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# Cloning, expression analysis and promoter structure of TBK1 (TANK-binding kinase 1) in Atlantic cod (*Gadus morhua* L.)

Heng Chi [a,b], Zuobing Zhang [b,c], Jarl Bøgwald [b], Wenbin Zhan [a], Roy A. Dalmo [b,*]

[a] Laboratory of Pathology and Immunology of Aquatic Animals, LMMEC, Ocean University of China, 266003 Qingdao, PR China
[b] Norwegian College of Fishery Science, University of Tromsø, N-9037 Tromsø, Norway
[c] Ministry of Education Key Laboratory of Biodiversity Science and Ecological Engineering, College of Life Sciences, Beijing Normal University, 100875 Beijing, PR China

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ABSTRACT

TBK1, also termed NAK or T2K, is a ubiquitous member of the IκB kinase (IKK) family that is required for innate and adaptive immune responses. We have identified and characterized the full-length TBK1 cDNA in Atlantic cod. The cod TBK1 gene consists of 2190 bp open reading frame encoding a polypeptide of 729 amino acids. According to a BLAST search, the cloned TBK1 gene has a high degree of sequence similarity (80.7–92%) to the various members of the TBK1 family, indicating that it is conserved during evolution. RT-PCR showed that the largest quantity of TBK1 transcripts was found in spleen, followed by the liver, gill, head kidney, gut, pyloric caeca, while the expression of TBK1 mRNA in muscle and skin was low. Both PMA, poly I:C and β-glucan promoted expression of TBK1 transcripts *in vivo*. Furthermore, we determined an 875 bp sequence upstream of the transcriptional start site (TSS) and found a number of sequence motifs that matched known transcription factor-binding sites. Activities of the presumptive regulatory regions of this gene were assessed by transfecting different 5′-deletion constructs in CHSE-214 cells. After the expression experiments, the results showed that the basal promoters and positive transcriptional regulator activities of cod TBK1 gene were dependent by sequences located from −875 to −425 bp and from −245 to +28 bp upstream of TSS. This study provides further insights into the transcriptional regulation of cod TBK1.



## 1. Introduction

Host organisms employ a multitude of innate defense mechanisms against invading microbial pathogens. Functions of the innate immune system include control and destruction of pathogens and instruction of the developing adaptive immune response through expression of cytokines, chemokines, and other proinflammatory molecules [1,2]. The expression of proinflammatory molecules is dependent on different transcription factors. Protein kinase function has been evolutionarily conserved from *Escherichia coli* to *Homo sapiens* [3]. Protein kinases mediate most of the signal transductions in eukaryotic cells by modification of substrate activity. Protein kinases also control many other cellular processes, including metabolism, transcription, cell cycle progression, cytoskeletal rearrangement and cell movement, apoptosis, and differentiation [4]. Also, protein kinases are among the largest families of genes in eukaryotes and have been intensively studied in mammals [5–8]. As such, they made an attractive target for an initial in-depth analysis of the gene distribution in the draft genome. Mutations and dysregulation of protein kinases play causal roles in disease, affording the possibility of developing agonists and antagonists of these enzymes for use in disease therapy [7,8].

TBK1 (TANK-binding kinase 1)/NAK (NF-κB activating kinase) is an IκB kinase (IKK)-activating kinase, and can activate IKK through direct phosphorylation [9,10]. TBK1 was identified through association with the TNF (tumor necrosis factors) receptor associated factor (TRAF) binding protein and found to function upstream of NF-κB-inducing kinase (NIK) and IKK in the activation of NF-κB [11,12]. TBK1 plays important roles in the regulation of interferon (IFN)-inducible genes during the immune response to bacterial and viral infections [13–16]. Cell stimulation with ssRNA virus, dsDNA virus or gram-negative bacteria leads to activation of TBK1 or IKK-i, which in turn phosphorylates the transcription factors, IFN-regulatory factor (IRF) 3 and IRF7, promoting their translocation in the nucleus [15,16]. In the past decade, DNA vaccines have emerged as a potential favored strategy for inducing immunity. TBK1 mediates the adjuvant effect of DNA vaccines and this effect occurs independently of the TLR (Toll-like receptor) signaling pathway [17].

* Corresponding author. Tel.: +47 77644482; fax: +47 77646020.
*E-mail address:* roy.dalmo@uit.no (R.A. Dalmo).

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TBK1 has been identified in the zebrafish genome [18], but functional studies of this kinase in fish are lacking. In the present study, we isolated full-length cDNA and 5′-flanking regions of TBK1 in Atlantic cod (*Gadus morhua* L.). Moreover, expression analysis and promoter genomic structure of cod TBK1 have been studied with the aim to delineate the regulation of TBK1 in teleosts for the first time.

## 2. Materials and methods

### 2.1. Cloning and sequencing of the cod TBK1 cDNA

A partial cod EST sequence homologous to vertebrate TBK1 was identified based on nucleotide and amino acid sequence homology to human, mouse and zebrafish TBK1 sequences deposited in GenBank using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST). Four Atlantic cod TBK1 ESTs (GenBank accession no: EX187388, EX735648, ES475123, EG633402) 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 g), 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. The gene specific primer (GSP) and nested GSP (NGSP) for 5′- or 3′-RACE are listed in Table 1. PCR products were purified using MinElute Gel Extraction kit (Qiagen) and cloned into a TOPO vector (Invitrogen). Plasmid DNA from at least five independent clones were purified using QIAprep Spin Miniprep kit (Qiagen) and sequenced using M13F and M13R primers (Invitrogen) and Big Dye Terminator v 3.1. The full-length nucleotide sequence obtained by 3′/5′-RACE was verified by PCR amplifications using the primers situated at the extreme ends (GmTBK1CFW1/GmTBK1CFW2 and GmTBK1CRV1/GmTBK1CRV2). The cDNA sequence and deduced amino acid sequence of Atlantic cod TBK1 sequences were further analyzed using the BLAST program, the ExPASy Molecular Biology server (http://us.expasy.org) and Pfamp [19]. Amino acid identity and similarity analysis were done with the Matrix Global Alignment Tool (MatGAT) program v 2.0 using default parameters [20].

### 2.2. Phylogenetic analysis

A multiple sequence alignment was created using CLUSTALW, while MEGA version 4.1 [21] was used to assess the similarities among the aligned sequences (www.ebi.ac.uk/clustalw/). 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 10,000 pseudo-replicates.

### 2.3. Isolation of 5′-flanking region of the cod TBK1 gene by genome walking

The 5′-flanking region of the TBK1 gene was isolated using the Universal GenomeWalker Kit (Clontech). Four GenomeWalker libraries were constructed according to the manufacturer's instruction. For each genomewalker experiment, three adjacent reverse primers 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 (GMTBK1gwFW1) was designed within this new sequence and used with a reverse primer (gmTBK1gwrv1) designed within the transcribed region of the TBK1 gene (Table 1). PCR from the Atlantic cod genomic DNA was performed, and the products obtained were cloned and sequenced. Identification of transcription factor-

**Table 1**
List of primers and their designated applications.

| Oligo name | Sequence (5′–3′) | Use |
|---|---|---|
| GmTBK1FW1 | AGCTGGAGGACGACGAGCAGTT | 3'-RACE |
| GmTBK1FW2 | AGTACCTGCACCCTGACATGTACGAG | 3'-RACE |
| GmTBK1RV1 | CCGGCAGGAGCGGCTAGTGGGCGT | 5'-RACE |
| GmTBK1RV2 | GCAGGAGCCCCTCCGGTATCCGC | 5'-RACE |
| GmTBK1CFW1 | ACATGGGGAGACCAAGGAAGCA | Full cDNA verification |
| GmTBK1CFW2 | CGGTCAGTTAGATTCGGTGTAGCTGG | Full cDNA verification |
| GmTBK1CRV1 | GAGGAGCAGTTGAATTGATATTTATT | Full cDNA verification |
| GmTBK1CRV2 | GTGACAGGATGCTGGGGCCG | Full cDNA verification |
| gmTBK1gwrv1 | GCAGTGGCCCCCTGACCCAGCAGGTCTGA | Genome walking |
| gmTBK1gwrv2 | ACGCAGGTCTTTGGTCGATGCGTGGATTAT | Genome walking |
| gmTBK1gwrv3 | AGCGCAACTCAAAACACCCCGTTCAACAA | Genome walking |
| AP1 | GTAATACGACTCACTATAGGGC | Genome walking |
| AP2 | ACTATAGGGCACGCGTGGT | Genome walking |
| GMTBK1gwFW1 | GGCCCGGGCTGGTATCAC | Promoter region verification |
| GMTBK1HindIIIfw1 | AGCTCAAGCTTGGCCCGGGCTGGTATCAC | Promoter cloning |
| GMTBK1HindIIIfw2 | AGCTCAAGCTTCGCGCTACTCAAGAAGCAAA | Promoter cloning |
| GMTBK1HindIIIfw3 | AGCTCAAGCTTATAAAATCAAATTATAAAGAAAGGA | Promoter cloning |
| GMTBK1sacIIRV | TAAGCCCGCGGCATCTTCCAACTTCTGCTTCCTTGGT | Promoter cloning |
| GmTBK1SFW1 | CAGTCTGTACACGGTGCTGG | Sequencing |
| GmTBK1SRV1 | CTGGAAGAGAAGGGTGGGGT | Sequencing |
| GmTBK1SFW2 | GGGGCTTCGACCAGTTCTTC | Sequencing |
| GmTBK1SRV2 | ATCTGCTCGGTCCTCTCCAG | Sequencing |
| M13 F | CAGGAAACAGCTATGAC | Sequencing |
| M13 R | GTAAAACGACGGGCCAG | Sequencing |
| pMetluc2-R | CACGATGTCGATGTTGGGG | Sequencing |
| GmTBK1RTFW1 | GCCGTCGAAGAAGAGGTGTGTACCAA | Real-time PCR |
| GmTBK1RTRV1 | GCCGTACTCCCTCAGGTGGTTCATC | Real-time PCR |
| COD β-actin FW | AGGTCATCACCATTGGAAACGA | Real-time PCR |
| COD β-actin RV | GTTGGCGTACAGGTCCTTGC | Real-time PCR |