

Identification and characterization of a novel gene *Saf* transcribed from the opposite strand of *Fas*

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Apoptosis is a morphologically distinct form of cell death involved in many physiological and pathological processes. The regulation of *Fas/Apo-1* involved in membrane-mediated apoptosis has also been known to play crucial roles in many systems. More and more naturally occurring antisense RNAs are now known to regulate, at least in part, a growing number of eukaryotic genes. In this report, we describe the findings of a novel RNA transcribed from the opposite strand of the intron 1 of the human *Fas* gene. Using orientation-specific RT-PCR and northern blot analysis, we show that this transcript is 1.5 kb in length and was expressed in several human tissues and cell lines. This transcript was cloned by 5'- and 3'-RACE (rapid amplification of cDNA ends) and the transcription start site was determined by primer extension. This novel gene was named *Saf*. To assess the functions of *Saf*, Jurkat cells transfected with human *Saf* or control vector was prepared. The stable *Saf*-transfectant was highly resistant to *Fas*-mediated but not to TNF- α -mediated apoptosis. Although the overall mRNA expression level of *Fas* was not affected, expression of some novel forms of *Fas* transcripts was increased in *Saf*-transfectant, especially the inhibitory soluble forms. These findings collectively suggest that *Saf* might protect T lymphocytes from *Fas*-mediated apoptosis by blocking the binding of *FasL* or its agonistic *Fas* antibody. *Saf* might regulate the expression of *Fas* alternative splice forms through pre-mRNA processing.

INTRODUCTION

Fas (Apo-1/CD95) is a receptor belonging to the tumor necrosis factor (TNF) and nerve growth factor receptor family. The human *Fas* gene consists of nine exons and is transcribed from chromosome 10q24.1. Once *Fas* is triggered by agonistic antibodies or its natural ligand (*FasL*), it induces apoptosis via direct and sequential activation of caspases. *Fas* is physiologically expressed in various tissues such as spleen, lymph nodes and on mature hematologic cells such as T- and B-cells, natural killer cells, monocytes and granulocytes (1). Thus, in both the central and the peripheral immune compartments, apoptosis plays an essential role in maintenance of tolerance and in the termination of an ongoing immune response (2). Deregulation of apoptosis contributes to many diseases including autoimmunity, cancer, stroke, drug resistance in tumors

and the acquired immunodeficiency syndrome (3). *Fas*-mediated apoptosis can be blocked by several mechanisms: (i) production of secreted form of soluble *Fas*, (ii) lack of cell-surface *Fas* expression, (iii) overexpression of inhibitory proteins in signal transduction pathways such as *Fas* associated phosphatase and FLICE-inhibitory protein and (iv) alterations of the primary structure of *Fas* due to mutations (4,5). In this study, we identified a novel endogenous antisense transcript of *Fas* and analyzed its role in the modulation of *Fas*-mediated apoptosis.

A growing number of endogenous antisense RNAs have been identified in many eukaryotic organisms over the last several years (6–8). Antisense transcripts often code for proteins involved in diverse biological functions. Non-coding antisense transcripts have also been reported. Their role appears to be mainly regulatory (9) and their transcription is often associated

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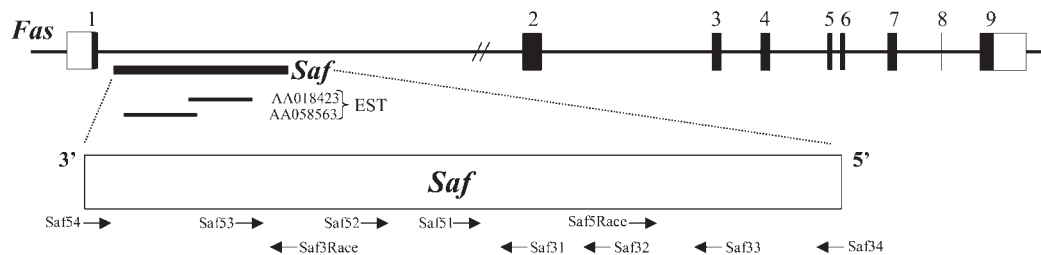


Figure 1. Genomic organization of human *Fas* and *Saf*. *Saf* is transcribed from the *Fas* intron 1 region but in the reverse orientation. The boxes represent the exons of *Fas* with coding regions in black. The thick black line is *Saf* with the two overlapping ESTs (GenBank accession nos AA018423 and AA058563) indicated underneath. The primers are shown in their respective positions and orientations (arrows).

with genomic imprinting (10). Although the effect of antisense RNAs on the corresponding sense RNAs are as yet mostly unclear, growing evidences have indicated an involvement in gene regulation (11). Naturally occurring long antisense RNAs described so far in the literature are *cis*-encoded, meaning that they are transcribed from the opposite strand and correspond to the complete or partial hnRNA sequence of the gene (12). In a small percentage of cases, the transcript initiates within an intron of its counterpart gene or entirely covers its counterpart, for example, *c-erba* (13). Mechanisms proposed for the regulation of gene expression by antisense RNA are numerous, and the discovery of novel sense/antisense RNA interactions would be insightful in understanding this mechanism of gene regulation.

Our unpublished data unexpectedly led to the discovery of an endogenous antisense RNA transcript of *Fas* in the human Jurkat T cell. This novel gene is named *Saf*. The aim of our study was to identify and characterize this novel antisense transcript. Our data showed that this transcript was 1.5 kb in length and expressed in the heart, placenta, liver, muscle, kidney and pancreas. In addition, apoptosis induced by the Fas agonistic antibody, CH11, was attenuated by *Saf* expression. These findings demonstrate that *Saf*, which is transcribed from the strand opposite to *Fas*, might play an important role in the regulation of the *Fas* gene expression and Fas-mediated apoptosis.

RESULTS

Identification of the novel antisense transcript

On the basis of the genomic sequence information corresponding to the human *Fas*, we found two entries in the database of human expressed sequence tags (ESTs) (GenBank accession nos AA018423 and AA058563) that had a transcription orientation opposite to that of *Fas* (Fig. 1). Sequence analysis was performed using the BLAST search algorithm through the NCBI WWW site. These two overlapping EST sequences are continuous and located within intron 1 of the *Fas* gene. This novel antisense gene was designated as *Saf*. Strand-specific RT-PCR was performed to verify the presence of the *Saf* transcript. It was found that the expected product of 415 bp was generated only when the reverse transcription was primed with the Saf53 primer (Fig. 2A) but not with the Saf33 primer (Fig. 2B). Taken together, these results confirmed the presence of the *Saf* transcript that was antisense to *Fas*.

Characterization of the *Saf* antisense transcript

RACE technique was used to clone both the 5' and the 3' ends of the *Saf* transcript from human placenta RNA. Gel analysis of the RACE products revealed one single band with 3'-RACE and several bands with 5'-RACE (Fig. 3A, upper panel). Southern blot with specific Dig-labeled probe generated by PCR using the Saf53/Saf33 primer pair revealed a specific band of 500 bp with 3'-RACE and a specific band of 550 bp with 5'-RACE (Fig. 3A, lower panel). Transcription start site of *Saf* was determined by primer extension. Three extension products from Jurkat cells were observed with primer Saf5Race (Fig. 3B, lane 4). Three possible transcription start sites at positions 350, 450 and 550 bp upstream of the Saf5Race primer were then determined. The longest 550 bp product was compatible with the 5'-RACE-PCR result implicating that the transcription start site was at position 550 bp upstream of the primer Saf5Race site. On the basis of the EST information and the RACE results, this *Saf* transcript was estimated to be 1.5 kb in length.

The 3'-RACE-PCR and 5'-RACE-PCR products were cloned and sequenced to obtain the full-length transcript sequences. It turned out that the full-length *Saf* antisense transcript was 1556 bp long and was entirely originated from intron 1 of the *Fas* gene. *Fas* and *Saf* are transcribed from the same locus but in opposite orientations (Fig. 1). After analysis with program TSSW (<http://www.softberry.com>), one promoter was predicted upstream of the transcription start site. Six transcription factor binding sites were predicted including one TATA box, two GATA-2 boxes, one MZF1 element, one SRY (testis determining factor binding) element and one c/EBP β element. The 3' end of the *Saf* transcript was located 23 bp downstream of the poly(A) signal (AATAAA) (Fig. 4). The *Saf* cDNA sequence exactly matched the genomic sequence, indicating that *Saf* is an intronless gene. In addition, the largest open reading frame predicted in this transcript was only 120 bp long, and we could not detect any signals using the coupled transcription-translation system (data not shown). Therefore, it suggests that *Saf* was not a protein-coding gene.

Northern blot analysis was used to examine the *Saf* expression in different tissues. A major transcript of 1.5 kb was detected (Fig. 5A). *Saf* was expressed in human heart, placenta, liver, muscle and pancreas. It was only slightly expressed in kidney and undetectable in brain and lung. As for cancer cell lines, *Saf* was expressed in Jurkat (acute T-cell leukemia), MES-SA (uterine carcinoma) and U-937 (Histocytic

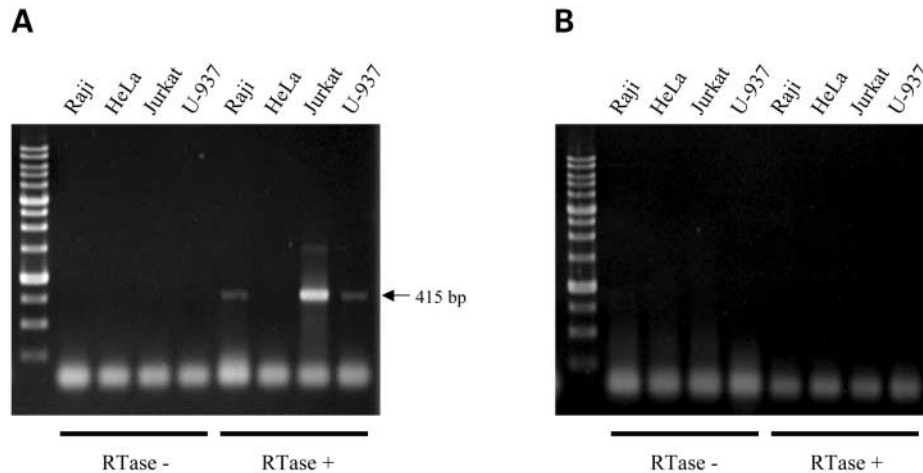


Figure 2. Strand-specific RT-PCR of *Saf*. Raji, HeLa, Jurkat and U-937 cells were used for the strand-specific RT-PCR analysis. cDNAs were synthesized from total RNA by using *Saf*-specific antisense primer Saf53 (A) or the sense primer Saf33 (B). cDNA was then amplified with the primer pair Saf52/Saf32 (RTase+). Negative controls of RT-PCR without reverse transcriptase (RTase-) were also included.

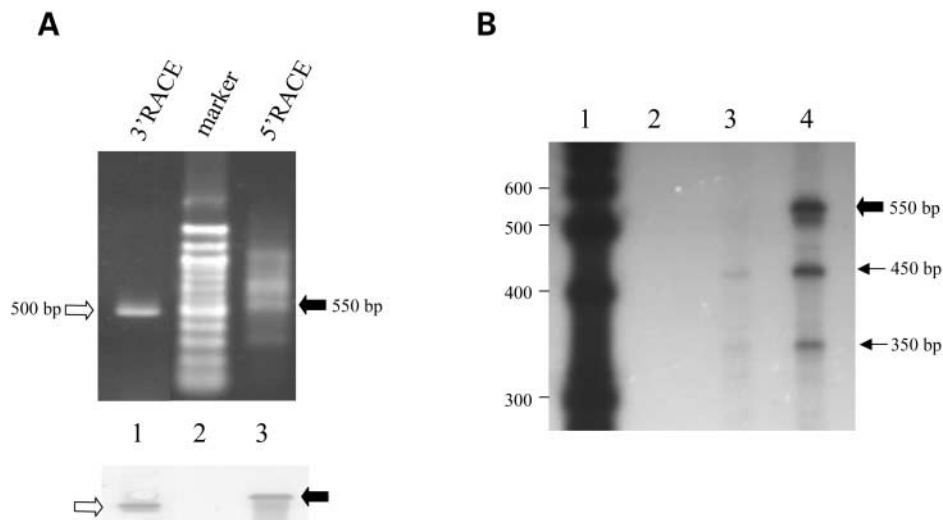


Figure 3. RACE-PCR and RNA primer extension. (A) Electrophoresis of the *Saf* RACE-PCR product (upper panel). RNA from human placenta was used for 5'-RACE and 3'-RACE with the primer Saf5Race and Saf3Race, respectively. Southern blot of the RACE-PCR products was performed with specific *Saf* probe generated by PCR (lower panel). Lane 1, 3'-RACE-PCR products; lane 2, marker and lane 3, 5'-RACE-PCR products. (B) Determination of the transcription start site by primer extension. The transcription start sites of *Saf* were mapped with Saf5Race primer using total RNA of HeLa and Jurkat cells. Three major primer extension products are indicated by black arrows. Lane 1, end-labeled 100 bp marker ladder; lane 2, yeast tRNA; lane 3, HeLa cells and lane 4, Jurkat cells.

Lymphoma); moderately expressed in CA46 (Burkitt's lymphoma), Raji (Burkitt's lymphoma) and only slightly expressed in MG-63 (osteosarcoma) (Fig. 5B). The expression of *Fas* was also examined. It was expressed in the heart, liver and Jurkat cells; moderately expressed in brain, MES-SA cells and MG-63 cells; slightly in placenta, kidney, U-937, A-431 and MDA-MD-453 cells (breast carcinoma).

Effect of *Saf* overexpression on the expression of *Fas*

To study the potential regulation of the *Fas* gene expression by the antisense *Saf*, overexpression of *Saf* was performed in Jurkat cells. The RNA expression levels of *Fas* in parental

Jurkat cells, vector-transfectant and *Saf*-transfectant were determined by quantitative, real-time RT-PCR. The overall *Fas* expression was not significantly affected in the *Saf*-transfectant in comparison to the parental Jurkat cells and the vector-transfectant. *Saf* expression in the *Saf*-transfectant was 1.9-fold that of the parental Jurkat and the vector-transfectant cells (Fig. 6).

Overexpression of *Saf* protects cells from Fas-mediated apoptosis

To examine the effect of *Saf* overexpression on the sensitivity of Jurkat cells to Fas-mediated apoptosis and to check whether

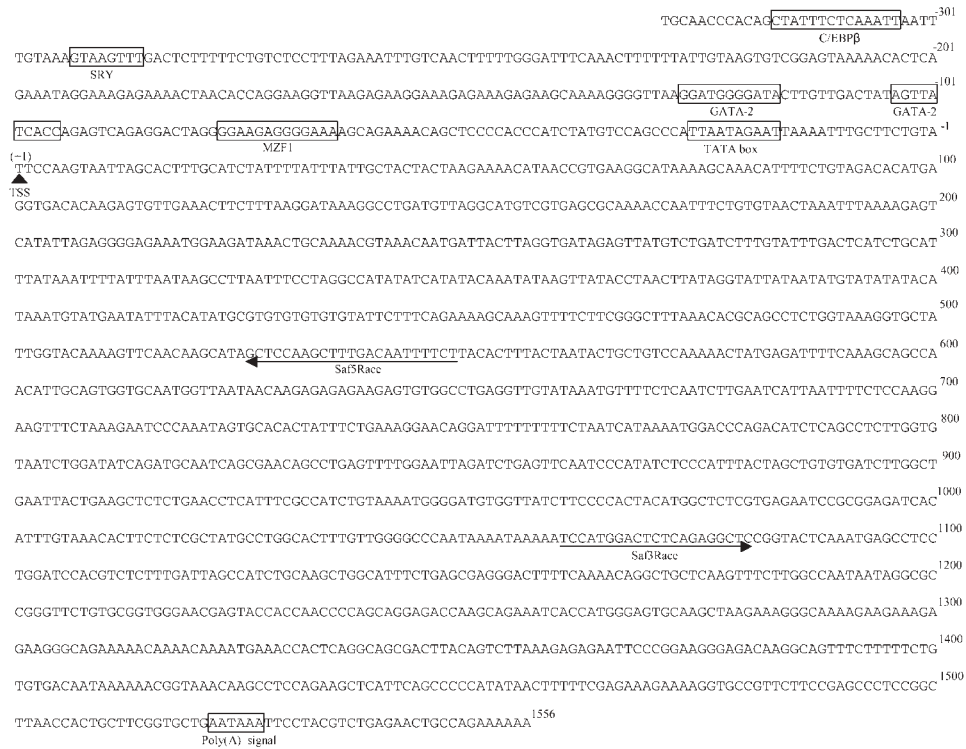


Figure 4. Nucleotide sequence of the human *Saf*. Nucleotide sequence is deduced from the full-length cDNA clone derived from the human placenta RNA. Potential transcription factor binding sites and poly(A) signal (AATAAA) are indicated (open box). TSS (transcription start site) is indicated by arrowhead. The promoter region of *Saf* contains one c/EBPβ (CCAAT-enhancer binding protein) element, one TATA box, two GATA-2 boxes, one MZF1 (myeloid-specific retinoic acid-responsive) element and one SRY (testis determining factor binding) element. The primers used in 5'-RACE (Saf5RACE) and 3'-RACE (Saf3RACE) are also indicated. The promoter region was predicted with the TSSW Program (<http://www.softberry.com>), which also successfully predicted the transcription start site of *Saf*.

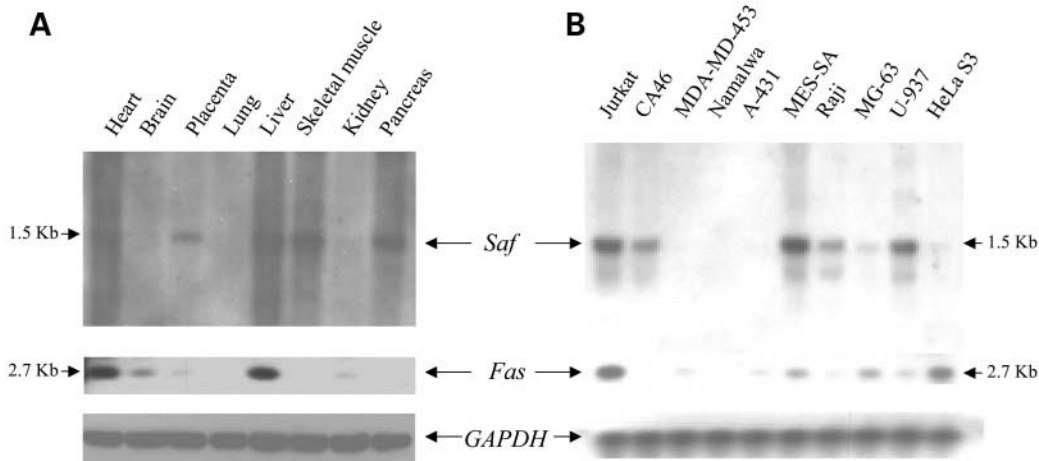


Figure 5. RNA expression of *Saf* and *Fas* in various tissues and cell lines. (A) Expression of *Saf* and *Fas* on multiple tissue northern blot (MTN blots, Clontech) containing 2 μg poly(A)⁺ RNA per lane. (B) Expression of *Saf* and *Fas* on multiple cancer cell northern blot (Human blot 3, Ambion) containing 2 μg poly(A)⁺ RNA per lane. The probes used were single-stranded *Saf* specific riboprobe (upper panel) and *Fas* specific riboprobe (middle panel), respectively. Hybridization with the control *GAPDH* riboprobe confirmed equal amount of RNA loaded in each lane. The same blot was used consecutively for all three probes.

Saf could protect cells from apoptosis induced by other ligands, we treated cells with the agonistic antibody CH11 (100 ng/ml), FasL (40 ng/ml) and TNF-α (100 ng/ml). Apoptotic cells were measured by using FITC-conjugated annexin V staining and flow cytometry. In the parental Jurkat cells

24 h after treatment by CH11, although the majority of cells have undergone apoptosis (86%), apoptosis was significantly attenuated in the *Saf*-transfectant (38%). Without the addition of CH11, both the parental Jurkat and the *Saf*-transfectant showed only basal levels of apoptosis (Fig. 7A). Although

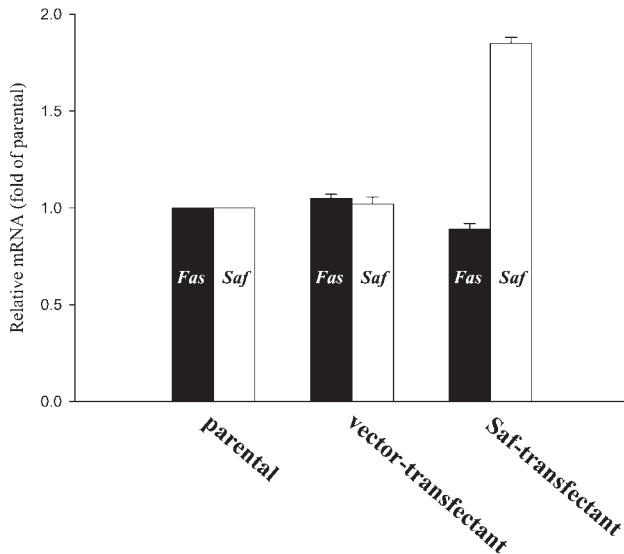


Figure 6. Effect of *Saf* overexpression on *Fas* expression. Relative levels of *Fas* (black bar) and *Saf* (white bar) transcripts were determined using real-time quantitative RT-PCR as described in Materials and Methods. cDNA was synthesized from 1 µg total RNA of parental Jurkat cells, vector-transfectant and *Saf*-transfectant. Aliquots of cDNA were used as template for quantitative PCR reactions containing primers for *Fas* (FasQ1/FasQ2), *Saf* (Saf51/Saf31) and *GAPDH* (GAPDHQ1/GAPDHQ2). Each reaction contained cDNA derived from 10 ng total RNA. Five replicates of each reaction were performed. The fold change in gene expression (*Fas* and *Saf*) of vector-transfectant and *Saf*-transfectant relative to the parental Jurkat cells was determined after normalization with the internal control gene (*GAPDH*).

the apoptotic effect exerted by FasL was almost identical to that by CH11, however, TNF- α -induced apoptosis was not protected by *Saf* (Fig. 7A), indicating that the effects of *Saf* is Fas-dependent. The CH11-induced apoptosis was further confirmed by the activation of caspase 8 and the cleavage of the poly ADP-ribose polymerase (PARP) protein. Although caspase 8 was fully activated and PARP was completely cleaved in the parental Jurkat and the vector-transfectant cells by CH11, caspase 8 activation and PARP cleavage were significantly attenuated in the *Saf*-transfectant (Fig. 7B).

Effect of *Saf* overexpression on expression of the *Fas* alternative splice forms

After analysis with AceView program (<http://www.ncbi.nlm.nih.gov/AceView/>), several *Fas* alternative splice forms retaining the *Fas* intron 1 sequences were found (Fig. 8A). RT-PCR was designed to verify the presence of these alternative splice forms of *Fas* (Fig. 8A). As shown in Figure 8B, by using various primer pairs, we confirmed the existence of these forms with expected sizes of 182, 353, 600 and 491 bp (*Fas* splice forms b, c, d and e, respectively). Upon alignment with the *Saf* sequence, the four transcript variants of *Fas* are found to overlap with *Saf* by 48, 88, 105 and 105 bp, respectively (Fig. 8A). Although the expression of b and c forms of *Fas* was not affected by *Saf*, expression of the other two *Fas* variants d and e forms was significantly elevated (Fig. 8B and C). It is noteworthy that the latter forms of *Fas* are both

inhibitory soluble Fas, and the two former forms of *Fas* are both membranous Fas. When focusing on the *Fas* transcripts that contain exon 5 and exon 9, the membrane form (367 bp) and the soluble form (304 bp, lacking exon 6) can also be distinguished with the Fas5/Fas6 primer pair. As a result, we found that these two forms of *Fas* transcript were expressed equally in the parental Jurkat cells and the vector-transfectant, but in the *Saf*-transfectant, the membrane form was repressed and the soluble form was increased (Fig. 8B).

DISCUSSION

In this study, we describe the findings of an endogenous transcript that is antisense to the *Fas* transcript. This antisense gene, named *Saf*, codes for an RNA that is 1556 bp in length. The *Saf* gene resides entirely in the 12.1 kb intron 1 of the *Fas* gene and is transcribed in the opposite direction. The *Saf* transcript ends at 521 bp downstream of the *Fas* exon 1. A probable functional TATA box is located upstream of the *Saf* transcription start site. Furthermore, in contrast to the parental Jurkat and vector-transfectant cells, the stable *Saf*-transfectant was highly resistant to Fas-mediated apoptosis. In order to check whether *Saf* could protect cells from apoptosis induced by other ligands, we also treated cells with TNF- α and FasL. For FasL-induced apoptosis, *Saf* could also exert its protection effect, however, TNF- α -induced apoptosis was not protected (Fig. 7A). These findings strongly suggest that Fas-mediated apoptosis in T lymphocytes was inhibited by *Saf*, and *Saf*'s protection effect was Fas/FasL-pathway specific.

Fas-mediated apoptosis is an important mechanism of immune homeostasis (14). Dysregulation of the Fas/FasL system leads to severe diseases (15,16). Our discovery of this antisense RNA may represent another level of regulation of the Fas system (17). However, the *Fas*'s exon/intron structure of human, chimpanzee and mouse are all strikingly similar, especially the presence of a relatively long intron 1, whereas sequences of the murine's intron 1 have no homology with those of the primate's. Interestingly, human and chimpanzee share high degree of similarity in intron 1 sequences (99% nucleotide identity in the whole 11.5 kb overlapping region) including the entire *Saf* region. Thus, this *Saf* gene appears to be conserved only among the primates and is absent in the rodents. The appearance of the *Saf* gene may, therefore, represent a relatively recent event in evolution.

Short endogenous antisense RNAs are generally *trans*-acting and produced from a locus different from that of their corresponding genes, whereas the transcription of long antisense RNA (generally *cis*-acting) often occurs in the same locus as the sense RNA (18). Our data showed that the *Saf* transcript did not code for proteins and the *Saf* gene is transcribed from *Fas* intron 1 in the opposite direction. Therefore, we propose that this transcript possibly belongs to the category of long antisense RNA and functional non-coding RNAs (8). Totally intronic antisense transcripts would have to target their complementary intronic sense message (in pre-mRNA) to play a regulatory role in processing protein-encoding RNA messages (19). We speculate that

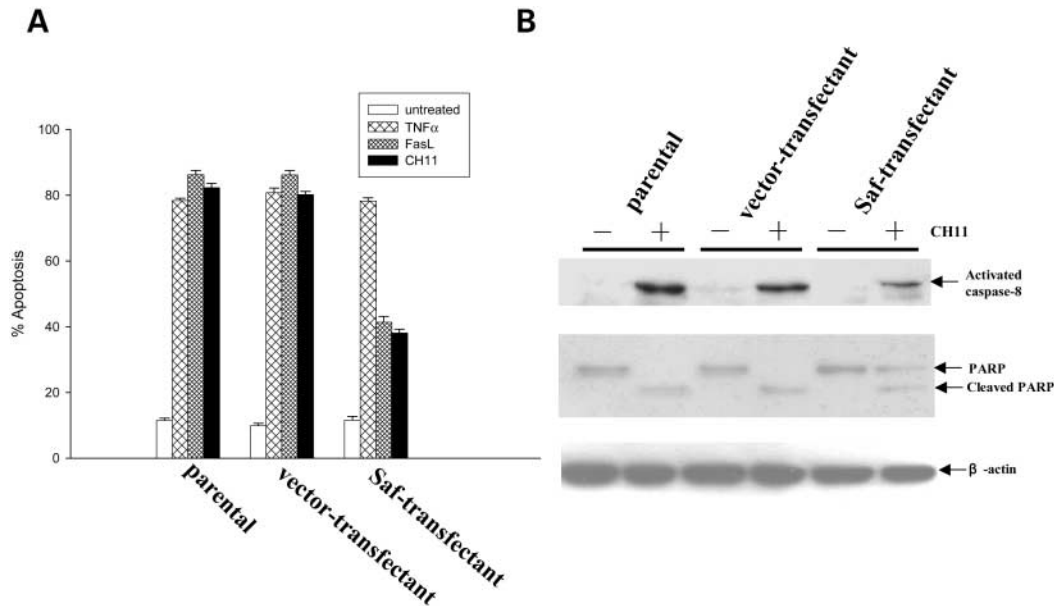


Figure 7. Protection of Saf-transfectant against Fas-mediated apoptosis. (A) Parental Jurkat cells, vector-transfectant and Saf-transfectant were treated with or without TNF- α (100 ng/ml), FasL (40 ng/ml) and the agonistic anti-Fas Ab (CH11, 100 ng/ml) for 24 h. Apoptotic cells were detected by annexin V-FITC staining and flow cytometry. Data were expressed as mean % apoptosis \pm SEM. (B) Inhibition of the CH11-induced activation of caspase 8 and cleavage of PARP by *Saf*. The parental Jurkat, vector-transfectant and Saf-transfectant cells were incubated with or without CH11 (100 ng/ml) for 24 h. At harvest, the cell lysates were analyzed by western blot using monoclonal antibodies against caspase 8, PARP and β -actin.

sense/antisense intronic transcript pairs may be part of a general regulatory mechanism of gene expression not yet fully understood in eukaryotes. *Cis*-acting and *trans*-acting antisense RNAs overlapping with corresponding sense RNAs have been reported for several eukaryotic genes, and they have been implicated in imprinting, alternative splicing regulation, post-transcriptional and translational regulation (20). As reported here, the possibility exists that the *Fas* gene may be regulated by an antisense mechanism. At least four alternative splice forms of *Fas* that contain intron 1 sequences overlapping with *Saf* were found in Jurkat cells. Among them, the two soluble forms (d and e) were upregulated by *Saf* and the two membrane forms (b and c) were not affected, resulting in an increased ratio of soluble forms to membrane forms (Fig. 8). As the *Saf*-overlapping regions of forms b and c are different from those of forms d and e, it is possible that either the length or the nature of the overlapping sequences or both are responsible for the difference. It is also worth to note that the 3'-extremity of the *Saf* transcript corresponds to the 5'-extremity of the d and e forms of the *Fas* transcript. To investigate the possible involvement of these overlapping sequences in the regulation of *Saf* on *Fas*, next we may plan to construct various expression vectors of *Saf* that have mutations in these overlapping regions to see how these mutations may affect the effects of *Saf*. The *Saf* transcript might modulate the splicing of the *Fas* pre-mRNA by the formation of a dsRNA duplex that favors or disfavors the production of these alternative splice forms. It is possible that these alternative splice forms might play crucial roles in modulating the functions of Fas, for example, soluble forms of Fas that neutralize FasL and/or dominant negative forms of Fas that lack the death domain (Fig. 8).

It is, therefore, apparent that intronic sequences may be of functional significance, as has been noted in the mechanism of antisense transcript at the *N-myc* locus and others (21–23) as well as the antisense *Saf* reported here.

More and more naturally occurring antisense RNAs are being discovered, and researchers have been engineering and expressing antisense transcripts as a means to control gene expression (24,25). Several research groups have been using libraries of genomic fragments to express large numbers of diverse antisense RNAs (26–28). In eukaryotes, there are now several examples where expressed microRNA (miRNA) can repress translation in an RNA interference independent manner (29). Hopefully, with more and more understanding of the antisense RNA structures, functions and their regulations, endogenous antisense RNA genes could potentially serve as a target for therapeutic applications. Splice variants have been associated with a variety of cancers and genetic diseases such as cystic fibrosis and thalassemia. The use of antisense oligonucleotides to modulate alternative splicing has been suggested to be a promising RNA repair-based approach to engender the production of therapeutic gene products (30).

It is apparent from the earlier discussion that although many examples of naturally occurring transcripts have been reported to date, no general rule of antisense RNA in the regulation of gene expression has been firmly established. We propose that the *Saf* transcript might form duplexes with the *Fas* pre-mRNA in the intron 1 regions and hence modulate gene expression at the RNA splicing level. We will be focusing on those alternative forms of *Fas* transcripts that have sequences overlapping with the *Saf* transcript, especially the first exon of the d and e forms of *Fas* transcripts.

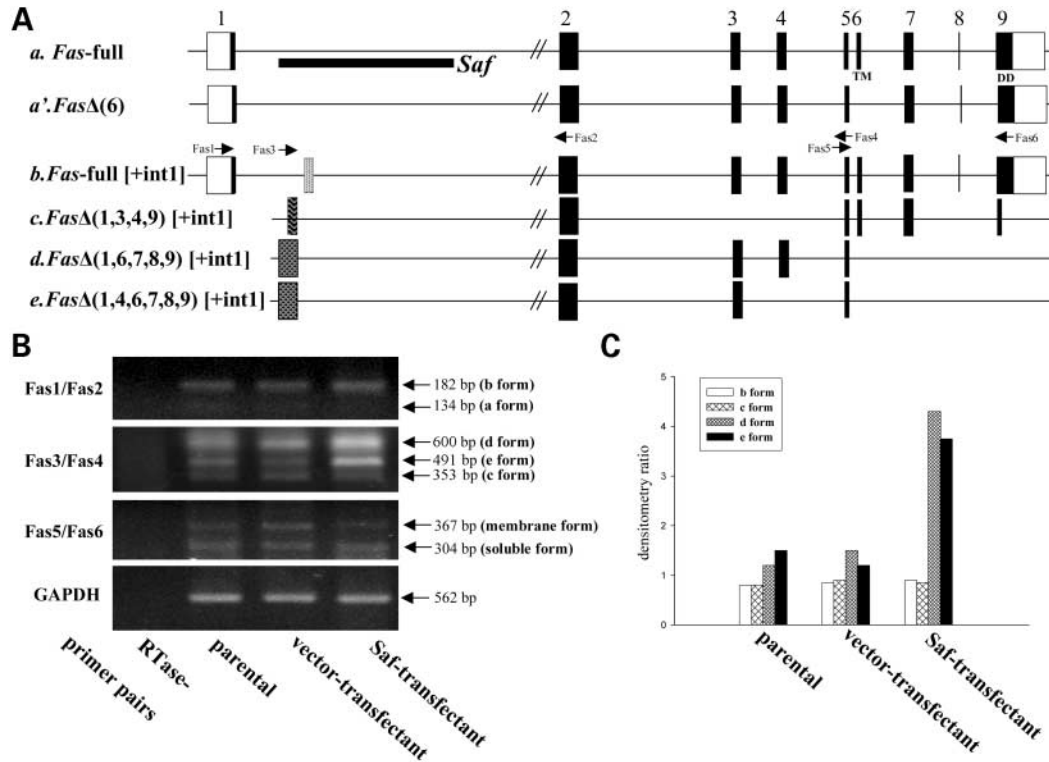


Figure 8. Effects of *Saf* overexpression on the alternative splice forms of *Fas*. (A) Exon–intron structure and alternative splice forms at the *Fas/Saf* locus. The boxes and thin lines represent *Fas* exons and introns, respectively. TM, transmembrane domain and DD, intracellular death domain. The thick line represents *Saf* at the bottom in black. The hatched areas indicate the parts of intron 1 sequences that are retained in the four splice forms (b, c, d and e) of *Fas* and these four variants overlap with *Saf* by 48, 88, 105 and 105 bp, respectively. The primers are shown in their respective positions and orientations (arrows). (B) RT–PCR with specific primer pairs was used to identify *Fas* variant transcripts expressed in parental Jurkat cells, vector-transfectant and *Saf*-transfectant. Transcripts of a/b forms, c/d/e forms, membrane forms, soluble forms and *GAPDH* were PCR amplified with primer pairs of Fas1/Fas2, Fas3/Fas4, Fas5/Fas6 and GAPDH1/GAPDH2, respectively. Reverse transcriptase negative controls (RTase–) for each sample were also included. (C) Quantification of *Fas* b/c/d/e forms by densitometry. The densitometry ratio changes in the expression of the b/c/d/e forms of parental Jurkat, vector-transfectant and *Saf*-transfectant cells were determined after normalization with *GAPDH*.

MATERIALS AND METHODS

Cell line

The human cell lines Jurkat (acute T cell leukemia), Raji (Burkitt's lymphoma), Hela (cervical adenocarcinoma) and U-937 (myeloid leukemia) cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (Gibco BRL, Rockville, MD, USA), 2 μ M glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin at 37°C in a humidified incubator with 5% CO₂.

RNA extraction

Total cellular RNA of cultured cells was isolated with the cytoplasmic RNA purification reagent ConcertTM (Invitrogen, Carlsbad, CA, USA). Total RNA was treated with DNase I (Ambion, Austin, TX, USA) for 30 min at 37°C to remove any contaminated genomic DNA before RT–PCR. The quality and quantity of the RNA preparations were tested by agarose gel electrophoresis and measurement of absorption at 260 and 280 nm. The ratio of optical density at 260 and 280 nm was ≥ 1.8 in all cases.

Strand-specific RT–PCR

First-strand cDNA was synthesized from 1 μ g total RNA at 50°C for 50 min with either sense (Saf33) or antisense (Saf53) primer by Superscript II reverse transcriptase (Gibco BRL), and then amplified with primers Saf52 and Saf32 by using Platinum *Taq* polymerase (Invitrogen). Conditions for PCR were 1 min each at 94, 55 and 72°C for 35 cycles. The designed primer Saf53 would prime cDNA synthesis only from the *Saf* transcript but not the *Fas* transcript.

Identification of *Fas* transcriptional variants by RT–PCR

cDNAs were synthesized from total RNA of parental Jurkat cells, vector-transfectant and *Saf*-transfectant, then amplified with specific primer pairs of Fas1/Fas2, Fas3/Fas4, Fas5/Fas6 and GAPDH1/GAPDH2, respectively. Reverse transcriptase negative controls (RTase–) were also included. The RT–PCR products were examined with agarose gel electrophoresis.

Table 1. Sequences of the primers

Primer	Sequence
Saf51	5'-ATG GGA GAT ATG GGA TTG AAC-3'
Saf52	5'-TCT CCG CGG ATT CTC ACG-3'
Saf53	5'-CTG TAA GTC GCT GCC TGA GTG G-3'
Saf54	5'-CGG AGG GCT CGG AAG AA-3'
Saf5Race	5'-AGA AAA TTG TCA AAG CTT GGA GC-3'
Saf31	5'-CAT CTC AGC CTC TTG GTG TAA-3'
Saf32	5'-GAG ATT TTC AAA GCA GCC AAC AT-3'
Saf33	5'-CTC TGG TAA AGG TGC TAT TGG TA-3'
Saf34	5'-ACT AAC ACC AGG AAG GTT GAG-3'
Saf3Race	5'-TCC ATG GAC TCT CAG AGG CTC-3'
Fas1	5'-ACG GAG TTG GGG AAG CTC TT-3'
Fas2	5'-TGT CAG TCA CTT GGG CAT TAA CA-3'
Fas3	5'-CGG AGG GCT CGG AAG AAC G-3'
Fas4	5'-TGG TGA GTG TGC ATT CCT TGA T-3'
Fas5	5'-TCA TAC AGG AAT GCA CAC TC-3'
Fas6	5'-ACT TTC TGT TCT GCT TGT TC-3'
FasQ1	5'-ATG CTG GGC ATC TGG ACC CTC CTA-3'
FasQ2	5'-AGT CTT AAA GAG AGA ATT CCC GGA-3'
GAPDH1	5'-GCA CCG TCA AGG CTG AGA A-3'
GAPDH2	5'-AGG TCC ACC ACT GAC ACG TTG-3'
GAPDHQ1	5'-TGG CGC TGA GTA CGT CGT G-3'
GAPDHQ2	5'-ATG GCA TGG ACT GTG GTC AT-3'

Rapid amplification of cDNA ends

RACE was performed by using a SMART RACE cDNA amplification kit (Clontech, Palo Alto, CA, USA) with 1 µg total RNA as the template according to the manufacturer's instructions. Gene-specific primers used in the 5'- and 3'-RACE reactions were Saf5Race and Saf3Race, respectively (Fig. 1 and Table 1). The RACE-PCR products were analyzed by agarose gel electrophoresis and Southern blotting. The products were also cloned into the pcDNA3.1-TOPO vector (Invitrogen).

Southern blot analysis

The DNA probes were synthesized by the incorporation of digoxigenin-11-dUTP during PCR using Saf52 and Saf32 as primers (Fig. 1 and Table 1). The RACE-PCR products were separated by gel electrophoresis and detected by the DIG non-radioactive detection system (Roche, Mannheim, Germany).

Primer extension reaction

Primer extension technique was used to determine the transcription start site of *Saf*. Reaction was carried out with 50 µg of total RNA from the HeLa cells or Jurkat cells with Saf5Race primer that is complementary to the 5' portion of *Saf* mRNA. The primer was ³²P end-labeled using T4 polynucleotide kinase and hybridized with total RNA in buffer containing 10 mM PIPES, 200 mM NaCl, 1 mM dithiothreitol pH 6.4 and 1 mM dNTPs. It was extended with 400 U MMLV reverse transcriptase (Gibco BRL) for 120 min at 50°C. After termination of the reaction, the reaction products were size fractionated by denaturing the polyacrylamide gel electrophoresis.

Northern blot analysis

To investigate the tissue distribution of *Saf* expression, northern blotting was performed. The *Saf* riboprobe used was generated by amplifying an 868 bp fragment from the human *Saf* cDNA with Saf33 and Saf53 and subcloning of the PCR product into the pcDNA3.1-TOPO vector. Antisense digoxigenin-labeled *Saf* RNA probe was transcribed from the T7 promoter with DIG RNA labeling kit (Roche). This strand-specific riboprobe was used to hybridize with a Human Multiple Tissue mRNA Blot 7760-1 (Clontech) and FirstChoice™ Human Blot 3 (Ambion) at 60°C overnight. Detection of RNA was performed with the DIG non-radioactive detection system (Roche). The blot was then washed twice with 2 × SSC/0.1% SDS for 45 min, followed by two washes with 0.1 × SSC/0.1% SDS at room temperature for 20 min. The RNA hybrids were detected by incubation with an anti-DIG/alkaline phosphatase conjugate and a subsequent chemiluminescence reaction with CSPD and then exposed to X-ray film. The membranes were stripped and reprobed with human *Fas* and *GAPDH*.

Construction of recombinant plasmids and transfection into Jurkat cells

Single-stranded *Saf* cDNA was synthesized using total RNA from normal placental tissue. Primers Saf54 and Saf34 (Fig. 1 and Table 1) were used in PCR to amplify *Saf* cDNA. The amplified 1681 bp *Saf* cDNA fragment was ligated into the pcDNA3.1-TOPO vector. The amplified *Saf* PCR product was DNA sequence verified. Lipofectamine 2000 (Invitrogen) was used to introduce the *Saf* expression vector into Jurkat cells. After 16 h of incubation, the cells were selected in complete medium containing 0.5 mg/ml G418 (Gibco BRL). Selected transfectants are cloned and maintained in medium containing 0.5 mg/ml G418 and screened for the expression of *Saf* mRNA by northern blot analysis. Jurkat cells were also transfected with pcDNA3.1-TOPO vector alone and similarly selected for G418 resistance to serve as an experimental control.

Real-time quantitative PCR

The primers used for analysis of *Fas* (FasQ1/FasQ2), *Saf* (Saf51/Saf31) and *GAPDH* (GAPDHQ1/GAPDHQ2) gene expression were shown in Table 1. The PCR reactions were performed in a LightCycler apparatus using LC-Fast Start Reaction Mix SYBR Green I (Roche). Thermocycling was performed in a final volume of 10 µl containing 1.5 µl of cDNA sample; 3 mM MgCl₂; 0.5 µM each of the *Fas*, *Saf* and *GAPDH* primers; 1 µl of LC-Fast Start Reaction Mix SYBR Green I and 1 µl of LC-Fast Start DNA Master SYBR Green I/Enzyme (containing *Taq* DNA polymerase, reaction buffer and deoxyribonucleotide triphosphate mixture). After 10 min at 95°C to denature the cDNA and to activate the *Taq* DNA polymerase, the cycling conditions were 40 cycles consisting of denaturation at 95°C for 15 s, annealing at 55°C for 15 s and extension at 72°C for 18 s. After PCR, a melting curve was constructed by increasing the temperature from 65 to 95°C with a temperature transition

rate of 0.1°C/s. To ensure that the correct product was amplified in the reaction, all samples were examined by 2% agarose gel electrophoresis. The LightCycler apparatus measured the fluorescence at every cycle at the end of the annealing step. The second derivative maximum method was used to automatically determine the crossing point (Cp). This was achieved by a LightCycler software algorithm (version 3.5.3) that identified the first turning point of the fluorescence curve, corresponding to the first maximum of the second derivative curve, which served as the Cp (31). The average Cp value of the *GAPDH* gene was subtracted from the average Cp value of the interest genes for each sample/primer combination. Five replicates of each reaction were performed. $Fas\Delta Cp = (Avg. Fas Cp - Avg. GAPDH Cp)$, $Saf\Delta Cp = (Avg. Saf Cp - Avg. GAPDH Cp)$, $Fas\Delta\Delta Cp = (Avg. Fas\Delta Cp - Avg. Fas\Delta Cp_{Parental Jurkat})$, $Saf\Delta\Delta Cp = (Avg. Saf\Delta Cp - Avg. Saf\Delta Cp_{Parental Jurkat})$. The fold change in the expression of the target genes (*Fas* and *Saf*) relative to the internal control gene (*GAPDH*) of various cells was calculated.

Apoptosis induction and measurement

For the induction of apoptosis, Jurkat cells with *Saf* expression vector or pcDNA 3.1-TOPO vector were cultured in six-well plates. Samples were treated with or without the following reagents: human recombinant TNF- α (100 ng/ml; Sigma, St Louis, MO, USA), soluble recombinant human Fas Ligand (40 ng/ml; Alexis, Carlsbad, CA, USA) and the agonistic anti-Fas antibody CH11 (100 ng/ml; Upstate Biotechnology, Lake Placid, NY, USA), for 24 h in complete medium. Cells were then stained with Annexin V-FITC Apoptosis Detection kit I (BD Pharmingen, San Diego, CA, USA) and immediately analyzed by a FACScan flow cytometer (BD Biosciences) using CELLQUEST software. Data were calculated as % apoptosis (% annexin V-FITC-positive cells) and reported as mean % apoptosis \pm SEM for a minimum of three independent experiments.

Western blot analysis

At harvest, cells were lysed in 100 μ l of cell lysis buffer (20 mM Tris, pH 7.4, 100 mM NaCl, 1% Triton, 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin and 10 μ g/ml aprotinin). After 30 min on ice, the lysate was clarified by centrifugation and the supernatant was collected. Protein concentration was determined by the bicinchoninic acid assay (Sigma). Equivalent amounts of protein (40 μ g/lane) were electrophoresed on 8% SDS-polyacrylamide gel. The gel was electroblotted onto PVDF membrane. Caspase-8, PARP and β -actin were visualized by probing with respective monoclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

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Conflict of Interest statement. None declared.

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