

A natural antisense transcript, BOKAS, regulates the pro-apoptotic activity of human Bok

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Received October 6, 2008; Accepted December 11, 2008

DOI: 10.3892/ijo_00000241

Abstract. Bok is a proapoptotic member in the Bcl-2 family and the expression of Bok is regulated by cellular stresses. In the present study, we have isolated and characterized a natural antisense transcript of Bok, BOKAS. The BOKAS gene consists of 2 exons, that expresses as a non-protein coding transcript. Both BOKAS and Bok are transcribed from the same locus but in opposite orientations. The mRNA expression of BOKAS was only detected in testis and certain cancer tissues but not in other normal adult tissues examined. Overexpression of BOKAS alone exhibited no significant anti- or pro-apoptotic activity but it was able to inhibit Bok-induced apoptosis in HeLa cells. Our results suggest that BOKAS may function specifically in the human testis, where it serves as an antisense molecule to regulate Bok-induced apoptosis. In addition, natural antisense transcripts were identified for BAD and BCL2L12.

Introduction

Bcl-2 family proteins are essential regulators of apoptosis and play an important role in embryonic development and in adult tissue homeostasis. Dysregulation of apoptosis is involved in the development of many diseases such as cancer, autoimmunity and various degenerative diseases (reviewed in refs. 1 and 2). Bok is one of the pro-apoptotic members consisting of BH1-3 domains (3,4). It has been demonstrated that the expression of hBok could be regulated by cis- and trans-acting elements (5-8).

Natural antisense transcripts are RNAs possessing transcript complementarity to other RNA transcripts within cells (9).

These RNAs can be either protein-coding or non-coding RNAs (10). It has been shown that natural antisense transcripts are important factors involved in gene regulation (9). Moreover, aberrant natural antisense transcription might contribute to major diseases, such as α -thalassemia, Parkinson's disease and some malignant cancers (11).

During our study of Bok expression, we identified a novel natural antisense transcript of Bok, denominated BOKAS. We demonstrated that it is a negative regulator of Bok-induced apoptosis being expressed only in testis and in some cancer cells.

Materials and methods

Cloning and chromosomal location of BOKAS. During our study of Bok expression, we found several alternative splicing variants of hBok by RT-PCR. The full length nucleotide sequence of Bok was then extensively subjected to homology search using the NCBI BLASTN algorithm (12) against the human EST database. Among the sequences identified, one novel sequence (IMAGE: 6173204) was found to contain nucleotide sequences with significant identity to that of Bok but in opposite orientation. Full length cDNA was assembled after 5'RACE and was termed BOKAS. The GenBank accession no. for BOKAS is FJ185168. For mammalian expression, the BOKAS cDNA was cloned in the NotI/Sall site of pSPORT VI. The genomic organization of BOKAS was found according to the BLAT:UCSC Human Genome Browser (Mar 2006 release).

Tissue expression pattern analysis by RT-PCR. RT-PCR was performed using the primers 5'-GTTCTGGCTTCAGGATC TGC-3' and 5'-TCCAAAATAGGCCTCAGTGG -3' on a panel of first-strand cDNAs from various human tissues (BD). The PCR products should be 245 bp in size and were sequenced.

Cell culture and cell viability assay. HeLa cells were seeded at 5×10^4 cells in 12-well plates with 10% FBS. Cells were transfected using Superfect (Qiagen, Hilden, Germany) with 2 μ g of plasmids of total in all settings. pcDNA3.Bok:GFP was transfected with pSPORT VI- BOKAS in the ratio of 1:1 μ g and 0.5:1.5 μ g. Green fluorescent cells were monitored, both normal flat and detached apoptotic cells were counted by microscopic examination after 48 h of serum deprivation.

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Key words: hBok, BOKAS, natural antisense transcripts, testis

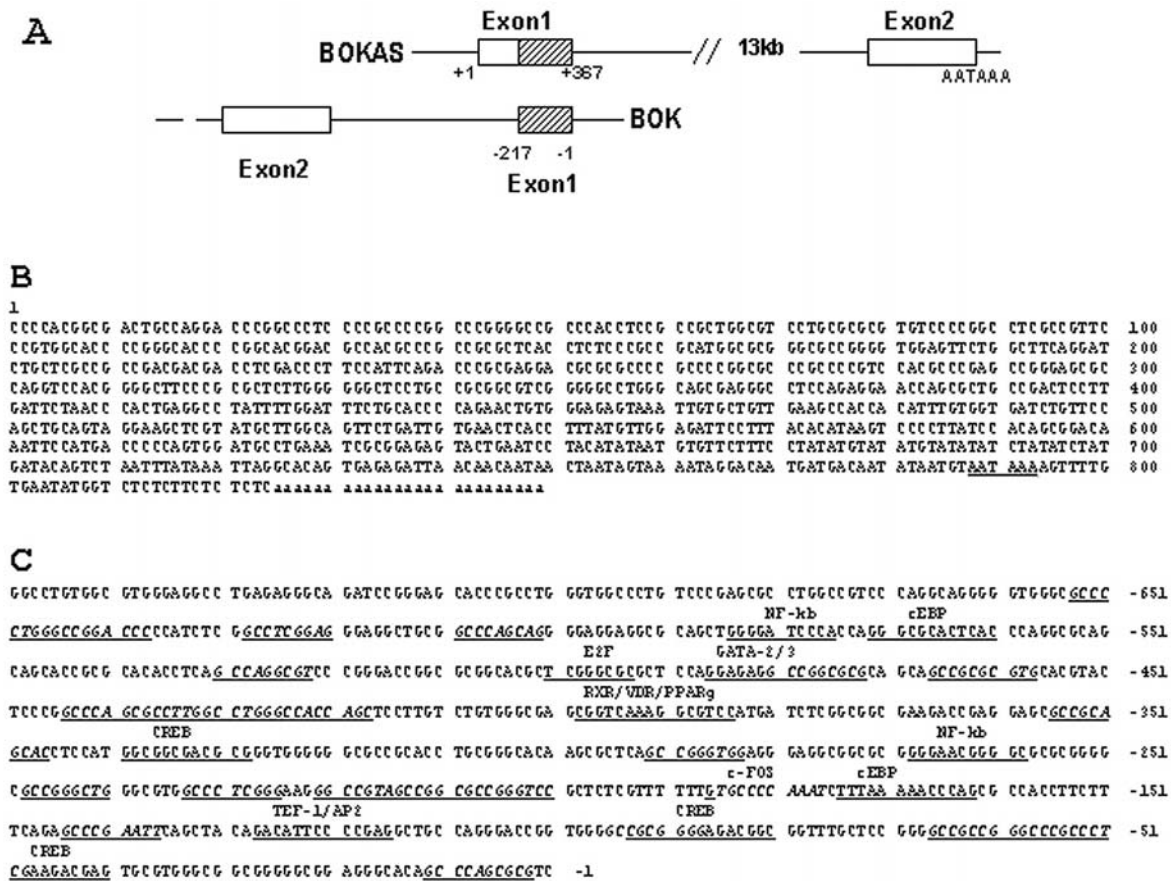


Figure 1. Identification of cDNAs for a novel human BOKAS. (A) Schematic representation of genomic organization of BOKAS in the relation with hBok. Exon regions are denoted by boxes. The black horizontal lines denote the introns. Black boxes indicate the regions with 100% identity in both genes but opposite orientation (B). The nucleotide sequence of BOKAS cDNA (Genbank accession no. FJ185168) demonstrate that it is a non-protein coding transcript. (C) The promoter region of BOKAS. Underlines represent transcriptional factor binding sites.

At least 100 green cells from each individual culture were analysed. Apoptosis is expressed as percentage of dying cells per total number of cells, given with the standard deviation of the assay (13).

Results and Discussion

BOKAS is a natural antisense transcript of the hBok gene. We have found several alternative splicing variants of hBok by RT-PCR (data not shown). Further screening of the GenBank™ ESTs database with the full length Bok nucleotide sequence led to isolation of a novel transcript that contains a 217-bp long sequence of Bok but in opposite orientation (Fig. 1A). The direction of transcription was examined through the PCR evaluation with primers spanned the intron-exon splice junctions (see Materials and methods). It was then termed BOKAS and considered as a natural antisense transcript of hBok gene (with GenBank accession no. FJ185168, Fig. 1B). The full-length BOKAS transcript has 855 nucleotides but does not contain a long open reading frame, giving 79 amino acids in conceptual translation, and we were not able to detect any signals using a coupled transcription-translation system (data not shown). Together, the results suggested that BOKAS is a non-protein coding transcript. Searching the BLAT:UCSC Human Genome Browser (Mar 2006 release) revealed that the BOKAS cDNA sequence matched exactly

to the genomic sequence in chromosome 2 and it contains 2 exons. Part of the first exon matched with 100% identity that of the Bok gene in opposite directions. The second exon located some 13 kb away from the 5'UTR of Bok. The polyadenylation signal (AATAAAA) was detected 30-bp upstream of the poly(A) (Fig. 1B). These results indicate that both BOKAS and Bok are transcribed from the same locus but in opposite orientations and that BOKAS represents a cis-natural antisense transcripts (Fig. 1A). Prediction of 750 bp in the promoter/enhancer region was preformed with program Matrix Search on upstream of the transcription start site (<http://rulai.cshl.edu/cgi-bin/TRED/tred.cgi?process=home>). Several transcription factor binding sites were predicted such as c-Fos and NF-κB binding elements (Fig. 1C). Strikingly, 28 AP-2 binding sites have been found with some overlapping by CREB, TEF-1 and cEBP.

BOKAS mRNA is differentially expressed in human tissues. We have used a semiquantitative PCR-based system to profile BOKAS mRNA expression in 24 different human organ tissues by using a pair of primers corresponding to sequences flanking to the two exons (Materials and methods). In contrast to the expression of *Bok* mRNA in colon, stomach, testis, placenta, pancreas, ovary and uterus (5), BOKAS expression could only be detected in the testis (Fig. 2). The study from the EST database revealed that BOKAS was also expressed

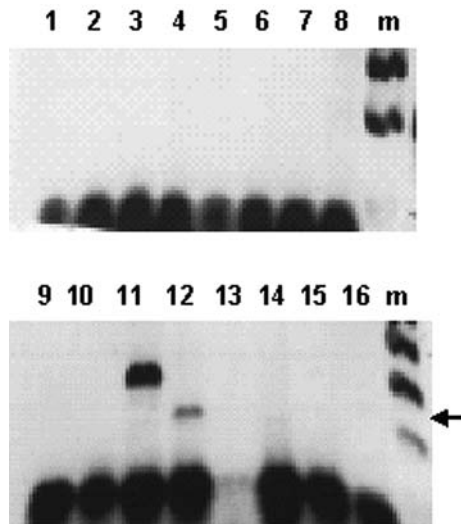


Figure 2. The expression of *BOKAS* mRNA in different tissues. PCR was performed on first strand cDNAs prepared from various human tissues (BD human multiple tissue cDNA panels). The numbers indicate tissues examined, 1 (heart), 2 (brain), 3 (placenta), 4 (lung), 5 (liver), 6 (muscle), 7 (kidney), 8 (pancreas), 9 (spleen), 10 (thymus), 11 (prostate), 12 (testis), 13 (ovary), 14 (small intestine), 15 (colon), 16 (peripheral blood leukocyte) and m (markers).

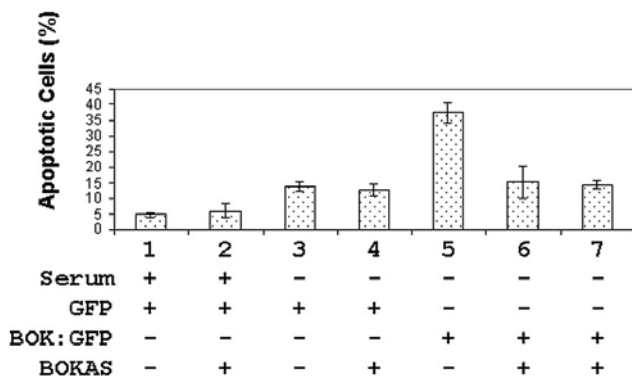


Figure 3. Suppression of Bok-induced apoptosis by BOKAS in HeLa cells. HeLa cells were cotransfected as pSPORT VI- BOKAS with plasmid pEGFP-C1 encoding GFP or pSPORT VI- BOKAS or pcDNA3-Bok:GFP carrying the genes for BOKAS together with Bok. After 48 h of serum deprivation, normal flat (survival) and round apoptotic cells were counted. Apoptosis is expressed (in %) as percentage of dying cells per total number of cells. Six is the transfectant with pcDNA3.Bok:GFP and pSPORT VI-BOKAS in the ratio of 1:1 μ g. Seven indicates the ratio of 0.5:1.5 μ g.

in fetal tissues and certain cancer cells, such as, fetal brain (EST AL534911, EST CR619343), and leiomyosarcoma uterus (BU184197). In addition, we also observed a longer PCR product in prostate. However, we could not confirm that the extra band corresponds to an alternative transcript since the sequence from the PCR product did not match to any known genomic sequences (data not shown) and sequences at the Chr.2 3q37.3 region have not yet been fully sequenced.

Overexpression of BOKAS reduced Bok-induced cell death. To assess the effect of BOKAS on Bok-induced apoptosis, we overexpressed ectopically BOKAS in HeLa cells. As shown in Fig. 3, expression of BOKAS alone exhibited no

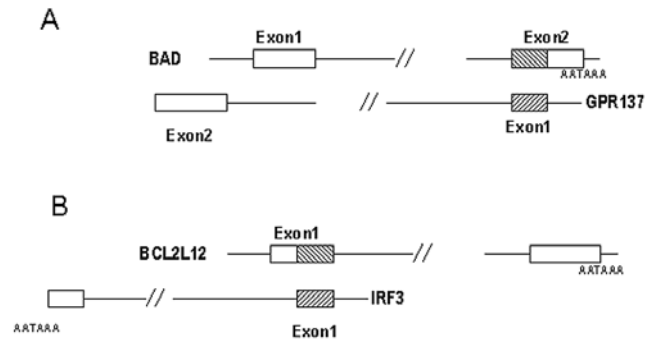


Figure 4. Natural antisense transcripts within the BCL2 family. Schematic representation of genomic organization of BAD in the relation with GPR137 (A) and BCL2L12 to interferon regulatory factor 3 (IRF3, B). Exon regions are denoted by boxes. The black horizontal lines denote the introns. Black boxes indicate the regions with 100% identity in both genes but opposite orientation.

significant anti- or pro-apoptotic activity when compared with control plasmids (pEGFP-C1). Forty-eight hours after transfection/serum deprivation, the number of apoptotic cells transfected with the control plasmid were 13% as compared to <5% of cells with serum in culture, whereas the expression of Bok:GFP encoding Bok-GFP fusion proteins (13) increased the number of apoptosis to ~40%, indicating that Bok expression sensitized cells to become apoptosis upon serum deprivation. The BOKAS construct transfected together with Bok reduced apoptotic cells close to 15%. These results indicated that BOKAS is a negative regulator of apoptosis induced by Bok.

Natural antisense transcripts within the BCL2 family. BOKAS is the first natural antisense transcript described in the Bcl2 family. The results prompted us to study *in silico* if any other natural antisense transcripts could be discovered in the Bcl2 family. Nucleotide sequences of full length and their alternative splicing variants were used to search the NCBI ESTs database, the results revealed that natural antisense transcripts could be identified for BAD and BCL2L12 (Fig. 4). BAD is gene encoding a BH3-only pro-apoptotic protein (14). Several EST clones (CN431725 from kidney cancer cells, BX415776 and CF541292) have been found to contain sequences of BAD 3'UTR in opposite directions. Sequence analysis revealed that these sequences code for putative G protein-couple receptor 137 with unknown function. BCL2L12 is an anti-apoptotic member of the family (15). Its exon I sequence matched in an opposite strand to numerous ESTs, such as DA559544 from heart and DB123802 from thymus. BLAST studies indicated that these clones are transcripts of interferon regulatory factor 3 (16). Thus 5' UTR of interferon regulatory factor 3 contains the natural antisense sequence matched to part of 5' UTR and encoding region of BCL2L12.

Taken together, all results describe a novel natural antisense transcript, BOKAS, being testis-specific and inhibiting Bok-induced apoptosis. Increasing numbers of natural antisense transcripts have been identified in various species (11,17). They play a potential role for gene regulation through several mechanisms. It remains to be determined whether BOKAS regulates expression of Bok via retention of Bok in the

nucleus by forming dsRNA duplexes or via degradation of Bok mRNA or through other mechanisms. Bok expression was detected in human, mouse and rat testis. The testis-specific expression of BOKAS would affect the known role of Bok during testicular development (5,18). Moreover, natural antisense transcripts were found in other members of the Bcl2 family, indicating that natural antisense transcripts may be regulatory factors in modulating apoptosis via the BCL2 family. Since they are present in cancer cells, it could be informative to examine whether upregulation of antisense transcripts in tumor cells would further enhance their malignancy.

Acknowledgements

We are grateful to The HUGO Gene Nomenclature Committee for providing the nomenclature of BOK antisense RNA (non-protein coding), BOKAS.

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