following intramuscular injection of DNA. Molecular Marine Biology and Biotechnology 1996;5:105–13.
[3] Lorenzen N, LaPatra SE. DNA vaccines for aquacultured fish. Review in Science Technology Office International Epizooties 2005;24:201–13.
[4] Tafalla C, Estepa A, Coll JM. New vaccination strategies for the improvement of DNA vaccines against virus in fish. In: Schwartz SH, editor. Aquaculture research trends. Nova Publishers; 2008. p. 213–35 [chapter 6].
[5] Helmick CM, Bailey JF, LaPatra S, Ristow S. The esophagus/cardiac stomach region: site of attachment and internalization of infectious hematopoietic necrosis virus in challenged juvenile rainbow trout *Onchorynchus mykiss* and coho salmon *O. kisutch*. Diseases Aquatic Organisms 1995;23:189–99.
[6] Harmache A, Leberre M, Droineau S, Giovannini M, Bremont M. Bioluminescence imaging of live infected Salmonids reveals that the fin bases are the major portal of entry for Novirhabdovirus. Journal of Virology 2006;103:3655–9.
[7] Bernard D, Six A, Rigottier-Gois L, Messiaen S, Chilmonczyk S, Quillet E, et al. Phenotypic and functional similarity of gut intraepithelial and systemic T cells in a teleost fish. Journal of Immunology 2006;176(7):3942–9.
[8] Cain KD, LaPatra SE, Baldwin TJ, Shewmaker B, Jones J, Ristow S. Characterization of mucosal immunity in rainbow trout *Oncorhynchuss mykiss* challenged with infectious hamatopoietic necrosis virus: identification of antiviral activity. Diseases Aquatic Organisms 1996;27:161–72.
[9] Fernandez-Alonso M, Rocha A, Coll JM. DNA vaccination by immersion and ultrasound to trout viral haemorrhagic septicaemia virus. Vaccine 2001;19:3067–75.
[10] Navot N, Kimmel E, Avtalion RR. Immunization of fish by bath immersion using ultrasound. Developmental Biology (Basel) 2005;121:135–42.
[11] Bearzotti M, Perrot E, Michard-Vanhee C, Jolivet G, Attal J, Theron MC, et al. Gene expression following transfection of fish cells. Journal Biotechnology 1992;26:315–25.
[12] Moav B, Liu Z, Groll Y, Hackett PB. Selection of promoters for gene transfer into fish. Molecular Marine Biology Biotechnology 1992;1:338–45.
[13] Lopez A, Fernandez-Alonso M, Rocha A, Estepa A, Coll JM. Transfection of epithelioma cyprini (EPC) carp cells. Biotechnology Letters 2001;23:481–7.
[14] Rocha A, Ruiz S, Coll JM. Improvement of transfection efficiency of epithelioma papulosum cyprini carp cells by modification of their cell cycle and using an optimal promoter. Marine Biotechnology 2005;6:401–10.
[15] Lorenzen E, Einer-Jensen K, Martinussen T, LaPatra SE, Lorenzen N. DNA vaccination of rainbow trout against viral hemorrhagic septicemia virus: a dose–response and time–course study. Journal of Aquatic Animal Health 2000;12(3):167–80.
[16] McLauchlan PE, Collet B, Ingerslev E, Secombes CJ, Lorenzen N, Ellis AE. DNA vaccination against viral haemorrhagic septicaemia (VHS) in rainbow trout: size, dose, route of injection and duration of protection-early protection correlates with Mx expression. Fish Shellfish Immunology 2003;15(1):39–50.
[17] Ivics Z, Izsvak Z, Hackett PB. Molecular reconstruction of sleeping beauty, a Tc1-like transposon system from fish and its transposition in human cells. Cell 1997;91:501–10.
[18] Izsvak Z, Ivics Z, Plasterk RH. Sleeping beauty, a wide host-range transposon vector for genetic transformation in vertebrates. Journal Molecular Biology 2000;302:93–102.
[19] Koga A, Suzuki M, Inagaki H, Bessho Y, Hori H. Transposable element in fish. Nature 1996;383:30.

[20] Koga A. Trnasposition mechanisms and biotechnology applications of the medaka fish Tol2 transposable element. Advances Biophysics 2004;38:161–80.
[21] Shen CH, Steiner LA. Genome structure and thymic expression of an endogenous retrovirus in zebrafish. Journal of Virology 2004;78:899–911.
[22] Holzschu DL, Fodor SK, Quackenbush SL, Earnest-Koons K, Bowser PR, Vogt VM, et al. Molecular characterization of a piscine retrovirus, walleye dermal sarcoma virus. Leukemia 1997;11(Suppl. 3):172–5.
[23] Hronek BW, Meagher A, Rovnak J, Quackenbush SL. Identification and characterization of cis-acting elements residing in the Walleye dermal sarcoma virus promoter. Journal of Virology 2004;78:7590–601.
[24] Quackenbush SL, Holzschu DL, Bowser PR, Casey JW. Transcriptional analysis of walleye dermal sarcoma virus (WDSV). Virology 1997;237(1):107–12.
[25] Cole AM, Darouiche RO, Legarda D, Connell N, Diamond G. Characterization of a fish antimicrobial peptide: gene expression, subcellular localization, and spectrum of activity. Antimicrobial Agents and Chemotherapy 2000;66:2039–45.
[26] Collet B, Secombes CJ. The rainbow trout (*Oncorhynchus mykiss*) Mx1 promoter. Structural and functional characterization. European Journal of Biochemistry 2001;268(6):1577–84.
[27] Gauvry L, Ennion S, Hansen E, Butterworth P, Goldspink G. The characterisation of the 5′ regulatory region of a temperature-induced myosin-heavy-chain gene associated with myotomal muscle growth in the carp. European Journal of Biochemistry 1996;236(3):887–94.
[28] Gauvry L, Fauconneau B. Cloning of a trout fast skeletal myosin heavy chain expressed both in embryo and adult muscles and in myotubes neoformed *in vitro*. Computational Biochemistry and Physiology B: Biochemistry and Molecular Biology 1996;115(2):183–90.
[29] Cheng C, Lu KL, Lau EL, Yang TY, Lee CY, Wu JL, et al. Growth promotion in ayu (*Plecoglossus altivelis*) by gene transfer of the rainbow trout growth hormone gene. Zoological Studies 2002;41:303–10.
[30] Hwang GL, Rahman A, Razak SA, Sohm F, Farahmand H, Smith A, et al. Isolation and characterisation of tilapia β-actin promoter comparison of its activity with carp β-actin promoter. Biochimica et Biophysica Acta 2003;1625:11–8.
[31] Hackett PB, Alvarez MC. The molecular genetics of transgenic fish. In: Fingerman M, Nagabhushanam R. editors. Recent advances in marine biotechnology. Aquaculture. part B. Fishes 2000;4:77–145.
[32] Brocal I, Falco A, Mas V, Rocha A, Perez L, Coll JM, et al. Stable expression of bioactive recombinant pleurocidin in a fish cell line. Applied Genetics and Molecular Biotechnology 2006;72:1217–28.
[33] Falco A, Brocal I, Perez L, Coll JM, Estepa A, Tafalla C. In vivo modulation of the rainbow trout (*Oncorhynchus mykiss*) immune response by the human alpha defensin 1, HNP1. Fish Shellfish Immunology 2008;24(1):102–12.
[34] Dai C, McAninch RE, Sutton RE. Identification of synthetic endothelial cell-specific promoters by use of a high-throughput screen. Journal of Virology 2004;78:6209–21.
[35] Wray GA, Hahn MW, Abouheif E, Balhoff JP, Pizer M, Rockman MV, et al. The evolution of transcriptional regulation in eukaryotes. Molecular Biology and Evolution 2003;20(9):1377–419.
[36] Cox RS, Surette MG, Elowitz MB. Programming gene expression with combinatorial promoters. Molecular Systems Biology 2007;3:145–56.
[37] Edelman GM, Meech R, Owens GC, Jones FS. Synthetic promoter elements obtained by nucleotide sequence variation and selection for activity. Proceedings of the National Academic Science USA 2000;97(7):3038–43.
[38] LeBerre M, De Kinkelin P, Metzger A. Identification sérologique des rhabdovirus des salmonidés. Bulletin Office International Epizooties 1977;87:391–3.
[39] Rocha A, Ruiz S, Tafalla C, Coll JM. Conformation and fusion defective mutants in the hypothetical phospholipid-binding and fusion peptides of the protein G of viral haemorrhagic septicemia salmonid rhabdovirus. Journal of Virology 2004;78:9115–22.
[40] Rocha A, Ruiz S, Coll JM. Improvement of transfection efficiency of epithelioma papulosum cyprini carp cells by modification of their cell cycle and using an optimal promoter. Marine Biotechnology 2004;6:401–10.
[41] Tsutsumi M, Koga A, Hori H. Long and short mRNAs transcribed from the medaka fish transposon Tol2 respectively exert positive and negative effects on excision. Genetical Research Cambridge 2003;82:33–40.
[42] Cui Z, Geurts AM, Liu G, Kaufman D, Hackett PB. Structure–function analysis of the inverted terminal repeats of the sleeping beauty transposon. Journal of Molecular Biology 2002;318:1221–35.
[43] Sanchez-Puig JM, Blasco R. Puromycin resistance (pac) gene as a selectable marker in vaccinia virus. Gene 2000;257:57–65.
[44] Fijan N, Sulimanovic D, Bearzotti M, Muzinic D, Zwillenberg LOZ, Chilmonczyk S, et al. Some properties of the epithelioma papulosum cyprini (EPC) cell line from carp *Cyprinus carpio*. Annals Virology (Institute Pasteur) 1983;134:207–20.
[45] Bearzotti M, Monnier AF, Vende P, Grosclaude J, DeKinkelin P, Benmansour A. The glycoprotein of viral hemorrhagic septicemia virus (VHSV): antigenicity and role in virulence. Veterinary Research 1995;26:413–22.
[46] Gaudin Y, DeKinkelin P, Benmansour A. Mutations in the glycoprotein of viral haemorrhagic septicaemia virus that affect virulence for fish and the pH threshold for membrane fusion. Journal General Virology 1999;80:1221–9.
[47] Estepa A, Coll JM. Pepscan mapping and fusion related properties of the major phosphatidylserine-binding domain of the glycoprotein of viral hemorrhagic septicemia virus, a salmonid rhabdovirus. Virology 1996;216:60–70.
[48] Lorenzo GA, Estepa A, Chilmonczyk S, Coll JM. Mapping of the G-regions and N-regions of viral hemorrhagic septicemia virus (Vhsv) inducing lymphoproliferation by pepscan. Veterinary Research 1995;26(5–6):521–5.
[49] Lee DH, Gershenzon N, Gupta M, Ioshikhes IP, Reinberg D, Lewis BA. Functional characterization of core promoter elements: the downstream core element is recognized by TAF1. Molecular in Cell Biology 2005;25:9674–86.
[50] Moldt B, Yant SR, Andersen PR, Kay MA, Mikkelsen JG. Cis-acting gene regulatory activities in the terminal regions of sleeping beauty DNA transposon-based vectors. Human Gene Therapy 2007;18:1193–204.
[51] Sawicki JA, Morris RJ, Monks B, Sakai K, Miyazaki JI. A composite CMV-IE enhancer/β-actin promoter is ubiquitously expressed in mouse cutaneous epithelium. Experimental Cell Research 1998;244:367–9.
[52] Garg S, Oran AE, Hon H, Jacob J. The hybrid cytomegalovirus enhanver/chicken β-actin promoter along with Woodchuck hepatitis virus posttranscriptional regulatory element enhances the protective efficacy of DNA vaccines. Journal of Immunology 2004;173:550–8.
[53] Wade EJ, Klucher KM, Spector DH. An Ap-1 binding-site is the predominant cis-acting regulatory element in the 1.2-kilobase early RNA promoter of human cytomegalovirus. Journal of Virology 1992;66:2407–17.
[54] Angel P, Szabowski A. Function of AP-1 target genes in mesenchymal–epithelial cross-talk in skin. Biochemical Pharmacology 2002;64:949–56.
[55] Rutberg SE, Adams TL, Glick A, Bonovich MT, Vinson C, Yuspa SH. Activator protein 1 transcription factors are fundamental to v-ras(Ha)-induced changes in gene expression in neoplastic keratinocytes. Cancer Research 2000;60:6332–8.
[56] Nagarajan P, Romano RA, Sinha S. Transcriptional control of the differentiation program of interfollicular epidermal keratinocytes. Critical Review in Eukarotic Gene Expression 2008;18:57–79.
[57] Jang SI, Karaman-Jurukovska N, Morasso MI, Steinert PM, Markova NG. Complex interactions between epidermal POU domain and activator protein 1 transcription factors regulate the expression of the profilaggrin gene in normal human epidermal keratinocytes. Journal of Biological Chemistry 2000;275:15295–304.
[58] Andreoli JM, Jang SI, Chung E, Coticchia CM, Steinert PM, Markova NG. The expression of a novel, epithelium-specific and transcription factor is restricted to the most differentiated layers in the epidermis. Nucleic Acids Research 1997;25:4287–95.
[59] Kurath G, Garver KA, Corbeil S, Elliott DG, Anderson ED, LaPatra SE. Protective immunity and lack of histopathological damage two years after DNA vaccination against infectious hematopoietic necrosis virus in trout. Vaccine 2006;24(3):345–54.
[60] Fickett JW, Hatzigeorgiou AG. Eukaryotic promoter recognition. Genome Research 1997;7(9):861–78.
[61] Papadakis ED, Nicklin SA, Baker AH, White SJ. Promoters and control elements: designing expression cassettes for gene therapy. Currents in Gene Therapy 2004;4(1):89–113.
[62] Prestridge DS, Burks C. The density of transcriptional elements in promoter and non-promoter sequences. Human Molecular Genetics 1993;2:1449–53.
[63] Meier LJ, Stinski MF. Regulation of human cytomegalovirus immediate-early gene expression. Intervirology Today 1996;39:331–42.
[64] Zhang Z, Kim E, Martineau D. Functional characterization of a piscine retroviral promoter. Journal of General Virology 1999;80:3065–72.
[65] Liu Z, Moav B, Faras A, Guise K, Kapuscinski AR, Hackett PB. Functional analysis of elements affecting expression of the β-actin gene of carp. Molecular Cellular Biology 1990;10:3432–40.