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T-box transcription factor eomesodermin/Tbr2 in Atlantic cod (*Gadus morhua* L.): Molecular characterization, promoter structure and function analysis

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ABSTRACT

Eomesodermin (Eomes) is a member of T-box transcription factor family and plays an important role in the regulation of a wide variety of developmental processes and immune response in animals. Here we report cloning and characterization of the full-length cDNA of Atlantic cod Eomes (GmEomes), which possesses a TBOX_3 domain similar to its counterpart in mammals. The regulated expression was observed in head kidney and spleen in response to live *Vibrio anguillarum* infection *in vivo*, and spleen leukocytes *in vitro* after PMA and poly I:C stimulation. Furthermore, we determined a 694 bp sequence, upstream of the transcriptional start site (TSS), to contain a number of sequence motifs that matched known transcription factor-binding sites. Activities of the presumptive regulatory gene were assessed by transfecting different 5′-deletion constructs in CHSE-214 cells. The results showed that the basal promoters and positive transcriptional regulator activities of GmEomes were dependent by sequences located from −694 to −376 bp upstream of TSS. Furthermore, we found that some Eomes binding sites were present in the 5′-flanking regions of the cod IFNγ gene predicted by bioinformatics. However, Co-transfection of eomesodermin overexpression plasmids with INFγ reporter vector into CHSE-214 cells determined that Atlantic cod eomesodermin played a minor role in activation of the INFγ promoter.

1. Introduction

T-box genes, a highly conserved family of transcription factors having a common DNA-binding sequence (the T-box), are important in the regulation of a wide variety of developmental processes and immune response in animals [1–4]. Eomesodermin (Eomes) and T-BRAIN1 together with T-box expressed in T-cells (T-bet/TBX21) all belong to TBR1 (T-box brain protein 1) sub-family of T-box genes in mammals [5–8]. Eomes has been shown to be involved in mesoderm formation in most vertebrates and in trophoblast differentiation in mammals [6,8–11]. Important roles of Eomes during early development have also been reported in fish [12–15]. In the mammalian immune system, Eomes is highly expressed in $CD8^+$ T cells and functions redundantly with T-bet in the induction of IFN-γ production to prevent $CD8^+$ T cells from differentiating into other T cell subtypes [16–18].

Atlantic cod (*Gadus morhua* L.) is an economically and ecologically important species in the northern Atlantic [19]. Recently, genomic analysis of Atlantic cod reveals an immune system that differs significantly from that in other vertebrates and most fish species. The major histocompatibility complex (MHC) II, CD4 and invariant chain (Ii) have been lost during evolution. However, there is an expanded number of MHCI genes and a unique composition of the toll-like receptor family. These compensatory changes in both adaptive and innate immunity suggest that cod may not be more susceptible to infectious disease than most other vertebrates [20], indicating a high relevance to study the importance of innate immunity and mechanisms leading to T-cell dependent cytotoxic responses. A comprehensive understanding of the molecular pathways involved in physiological and immunological responses of Atlantic cod may help to overcome the challenges in health management (e.g. viral diseases) [20,21]. While MHCI, present in all nucleated cells, has long been thought to be exclusively involved in the presentation of endogenous antigens, this classical view has been

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Table 1
List of primers and their designated applications.

Oligo Name	Sequence 5′ to 3′	Use
GmEomefw1	ccccggctgcacatcgtggaggttaccga	3′-RACE
GmEomefw2	cgtggaggttaccgaggagggcgtggagg	3′-RACE
GmEomefw3	agcaacgaggcgcgagacacagaccttca	3′-RACE
GmRomerv1	tttcgcgaaggggttgtggtctatcttcag	5′-RACE
GmRomerv2	tggtaggcggtcacggcgatgaactggtt	5′-RACE
GmRomerv3	aggtctgtgtctcgcgcctcgttgctcat	5′-RACE
AP1	gtaatacgactcactatagggc	Genome walking
AP2	actatagggcacgcgtggt	Genome walking
GmEomes gwrv1	atactcgatctgtgaggagccggggctgt	Genome walking
GmEomes gwrv2	ttgcgctgtcggacgacgagaggttataaa	Genome walking
GmEomesEcoRIfw1	gcttcgaattcaaattgtgaactaaatgccc	Promoter cloning
GmEomesEcoRIfw2	gcttcgaattctcaatcttccgagaatcctattta	Promoter cloning
GmEomesEcoRIfw3	gcttcgaattcctatgtcgataaggcaagtgcattt	Promoter cloning
GmEomesSacIIrv	taagcccgcggatgtgcaactccgattatgatct	Promoter cloning
GmEomesRTfw1	cgacatggccaacccgtgct	Real-time PCR
GmEomesRTrv1	ccgatgctggatcccgtcgc	Real-time PCR
GmIFNγ F	tggtctgcatgtcagtttgtctg	Real-time PCR
GmIFNγ R	ttctgtggatgttgttggctaaga	Real-time PCR
GmEF1α F	atgtgagcggtgtggcaatc	Real-time PCR
GmEF1α R	tcatcatcctgaaccaccctg	Real-time PCR
GmEomesORFfw	cagatctcgagatgcagttggagaacatccttcct	Plasmid construction
GmEomesORFrv	ttgagctcgagggggctcgtgtagaacgcatagta	Plasmid construction
GmIFNγpromfw	cagatctcgagtctgccttcctagtaagtgaggctgc	Plasmid construction
GmIFNγpromrv	ttgagctcgagccctgcagctgtacacgctgaaagtcgc	Plasmid construction

progressively replaced by a more complete understanding of the cell biology of antigen-presenting cells. Indeed, exogenous antigens can also be processed by the proteasome and loaded on MHCI molecules by an alternative pathway called cross-presentation, which ultimately activates CD8$^+$ T-cells [22]. The presence of such a high number of MHCI loci in cod has therefore led to the hypothesis that different subsets of CD8$^+$ T cells have been generated to compensate the absence of CD4$^+$ T-cells [23].

Eomes is an important transcription factor during the immune response of CD8$^+$ T-cells, and has been identified in some teleost species such as Atlantic salmon, rainbow trout, ginbuna crucian carp and zebrafish [24–26], while studies of this transcription factor in Atlantic cod still are lacking. The main objective of this study was to characterise the expression pattern of Eomes in healthy and *V. anguillarum* infected cod, the promoter structure analysis and ability of GmEomes to activate the expression of IFN-γ.

2. Materials and methods

2.1. Cloning and sequencing of GmEomes cDNA

A partial cod EST sequence similar to vertebrate Eomes was identified based on nucleotide and amino acid sequence homology to annotated zebrafish and Atlantic salmon T-bet and Eomes sequences deposited in GenBank using the BLAST software (http://www.ncbi.nlm.nih.gov/BLAST). Two Atlantic cod Eomes ESTs (GenBank accession no: ES786771.1 and ES786391.1) were retrieved. Internal primers were designed from the cod EST sequences and PCR products were obtained from the cDNA library of the stimulated spleen tissue and sequenced. Total RNA (1 μg) isolated from Atlantic cod spleen (~30 mg), using TRIZOL® Reagent (Invitrogen), was used as a template and reverse transcribed to cDNA for RACE (rapid amplification of cDNA ends) with a SMART RACE cDNA Amplification kit (Clontech) according to the manufacturer's instruction and as described previously [27]. The cDNA sequence and deduced amino acid sequence of Atlantic cod Eomes sequences were further analyzed using BLAST and the ExPASy Molecular Biology server (http://us.expasy.org) and Pfamp [28]. Amino acid identity and similarity analysis were done with the Matrix Global Alignment Tool (MatGAT) program v 2.0 using default parameters [29].

2.2. Phylogenetic analysis

Multiple sequence alignment was created using CLUSTALW, while MEGA version 4.1 was used to assess the similarities among the aligned sequences (www.ebi.ac.uk/clustalw/) [30]. A phylogenetic tree, based on the deduced amino acid sequences, was constructed using the neighbor-joining (NJ) algorithm, and the reliability of the branching was tested using bootstrap re-samplings with 1000 pseudo-replicates.

2.3. Isolation of 5′-flanking region of the GmEomes gene by genome walking

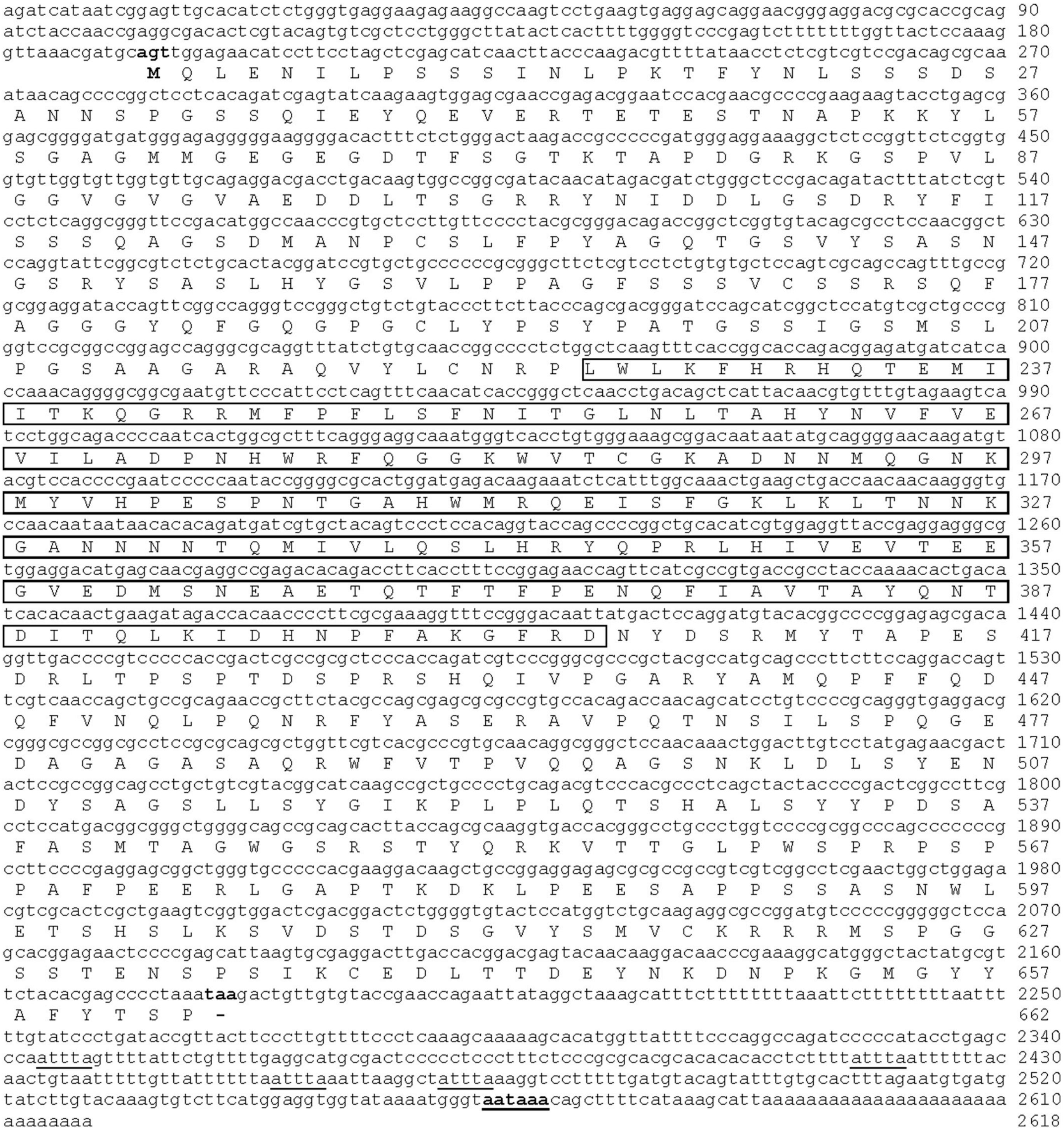
The 5′-flanking region of the GmEomes gene was isolated using the Universal GenomeWalker Kit (Clontech). Four GenomeWalker libraries were constructed according to the manufacturer's instruction. For each genome walker experiment, two adjacent reverse primers (GmEomes gwrv1 and GmEomes gwrv2) were designed near 5′-UTR region of the target gene (Table 1), and used in two PCRs in combination with the forward adaptor primers AP1 and AP2 (Clontech) for each library. The resulting PCR products from four different libraries were cloned in TOPO vector (Invitrogen), sequenced and analyzed as described above.

In order to verify this new sequence, a forward primer (GmEomesEcoRIfw1) was designed within this new sequence and used with a reverse primer (GmEomesSacIIrv) designed within the transcribed region of the Eomes gene (Table 1). PCR from the Atlantic cod genomic DNA was performed, and the products obtained were cloned and sequenced. Identification of transcription factor binding motifs was predicted with TRANSFAC® (Biobase International) and MatInspector version 6.2 [31,32].

2.4. Construction of GmEomes reporter gene plasmids

Deletion constructs with successive removal of the 5′-region were generated by PCR using the forward primers GmEomesEcoRIfw1, GmEomesEcoRIfw2, and GmEomesEcoRIfw3 having recognition sequences for restriction endonuclease EcoRI, while the reverse primer GmEomesSacIIrv (Table 1) had a SacII restriction site to generate the constructs p(-694/+23)Luc, p(-376/+23)Luc, and p(−216/+23)Luc respectively. The promoterless pMet Luciferase Reporter (Clontech) was used as reporter plasmids for cloning.

Both the PCR products of different 5′-deletion constructs and the



```
agatcataatcggagttgcacatctctgggtgaggaagagaaggccaagtcctgaagtgaggagcaggaacgggaggacgcgcaccgcag 90
atctaccaaccgaggcgacactcgtacagtgtcgctcctgggcttatactcacttttggggtcccgagtctttttttggttactccaaag 180
gttaaacgatgcagttggagaacatccttcctagctcgagcatcaacttacccaagacgttttataacctctcgtcgtccgacagcgcaa 270
            M  Q  L  E  N  I  L  P  S  S  S  I  N  L  P  K  T  F  Y  N  L  S  S  S  D  S   27
ataacagccccggctcctcacagatcgagtatcaagaagtggagcgaaccgagacggaatccacgaacgccccgaagaagtacctgagcg 360
 A  N  N  S  P  G  S  S  Q  I  E  Y  Q  E  V  E  R  T  E  T  E  S  T  N  A  P  K  K  Y  L  57
gagcggggatgatgggagagggggaaggggacactttctctgggactaagaccgcccccgatgggaggaaaggctctccggttctcggtg 450
 S  G  A  G  M  M  G  E  G  E  G  D  T  F  S  G  T  K  T  A  P  D  G  R  K  G  S  P  V  L  87
gtgttggtgttggtgttgcagaggacgacctgacaagtggccggcgatacaacatagacgatctgggctccgacagatactttatctcgt 540
 G  G  V  G  V  G  V  A  E  D  D  L  T  S  G  R  R  Y  N  I  D  D  L  G  S  D  R  Y  F  I  117
cctctcaggcgggttccgacatggccaacccgtgctccttgttcccctacgcgggacagaccggctcggtgtacagcgcctccaacggct 630
 S  S  S  Q  A  G  S  D  M  A  N  P  C  S  L  F  P  Y  A  G  Q  T  G  S  V  Y  S  A  S  N  147
ccaggtattcggcgtctctgcactacggatccgtgctgccccccgcgggcttctcgtcctctgtgtgctccagtcgcagccagtttgccg 720
 G  S  R  Y  S  A  S  L  H  Y  G  S  V  L  P  P  A  G  F  S  S  S  V  C  S  S  R  S  Q  F  177
gcggaggataccagttcggccagggtccgggctgtctgtacccttcttacccagcgacgggatccagcatcggctccatgtcgctgcccg 810
 A  G  G  G  Y  Q  F  G  Q  G  P  G  C  L  Y  P  S  Y  P  A  T  G  S  S  I  G  S  M  S  L  207
ggtccgcggccggagccagggcgcaggtttatctgtgcaaccggcccctctggctcaagtttcaccggcaccagacggagatgatcatca 900
 P  G  S  A  A  G  A  R  A  Q  V  Y  L  C  N  R  P  L  W  L  K  F  H  R  H  Q  T  E  M  I  237
ccaaacaggggcggcgaatgttcccattcctcagtttcaacatcaccgggctcaacctgacagctcattacaacgtgtttgtagaagtca 990
 I  T  K  Q  G  R  R  M  F  P  F  L  S  F  N  I  T  G  L  N  L  T  A  H  Y  N  V  F  V  E  267
tcctggcagaccccaatcactggcgctttcagggaggcaaatgggtcacctgtgggaaagcggacaataatatgcaggggaacaagatgt 1080
 V  I  L  A  D  P  N  H  W  R  F  Q  G  G  K  W  V  T  C  G  K  A  D  N  N  M  Q  G  N  K  297
acgtccaccccgaatcccccaataccggggcgcactggatgagacaagaaatctcatttggcaaactgaagctgaccaacaacaagggtg 1170
 M  Y  V  H  P  E  S  P  N  T  G  A  H  W  M  R  Q  E  I  S  F  G  K  L  K  L  T  N  N  K  327
ccaacaataataacacacagatgatcgtgctacagtccctccacaggtaccagccccggctgcacatcgtggaggttaccgaggagggcg 1260
 G  A  N  N  N  N  T  Q  M  I  V  L  Q  S  L  H  R  Y  Q  P  R  L  H  I  V  E  V  T  E  E  357
tggaggacatgagcaacgaggccgagacacagaccttcacctttccggagaaccagttcatcgccgtgaccgcctaccaaaacactgaca 1350
 G  V  E  D  M  S  N  E  A  E  T  Q  T  F  T  F  P  E  N  Q  F  I  A  V  T  A  Y  Q  N  T  387
tcacacaactgaagatagaccacaaccccttcgcgaaaggtttccgggacaattatgactccaggatgtacacggccccggagagcgaca 1440
 D  I  T  Q  L  K  I  D  H  N  P  F  A  K  G  F  R  D  N  Y  D  S  R  M  Y  T  A  P  E  S  417
ggttgaccccgtcccccaccgactcgccgcgctcccaccagatcgtcccgggcgcccgctacgccatgcagcccttcttccaggaccagt 1530
 D  R  L  T  P  S  P  T  D  S  P  R  S  H  Q  I  V  P  G  A  R  Y  A  M  Q  P  F  F  Q  D  447
tcgtcaaccagctgccgcagaaccgcttctacgccagcgagcgcgccgtgccacagaccaacagcatcctgtccccgcagggtgaggacg 1620
 Q  F  V  N  Q  L  P  Q  N  R  F  Y  A  S  E  R  A  V  P  Q  T  N  S  I  L  S  P  Q  G  E  477
cgggcgccggcgcctccgcgcagcgctggttcgtcacgcccgtgcaacaggcgggctccaacaaactggacttgtcctatgagaacgact 1710
 D  A  G  A  G  A  S  A  Q  R  W  F  V  T  P  V  Q  Q  A  G  S  N  K  L  D  L  S  Y  E  N  507
actccgccggcagcctgctgtcgtacggcatcaagccgctgcccctgcagacgtcccacgccctcagctactaccccgactcggccttcg 1800
 D  Y  S  A  G  S  L  L  S  Y  G  I  K  P  L  P  L  Q  T  S  H  A  L  S  Y  Y  P  D  S  A  537
cctccatgacggcgggctggggcagccgcagcacttaccagcgcaaggtgaccacgggcctgccctggtccccgcggcccagcccccccg 1890
 F  A  S  M  T  A  G  W  G  S  R  S  T  Y  Q  R  K  V  T  T  G  L  P  W  S  P  R  P  S  P  567
ccttccccgaggagcggctgggtgcccccacgaaggacaagctgccggaggagagcgcgccgccgtcgtcggcctcgaactggctggaga 1980
 P  A  F  P  E  E  R  L  G  A  P  T  K  D  K  L  P  E  E  S  A  P  P  S  S  A  S  N  W  L  597
cgtcgcactcgctgaagtcggtggactcgacggactctggggtgtactccatggtctgcaagaggcgccggatgtcccccggggctcca 2070
 E  T  S  H  S  L  K  S  V  D  S  T  D  S  G  V  Y  S  M  V  C  K  R  R  R  M  S  P  G  G  627
gcacggagaactccccgagcattaagtgcgaggacttgaccacggacgagtacaacaaggacaacccgaaaggcatgggctactatgcgt 2160
 S  S  T  E  N  S  P  S  I  K  C  E  D  L  T  T  D  E  Y  N  K  D  N  P  K  G  M  G  Y  Y  657
tctacacgagcccctaaataagactgttgtgtaccgaaccagaattataggctaaagcatttctttttttaaattctttttttttaattt 2250
 A  F  Y  T  S  P  -                                                                         662
ttgtatccctgataccgttacttcccttgttttccctcaaagcaaaaagcacatggttattttcccaggccagatcccccatacctgagc 2340
ccaatttagttttattctgttttgaggcatgcgactccccctccctttctcccgcgcacgcacacacacctcttttatttaatttttac 2430
aactgtaattttgttatttttaatttaaattaaggctatttaaaggtcctttttgatgtacagtatttgtgcactttagaatgtgatg 2520
tatcttgtacaaagtgtcttcatggaggtggtataaaatgggtaataaacagcttttcataaagcattaaaaaaaaaaaaaaaaaaa 2610
aaaaaaaa                                                                                  2618
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Fig. 1. Nucleotide and deduced amino acid sequences of GmEomes cDNA. Start and stop codons are in bold. The TBOX_3 domain is bordered. The RNA instability motifs (attta) are marked with underlines. The putative polyadenylation (aataaa) signal is in bold and underlined.

basic reporter vectors were digested with their respective restriction enzymes (New England) and ligated (T4 DNA ligase) to generate the above-mentioned constructs for each basic reporter vector (pMet Luciferase) in parallel. All plasmid DNA constructs were isolated using Endo-free Plasmid Mini Kit (Qiagen) to have high quality plasmid for transfection. All plasmid constructs were verified by restriction map analysis and DNA sequencing.

2.5. Cell culture, transfection and reporter activity assay

Chinook salmon embryonic cells (CHSE-214) were seeded in a flask (Nunc) containing L-15 medium (Invitrogen), penicillin (60 μg ml^{-1}), streptomycin (100 μg ml^{-1}), 1% non-essential amino acid (NEAA, Gibco) and 8% fetal calf serum (FCS) at 20 °C in an incubator for one week. Cells were washed twice in 10 ml phosphate buffered saline (PBS), and then 1.5 ml trypsin (1.25%) was added. Cells were re-suspended in L-15 medium (8% FCS, 1% NEAA, without antibiotics) and the cell number was adjusted to give a proper density. Cells were

Table 2
Identities (%) of Eomes in 10 vertebrate species at nucleotide and amino acid levels. The percentage identity values of nucleotides are marked with numbers in bold and the percentage identity of amino acids are marked as numbers in italic font. Matrix Global Alignment Tool (MatGAT) was applied in the calculation.

	1	2	3	4	5	6	7	8	9	10
1. Human		**97.9**	**72.7**	**66.3**	**69.6**	**59.8**	**59.2**	**59.8**	**55.7**	**56.5**
2. Monkey	*98.3*		**73.3**	**66.1**	**70.3**	**59.8**	**59.2**	**60.9**	**56.4**	**55.9**
3. Dog	*72.8*	*73.6*		**80.9**	**89.8**	**63.4**	**63.5**	**66.9**	**59.1**	**59.9**
4. Norway rat	*69.8*	*70.1*	*85*		**88.4**	**60.9**	**60.4**	**62.5**	**55.9**	**57.1**
5. Mouse	*70.9*	*71.4*	*92.8*	*90.4*		**63.3**	**62.8**	**65**	**58.5**	**59.5**
6. Gray bichir	*67.8*	*67.6*	*72.4*	*73.3*	*72.4*		**64.7**	**67.8**	**73.6**	**74.4**
7. Newt	*65.8*	*65.9*	*73.7*	*72.8*	*73.1*	*75.9*		**70.9**	**62.7**	**62.5**
8. Frog	*66.4*	*67.9*	*76*	*74.6*	*74.7*	*79.4*	*79.8*		**61.7**	**64.4**
9. Zebrafish	*64.1*	*65.1*	*71.7*	*72.1*	*71.3*	*85.8*	*73.8*	*76.1*		**78.1**
10. Cod	*64.8*	*64.4*	*72.3*	*73*	*71.9*	*84.7*	*74.7*	*78.5*	*89.9*	

washed twice with PBS and re-suspended in buffer R (Invitrogen) and divided into tubes (10 μl per tube). For each tube, 2×10^5 cells and plasmids {300 ng luciferase vector and 50 ng pSEAP2 control vector (Clontech)} were mixed and transfection was performed using electroporation by the Neon Transfection System (Invitrogen): 10 μl volume using D1 program (voltage 1100, pulse width 30, pulse no. 2) in line with the protocol supplied by the manufacturer. The pSEAP2-control vector for normalizing transfection efficiency was included in all assays to standardize protein expression levels.

The transfected CHSE-214 cells were seeded in 24 well plates (Nunc) at a density of 2×10^5 cells well^{-1}. 12 h after transfection, the medium was removed and replaced by fresh medium. The analysis for Metridia luciferase activity using Ready-To-Glow™ Secreted Luciferase Reporter System (Clontech) and SEAP activity using Great EscAPe™ SEAP Chemiluminescence Detection Kit (Clontech), and then the luciferase and SEAP activity were assayed using a plate Luminometer, Luminoskan Ascent (Thermo). The assay was performed thrice.

2.6. Tissue specific expression of GmEomes in healthy cod

Atlantic cod (~50 g) were supplied from the Aquaculture Research Station (Tromsø, Norway). Six fish were killed by immersion the fish in 100 mg L^{-1} Metacaine, and then, gill, head kidney, liver, heart, gut, pyloric caeca, skin and muscle were sampled and quickly immersed in RNAlater (Invitrogen). Total RNA was isolated by using RNeasy Mini Kit (Qiagen). RNase-Free DNase Set (Qiagen) was additionally used to remove genomic DNA contamination and QuantiTect Reverse Transcription Kit (Qiagen) was applied for cDNA synthesis. The synthesized cDNA was diluted 10-fold with MilliQ water and 1 μl of this dilution was used as template in a 20 μl reaction volume. Fast SYBR® Green Master Mix (Applied Biosystems) was used as reagents. qPCR was carried out in ABI PRISM 7500 Fast Real-Time PCR System (Applied Biosystems). Primers for qPCR have been listed in Table 1. An evaluation of gene expression of the two endogenous controls, β-actin and eF1α, No significant differences in gene expression ($P > 0.05$) were detected between the two endogenous controls, and eF1α was used as endogenous control in this study [33,34]. The results were expressed as fold change in comparison with the lowest expression level. All experiments on fish were approved by the Norwegian Food Safety Authority, and experiments were in accordance the animal welfare act.

2.7. Expression of GmEomes in spleen leucocytes after poly I:C and PMA stimulation

Isolation of spleen primary cells was performed in line with the previously published protocol [27]. Briefly, the spleen was removed and minced through a 100 μm nylon Falcon cell strainer (BD Bioscience) in L-15 culture medium (Invitrogen) supplemented with heparin (20 U ml^{-1}). The cell suspension was loaded on a discontinuous 25/50% Percoll (Amersham Pharmacia Biotec) gradient, and centrifuged at 850 *g* for 40 min at 4 °C. The cells at the interface were collected and washed twice with L-15 medium. Cells were seeded at a density of 5×10^6 cells per well in 24-well cell culture plates in L-15 with 1% FCS. The cells were divided into 3 groups with 6 replications for each group and incubated with polyinosinic-polycytidylic acid (poly I:C; Sigma) (100 ng ml^{-1}), phorbol 12-myristate 13-acetate (PMA; Sigma) (100 ng ml^{-1}) or left unstimulated, respectively. After incubation for 0, 6, 12, 24 and 48 h, cells were harvested for qPCR analysis, and the results were expressed as fold change in comparison with the unstimulated group at 0 h. The methods of RNA isolation and cDNA synthesis have been described in 2.6.

2.8. Expression of GmEomes in head kidney and spleen after infection by V. anguillarum

V. anguillarum (serotype O2b; isolate 4299) [35] were inoculated on Tryptic Soya Agar (Oxoid) supplemented with 5% human blood and 1.5% NaCl and incubated for 3 days and grown in Marine Broth (MB-2216, Difco) at 12 °C with gentle shaking until optical density (OD$_{600\ \mathrm{nm}}$) was = 0.5–0.6. The culture was washed, centrifuged and diluted with 0.9% NaCl (saline) giving OD$_{600\ \mathrm{nm}}$ = 0.2 corresponding to approximately 10^8 bacteria ml^{-1}. Infection doses were adjusted by diluting the bacteria suspension to ~10^7 bacteria ml^{-1}. Colony forming units (CFU) of the infection doses were determined using plates which were incubated at 22 °C for five days. 18 fish (~50 g) were intraperitoneally (i.p) injected with 0.1 ml bacterial suspension after being anesthetized, whereas the time-control fish received 0.1 ml of saline. Fish in these two treatment groups were kept apart in two tanks (500 L) with continuous supply of sea water (10 °C). Additional six untreated fish were sampled as time zero control fish at the start of the experiment. The head kidney and spleen from six fish of each group were sampled at 1, 2 and 4 days after injection and immersed in RNAlater (Thermo) for qPCR analysis, and the results were expressed as fold change in comparison with the control group at 0 day. The methods of RNA isolation and cDNA synthesis have been described in 2.6. The experiment with *V. anguillarum* infection was approved by the Norwegian Food Safety Authority.

2.9. Overexpression plasmid construction of pGmEomes-RFP and promoter-reporter plasmid construction of pLuc2-IFNγ

To construct pGmEomes-RFP which express GmEomes fused to red fluorescent protein (RFP), the coding sequences of GmEomes were amplified with primers GmEomesORFfw/GmEomesORFrv (Table 1), and the PCR products were inserted into pTagRFP-N (Evrogen). The construction of pSsT-bet-RFP plasmid which expresses salmon T-bet fused to RFP has been described previously [36]. Genomic DNA was isolated from Atlantic cod spleen with the DNA isolation kit (Qiagen). About 588 bp of the 5' flanking region sequences of the cod IFNγ (GenBank No: FJ356236.1) were obtained from the genomic DNA by PCR using the primers GmIFNrpromfw/GmIFNrpromrv (Table 1), and the PCR products were inserted into pMetLuc-2 vector (Clontech). All

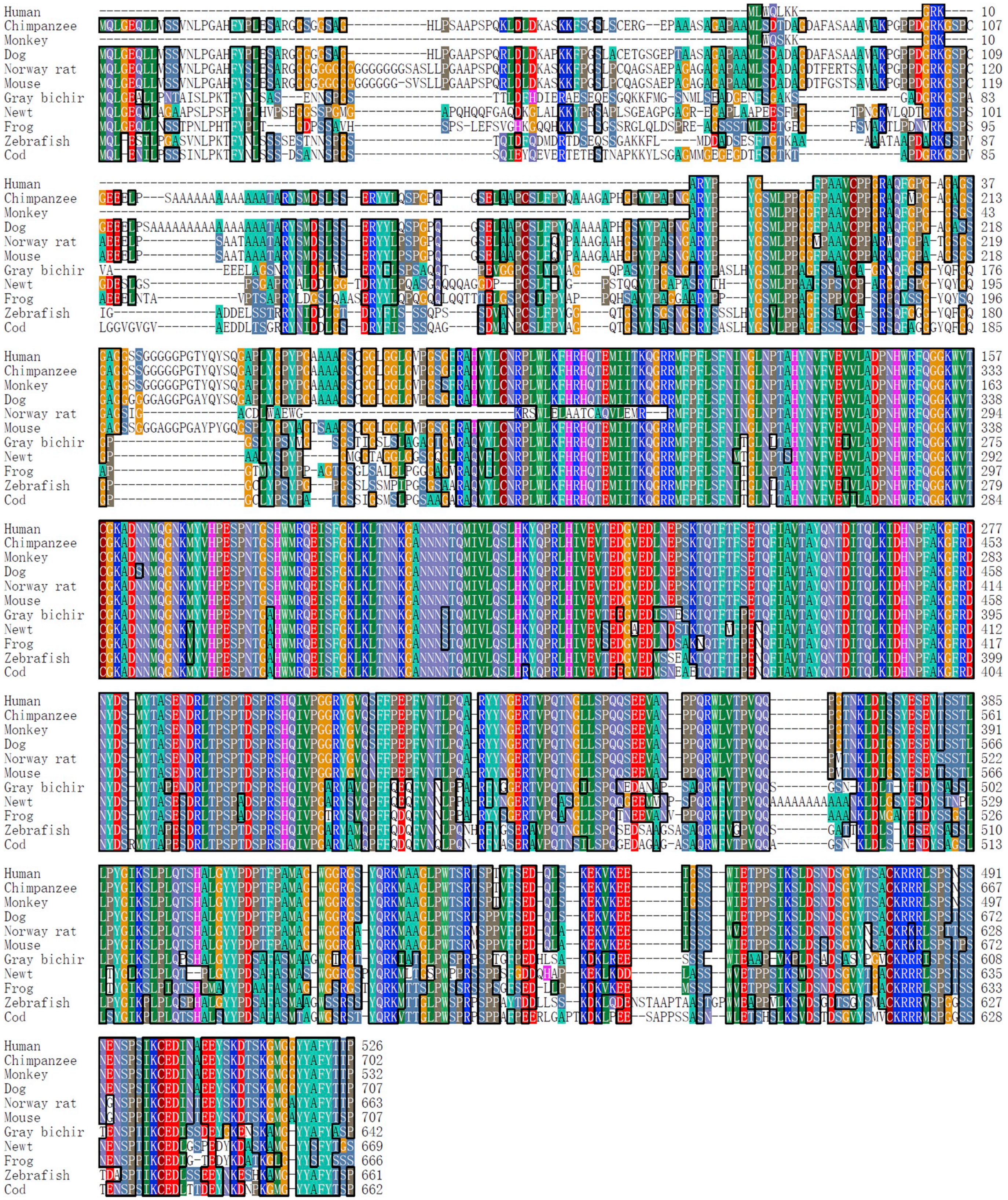

Fig. 2. Multiple alignment of the deduced amino acid sequences of Eomes in cod and other vertebrates by the ClustalW program. Residues shaded in the same colour are completely conserved across all species aligned, and residues shaded in different colour to 50–90% identity. Dashes indicate gaps. The GenBank accession numbers of the Eomes sequences are as follows: human: BAH13105.1; monkey: XP_002803087.1; chimpanzee: XP_526157.2; dog: XP_542755.2; Norway rat: XP_001061749.2; mouse: NP_034266.2; newt: BAA84718.1; frog: AAI25987.1; gray bichir: BAH58788.1; Zebrafish: NP_571754.3. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

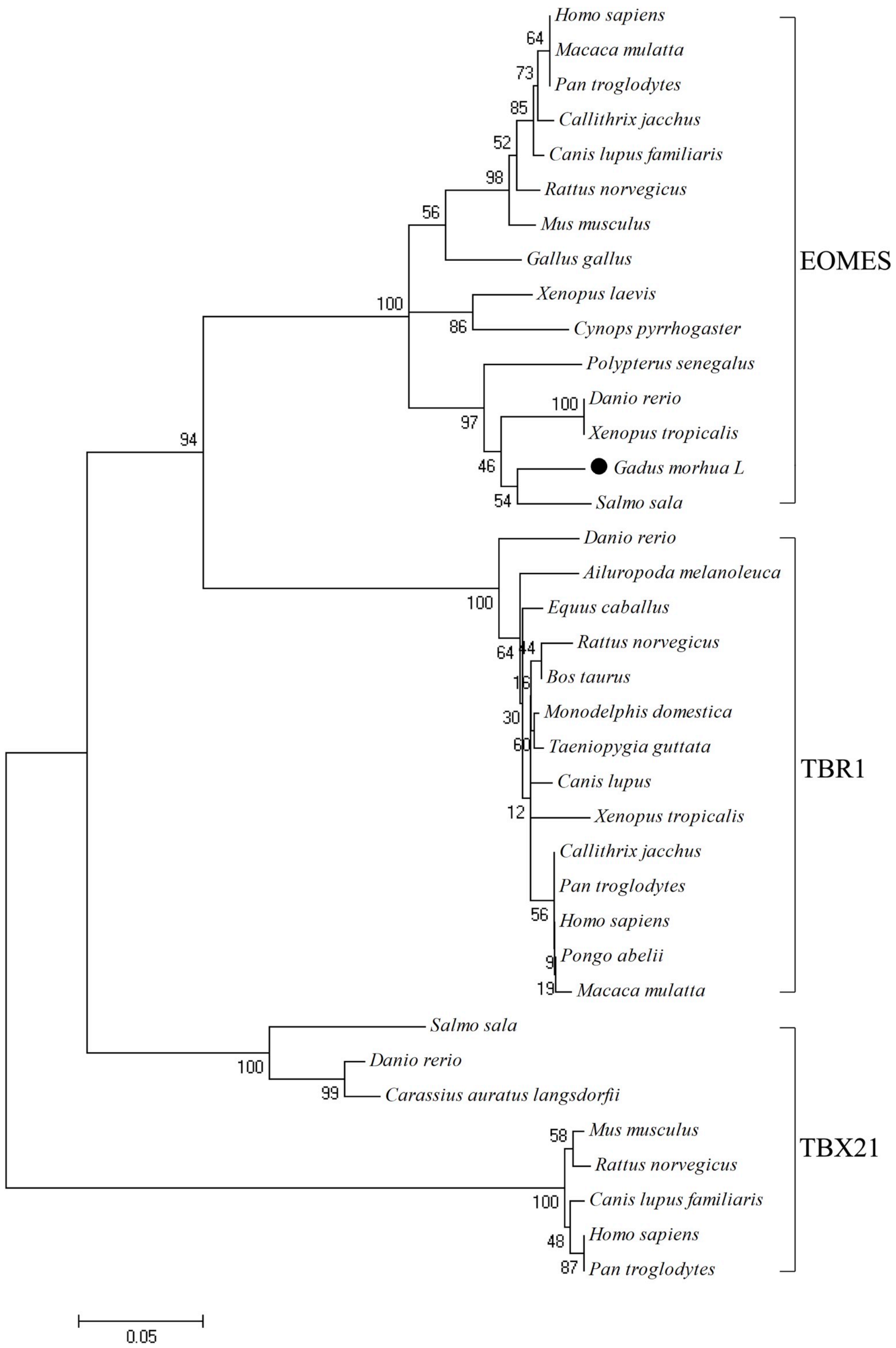

Fig. 3. Phylogenetic tree showing the relationship between GmEomes and other vertebrate amino acid sequences. The phylogram was constructed with the MEGA 4.0 software using the neighbor-joining method based on an amino acid alignment (ClustalW) of the full-length protein. Numbers beside the internal branches indicate bootstrap values based on 1000 replications. The 0.05 scale indicates the genetic distance. The position of Atlantic cod (*Gadus morhua* L.) Eomes was marked with "●". GenBank accession numbers for some Eomes sequences are listed in the legend of Fig. 2, while the others are listed as follows: Eomes: *Salmo salar*: NP_001191029.1; *Gallus gallus*: XP_426003.2; TBR1: *Danio rerio*: AAG_48249.1; *Xenopus tropicalis*: AAI36087.1; *Ailuropoda melanoleuca*: XP_002924534.1; *Canis lupus*: XP_545492.2; *Callithrix jacchus*: XP_002798955.1; *Pan troglodytes*: XP_001150172.1; *Homo sapiens*: AAI13419.1; *Rattus norvegicus*: NP_001177999.1; *Bos taurus*: NP_ 001178978.1; *Equus caballus*: XP_001493157.2; TBX21: *Salmo salar*: ADP36855.1; *Carassius auratus langsdorfii*: BAF73805.1; *Danio rerio*: NP_001164070.1; *Mus musculus*: NP_062380.2; *Rattus norvegicus*: NP_001100513.1; *Canis lupus familiaris*: XP_548164.1; *Homo sapiens*: BAJ21009.1; *Pan troglodytes*: XP_001173500.1.

plasmid DNA constructs were isolated using EndoFree Plasmid Kit (Qiagen) to have high quality plasmid for transfection. All plasmid constructs were verified by restriction map analysis and DNA sequencing. The cell culture, transfection and reporter activity assay have been described above in 2.5. The reporter assay experiment was done in triplicate fashion.

At 48 h after transfection, the medium in the wells was used for Metridia luciferase and SEAP analysis. The cells transfected by pGmEomes-RFP and pTagRFP-N expressing plasmids were fixed with 4% formaldehyde (w/v) (Thermo) for a half hour, then DAPI (Invitrogen) was used for nucleic acid (nucleus) staining in line with the protocol supplied by the manufacturer. Micrographs were obtained by inverted fluorescence microscope (Zeiss).

2.10. Statistical analysis

Statistical analyses were performed using one-way ANOVA followed by LSD multiple group comparisons in the SPSS 18.0 software package (SPSS Inc., Chicago, IL, USA). Data are presented as means ± SD, and statistical significance was defined as $P < 0.05$.

A

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-694    AAATTGTGAACTAAATGCCCCACAACCTGTCCTAATAATAAAATATCATAAGGTTTCAGG
                                          Oct-1
-634    TTGTTCATTCTAAAGTGGGTATTGGCACACCGGTCACCTGCGGTGCGGAGGTGGGCGCGT
                                         E47(-)
-574    TCAGCGACATGAGGAGGCCAATTAGAAAGTTCCACATTTCTTGTCGGAAGTCTTGCACAT
                                  HSF2
-514    GTGTTGTCTAATTGCGTCGCTGCATGAACATCTGATCATTGTATGTGACGGCACCGAAAA
         MyoD
-454    TGCTGAACTATGCTACTGGTTTAGTTTCTTTTTTAAGTTTTACGTTTTAATTTATCTACT
                                SRY
-394    AATTATGTAGGATTGATTCAATCTTCCGAGAATCCTATTTATCTGAAACATTTTAATGGA
          S8(-)                               GATA-1(-)
-334    CTTGCTTTGTGGATTTCAATCTGTAAATAAATGTAGACTAAACACTTGCTCTCGAAATGC
                                   CdxA
-274    CTGTCCAGGATCGGGGTAAGTCTGACATCCTTCTTATAAAACATGTGTAATTTAGTGCTA
              GATA-1/2                        TATA
-214    TGTCGATAAGGCAAGTGCATTTAGTTCGACTAATTGATTGGGAGAAATATGACCATCGGA
             GATA-x                       S8(-)
-154    CGTGCAGAGGACTGGAACCAATTCCAAGGATTCAGAAAGCTCCTCCCATGTCGCTTGCAG

 -94    CGAGTCGTGGGTGTGTGTGACTGAGCGTGTGTGTGTGAGAGTGTCTGTGTGTTGAGAACA
                         NF-E2(-)
 -34    GCGTCTTATAAGAGCCATTGAACCACCCCTGACCAGATCATAATCGGAGTTGCACATCTC
                                         +1
 +27    TGGGTGAGGAAGAGAAGGCCAAGTCCTGAAGTGAGGAGCAGGAACGGGAGGACGCGCACC
 +87    GCAGATCTACC
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B

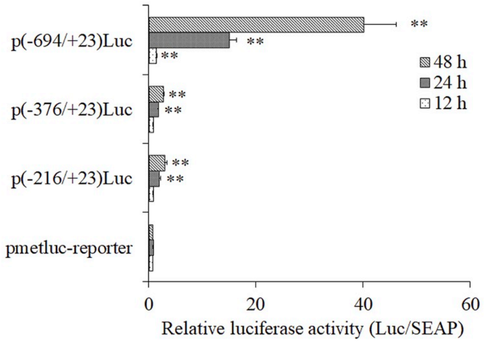

Fig. 4. The sequence and activity of Eomes promoter. A. The nucleotide sequence of promoter regions (694 bp) was determined. The transcription start site is designated as +1 and boxed. Transcription factor binding sites were predicted by MatInspector and TRANSFAC®. Consensus elements of transcription factor binding sites are underlined, while (−) sign indicates the binding sites identified on the negative strand. B. CHSE-214 cells were transiently transfected with the promoter constructs plus pSEAP2 internal control vector in 24-well plates. Luciferase activity is expressed relative to SEAP (mean ± SD from six wells). Double asterisks (**) above the bars show significant differences ($P < 0.01$) compared to the different promoter constructs with the same treatment. The data are from six cell wells per treatment in one experiment and are representative of three independent experiments.

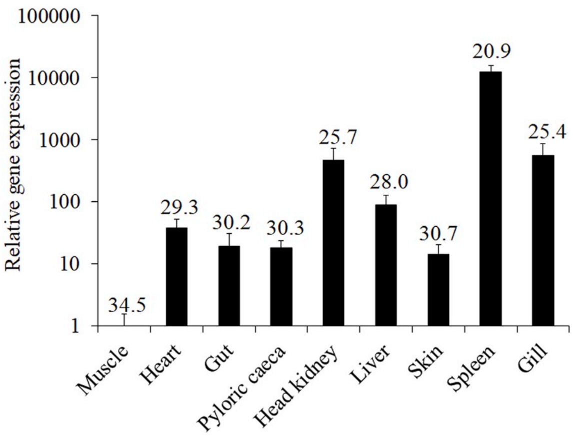

Fig. 5. Tissue distribution of GmEomes transcripts. The expression of GmEomes mRNA was determined by qRT-PCR in different organs. The results were calculated by relative expression with eF1α as the house keeping gene and muscle as a calibrator. The value above the bars shows average real-time CT values of six fish. Data are represented as mean ± SD (N = 6).

3. Results

3.1. Characterization of the nucleotide and amino acid sequences of Eomes

The GmEomes cDNA (GenBank accession no. JF968408) consisted of 2618 bp in length with an open reading frame of 1989 bp encoding a polypeptide of 662 amino acids, a 278 bp 5′ un-translated region (UTR) and a 437 bp3′ UTR. Within the 3’ UTR, one polyadenylation signals (AATAAA) and four mRNA instability motifs (ATTTA) were found (Fig. 1). The putative protein had a molecular weight of 72321.9 Da and an estimated pI of 6.15.

According to a BLAST search, the cloned GmEomes gene possessed a high degree of sequence similarity (64.8–89.9%) (Table 2) to various members of the Eomes family in different animals, indicating that it has been conserved during evolution (Fig. 2). Alignment of the predicted protein of GmEomes revealed the presence of conserved TBOX_3 domain (219–414). A phylogenetic tree was developed based on multiple alignments of Eomes from various species, including fish, frog, chicken and mammals (Fig. 3). GmEomes was more closely related to Atlantic salmon Eomes than to the other species.

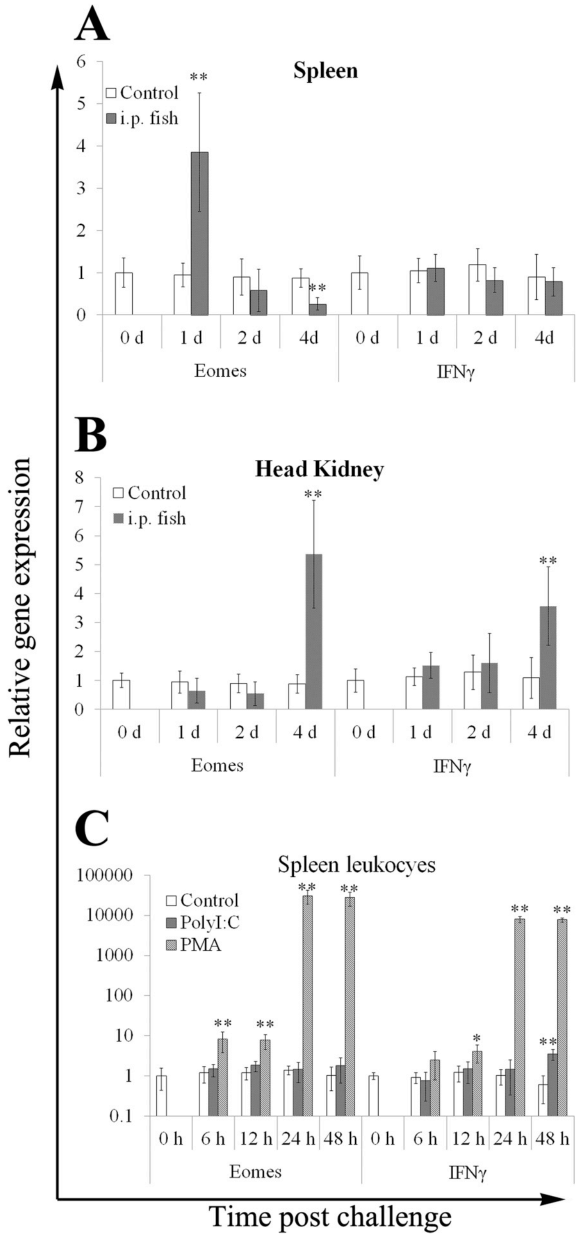

Fig. 6. Specific expression of GmEomes and GmIFNγ in Atlantic cod spleen (A) and head kidney (B) during *V. anguillarum* infection and spleen leukocytes (C) at different time-points upon PolyI:C or PMA stimulation. (A) Spleen. Data are presented as means ± SD (N = 6). Untreated fish spleen as calibrator for GmEomes or GmIFNγ. (B) Head kidney. Data are presented as means ± SD (N = 6). Untreated fish head kidney as calibrator for GmEomes or GmIFNγ. (C) Head kidney leucocytes. Data are presented as means ± SD (N = 6). Untreated fish spleen leucocytes as calibrator for GmEomes or GmIFNγ. $^{**}P < 0.01$ and $^{*}P < 0.05$ show the significantly differences compared the control group.

3.2. Structure of 5′-flanking region of GmEomes gene

As the first step towards understanding the transcriptional regulation of the GmEomes gene, a sequence of 694 bp lying 5′ to the transcription start site (TSS) was determined (GenBank accession no. JF968407). Sequence analysis using the transcription factor binding site prediction program MatInspector and TRANSFAC® revealed several notable features. The TATA-box, considered being the core promoter sequence, was present at position −309 relative to the putative GmEomes transcription start site (Fig. 4). In addition, we found two potential GATA consensus sequences present within this region at −211, and −269 which are known to be enriched in specific gene regulatory regions of immune cells. Oct-1, HSF2, MyoD, CdxA and SRY, known to be important for transcriptional regulation of genes were also found in this region. Lastly, the NF-E2 was found close to the TSS. Other putative transcription factor binding sites are shown in Fig. 4.

3.3. Activity of the GmEomes promoter

To precisely define the 5′-end of TSS of GmEomes that was required for the induced activation, progressive deletion constructs of the GmEomes promoter region were generated and transiently transfected into CHSE-214 cells (Fig. 4). All promoter constructs were active at 12 h, and induced increasing luciferase activities from 24 h to 48 h ($P < 0.001$). The minimal promoter construct induced low luciferase activity at all time points and similar time kinetics as the p(-376/+23)Luc ($P > 0.05$), but showed significant difference compared to p(-694/+23)Luc ($P < 0.001$). The full-length p(-694/+23)Luc promoter construct induced relative luciferase activity 40.16-fold higher than the promoterless controls at 48 h, whereas the p(-376/+23)Luc construct and minimal Eomes promoter induced only 2.97-fold and 2.65-fold, compared to promoterless controls, respectively (Fig. 4).

3.4. Tissue distribution of Eomes mRNA

As shown in Fig. 5, the GmEomes gene was widely expressed in all the sampled tissues of healthy fish. The largest quantity of GmEomes mRNA was found in spleen, followed by gill, head kidney, liver, heart, gut, pyloric caeca and skin. The level of GmEomes transcripts in muscle was relatively low.

3.5. Expression profile of GmEomes in head kidney and spleen in vivo after *V. anguillarum* infection and in spleen leukocytes in vitro after PMA and poly I:C stimulation

No mortality and abnormal behavior were observed during the experimental challenge with *V. anguillarum*. In spleen, the expression of GmEomes went 3.85-fold higher than in the control group at day 1 ($P < 0.01$), whereas it was decreased at day 4 post infection (Fig. 6A). No statistically significant changes of GmIFNγ mRNA levels were observed in the saline injected fish in the control group or during challenge with *V. anguillarum*. In head kidney, both GmEomes and GmIFNγ expressions were significantly upregulated at 4 d post-infection, with 5.36 and 3.56-fold increase, respectively ($P < 0.01$) (Fig. 6B). No statistically significant differences in the expression of GmEomes and GmIFNγ between fish in the control group at the other time points was found.

The spleen leukocytes stimulated with PMA showed significantly increased expression of GmEomes at 6, 12, 24 and 48 h ($P < 0.01$), compared to non-stimulated cells. Similarly, the expression of GmIFNγ was also significantly upregulated at 12, 24 and 48 h after PMA stimulation. The GmIFNγ expression was significantly higher at 48 h post poly I:C stimulation compared to control cells ($P < 0.01$), whereas no statistically significant changes of GmEomes mRNA levels was observed in control cells and during poly I:C stimulation ($P > 0.05$) (Fig. 6C).

3.6. Localization of over expressed GmEomes in CHSE-214 cells

To obtain information from overexpression of GmEomes, microscopic analysis of cells transfected with GmEomes encoding plasmid fused to red fluorescent protein (RFP) were carried out. The complete coding sequence of GmEomes was inserted into the plasmids encoding red fluorescent protein fused to the 3′-terminal ends. Approximately 15–20% of RFP positive CHSE-214 cells were observed after transfection with the plasmids, as shown in Fig. 7A. GmEomes fused with RFP were identified in or close to the nuclei at 48 h post transfection, whereas the cells transfected with empty control vector (pTagRFP-N)

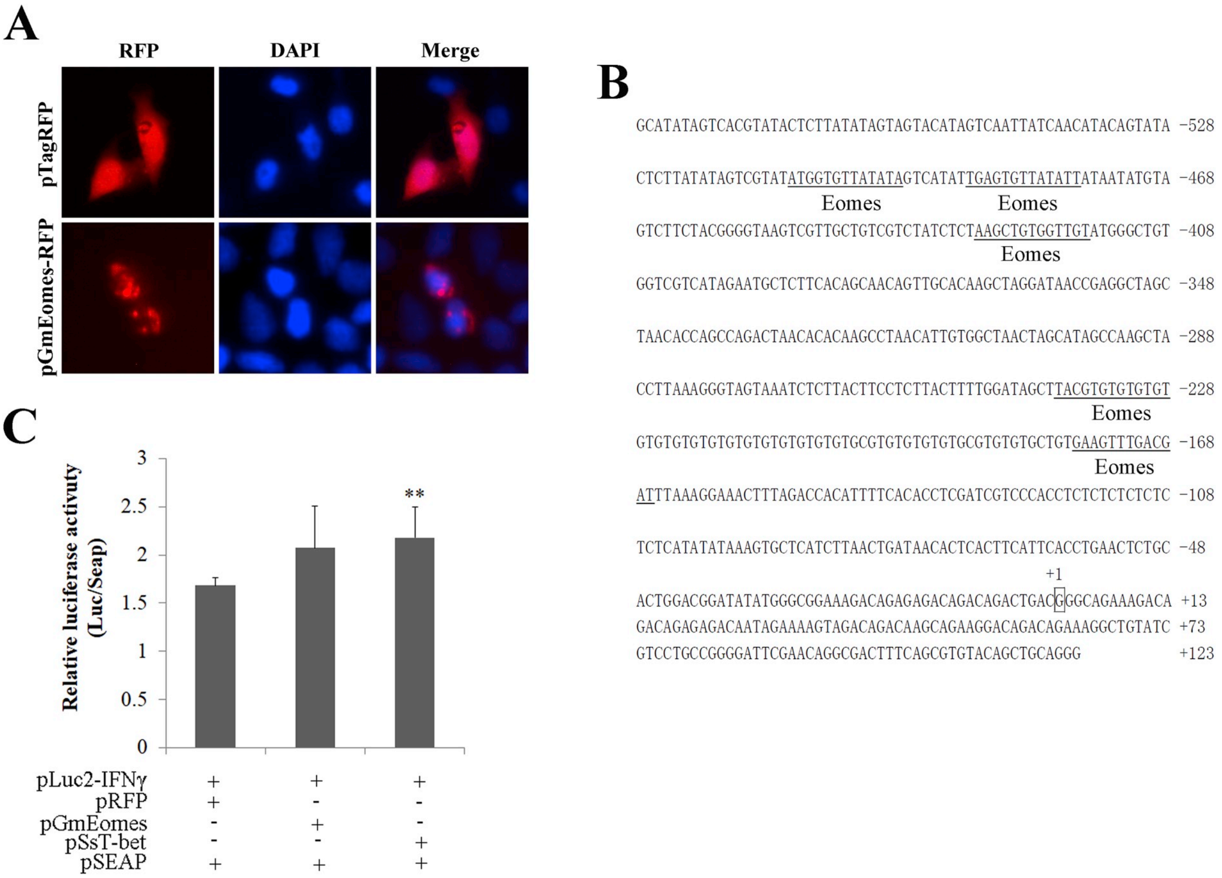

Fig. 7. The regulation of GmEomes on Atlantic cod IFNγ (GmIFNγ) promoter. A. The micrograph of overexpression of RFP and GmEomes-RFP (red) in CHSE-214 cells. The colour of blue showed nuclei stained by DAPI (blue). B. Gene sequence and schematic representation of the cod IFNγ promoter. The transcription start site is designated as +1 and boxed. Eomes binding sites were predicted by MatInspector and TRANSFAC®. C. GmIFNγ promoter activity in CHSE-214 cells. Luciferase and SEAP activities were measured at 48 h after the transfection. Luciferase activity is expressed relative to SEAP (mean ± SD, n = 6). Double asterisks (**) above the bars show significant differences ($P < 0.01$) compared to the control group. The data are from six cell wells per treatment in one experiment and are representative of at least three independent experiments.

but still containing a RFP gene, showed RFP widespread in the cytoplasms.

3.7. The effect from over expression of GmEomes on Atlantic cod IFNγ promoter

Approximately 588 bp 5' flanking region of GmIFNγ were cloned, in which Eomes binding sites were found (Fig. 7B). Overexpression of GmEomes in CHSE-214 cells slightly enhanced the activity of GmIFNγ promoter region, but not with statistically significant difference compared to controls ($P = 0.083$), while an overexpression of salmon T-bet significantly enhanced the activity of the IFNγ promoter ($P < 0.01$) (Fig. 7C).

4. Discussion

In this study, we identified an Eomes homologue, GmEomes, from Atlantic cod and analyzed its structure, expression, and regulatory property. We found that GmEomes shared 64.8 and 89.9% overall amino acid sequence identities with human and zebrafish Eomes, respectively. This observation indicated that Eomes is considerably conserved among lower and higher vertebrates, which may be consistent with the fundamental role of Eomes. GmEomes contains a T-box domain which was defined as the minimal region within the T-box family proteins that is both necessary and sufficient for sequence-specific DNA binding. The presence of this domain in GmEomes suggested a potential capacity of the translated protein to bind DNA as a transcription factor.

Sequence analysis of 5' flanking region of GmEomes revealed that the presence of TATA box in the proximal promoter (694 bp to the TSS) are in line with the previous study on the Xenopus and salmon Eomes promoters [9,24]. By PCR-aided deletion of the putative promoter region we detected a strong positive regulatory element between positions −694 and-377 bp in the GmEomes promoter. Further analysis of this element indicated that it shared a number of consensus transcription factor binding sites, such as binding sites for MyoD, Oct-1, HSF and SRY. Recent reports have shown that MyoD is a transcription factor involved in regulating muscle differentiation, whereas both Oct-1 and Cdx are involved in regulation of the genes essential for growth and embryonic development [37,38]. The sex-determining region Y (SRY) is responsible for the initiation of male sex determination in humans. HSF is needed for proper animal development and the survival of cancer cells [39,40]. The presence of MyoD, Oct-1, Cdx and SRY transcription factor binding sites in the promoter of GmEomes indicated similar mechanisms of development and cell differentiation in cod as in mammals. By PCR-aided deletion of the putative promoter region we detected a weak positive regulatory element between positions −376 and + 23 bp in the GmEomes promoter. Further analysis of this element indicated that it shared a number of consensus transcription factor binding sites, including GATA and Cdx for positive control transcription factor binding sites, and S8 and HF-E2 negative control transcription factor binding sites. This suggests that GATA and Cdx are not the main transcription factors for the expression of GmEomes.

In mammals, Eomes is expressed highly at the certain stages during development [41] and has been well defined as key drivers of neuronic, cardiac and immune cell development associated with effector function [41–43]. In the immune system, Eomes expression has been reported to be present in different lymphoid tissues, and in cells and their subsets, including CD8$^+$ T-cells, γδ T-cells, invariant NKT cells, natural killer cells, B-cells, and dendritic cells [44]. In this study, we found that the expression of GmEomes occurred in multiple tissues and was highly expressed in spleen, head kidney and gill. This is not in full consistence with the reports on Atlantic salmon, trout and zebrafish [24,26]. The expression of Eomes mRNA in gills is rather at low levels when compared to head kidney in trout and ginbuna carp [25,26]. However, the levels of GmEomes transcripts were similar in gill and head kidney of Atlantic cod. In salmonid fishes and ginbuna carp, constitutive high expression of GATA-3 and IL-4 in gill suggests that this tissue may be capable to form a Th2-skewed immune environment [45,46]. A presence of high level of Eomes in the gills of cod may also lead to differentiation of naïve T-cells into CD8$^+$ T cells to compensate the absence of CD4$^+$T cells and subsets.

V. anguillarum is an abundant pathogen in aquaculture, and causes vibriosis in a range of different fish species [47–51]. In our study, the expression of GmEomes was enhanced in spleen (day 2) and in the head kidney (day 4) after experimental infection with the *V. anguillarum*. These results suggest a role for GmEomes in host immune defence against microbial pathogens. PMA, through its activation of protein kinase C, is often used experimentally to induce leucocyte activation - especially in T-cells [52,53]. In this study, the expression of GmEomes was indeed induced in spleen leucocytes following PMA stimulation (24 h and 48 h).

In mammals, Eomes is highly expressed in CD8$^+$ T cells, but not in CD4$^+$ T cells. Even though Eomes/Tbr2 plays a role in the ability of CD8$^+$ T cells to penetrate tumors, it only plays a small role in production of IFNγ [16,17,54]. In our study, the transcripts levels of GmIFNγ were increased with the increase of GmEomes expression at some time points after PMA stimulation *in vitro* or during *V. anguillarum* infection *in vivo*. In addition, we found that some Eomes binding sites were present in the 5′-flanking regions of the cod IFNγ gene predicted by bioinformatics. In CHSE-214 cells transfected with pGmEomes-RFP, the fusion protein was detected mainly in the nucleus, which is consistent with the known function of Eomes in regulation of gene expression. However, GmEomes only slightly increased the activity of this IFNγ promoter suggesting that GmEomes does not significantly contribute to IFNγ production, while the SsT-bet significantly enhanced the activity of GmIFNγ promoter. These results were in line with some reports from mice, where similar numbers of IFNγ producing cells were observed in WT and Eomes KO effector CD8$^+$T cells. T-bet KO CD8$^+$ T cells and T-bet/Eomes double KO CD8$^+$ T cells showed only a slight reduction (approx. 10%) of IFN-γ$^+$ cells [54]. As such, Eomes is required, but not crucial, for IFN-γ production by T cells. Mechanistic studies on the interplay between Eomes and T-bet should be performed to find out their relative importance to induce IFNγ production in fish lymphocytes.

In summary, we identified and characterized GmEomes which is a T-box transcription factor molecule and its expression pattern during homeostasis and during contains of PAMPs and pathogen challenge in A. cod. This work presents new knowledge about the promoter region and the promoter activity of the GmEomes. Furthermore, we found that GmEomes mainly was localized in the nucleus, but played a minor role in activation of the INFγ promoter.

Acknowledgement

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